

CFTR mediates bicarbonate-dependent activation of miR-125b in preimplantation embryo development

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Although HCO_3^- is known to be required for early embryo development, its exact role remains elusive. Here we report that HCO_3^- acts as an environmental cue in regulating miR-125b expression through CFTR-mediated influx during preimplantation embryo development. The results show that the effect of HCO_3^- on preimplantation embryo development can be suppressed by interfering the function of a HCO_3^- -conducting channel, CFTR, by a specific inhibitor or gene knockout. Removal of extracellular HCO_3^- or inhibition of CFTR reduces miR-125b expression in 2 cell-stage mouse embryos. Knockdown of miR-125b mimics the effect of HCO_3^- removal and CFTR inhibition, while injection of miR-125b precursor reverses it. Downregulation of miR-125b upregulates p53 cascade in both human and mouse embryos. The activation of miR-125b is shown to be mediated by sAC/PKA-dependent nuclear shuttling of NF- κ B. These results have revealed a critical role of CFTR in signal transduction linking the environmental HCO_3^- to activation of miR-125b during preimplantation embryo development and indicated the importance of ion channels in regulation of miRNAs.

Keywords: embryo; HCO_3^- ; miR-125b; CFTR; sAC

Cell Research (2012) 22:1453-1466. doi:10.1038/cr.2012.88; published online 5 June 2012

Introduction

After fertilization, mammalian embryos quickly undergo cleavage and differentiation while traveling along the oviduct and making their way to the uterus before implantation. It has been known for decades that female

reproductive tract, including the oviduct and uterus, contains high concentrations of HCO_3^- (up to 90 mM), which is much higher than that in most other tissues [1-3]. Thus, preimplantation embryo development occurs in a HCO_3^- -rich environment. Indeed, our recent study and previous reports have shown that the absence of HCO_3^- is detrimental beyond two-cell stage. The effect of HCO_3^- is independent of CO_2 and pH, since embryos successfully grew to blastocysts in HEPES-buffered HCO_3^- medium without CO_2 (mouse) [4], but failed to develop beyond two-cell (mouse) [4, 5] or morula (rabbit) [6] stage in HEPES-buffered HCO_3^- -free medium, suggesting that HCO_3^- is required for early embryo development. However, the question as to how HCO_3^-

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Received 20 December 2011; revised 28 March 2012; accepted 9 April 2012; published online 5 June 2012

affects preimplantation embryo development remains unanswered.

MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene expression primarily through base pairing to the 3'UTR of target mRNAs. A number of studies have indicated that miRNAs are essential to embryo development [7, 8]. In particular, miR-125b, which is known to regulate cell proliferation [9, 10] and differentiation [11-13], has been shown to be required for the development of lower organisms, such as *Drosophila* [14] and zebrafish [15]. The expression of miR-125b is also found in mouse preimplantation embryos although its exact role has not been elucidated [16]. Several proteins, including p53, have been reported to be the targets of miR-125b and mediate its action in various physiological and pathological events, including neuronal differentiation [12, 17, 18], immune response [19, 20] and cancer [10, 21-23]. Particularly, p53 has been reported to mediate the effect of miR-125b on embryo development [15]. However, compared to the well-demonstrated downstream pathway of miR-125b, the molecular mechanism and signaling pathway in regulating the expression of miR-125b remain largely unknown. Interestingly, recent studies have suggested that the expression of miRNAs are triggered and/or regulated by microenvironments, such as hypoxia and pH [24-27]. Therefore, it is plausible that the high HCO₃⁻ content in the female reproductive tract may act as an environmental cue in regulating miR-125b expression in the transiting preimplantation embryo.

We have previously demonstrated that HCO₃⁻-rich uterine environment triggers sperm capacitation [28], a process by which sperms acquire their fertilizing capacity. The effect of HCO₃⁻ in triggering downstream signaling requires CFTR (cystic fibrosis transmembrane conductance regulator), a cAMP-activated anion channel known to conduct Cl⁻ [29, 30] and HCO₃⁻ [31], as the necessary HCO₃⁻ transport mechanism, either directly or indirectly [28, 32]. This suggests that high HCO₃⁻ concentration may act as an environmental stimulus in initiating cellular responses through CFTR-mediated entry. After its entry, HCO₃⁻ has been shown to activate the downstream cAMP-dependent pathway in a number of cell types via its sensor, the soluble adenylate cyclase (sAC) [33]. Interestingly, it has been shown that cAMP activation is essential for embryo development [34] and both CFTR and sAC are expressed in preimplantation embryos [5] (Supplementary information, Figures S1 and S2), suggesting their possible involvement in mediating HCO₃⁻-dependent cAMP signaling in preimplantation embryo. Given the recently revealed role of miR-125b in embryo development of lower organisms, we proposed

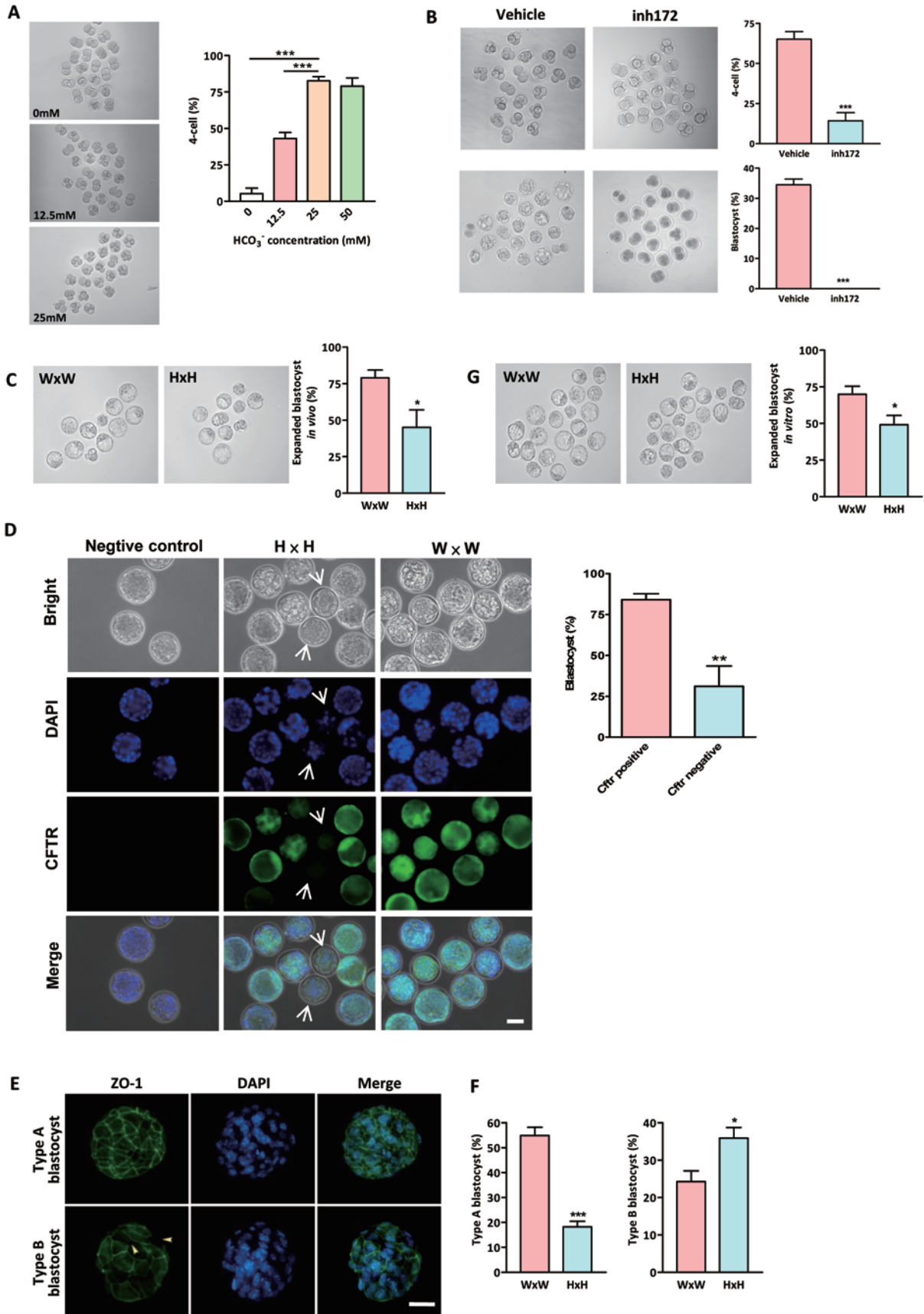
that the high HCO₃⁻ content present in the female reproductive tract may exert its effect on embryo development by regulating miR-125b expression via CFTR-mediated cAMP pathway. We undertook the present study to test this hypothesis.

