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A novel role of SIRT2 in regulating gap junction communications via connexin-43 in bovine cumulus-oocyte complexes

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Abstract

SIRT2, the predominantly cytosolic sirtuin, plays important role in multiple biological processes, including metabolism, stress response, and aging. However, the function of SIRT2 in gap junction intercellular communications (GJICs) of cumulus-oocyte complexes (COCs) is not yet known. The purpose of the present study was to evaluate the effect and underlining mechanism of SIRT2 on GJICs in COCs. Here, we found that treatment with SIRT2 inhibitors (SirReal2 or TM) inhibited bovine oocyte nuclear maturation. Further analysis revealed that SIRT2 inactivation disturbed the GJICs of COCs during in vitro maturation. Correspondingly, both the Cx43 phosphorylation levels and MEK/MER signaling pathways were induced by SIRT2 inhibition. Importantly, SIRT2-mediated Cx43 phosphorylation was completely abolished by treatment with MEK1/2 inhibitor (Trametinib). Furthermore, treatment with SIRT2 inhibitors resulted in the high levels of MEK1/2 acetylation. Functionally, downregulating the MER/ERK pathways with inhibitors (Trametinib or SCH772984) could attenuate the closure of GJICs caused by SIRT2 inactivation in partly. In addition, inhibition of SIRT2 activity significantly decreased the membrane and zona pellucida localization of Cx43 by upregulating the levels of Cx43 acetylation. Taken together, these results demonstrated a novel role that SIRT2 regulates GJICs via modulating the phosphorylation and deacetylation of Cx43 in COCs.

KEYWORDS
acetylation, connexin-43, gap junction intercellular communications, phosphorylation, SIRT2

1 | INTRODUCTION

Gap junctions contain intercellular channels that connect neighboring cells and allow passage of small molecules (Nicholson, 2003). The gap junction intercellular communications (GJICs) are strongly involved in multiple physiological processes such as tumorigenesis (Z. Khan et al., 2014), stress response (Ge et al., 2018), adipogenic differentiation (Wiesner, Berberich, Hoefner, Blunk, & Bauer-Kreisel, 2018), and nervous system functions (Bosone, Andreu, & Echevarria, 2016). In the mammalian follicle, the GJICs are observed between cumulus cells and oocytes (Gilula, Epstein, & Beers, 1978). There are abundant evidence showing that GJICs play a central role in oocyte maturation and fertilization (Carabatsos, Sellitto, Goodenough, & Albertini, 2000; Sela-Abramovich, Edry, Galiani, Nevo, & Dekel, 2006). The presence of cumulus cells is thought to be essential for oocyte maturation and development. An alternative possibility is that cumulus cells transmit a maturation-promoting signal to oocytes via gap junctions (Eppig, 2001). Numerous studies have shown that cumulus cells are depended on GJICs supplying some small nutrients (such as glucose metabolites, glutamate, ions) or regulatory molecules (such as ATP or cAMP) for oocyte by supporting the cytoplasmic maturation or

fertilization (Carvacho, Piesche, Maier, & Machaca, 2018; Nagai, Ding, & Moor, 1993; Van-Soom, Tanghe, De-Pauw, Maes, & de-Kruif, 2002).

Gap junctions are composed of connexins, a multigene family of integral membrane proteins. There are at least eight types of connexin proteins, including Cx26, Cx30.3, Cx32, Cx37, Cx40, Cx43, Cx45, and Cx57 that have been identified in the mammalian ovarian (Kidder & Mhawi, 2002). Among these connexins, Cx43 is known as the most abundantly expressed in granulosa cells throughout all stages of follicle development, which has been proposed as a major mediator of gap-junction channels (Grazul-Bilska, Reynolds, & Redmer, 1997). A decrease of Cx43 protein is observed in cumulus cell-enclosed oocytes of diabetic mice, which may have a detrimental effect on oocyte maturation and development (Chang, Dale, & Moley, 2005). In the mouse model, Cx43-deficient interrupts folliculogenesis, and prevents cumulus cells from forming more than one layer around the oocyte, thereby resulting in severe retardation of oocyte development and failure of meiotic maturation (Ackert, Gittens, O'Brien, Eppig, & Kidder, 2001). These findings suggest that Cx43 appears to have an indispensable role in oocyte development and maturation. Cx43 is a multiphosphorylated protein, which can determine the conductance and permeability of gap junctions at Ser-279/282/262/368 and Tyr-265 positions in a short period of time (Boeldt, Grummer, Yi, Magness, & Bird, 2015; Lampe & Lau, 2004). Evidence support that phosphorylation of Cx43 can close the gap-junction channels between cumulus cells and oocytes (Richards et al., 2004; Zhang et al., 2019). Previous studies indicated that Cx43 can be phosphorylated by some protein kinases and signaling pathways, including protein kinase C (PKC; Yang, Peng, Wu, Li, & Liu, 2017), mitogen-activated protein kinase (MAPK) (Hossain, Ao, & Boynton, 1998), extracellular signal-regulated kinase (ERK; Cho et al., 2002), and inositol 1,4,5-trisphosphate (IP3; Kang et al., 2014). Interestingly, recent studies have demonstrated that lysine acetylation of Cx43 determines its subcellular localization, thereby affecting gap junctions in cardiomyocytes and Hela cells (Colussi et al., 2011; Laguesse et al., 2017). Although the posttranslational modification of Cx43 has been implicated as a regulatory pathway for the gap junction channels, the mechanisms that regulate GJICs between cumulus cells and oocytes are still poorly understood.

Sirtuins are an evolutionarily conserved family of NAD^+-dependent deacetylases that have been implicated in diverse biological events, including aging (I. Khan et al., 2017), metabolism (Gomes, Fleming-Outeiro, & Cavadas, 2015), stress response (Bi et al., 2019), and apoptosis (Veiga-Santos et al., 2014). There are seven mammalian Sirtuins (SIRT1–7), which display diversity in subcellular localization and function (Frye, 2000). Of these seven, SIRT2 is the only member of Sirtuins that predominantly located in the cytoplasm (Vaquero et al., 2006). Several studies showed that SIRT2 mediated MEK kinase activity (Bajpe et al., 2015; Yeung et al., 2015), which might affect Cx43 phosphorylation levels. Furthermore, a recent report has shown that Cx43 is deacetylated by HDAC6 in apical progenitors of the cerebral cortex (Laguesse et al., 2017). HDAC6 is a cytoplasmic localization deacetylase, and has been shown to acetylate α-tubulin. Similar to HDAC6, SIRT2 as one deacetylase of cytoplasm also may mediate the same cytoplasmic target Cx43. Specially, we have previously discovered that SIRT2 is abundantly expressed in bovine cumulus cells and oocytes (Xu et al., 2019).

These findings emerge that SIRT2 might regulate the post-translational modifications of Cx43. It would be interesting to test whether SIRT2 is involved in Cx43-mediated gap junctions in cumulus-oocyte complexes (COCs) during in vitro maturation. To verify this hypothesis, we investigated the possible function of SIRT2 on GJICs, as well as the mechanisms underlying SIRT2′s actions on Cx43 in COCs.