Results

Involvement of CFTR in HCO₃⁻-dependent preimplantation embryo development

In our previous study, we have compared the effect of HCO₃⁻ and pH on early embryo development and found that pH is dispensable for development from two-cell stage to blastocyst [5], suggesting that HCO₃⁻ ion itself is required for early embryo development. Consistent with our previous observation, the present results showed that the percentage of embryos undergoing the second zygotic division increased in a HCO₃⁻ concentration-dependent manner (Figure 1A). Formation of blastocysts also depended on HCO₃⁻ concentrations (Supplementary information, Figure S3). The effect of HCO₃⁻ on embryo development could be inhibited by a specific inhibitor for CFTR, CFTRinh172 (10 μM), which inhibited HCO₃⁻ influx in embryos (Supplementary information, Figure S4). As shown in Figure 1B, inhibition of CFTR reduced the cleavage from two-cell stage to four-cell stage by 78% and completely abolished the formation of blastocysts (Figure 1B), suggesting a critical role of CFTR in mediating the effect of HCO₃⁻ on embryo development.

To confirm the inhibition of embryo development did not result from the nonspecific effects of CFTRinh172, we further retrieved embryos that had undergone preimplantation development *in vivo* from *Cftr* wild-type and knockout (*Cftr*^{tm1Unc}) mice. Since adult *Cftr*^{-/-} mice are rare due to high mortality rate, *Cftr*^{-/-} embryos could only be obtained through *Cftr*^{+/-} × *Cftr*^{+/-} mating, which should, theoretically, yield 25% *Cftr*^{+/+}, 50% *Cftr*^{+/-} and 25% *Cftr*^{-/-} embryos. Examination of the embryos retrieved on 3.5 dpc (days post coitum) showed that only 45% of the embryos (31 out of 68) from *Cftr*^{+/-} mating formed expanded blastocysts while 79% of the embryos (60 out of 76) from *Cftr*^{+/+} mating formed expanded blastocysts (Figure 1C). To confirm that the developmental defects were specific to *Cftr*^{-/-} embryos, we examined the expression of CFTR in embryos obtained from *Cftr*^{+/-} × *Cftr*^{+/-} crosses. CFTR immunoreactivity should only be observed in wild-type and heterozygous embryos but not in homozygous knockout embryos. Indeed, embryos lacking *Cftr* immunoreactivity exhibited a remarkable decrease in blastocyst formation rate (Figure 1D), suggesting that CFTR is required for blastocyst formation. Further examination of the tight junction protein, ZO-1,



a differentiation marker of preimplantation embryos that involved in morula to blastocyst formation, showed that embryos from *Cftr*^{+/-} mating had a significantly lower number of blastocysts exhibiting well-organized ZO-1 staining pattern as compared to those obtained from wild type (Figure 1E and 1F), indicating that normal embryo development requires CFTR.

To exclude possible influence of maternal effect of cystic fibrosis (CF) mice due to impaired CFTR-mediated oviductal HCO₃⁻ secretion [5], two-cell embryos were obtained on 1.5 dpc from both *Cftr*^{+/+} mating and *Cftr*^{+/-} mating, then cultured in the bicarbonate-containing medium for 72 h. The result showed that 70% of the embryos (87 out of 126) collected from *Cftr*^{+/+} mating, but only 49% of the embryos (60 out of 122) collected from *Cftr*^{+/-} mating, developed to expanded blastocyst (Figure 1G). Taken together, these results suggest that CFTR is involved in mediating the effect of extracellular HCO₃⁻ on early-embryo development.

Regulation of miR-125b by CFTR-mediated HCO₃⁻ influx in mouse embryo development

Since miR-125b, which is known to be involved in cellular differentiation and the development of lower organisms [15, 35], is expressed in mouse preimplantation embryo [16], we attempted to test whether HCO₃⁻ may act as an environmental cue regulating miR-125b levels and hence embryo development. Using real-time PCR, we first examined the expression profile of miR-125b at different stages of mouse preimplantation embryo development and the results showed a relatively high level of miR-125b at two-cell and four-cell stages, but a significantly lower level in morula and blastocyst (Figure 2A), indicating that miR-125b may be required for early cleavage of embryos, but no longer induced at higher

differentiation stage. Strikingly, miR-125b expression in two-cell embryos was significantly reduced by removal of HCO₃⁻ and the addition of CFTR inhibitor, CFTRinh172 (10 μM) (Figure 2B), suggesting that miR-125b expression may be regulated by CFTR-mediated HCO₃⁻ entry. This result also suggests that miR-125b may be important in executing the effect of HCO₃⁻ on embryo development. To test this, we knocked down endogenous miR-125b by transfecting anti-miR-125b oligo into embryos. The results showed that knockdown of miR-125b significantly decreased the number of four-cell embryos formed compared to anti-miR negative control, which does not target any known miRNAs (Figure 2C). The inhibition rate was similar to that obtained using CFTRinh172 (Figure 1B). We also attempted to rescue the impaired embryo development induced by either HCO₃⁻ removal or CFTR inhibition by injecting miR-125b precursor (pre-miR-125b) into two-cell embryos. Indeed, the four-cell and blastocyst formation rates were significantly increased by pre-miR-125b, but not by pre-miR-nc control, in HCO₃⁻-free and CFTR-inhibited conditions (Figure 2D), further confirming the involvement of miR-125b in CFTR/HCO₃⁻-regulated embryo development.

Downregulation of miR-125b by blocking CFTR-HCO₃⁻ pathway leads to upregulation of p53 and p21

We next sought to identify the downstream effector of miR-125b in mouse embryo. A recent study has found that miR-125b is a novel negative regulator of p53 [15]. Preimplantation embryo development has been shown to require the latency of p53 [36] and increased expression of p53 is associated with poor developmental potential of preimplantation embryos [37]. Therefore, we tested whether downregulation of miR-125b by removal

Figure 1 Involvement of CFTR in HCO₃⁻-dependent preimplantation embryo development. **(A)** Effects of HCO₃⁻ on preimplantation embryo development. Embryos were cultured in different concentrations of HCO₃⁻. Transition from two-cell to four-cell embryos were significantly inhibited by removing (0 mM) or reducing extracellular (12.5 mM) HCO₃⁻ (0 mM group: 6/108 embryos; 12.5 mM group: 46/106 embryos; 25 mM group: 95/110 embryos; 50 mM group: 83/105 embryos). **(B)** CFTRinh172 (inh172) significantly reduced four-cell ($n = 4$, 12/85 embryos) and blastocyst formation (0/85 embryos) in embryo culture containing 25 mM HCO₃⁻ compared to the DMSO-treated vehicle control (four-cell: 57/88 embryos; blastocyst: 30/88). **(C)** Embryos obtained from *Cftr*^{+/-} mice (H × H) on 3.5 dpc have reduced percentage of expanded blastocysts (31/68 embryos) as compared to those obtained from *Cftr*^{+/+} mice (W × W) (60/79 embryos). **(D)** *Cftr*^{+/-} embryos, as indicated by lacking CFTR immunoreactivity (arrow), exhibit a remarkable decrease in blastocyst formation rate (5/13 embryos) as compared to CFTR-positive embryos (38/48 embryos). **(E)** Embryos were categorized into two types according to the developmental stages and ZO-1 fluorescence: type A blastocysts showed continuous and well-organized ZO-1 expression only at the cell junction, while type B blastocyst showed disrupted expression of ZO-1 at the cell junction (arrow head), and diffused localization in cytoplasm. **(F)** Embryos from *Cftr*^{+/+} and *Cftr*^{+/-} mice were classified by ZO-1 expression patterns (green) in blastocyst stages. Nuclei were counterstained by DAPI. Embryos obtained from *Cftr*^{+/-} mice have a significantly reduced percentage of type A blastocyst. **(G)** Embryos obtained from *Cftr*^{+/-} mice (H × H) on 1.5 dpc have reduced expanded blastocyst formation (60/122 embryos) after *in vitro* culture for 72 h as compared to those obtained from *Cftr*^{+/+} mice (W × W) (87/126 embryos). Data are presented as mean ± SEM; one-way ANOVA **(A)**; *t*-test **(B-E)**; **P* < 0.05 and ****P* < 0.001. Scale bar, 50 μm.

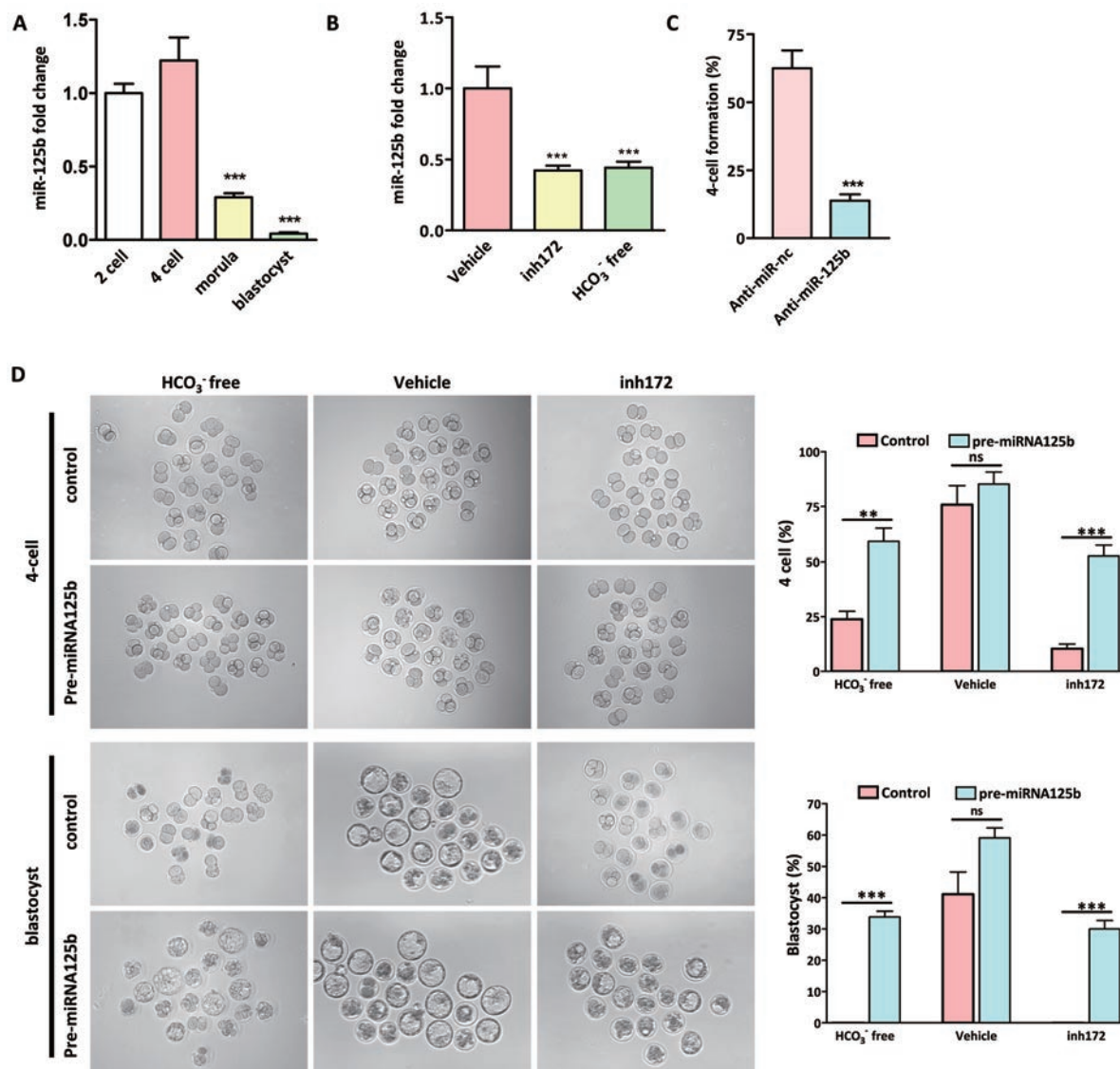


Figure 2 Involvement of miR-125b in CFTR-regulated embryo development. **(A)** Real-time PCR results showing high miR-125b expression in two-cell and four-cell embryos, but significantly reduced in morula and blastocyst stages ($n = 4$, 100 embryos/group). **(B)** MiR-125b expression in two-cell embryo was inhibited by CFTRinh172 treatment (compared to DMSO-treated vehicle control) and in HCO₃⁻ free condition ($n = 4$, 100 embryos/group). **(C)** Knockdown of miR-125b inhibited four-cell embryo formation ($n = 4$, 80 embryos/group). **(D)** Impaired four-cell and blastocyst formation by removal of HCO₃⁻ and CFTR inhibition was rescued by injection of miR-125b precursor (pre-miR-125b) ($n = 3$); HCO₃⁻ free + pre-miR-nc group: four-cell (15/62 embryos), blastocyst (0/62 embryos); HCO₃⁻ free + pre-miR-125b group: four-cell (36/61 embryos), blastocyst (21/60 embryos); Vehicle + pre-miR-nc group: four-cell (46/60 embryos), blastocyst (25/60 embryos); Vehicle + pre-miR-125b group: four-cell (55/64 embryos), blastocyst (38/64 embryos); CFTRinh172 + pre-miR-nc group: four-cell (10/60 embryos), blastocyst (0/60 embryos); CFTRinh172 + pre-miR-125b group: four-cell (33/63 embryos), blastocyst (19/63 embryos). Data are presented as mean \pm SEM; One way ANOVA (**A**, **B**); *t*-test (**C**, **D**); ** $P < 0.01$, *** $P < 0.001$, ns – not significant. Scale bar, 50 μ m.