2 | MATERIALS AND METHODS

2.1 | Chemicals and reagents

SirReal2 (Cat#: S7845), Thiomyristoyl (TM, Cat#: S8245), Trametinib (Cat#: S2673), and SCH772984 (Cat#: S7101) were purchased from Selleck chemicals (Houston, TX). Rabbit polyclonal anti-Cx43 (Cat#: 15386-1-AP) antibody was purchased from Proteintech Group, Inc (Wuhan, China). Rabbit polyclonal anti-pCx43 (Phospho-Ser368, Cat#: 11258) antibody was purchased from Signalway antibody LLC (College park, MD). Rabbit polyclonal anti-ERK1/2 (Cat#: bs-0022R), anti-pERK1/2 (Phospho-Thr202/Tyr204; Cat#:bs3016R), anti-MEK1/2 (Phospho-Ser218/Ser222, Cat#: bs3270R) antibodies, and goat anti-rabbit IgG/HRP (bs-0295G) were purchased from Bioss (Beijing, China). Rabbit monoclonal anti-GAPDH (Cat#: ab181603), anti-acetyl lysine (Cat#: ab190479) antibodies, and anti-MEK1/2 (Cat#: ab178876) were purchased from Abcam (Cambridge, UK). Unless otherwise indicated, the other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

2.2 | Isolation and culture of bovine COCs and treatment

Bovine ovaries were obtained from a local abattoir (Shaanxi, China), and delivered to the laboratory in phosphate-buffered saline (PBS) containing penicillin (100 IU/ml) and streptomycin (100 mg/ml) at 27–30°C within 8 hr. Follicles (2–8 mm in diameter) were aspirated using an 18-Gauge needle on a 10 ml disposable syringe. The cumulus-oocyte complexes (COCs) with three or more layers of cumulus cells were chosen for in vitro maturation (IVM). After washing with TCM199 (Gibco, Waltham, MA) containing 5% (v/v) fetal bovine serum (FBS), approximately 50 COCs were randomly selected as a group, and cultured in 750 μl of IVM medium at 38.5°C in a humidified atmosphere containing 5% CO_2 for 0, 6, 12, and 24 hr, respectively. The IVM medium was TCM-199, supplemented with 10% (v/v) FBS (Gibco), 1% (v/v) ITS (containing 1.0 mg/ml recombinant human insulin, 0.55 mg/ml human transferrin, and 0.5 μg/ml sodium selenite at the 100× concentration), 0.1 IU/ml human menopausal gonadotropin (HMG; Livzon Pharm, Zhuhai, China), 0.2 mM sodium pyruvate, 10 ng/ml epidermal

growth factor (EGF), 0.2 IU/ml follicle-stimulating hormone (FSH; Ningbo Second Hormone Factory, Ningbo, China), 2 μg/ml 17β-estradiol, 100 IU/ml penicillin, and 100 mg/ml streptomycin. According to previous reports (Xu et al., 2019; Hong et al., 2018; Zhao et al., 2017) and viability assay, the optimal concentrations of SIRT2 inhibitors (5 μM SirReal2, 2 μM TM), and/or MEK1/2 inhibitor (10 nM Trametinib), ERK/2 inhibitor (1 μM SCH772984) were supplemented in IVM medium, respectively.

2.3 | Viability and maturation assessment of oocytes

The viability and maturation of bovine oocytes were evaluated following a previously described procedure with some modifications (Casas, Bonilla, Ducolomb, & Betancourt, 2010; Domínguez et al., 2019). After treatment with various concentrations of SirReal2 (1.0, 2.0, 5.0, and 10.0 μM) or TM (0.5, 1.0, 2.0, and 4.0 μM) for 24 hr, the viability of bovine oocytes was evaluated using a methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay kit (Beyotime, China). Briefly, oocytes were mechanically denuded and incubated for 30 min in 0.5 mg/ml MTT. Viable oocytes contain NAD(P)H-dependent oxidoreductase enzymes, which reduce the MTT reagent to formazan, an insoluble crystalline product with a deep purple color. Purple stained oocytes were considered alive when evaluated under a light microscope. For the evaluation maturation, oocytes were incubated with 10 μg/ml Hoechst 33342 (Beyotime) for 10 min and fixed for 30 min with 5% paraformaldehyde. After washed with PBS, only oocytes showing second metaphase (MII) and a polar body were judged to be maturation using a confocal epifluorescence microscope. Then the percentages of viability and maturation oocytes were calculated.

2.4 | GJIC assay of oocyte-cumulus cells

Calcein-AM (calcein-acetoxymethyl; Invitrogen) was used to examine the GJICs between cumulus cells and oocyte, as previously described (Santiquet, Develle, Laroche, Robert, & Richard, 2012; Zhang et al., 2019). Carbenoxolone disodium (CBX; Sigma-Aldrich), an uncoupling agent of gap junctions as a blocker, was used as a positive control at 100 μM (Domínguez et al., 2016; Santiquet et al., 2012). COCs were exposed to calcein-AM (1 mM) for 15 min after treatment with SIRT2 inhibitors and/or MEK1/2 inhibitor, ERK/2 inhibitor for 0, 6, and 12 hr, then were transferred to calcein-AM free IVM media. For fluorescent calcein exchange between the cumulus cells and the oocyte, the preincubated COCs were cultured for a further 25 min at 38.5°C in a humidified atmosphere containing 5% CO_2. COCs were then washed in PBS containing 0.01% (w/v) PVA at least three times to remove the unincorporated dye. The stained COCs were photographed under a confocal epifluorescence microscope (Nikon, JP), and the fluorescence intensity of calcein in COCs were obtained using Image-Pro Plus 6.0 software. For evaluating the GJIC between the oocyte and cumulus cells, the ratio of mean integrated optical density in the oocyte to mean integrated optical density in cumulus cells was calculated.

2.5 | Immunofluorescence staining

COCs were fixed in 4% paraformaldehyde in PBS for 30 min at room temperature. After washing, the COCs were permeabilized for 30 min at room temperature in PBS containing 0.5% (v/v) Triton X-100. Then, the permeabilized COCs were blocked for 1 hr at room temperature with QuickBlock™ blocking buffer (Beyotime), and incubated with rabbit polyclonal anti-Cx43 (1:100), rabbit polyclonal anti-pCx43 (1:100) antibodies at 4°C overnight, respectively. After washing with PVA–PBS, COCs were incubated for 1.5 hr at room temperature in the dark with Alexa Fluor 555-labeled goat anti-rabbit IgG (1:200; Beyotime). The chromosomes were counterstained with 4′,6-diamidino-2-phenylindole, and the stained COCs were mounted and observed with a confocal epifluorescence microscope (Nikon, JP) or Leica confocal microscope (Leica, Germany). Then, the fluorescence intensities of COCs were analyzed by Image-Pro Plus 6.0 software (Media Cybernetics).