of HCO₃⁻ or addition of CFTR inhibitor could lead to the increase in p53 and its downstream target p21 in preimplantation embryos, resulting in impaired development. Immunofluorescence results showed that p53

and p21 signals were enhanced after two-cell embryos were treated with CFTRinh172 or cultured in HCO₃⁻ free condition (Figure 3A). The increase in p53 protein level is consistent with the downregulation of miR-125b

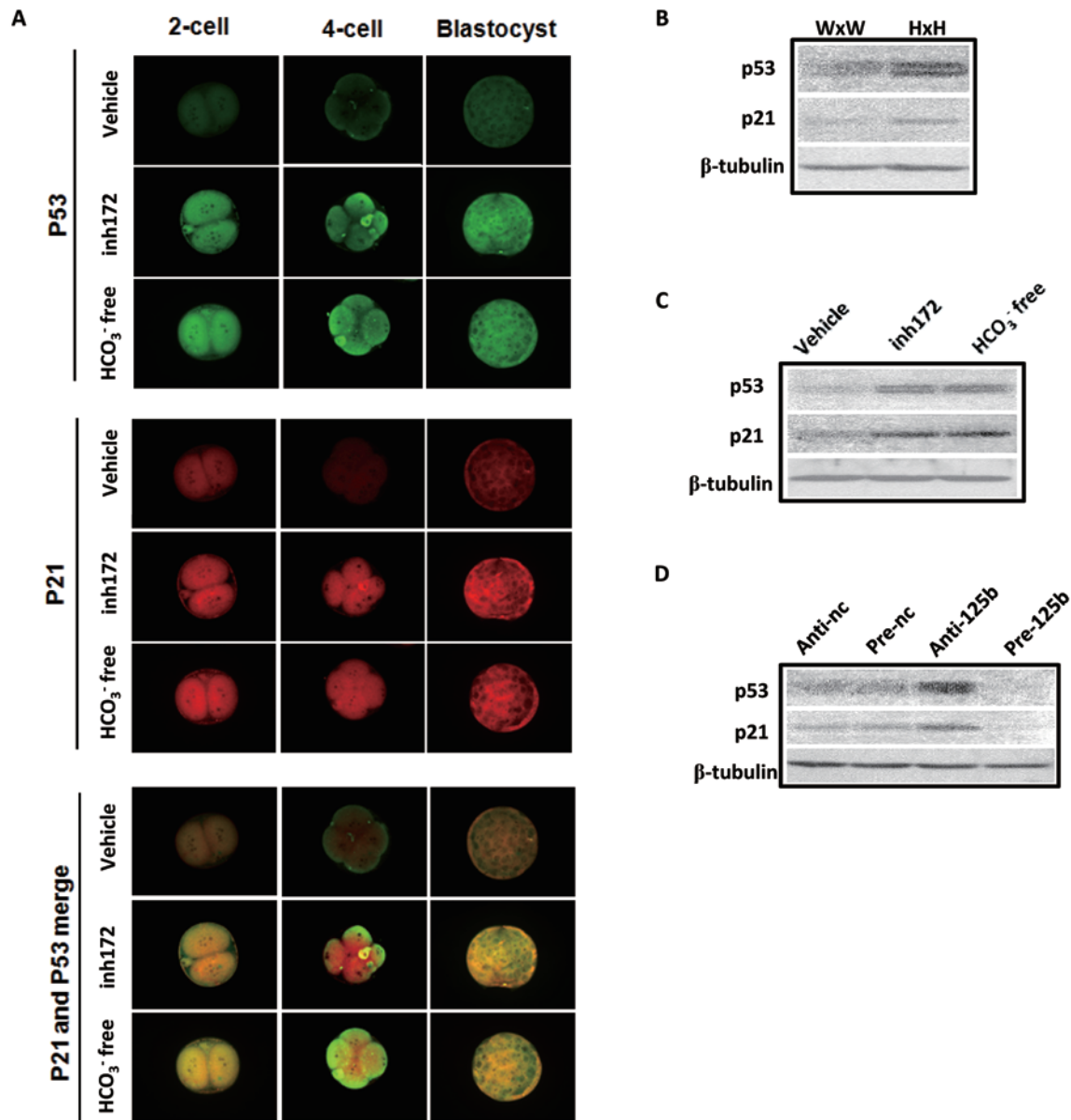


Figure 3 p53 and p21 are the downstream targets of CFTR-HCO₃⁻-miR-125b pathway. **(A)** Immunofluorescent results showing increased expression of p53 (green) and p21 (red) in HCO₃⁻-free condition or after CFTRinh172 treatment (5-10 embryos/group). **(B)** Western blot results showing increased expression of p53 and p21 in two-cell embryos from *Cftr*^{-/-} mice (H × H) compared to those from *Cftr*^{+/+} (W × W) mice. **(C)** Western blot results showing upregulation of p53 and p21 by CFTRinh172 or HCO₃⁻-free condition in two-cell embryos. **(D)** Western blot results showing that transfection of anti-miR-125b oligo leads to increased expression of p53 and p21, while transfection of miR-125b precursor leads to decreased expression of p53 and p21 in two-cell embryos. Scale bar, 50 μm.

by HCO₃⁻ removed or CFTRinh172 treatment in two-cell embryos. The upregulation of p53 and p21 proteins was confirmed by western blot in embryos collected from CFTR-knockout mice (Figure 3B), treated with CFTRinh172 or under HCO₃⁻-free condition (Figure 3C). We further examined the effect of miR-125b on p53 and p21 expression. We knocked down miR-125b

by anti-miR-125b or ectopically overexpressed the precursor (pre-miRNA-125b) in mouse embryos and found that knockdown of miR-125b upregulated p53 and p21 while overexpression of the miR-125b precursor decreased the protein expression of both p53 and p21 (Figure 3D). Taken together, these results indicate an important role of CFTR-mediated HCO₃⁻ entry in

regulation of embryo development involving miR-125b-regulated p53 cascade.

Removal of HCO_3^- and inhibition of CFTR suppresses human embryo development with altered miR-125b and p53

We then explored whether the CFTR/ HCO_3^- -dependent regulation of embryo development involving

miR-125b and p53 is conserved in human embryos. As shown in Figure 4A, removal of HCO_3^- or treatment with CFTRinh172 significantly inhibited human-embryo development from two-cell stage to four-cell stage and formation of blastocyst. Removal of HCO_3^- (Figure 4B) or treatment with CFTRinh172 (Figure 4C) also downregulated miR-125b expression in human embryos at two-cell

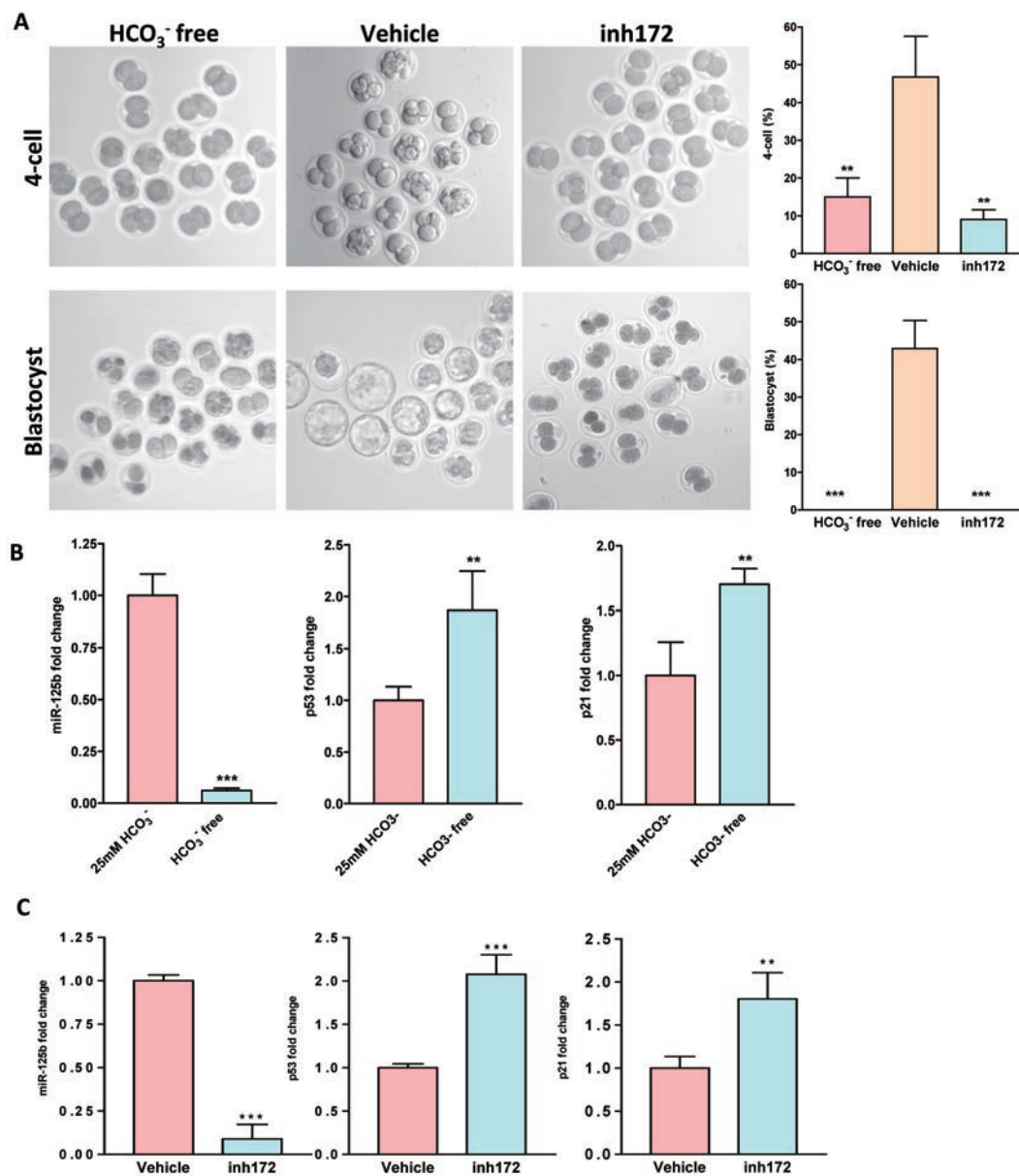


Figure 4 Involvement of CFTR and HCO_3^- in human embryo development and regulation of miR-125b-p53 cascade. **(A)** Decreased four-cell and blastocyst formation in HCO_3^- -free condition or after CFTRinh172 (inh172) treatment ($n = 3$; HCO_3^- -free group: four-cell (9/60 embryos), blastocyst (0/60 embryos); Vehicle group: four-cell (30/63 embryos), blastocyst (27/63 embryos); CFTRinh172 group: four-cell (6/62 embryos), blastocyst (0/62)). **(B, C)** Real-time PCR showed expression of miR-125b, p53 and p21 under HCO_3^- -free condition **(B)** or after CFTRinh172 treatment **(C)** ($n = 4$ 100 embryos/group). Inhibition of CFTR or cultured in HCO_3^- -free condition reduced the expression of miR-125b and increased the expression of p53 and p21. Data are represented as mean \pm SEM; One way ANOVA **(A)**; *t*-test **(B, C)**; ** $P < 0.01$, *** $P < 0.001$.

to four-cell stages, while p53 and p21 expression were upregulated, which is consistent with that observed in mouse embryos. These results indicate that the effect of HCO₃⁻ on mouse and human-embryo development may be mediated by the same conserved mechanism involving CFTR and miR-125b-p53 cascade.

HCO₃⁻-dependent activation of miR-125b involves sAC-PKA-NF-κB cascade

We have established that HCO₃⁻ acts as an environmental cue in regulating miR-125b expression and downstream p53 cascade. Next, we set out to explore the signaling mechanism involved. Since HCO₃⁻ is an activator of sAC, the effect of CFTR-mediated HCO₃⁻ influx on miR-125b expression and embryo development may be mediated by sAC and its downstream cAMP/PKA-dependent pathway [28, 38]. We therefore tested the involvement of sAC and PKA in HCO₃⁻-dependent embryo development. Two-cell embryos were cultured in TALP medium containing 25 mM HCO₃⁻. Application of sAC-specific inhibitor KH7 (10 μM) or PKA inhibitor H89 (20 μM) in embryo culture drastically inhibited the transformation from two-cell stage to four-cell stage compared to DMSO-treated vehicle control (Figure 5A). In line with the effect on embryo development, miR-125b expression was significantly downregulated by both sAC and PKA inhibitors compared to DMSO-treated vehicle control (Figure 5B). These results suggest the involvement of sAC and its downstream PKA signaling pathway in HCO₃⁻-dependent miR-125b expression and embryo development.