2.6 | Western blot analysis

After treatment for 6 hr, COCs were lysed with cold radioimmunoprecipitation assay buffer supplemented with 1 mM phenylmethylsulphonyl fluoride (Beyotime) on ice for 30 min. The total protein concentrations of COCs were measured using a BCA protein assay kit (Beyotime). Equal amounts of protein were mixed with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample loading buffer and boiled for 5 min. About 20 μg of total proteins were submitted to each gel electrophoresis and separated by 15% SDS-PAGE gel. After transferring onto nitrocellulose membranes (Beyotime), nonspecific binding sites were blocked for 1 hr with blocking buffer (TBST containing 5% (w/v) nonfat dry milk), and incubated overnight at 4°C with primary antibodies (anti-pMEK1/2, 1:500; anti-MEK1/2, 1:500; anti-pERK1/2, 1:500; anti-ERK1/2, 1:500; anti-pCx43, 1:500; anti-Cx43, 1:500; anti-GAPDH, 1:10,000) antibodies, respectively. After washed with TBST, the membranes were incubated with slight shaking for 2 hr at room temperature with HRP-conjugated goat anti-rabbit IgG (H+L; 1:5,000; Sungene Biotech, China). After washed at least four times with TBST, the membranes were exposed to X-ray film for visualization with ECL Plus (Millipore), and band intensities were quantified with Quantity One software (v. 4.52; Bio-Rad Laboratories).

2.7 | Coimmunoprecipitation

The freshly lysed proteins of COCs (a total of 200 COCs each treatment) were used for immunoprecipitation assays. The 10%

lysates were analyzed by western blot analysis with anti-MEK1/2 (1: 500) and anti-Cx43 (1:500) antibodies, respectively. Nonspecific background was eliminated with protein A agarose and normal rabbit IgG (Beyotime). The anti-MEK1/2 (1:50) and anti-Cx43 (1:50) antibodies were added to the lysate samples, respectively, and incubation occurred overnight at 4℃ with slight shaking. The immune complexes were captured by protein A agarose with slight shaking for 3 hr at 4℃. Then the IPKine™ HRP-mouse anti-rabbit IgG LCS (Abbkine, China; 1:5,000) was used to clear heavy chain blotting contamination from the immunoprecipitation. The immune complexes were performed by western blot analysis with anti-MEK1/2 (1:500), anti-Cx43 (1:500), anti-Ac-Lys (1:1,000) as previously described, respectively.

2.8 | Statistical analysis

All of these experiments were repeated at least three times unless specified otherwise. Data were represented as mean ± standard error of the mean. Statistical analyses were performed using SPSS 20.0 statistical software (SPSS Inc.). Statistical comparisons were analyzed by one-way analysis of variance followed by Duncan's test, and differences between treatment groups were assessed with Student's *t*-test (the means of two groups) using A $p < .05$ was considered as a statistically significant difference.

3 | RESULTS

3.1 | Effect of SIRT2 inhibitors on oocyte viability and nuclear maturation

After treatment with various concentrations of SIRT2 inhibitors (SirReal2, TM), the viability of oocyte was evaluated by the MTT assay. As shown in Figure 1a,b, treatment with 1.0, 2.0, and 5.0 μM SirReal2 or 0.5, 1.0, and 2.0 μM TM hardly affected the viability of oocyte ($p > .05$), whereas a significant decrease in oocyte viability was observed at a higher concentration of

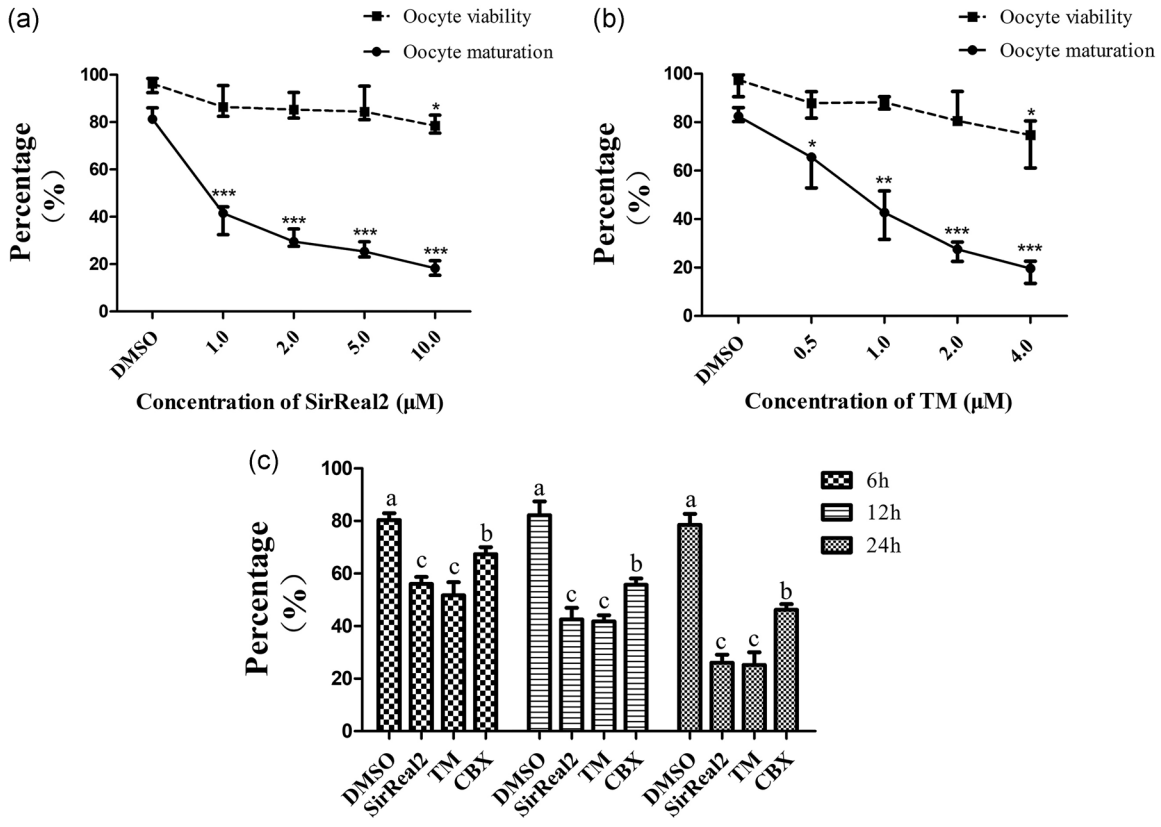


FIGURE 1 SIRT2 inactivation inhibited oocyte nuclear maturation. (a) Effect of SirReal2 (1.0, 2.0, 5.0, and 10.0 μM) on the viability (open curve: viability percentage) and in vitro maturation (closed curve: maturation percentage). (b) Effect of TM (0.5, 1.0, 2.0, and 4 μM) on the viability (open curve: viability percentage) and in vitro maturation (closed curve: maturation percentage). (c) The percentage of oocyte maturation in SirReal2 (5.0 μM)-, TM (2.0 μM)-, and CBX (100.0 μM)-exposed COCs at 6, 12, and 24 hr of incubation. For viability and maturation, more than 600 oocytes in three independent experiments were analyzed. The graph shows the mean ± SEM of the results obtained in three independent experiments. CBX, carbenoxolone disodium; COC, cumulus-oocyte complexes; DMSO, dimethyl sulfoxide; SEM, standard error of mean; *$p < .05$; **$p < .01$; ***$p < .001$, comparing the indicated groups. Bars with different letters (a, b, and c) indicate significant differences, $p < .05$