Activation of PKA has been shown to stimulate the transcriptional activity of NF-κB [39]. Interestingly, activation of NF-κB has been observed in early single-cell stage embryo and postulated to be required for the development of mouse embryos beyond the two-cell stage [40]. More importantly, NF-κB can bind to the promoter element of miR-125b and upregulate its expression [20]. Therefore, we proposed that CFTR-mediated HCO₃⁻ influx activates sAC, which in turns triggers PKA-dependent NF-κB activation, leading to upregulation of miR-125b expression. To test this, we studied the activation of NF-κB by examining the NF-κB p65 shuttle between cytoplasm and nucleus using immunofluorescence staining. As shown in Figure 5C, NF-κB p65 translocated to the nucleus in two-cell embryos in the presence of 25 mM HCO₃⁻. The translocation was inhibited by removal of HCO₃⁻ or treatment with CFTRinh172, sAC inhibitor KH7, PKA inhibitor H89 (Figure 5C) and NF-κB inhibitor AKβBA (10 μM) (Figure 5D). These results suggest that the translocation or activation of NF-κB in mouse em-

bryos depends on HCO₃⁻ and CFTR, which are known to activate sAC/cAMP/PKA pathway. Blocking NF-κB activation by its inhibitor, AKβBA, also resulted in downregulation of miR-125b in two-cell embryos in the presence of 25 mM HCO₃⁻ as demonstrated by real-time PCR (Figure 5E). These results indicate that the CFTR/HCO₃⁻-dependent activation of miR-125b is mediated by sAC/PKA-dependent NF-κB activation.

Discussion

Although it has been reported that miRNAs undergo dynamic changes during preimplantation embryo development [41], the exact nature of the environmental cues that lead to such alterations in embryos and the underlying mechanism have not been elucidated. The present study has demonstrated for the first time that extracellular HCO₃⁻ can regulate embryonic miR-125b through CFTR-mediated entry and subsequent sAC, PKA and NF-κB activation, which is important for preimplantation embryo development (Figure 6). This provides the first explanation to the long-observed dependency of early embryo development on HCO₃⁻ and suggests that HCO₃⁻ acts as an environmental cue for the regulation of miRNAs during early embryo development.

MiR-125b appears to be dynamically regulated during preimplantation embryo development. The expression levels of miR-125b in two-cell and four-cell embryos were significantly higher than that in morula and blastocyst stage (Figure 2A), which coincides with the transit of embryo from oviduct (high bicarbonate concentration) to the uterus (lower bicarbonate concentration) [42]. This suggested that the window for HCO₃⁻-dependent miR-125b activation occurs from fertilization to four-cell stage but not in subsequent stages. The HCO₃⁻-dependent miR-125b activation is physiologically important for early embryo development by targeting p53 and its downstream target p21. It has been previously demonstrated that the latency of p53 is required for normal preimplantation embryo development [43]. Stress-induced upregulation of p53 has been shown to result in developmental arrest of preimplantation embryo [44]. The latency of p53 in preimplantation embryo is well-documented to be maintained by autocrine stimulation through PI3K/Akt/Mdm2 pathway [43]. In this study, we have demonstrated an alternative pathway regulating the latency of p53 through HCO₃⁻/CFTR-dependent miR-125b activation. This p53 latency appears to be dynamically regulated, as shown by the expression profile of miR-125b in embryos of different stages (Figure 2A). The presently demonstrated epigenetic regulation of p53 latency may provide a fine-tuning mechanism

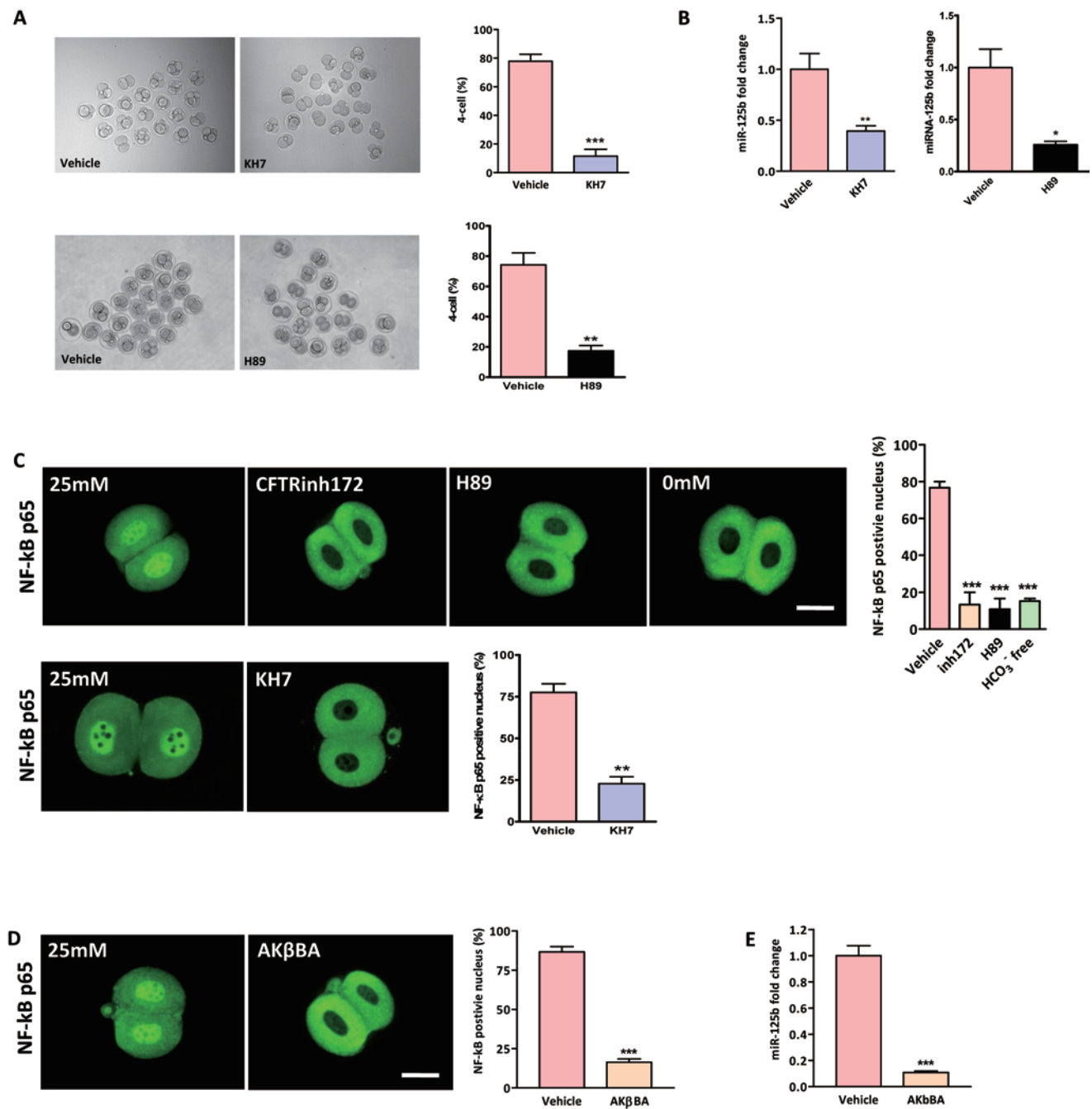


Figure 5 Involvement of sAC/PKA/NF-κB cascade in HCO₃⁻-dependent miR-125b activation. **(A)** Involvement of sAC in the HCO₃⁻-dependent embryo development. HCO₃⁻-dependent four-cell formation was significantly inhibited by addition of sAC inhibitor KH7 (10/86 embryos) and PKA inhibitor H89 (14/80 embryos) compared to DMSO-treated vehicle control (60/77 embryos (KH7); 56/75 embryos (H89)). **(B)** miR-125b expression in two-cell embryo was inhibited by both KH7 and H89 treatments compared to DMSO-treated vehicle control. **(C, D)** Immunofluorescent staining showing the localization of NF-κB p65 subunit in both nucleus and cytoplasm in 25 mM HCO₃⁻ condition (23/30 embryos). HCO₃⁻-free (3/20 embryos), 10 μM CFTRinh172 (4/28 embryos), 10 μM KH7 (7/30 embryos), 20 μM PKA inhibitor H89 (3/27 embryos) **(C)** and 10 μM NF-κB inhibitor AKβBA (4/24 embryos) **(D)** treatments triggered the extrusion of NF-κB p65 from nucleus to the cytoplasm compared to DMSO-treated vehicle control group (20/26 embryos (KH7); 26/30 embryos (AKβBA)); statistics showing HCO₃⁻-free condition, CFTRinh172, H89 and AKβBA significantly reduced the percentage of p65 nuclear positive embryos. **(E)** Real-time PCR results showing that AKβBA inhibited miR-125b expression in two-cell embryos (*n* = 4 100 embryos/group). Data are represented as mean ± SEM. One way ANOVA **(C)**; *t*-test **(A, B, D)**; ***P* < 0.01, ****P* < 0.001. Scale bar, 50 μm.