10.0 μM SirReal2 or 4.0 μM TM ($p < .05$). Interestingly, SIRT2 inhibitors significantly decreased the percentage of oocyte maturation in a dose-dependent manner ($p < .05$), indicating that inhibition effect of SIRT2 inactivation on oocyte maturation might depend on its deacetylation activity, but not because of cytotoxicity-induced viability issues. To analyze the potential underlying mechanism of oocyte maturation caused by SIRT2 inhibitors, we incubated bovine cumulus-oocyte complexes (COCs) with CBX, a known blocker of gap junctions. Consistent with SIRT2 inhibitors, gap junction communications (GJC) breakdown with CBX treatment significantly inhibited oocyte maturation (Figure 1c; $p < .05$), suggesting that redundancy of functions might exist between SIRT2 and GJC in vitro maturation.

3.2 | SIRT2 regulated GJICs during in vitro maturation

It is well known that GJICs are necessary for oocyte development and maturation. To determine whether SIRT2 mediated the GJICs, the COCs were treated with SIRT2 inhibitors (5 μM SirReal2, 2 μM TM) for 0, 6, and 12 hr, respectively, then GJICs were assessed using calcein-AM. As shown in Figure 2a, significant fluorescence intensity was observed in oocytes and cumulus cells in the control, CBX, and treatment groups at 0 hr of incubation, suggesting good gap communications between COCs. After 6 and 12 hr of incubation, although a strong fluorescence was still observed in cumulus cells, the fluorescence was weak inside the oocytes in the SirReal2-, TM-, and CBX- exposed groups (Figure 2a,b). The results showed that SIRT2 inhibitors (SirReal2, TM) prevented calcein transfer from cumulus cells to oocytes significantly ($p < .05$), indicating that SIRT2 inactivation could disrupt the GJICs in COCs.

3.3 | SIRT2 inactivation increased phosphorylated Cx43 via upregulating MER/ERK signaling pathways

The importance of Cx43 as a major contributor to gap junctions was confirmed in mammalian COCs (Kalma, Granot, Galiani, Barash, & Dekel, 2004; Winterhager & Kidder, 2015). To analyze the underlining mechanism that SIRT2 inactivation induced disruption of GJICs, the role of SIRT2 on Cx43 was determined in COCs. As shown in Figure 3a, Cx43 was abundantly expressed in bovine cumulus cells, indeed. By performing immunofluorescence staining and western blot analysis, the results showed that the protein levels of Cx43 were unaltered in the control, SirReal2-, and TM-exposed groups ($p > .05$; Figure 3a,b,d,e). In most cases, gap junction channel gating is regulated by phosphorylation and dephosphorylation of connexins (Moreno & Lau, 2007). Thus, the levels of phosphorylated Cx43 in COCs were evaluated after pre-incubation with SirReal2 or TM for 6 hr, respectively. As documented by immunofluorescence and western blot analysis, the levels of phosphorylated Cx43 on S368 was significantly increased by treatment with SirReal2 or TM ($p < .01$; Figure 3a,c,d,f), suggested that SIRT2 inactivation results in hyperphosphorylation of Cx43.

To determine the possible SIRT2-mediated pathways of phosphorylated Cx43, the COCs were challenged by MEK1/2 antagonist (Trametinib) for 6 hr with or without pretreatment with SIRT2 inhibitor. The data showed that inhibition of SIRT2 activation enhanced the phosphorylation levels of MEK1/2 and ERK1/2 in COCs ($p < .05$; Figure 3d,g,h). Moreover, treatment with Trametinib could prevent phosphorylation of MEK/ERK in the response to SirReal2 or TM exposure (Figure 3d,g,h), indicating that SIRT2 inhibition increased the activity of MEK/ERK signaling pathways. Importantly, the upregulation effect of SIRT2 inactivation on phosphorylated Cx43 was abrogated by treatment with Trametinib (Figure 3d,f). Considered together, these

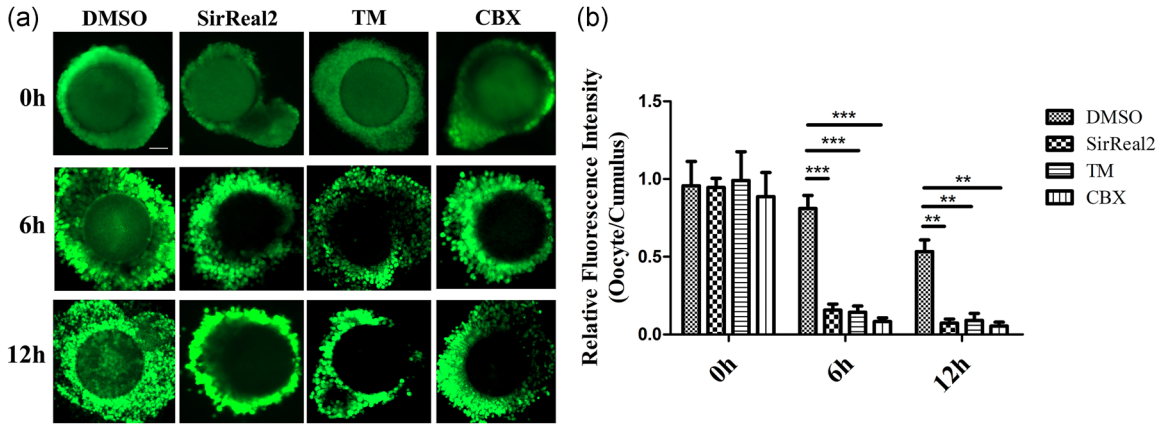
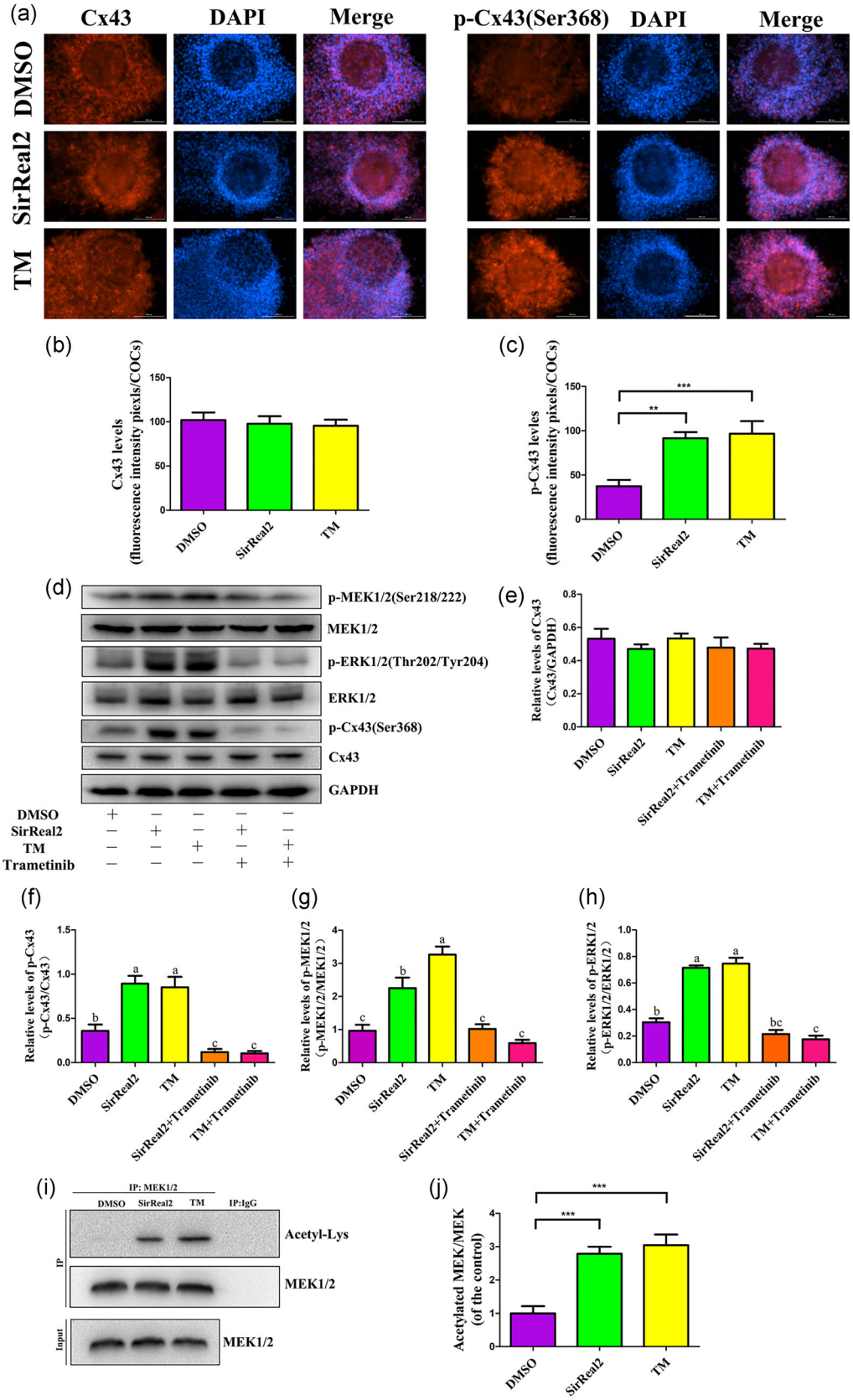


FIGURE 2 SIRT2 inhibition disturbed gap junction communication in bovine cumulus-oocyte complexes (COCs). (a) Representative images showing the calcein-AM transfer from cumulus cells to oocytes in DMSO-, SirReal2 (5.0 μM)-, TM (2.0 μM)-, and CBX (100.0 μM)-exposed COCs at 0, 6, and 12 hr of incubation. Scale bar: 100 μm. (b) The ratio of oocytes/cumulus cells calcein fluorescence intensity to reflect permeability of gap junction in different treatment groups. The graph shows the mean ± SEM of the results obtained in three independent experiments, with a total of 261 COCs (pictures). CBX, carbenoxolone disodium; DMSO, dimethyl sulfoxide; SEM, standard error of mean; $^{**}p < .01$; $^{***}p < .001$, comparing the indicated groups


(a)
Cx43
DAPI
Merge
p-Cx43(Ser368)
DAPI
Merge
DMSO
SirReal2
TM
(b)
Cx43 levels (fluorescence intensity pixels/COCs)
DMSO
SirReal2
TM
(c)
p-Cx43 levles (fluorescence intensity pixels/COCs)
DMSO
SirReal2
TM
(d)
p-MEK1/2(Ser218/222)
MEK1/2
p-ERK1/2(Thr202/Tyr204)
ERK1/2
p-Cx43(Ser368)
Cx43
GAPDH
DMSO
SirReal2
TM
Trametinib
(e)
Relative levels of Cx43 (Cx43/GAPDH)
DMSO
SirReal2
TM
SirReal2+Trametinib
TM+Trametinib
(f)
Relative levels of p-Cx43 (p-Cx43/Cx43)
DMSO
SirReal2
TM
SirReal2+Trametinib
TM+Trametinib
(g)
Relative levels of p-MEK1/2 (p-MEK1/2/MEK1/2)
DMSO
SirReal2
TM
SirReal2+Trametinib
TM+Trametinib
(h)
Relative levels of p-ERK1/2 (p-ERK1/2/ERK1/2)
DMSO
SirReal2
TM
SirReal2+Trametinib
TM+Trametinib
(i)
IP: MEK1/2
DMSO
SirReal2
TM
IP:IgG
Acetyl-Lys
MEK1/2
IP
Input
MEK1/2
(j)
Acetylated MEK/MEK (of the control)
DMSO
SirReal2
TM

results revealed that SIRT2 regulated phosphorylated Cx43 via MEK/ERK pathways. To further confirm how SIRT2 mediated kinase activity, we performed western blot to detect acetylated levels in MEK1/2 immunoprecipitate. The results showed that the levels of acetylated MEK1/2 were significantly increased by treatment with SirReal2 or TM ($p < .001$; Figure 3i,j). These findings indicated that the kinase activity of MEK/ERK pathways is induced by SIRT2 inactivation-mediated MEK1/2 hyperacetylation.

3.4 | MER/ERK signaling pathways contributed to SIRT2-mediated GJICs

To further confirm whether SIRT2-mediated GJICs were dependent on MER/ERK signaling pathways, we exposed COCs to MEK1/2 antagonist (Trametinib), ERK1/2 antagonist (SCH772984) for 6 hr with pretreatment with SIRT2 inhibitors (SirReal2, TM), respectively, and subsequently assessed GJICs using calcein-AM. As shown in Figure 4a,b, the blocking effect of SIRT2 inactivation on GJICs was partly attenuated by treatment with Trametinib or SCH772984 ($p < .05$). Interestingly, MER/ERK-mediated Cx43 dephosphorylation is not sufficient to absolutely abolish the response to SIRT2 inhibitors on GJICs (Figure 4a,b). The results indicated that SIRT2 not only regulates GJICs by ERK1/2-mediated Cx43 phosphorylation but also through other pathways.