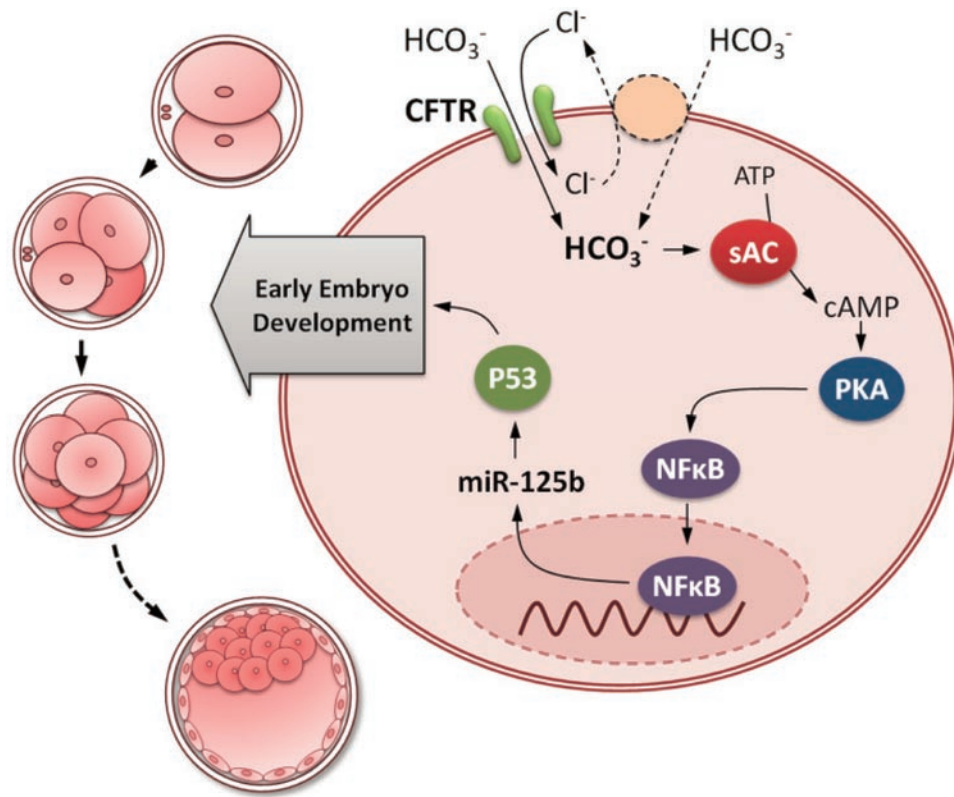


Figure 6 Working model for the regulation of early embryo development by CFTR/HCO₃⁻ dependent activation of miR-125b. CFTR mediates the influx of HCO₃⁻ ion directly and/or indirectly by cooperating with an anion exchanger. The influx of HCO₃⁻ activates sAC, an enzyme that converts ATP to cAMP, which in turn activates PKA, triggering the nuclear shuttle of NF-κB a transcription factor known to regulate the expression of miR-125b. Induction of miR-125b expression by CFTR-mediated HCO₃⁻ influx maintains the dormancy of p53, which is required for early embryo development.

for embryo development on top of the well-established PI3K/Akt/Mdm2 autocrine feedback loop.

The observed inhibitory effect of HCO₃⁻ deprivation, CFTR inhibitor and sAC inhibitor on two-cell to four-cell transition is interesting, since two-cell stage is the time for mouse zygotic genome activation (ZGA), failure of which results in two-cell arrest. ZGA is the global reprogramming of gene expression in early embryos, which transforms the genome from transcription quiescence at fertilization to robust transcriptional activity [45]. The initiation of transcription requires the activation of a variety of transcription factors. It has been reported that the cAMP-PKA pathway is involved in ZGA and also the subsequent embryo development [46, 47]. A recent report has also demonstrated the increase in DNA binding activity of NF-κB in bovine embryo, suggesting that NF-κB might be involved in ZGA [48]. In the present study, we have shown that HCO₃⁻ activates cAMP-PKA and NF-κB through CFTR and sAC, suggesting that HCO₃⁻ may be involved in the regulation of ZGA via the CFTR/sAC/PKA/NF-κB pathway, apart from its involvement in

the epigenetic regulation of p53 latency.

While the present results show that CFTR inhibitor can almost completely retard the embryo development in vitro, some *Cftr*^{-/-} embryos, if not all, from *Cftr*^{+/-} × *Cftr*^{+/-} mating can survive and develop to birth. If CFTR is required for preimplantation embryo development, how can some CFTR-deficient embryos survive and undergo further development? Ben-Chetrit et al.[49] have suggested that the maternal CFTR mRNA stored in the heterozygous mother's oocytes can be translated into protein and rescue the *Cftr*^{-/-} embryos from developmental block in early embryo stage. In fact, some maternal derived protein can persist until expanded blastocyst stage and have epigenetic effect on the early embryo development [50]. The possible presence of maternal CFTR mRNA in *Cftr*^{-/-} embryos may partially explain the survival of some homozygous CFTR-deficient mice. Similar argument can be used to explain the survival of CF patients. It should be noted that the ΔF508 CFTR mutation most commonly seen in CF patients affects the processing of the protein, but retains residual channel

function on the plasma membrane, which may still support some CF embryo development. The survival of CF patients through development may present a false picture since the incidence of preimplantation loss of CF embryos in humans is unknown. According to our long-term animal breeding data, the percentage of offspring with *Cftr*^{-/-} genotype from *Cftr*^{+/-} mice mating is only 0.23/4, significantly less than the expected Mendelian ratio 1/4 (Supplementary information, Table S1), suggesting developmental disadvantage of *Cftr*^{-/-} embryos. Thus, poor or delayed embryo development due to defective CFTR, which may miss the implantation window, may contribute to the reduced fertility in CF.