3.5 | SIRT2-mediated Cx43 deacetylation drove its membrane localization

Furthermore, we analyzed the cellular localization of Cx43 in COCs after SIRT2 inhibitors treatment using confocal scanning. As shown in Figure 5a,b, the membrane localization of Cx43 was significantly decreased between cumulus cells by treatment with SirReal2 or TM ($p < .01$). Furthermore, a significantly decreased fluorescence intensity of Cx43 was observed at the surface of zona pellucida after treatment with SirReal2 or TM ($p < .001$; Figure 5a,c), suggesting that Cx43 formed connexons at the contact points between cumulus cells and oocyte were closed by SIRT2 inactivation. Previous studies have shown that acetylated Cx43 delocalizes from the membrane and is mainly found in the cytoplasm or nucleus (Colussi et al., 2011; Laguesse et al., 2017). To identify SIRT2 responsible for Cx43 deacetylation, Cx43 acetylation levels were tested using coimmunoprecipitation assays in SIRT2 inhibitor-exposed COCs. As shown in Figure 5d,e, treatment with SirReal2 or TM significantly increased the Cx43 acetylation levels of COCs ($p < .01$), suggesting that SIRT2 can directly target Cx43 as a deacetylase. Altogether, these results indicated that SIRT2 inactivation-mediated Cx43 acetylation leads to its delocalization from the membrane, thereby disturbing the GJICs of COCs.

4 | DISCUSSION

In some reports, rupture of gap junction will induce germinal vesicle breakdown (GVBD) in mouse oocyte via downregulation cGMP/cAMP levels (Norris et al., 2009; Sela-Abramovich et al., 2006), that causes meiotic resumption in mouse oocytes, whereas these studies have not demonstrated whether gap junction breakdown further promotes oocyte growth to the M phase and nuclear maturation. In fact, gap junctions are the main connection between cumulus cells and oocytes during bovine oocyte nuclear maturation (Feng, Shi, Yang, & Wang, 2013), and a rapid transfer of small metabolites from the cumulus cells into the oocyte is essential for oocyte nuclear maturation events (Van-Soom et al., 2002). Moreover, oocytes from connexin-deficient mice fail to undergo the M phase and meiotic maturation (Ackert et al., 2001; Carabatsos et al., 2000). In this study, SIRT2 inactivation-induced gap junction breakdown blocked nuclear maturation in the bovine oocyte. This is in agreement with Vozzi et al. (2001) confirm that the absence of gap junction communication in bovine COCs prevents oocyte nuclear maturation.

It is well known that gap junctions are required for oocyte growth, enabling nutrients, and other small molecules to transfer between cumulus cells and oocytes (Campen et al., 2018; Gilchrist, 2011), but the regulated mechanism for GJICs remains poorly understood during

FIGURE 3 SIRT2 inhibition increased Cx43 phosphorylation via MEK/ERK signaling pathways. (a) Immunofluorescence of Cx43 and p-Cx43 (Ser368) expressed in DMSO-, SirReal2-, and TM-exposed COCs. After different treatment conditions for 6 hr, COCs were stained with anti-Cx43 or anti-p-Cx43 antibody (red), and counterstained with DAPI (blue). Scale bar: 100 μm. (b) Fluorescence intensity of Cx43 in DMSO-, SirReal2 (5.0 μM)-, and TM (2.0 μM)-exposed COCs. The graph shows the mean ± SEM of the results obtained in three independent experiments, with a total of 127 COCs. (c) Fluorescence intensity of p-Cx43 (Ser368) in different treatment groups. The graph shows the mean ± SEM of the results obtained in three independent experiments, with a total of 114 COCs. (d) Western blot analysis analysis for phosphorylation of MER1/2, ERK1/2 and Cx43 levels in COCs under different treatment conditions for 6 hr. (e–h) The ratios of Cx43 to GAPDH, p-Cx43 to Cx43, p-MEK1/2 to MEK1/2, and pERK1/2 to ERK1/2 expression were normalized and the values are shown, respectively. (i) SIRT2 inhibition resulted in the increased MEK1/2 acetylation levels. COCs were treated with DMSO (control), SirReal2, TM for 6 hr, respectively. Immunoprecipitation (IP) was carried out using anti-MEK1/2 antibodies or nonspecific IgG as control. The immunoprecipitates were analyzed by western blot analysis with anti-Acetyl-Lys antibody or anti-MEK1/2 antibodies. Corresponding western blot with anti-MEK1/2 antibodies were performed on crude cell extracts (inputs). (j) Data are presented as the average ratio of acetylated MEK1/2 to MEK1/2 ± SEM, and are expressed as the ratio of control. Data are expressed as the mean ± SEM from three independent experiments. Bars with different letters (a, b, and c) indicate significant differences, COC, cumulus-oocyte complexes; DAPI, 4′,6-diamidino-2-phenylindole; DMSO, dimethyl sulfoxide; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; SEM, standard error of mean; $p < 0.05$; $^{***}p < .001$, comparing the indicated groups

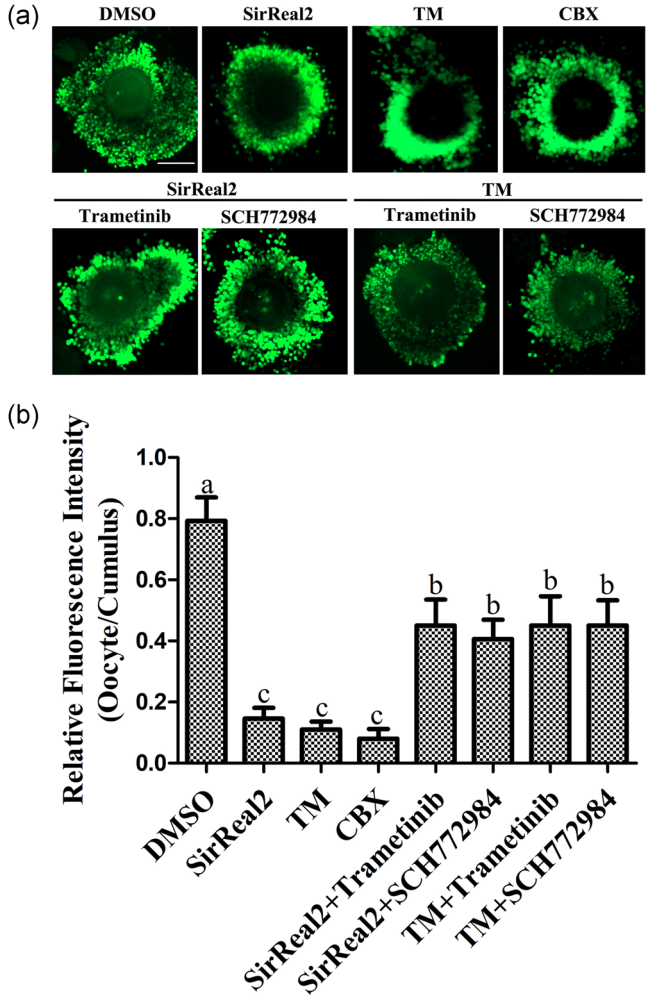


FIGURE 4 MER/ERK pathways regulated SIRT2-mediated gap junction communication between cumulus cells and oocytes. (a) Representative images showing the calcein-AM transfer from cumulus cells to oocytes under different treatment conditions for 6 hr. Scale bar: 100 μm. (b) The ratio of oocytes/cumulus cells calcein fluorescence intensity to reflect the permeability of gap junction in different treatment groups. The graph shows the mean ± standard error of mean of the results obtained in three independent experiments, with a total of 246 COCs. Bars with different letters (a, b, and c) indicate significant differences; COC, cumulus-oocyte complexes; DMSO, dimethyl sulfoxide; $p < .05$

oocyte maturation. In mammal COCs, Cx43 plays a major function on GJICs, which is primarily expressed in granulosa cells, theca cells, and the surface of zona pellucida (Lee et al., 2016; Sommersberg et al., 2000; Zhang et al., 2019). Similar to this finding, our results showed that Cx43 was abundantly observed in cumulus cells and the surface of zona pellucida. We previously reported that SIRT2 also is abundant in cumulus cells (Xu et al., 2019). In addition, treatment with SIRT2 inhibitors (SirReal2 or TM) disturbed the GJICs between COCs in this study, thereby affecting oocyte maturation. Those findings hinted that redundancy of function might exist between SIRT2 and Cx43 in GJICs of oocytes. Thus, we focused on the role of SIRT2 on Cx43-mediated GJICs in the present study.

In the swine model, the gap-junctional communications remain high during early in vitro oocyte maturation until 18 hr, when they fall simultaneously with the oocyte germinal vesicle breakdown (Sasseville et al., 2009). Whereas our results showed that GJICs begin to decrease in vitro maturation at 12 hr in bovine COCs. Compared with a genetic knockdown, inhibitors exhibit a well inhibitory effect in a short time, special in bovine, the gap communications of COCs usually begin to decrease rapidly at the later stage of oocyte maturation in vitro (Lodde, Modina, Galbusera, Franciosi, & Luciano, 2007). Therefore, we used different small molecule inhibitors (SirReal2, TM) to inhibit SIRT2 activity. Because SirReal2 and TM belong to different classes (H. Jing et al., 2016; Rumpf et al., 2015), it is unlikely for them to have the same off-target effects. Interestingly, the GJICs of COCs were disrupted by SIRT2 inactivation at 6 hr of maturation, and this effect remained until 12 hr in this study. Phosphorylation of Cx43 has been shown to an important regulatory mechanism in the gap-junction channels of COCs (Richards et al., 2004; Zhang et al., 2019). The reduced permeability of connexon channels via phosphorylated Cx43 of Ser368 has been well demonstrated (Bao, Reuss, & Altenberg, 2004). Our study showed that SIRT2 inactivation with inhibitors increased the phosphorylation of Cx43 at Ser 368. Previous studies have suggested that Cx43 can be phosphorylated by ERK kinase activity at Ser368 (Pahujaa, Anikin, & Goldberg, 2007; Zhang et al., 2019). Certainly, some kinases (e.g., PKC, MAPK) also phosphorylated Cx43 at Ser 279/282/368, which have been shown to reduce the permeability of Cx43 channels (Dyce, Norris, Lampe, & Kidder, 2012; Lampe et al., 2000). Further, our work has revealed that SIRT2 inhibition induced activation of MER/ERK signaling pathways, and down-regulating MER1/2 kinase activity with inhibitors (Trametinib) could abolish the SIRT2-mediated Cx43 phosphorylation, suggesting that SIRT2 inactivation increases phosphorylated Cx43 via the activation of MER/ERK. It would be interesting to test whether SIRT2 is also involved in the regulation of MER acetylation. As expected, an increase in MEK acetylation was observed in SIRT2 inhibitors-exposed COCs. Yeung et al. (2015) had well demonstrated a novel post-translational regulation of MEK by showing that acetylation of MEK activates its kinase activity. Consistent with our results, Bajpe et al. (2015) found that loss of SIRT2 expression resulted in an increase in MEK acetylation and activation of its kinase activity, thereby upregulating ERK activity. These evidence support that SIRT2 regulates phosphorylated Cx43 via MER deacetylation.

Previous reports have shown that ERK1/2-mediated Cx43 phosphorylation is an important mechanism for gap junctions (Solan, Marquez-Rosado & Lampe, 2019; Zhang et al., 2019). Interestingly, although our work indicated that blocking the MER/ERK pathways attenuated the closure of GJICs caused by SIRT2 inactivation in part, these effects could not be completely restored. These results raised the possibility that SIRT2 might not only regulate GJICs by ERK1/2-mediated Cx43 phosphorylation but also through other pathways in COCs. By performing confocal scanning, our data showed that the membrane localization of Cx43 was significantly reduced in both cumulus cells and zona pellucida of oocytes by SIRT2 inhibition.