The present study has demonstrated a critical role of CFTR in mediating the regulatory effect of HCO₃⁻ on embryo development. The altered miR-125b levels in response to changes in HCO₃⁻ concentrations in embryos and the demonstrated ability of CFTR in transporting HCO₃⁻ [28] suggest an important role of CFTR, as an ion channel, in linking the extracellular environment, such as the HCO₃⁻-rich oviductal/uterine fluid, to the intracellular response, such as the changes in miR-125b and its downstream targets p53 and p21, for early embryo development. More importantly, CFTR is expressed in many different tissues, including the epithelium [51], endothelium [52], heart [53], neurons [54] and germ cells [55]. On the other hand, HCO₃⁻ is also abundant in extracellular fluid and acts as a bioactive molecule, with sAC as its sensor, which is also known to be evolutionally conserved and widely expressed in different tissues [56]. The conserved wide distribution of CFTR and sAC in various cell types suggests that the presently demonstrated CFTR-HCO₃⁻-dependent signaling pathway may have implications in other cellular processes far beyond embryo development. Of interest, CFTR expression and its channel activity are also found to be regulated by various environmental factors, such as hypoxia [57], ion concentrations [58, 59] and mechanical stimulation [60], suggesting its potential in sensing a wide spectrum of environmental changes and converting these changes into epigenetic changes, such as miRNA alteration, in the cell under various physiological and pathological conditions.

Materials and Methods

Mouse embryos

Mice were obtained from the laboratory animal service center (LASEC) of the Chinese University of Hong Kong (CUHK). *Cftr*^{miUnc} (CFTR knock-out) mice, with a neomycin selection cassette inserted into exon 10 at sequences corresponding to codon 489 of the encoded CFTR protein [61], were obtained from the Jackson Laboratory and maintained in LASEC of CUHK. To obtain preimplantation embryos, female ICR mice of 8-10 weeks old

were superovulated by intraperitoneal injections of 10 IU pregnant mares' serum gonadotropin (PMSG), followed by 10 IU human chorionic gonadotropin (hCG) and caged with male mice at 48 h post PMSG. *Cftr*^{miUnc}(+/-) or *Cftr*(+/+) mice were mated without superovulation. Successful mating was confirmed by the appearance of vaginal plug. Two-cell embryos were flushed out from the oviducts or uteri with TALP-HEPES medium and cultured in TALP medium [62] in 5% CO₂/95% air at 37 °C. Unless otherwise specified, all embryos were cultured in the presence of 25 mM HCO₃⁻. For development assessment, embryos were cultured for 72 h till expanded blastocyst stage and stages of development were monitored and recorded at different time points. For analysis of miR-125b, p53, p21, embryos were collected after various treatments for 6 h. For NF-κB analysis, embryos were treated for 10 and 30 min. All animal handling protocols were approved by the Animal Research Ethics Committee of the Chinese University of Hong Kong.

Human embryo collection and manipulation

Human embryos were obtained by intracytoplasmic sperm injection (ICSI) and stored in liquid nitrogen in Henan Institute of Population and Family Planning Research. Written consents were obtained from patients who got pregnant after the assisted reproduction program and agreed to donate the spare frozen embryos to research. Human embryos were cultured in TALP medium and treated in the same conditions as that for the mouse embryos. All procedures were approved by the Ethics Committee of Henan Institute of Population and Family Planning Research.

Anti-miR-125b and pre-miR-125b transfection

Zona pellucida of the embryos was dissolved in TALP medium (pH 7.35). The embryos were washed in normal TALP-HEPES medium for three times and transfected with 5 μL Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) and 2 μL of 50 nM anti-miRNA negative control, anti-miR-125b inhibitor (AM17000, Ambion, Austin, TX, USA), pre-miRNA negative control or pre-miR-125b (AM17100, Ambion, Austin, TX, USA) as described. The embryos were collected 12 h post-transfection for RT-PCR or western blot analysis.

Pre-miR-125b microinjection

Pre-miRNA negative control or pre-miR-125b was dissolved in RNase-free water. Each blastomere of two-cell embryos was injected with 50 fmol of pre-miRNA solution at the two-cell stage using the micromanipulation system. Formation rate of four-cell and blastocyst was accessed 12 and 48 h after injection.

Immunofluorescent staining

Embryos were fixed in 4% paraformaldehyde, blocked with 10% goat serum and incubated at 4 °C overnight with primary antibodies: rabbit anti-CFTR-polyclonal antibody (1:500, Alomone labs, Jerusalem, Israel); rabbit anti-sAC polyclonal antibody (1:500, a gift from Dr Ping Bo Huang); rabbit anti-ZO-1 polyclonal antibody (1:500, Zymed, San Francisco, CA, USA); goat anti-p53 polyclonal antibody (1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA); mouse anti-p21 monoclonal antibody (1:100, Cell Signaling Technology, Danvers, MA, USA); and rabbit anti-NF-κB p65 (1:200, Cell Signaling Technology, Danvers, MA, USA). After intensive wash with PBS with 0.1% Triton X-100

(PBST), embryos were incubated with Alexa 488 goat anti-rabbit IgG (1:500), Alexa 568 goat anti-mouse IgG (1:500) or Alexa 488 rabbit anti-goat IgG (1:500) for 30 min, followed by nuclei counterstaining with DAPI (1:1 000) for 20 min.

RT-PCR and real-time PCR

Embryos were washed in PBS, frozen in liquid nitrogen and stored at -80 °C. Total RNA extraction and reverse transcription (RT) were performed using Cells-to-cDNA™ II Kit as described (Ambion, Austin, TX, USA). Negative control was performed with no reverse transcriptase added. Expression of Cfr and sAC were determined by RT-PCR using the following primers: mCfr F 5'-CAT CTT TGG TGT TTC CTA TGA TG-3' and mCfr R 5'-GTA AGG TCT CAG TTA GAA TTG AA-3'; msAC F 5'-CAT GAG CAA GGA ATG GTG GTA CTC A-3' and msAC R 5'-AAG GGT TAC CTT GCC TGA TAC AAT T-3'; mGapdh F 5'-GAC CAC AGT CCA TGA CAT CAC TGC-3' and mGapdh R 5'-GCT GTT GAA GTC GCA GGA AAC-3'. Real time PCR for mir-125b was carried out with microRNA assay kit (Assay ID: 000449, Applied Biosystems, Carlsbad, CA, USA). snoRNA202 was used as miRNA control (Assay ID: 001232, Applied Biosystems, Carlsbad, CA, USA).

Western blot analysis

Embryos (200-400 embryos/group) were pooled, lysed by loading buffer. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane. After blocking with 4% milk, the blots were probed overnight at 4 °C with the following antibodies: anti-CFTR (1:500, Alexis); anti-sAC (1:500); anti-β-tubulin (1:2 000); anti-p53 (1:200); anti-p21 (1:200) (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA). The signal was detected with HRP-conjugated secondary antibody and visualized using ECL Western Blot Detection Reagent (GE Healthcare).

Statistical analysis

Results were presented as mean ± SEM. Student's unpaired t-test was used for two-group comparison. One-way ANOVA followed by Tukey's *post-hoc* test was used when comparing three or more groups. A probability of $P < 0.05$ was considered to be statistically significant.

Acknowledgments

This work was supported by the National Basic Research Program of China (973 program; 2012CB944900), the National Natural Science Foundation of China (30973209), the Focused Investment Scheme and Li Ka Shing Institute of Health Sciences of The Chinese University of Hong Kong, Fundamental Research Funds for the Central Universities (Jinan University) and the Morning-side Foundation. We would like to thank Dr YC Ruan for her help in the artwork.

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