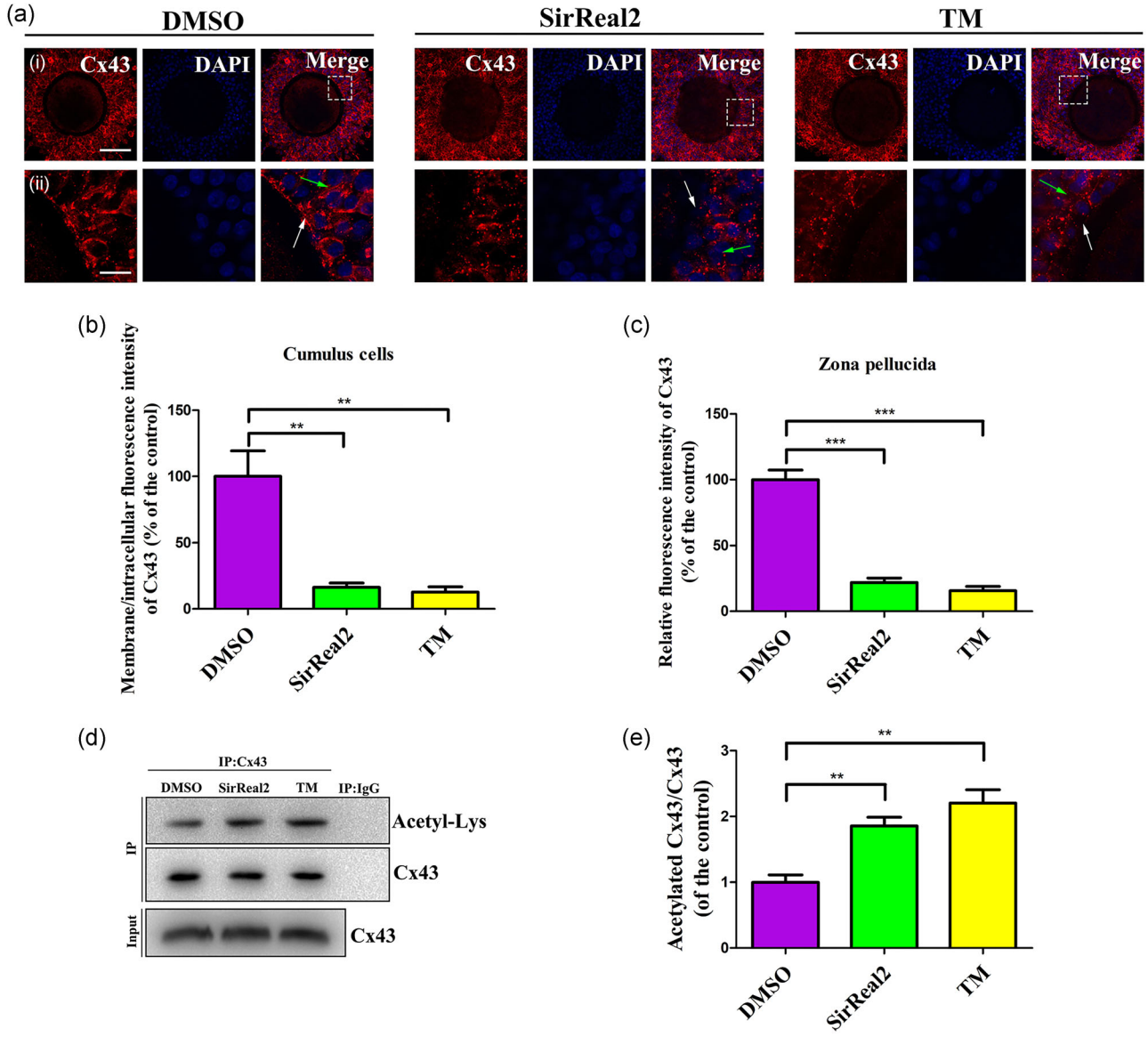


FIGURE 5 SIRT2 regulated Cx43 localization via deacetylating Cx43. (a) Representative images showing the Cx43 localization in DMSO-, SirReal2-, and TM-exposed COCs using a confocal scanning. After different treatment conditions for 6 hr, COCs were stained with anti-Cx43 antibody (red), and counterstained with DAPI (blue). Green arrows indicated cellular localization of Cx43 in cumulus cells, whereas white arrows indicated localization of Cx43 at the surface of zona pellucidae. (a) Scale bar: 50 μm. (b) Scale bar: 10 μm. (b) Cx43 membrane localization was determined by measuring the ratio of membrane and intracellular fluorescence intensity, and were expressed as percentage of control in DMSO-, SirReal2 (5.0 μM)-, and TM (2.0 μM)-exposed COCs. The graph shows the mean ± SEM of the results obtained in three independent experiments, with a total of 91 COCs. (c) Fluorescence intensity of Cx43 at the surface of zona pellucidae in different treatment groups. The graph shows the mean ± SEM of the results obtained in three independent experiments, with a total of 105 COCs. (d) SIRT2 inhibition resulted in the increased Cx43 acetylation levels. COCs were treated with DMSO (control), SirReal2, TM for 6 hr, respectively. Immunoprecipitation (IP) was carried out using anti-Cx43 antibodies or nonspecific IgG as control. The immunoprecipitates were analyzed by western blot analysis with anti-Acetyl-Lys antibody or anti-Cx43 antibodies. Corresponding western blot with anti-Cx43 antibodies were performed on crude cell extracts (inputs). (e) Data are presented as the average ratio of acetylated Cx43 to Cx43 ± SEM, and are expressed as the ratio of control. Data are expressed as the mean ± SEM from three independent experiments; COC, cumulus-oocyte complexes; DAPI, 4′,6-diamidino-2-phenylindole; DMSO, dimethyl sulfoxide; SEM, standard error of mean; $^{**}p < .01$; $^{***}p < .001$, comparing the indicated groups

Numerous studies have proved that the function of Cx43 is regulated by several posttranslational modifications, including phosphorylation (Alstrom, Stroemlund, Nielsen, & MacAulay, 2015), acetylation (Meraviglia et al., 2015), and ubiquitination (Leithe, 2016). It is worth noting that SIRT2 is an NAD^+-dependent class I deacetylase, which has a predominantly cytoplasmic localization and has been shown to acetylate cytoplasmic targets such as PEPCK1 (Jiang et al., 2011) and FoxO1 (E. Jing, Gesta, & Kahn, 2007). In addition, Colussi et al. (2011) have shown that Cx43 is localization in the membrane but also in cytoplasmic and nuclear compartments. It would thus be of interest

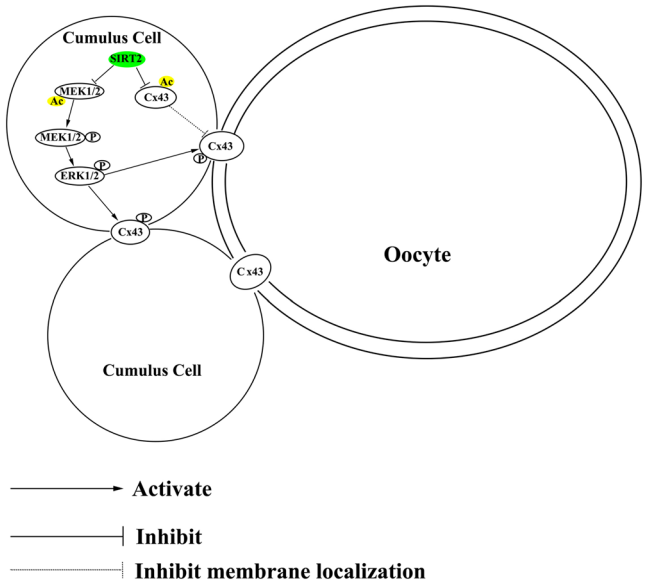


FIGURE 6 Possible mechanism by which SIRT2 mediated GJICs in bovine COCs. SIRT2 restrained Cx43 phosphorylation via downregulating the MEK/ERK signal pathway, thereby upregulating the permeability of Cx43 channels. On the other hand, SIRT2 directly deacetylated Cx43, thereby driving Cx43 membrane localization. COC, cumulus-oocyte complexes; GJIC, gap junction intercellular communications

to test whether SIRT2 could also regulate Cx43 acetylation in COCs. Recently, histone deacetylases (HDACs) as class I, II, and IV deacetylases were reported to play a deacetylation effect on Cx43 (Laguesse et al., 2017). Similar to HDACs, we found here a novel SIRT2-mediated posttranslational regulation of Cx43 by deacetylation. Previous studies have revealed that Cx43 acetylation leads to its delocalization from the membrane toward the intracellular compartment (Colussi et al., 2011; Laguesse et al., 2017). These findings supported our results that SIRT2 might maintain membrane localization of Cx43 via deacetylating Cx43 in COCs, thereby regulating GJICs.

In summary, our results demonstrated a novel role and mechanism for SIRT2 in the regulation of Cx43-mediated GJICs by affecting the posttranslational modifications of Cx43 in COCs during IVM (Figure 6). Specifically, SIRT2 inhibited ERK1/2-mediated Cx43 phosphorylation via deacetylating MEK, thereby upregulating the permeability of Cx43 channels. On the other hand, SIRT2 directly deacetylated Cx43 and contributed its membrane localization. These results demonstrate that SIRT2-dependent deacetylation activity is necessary for GJICs in COCs during in vitro maturation.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

Q. L. provided financial support for this study. D. X. wrote and drafted the manuscript. D. X. and H. H. performed the experiments. D. L. and G. G participated in guiding the design of this research work and supervised the whole experimental work. All authors read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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