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Impaired imprinted X chromosome inactivation is responsible for the skewed sex ratio following in vitro fertilization

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Dynamic epigenetic reprogramming occurs during normal embryonic development at the preimplantation stage. Erroneous epigenetic modifications due to environmental perturbations such as manipulation and culture of embryos during in vitro fertilization (IVF) are linked to various short- or long-term consequences. Among these, the skewed sex ratio, an indicator of reproductive hazards, was reported in bovine and porcine embryos and even human IVF newborns. However, since the first case of sex skewing reported in 1991, the underlying mechanisms remain unclear. We reported herein that sex ratio is skewed in mouse IVF offspring, and this was a result of female-biased peri-implantation developmental defects that were originated from impaired imprinted X chromosome inactivation (iXCI) through reduced ring finger protein 12 (*Rnf12*)/X-inactive specific transcript (*Xist*) expression. Compensation of impaired iXCI by overexpression of *Rnf12* to up-regulate *Xist* significantly rescued female-biased developmental defects and corrected sex ratio in IVF offspring. Moreover, supplementation of an epigenetic modulator retinoic acid in embryo culture medium up-regulated *Rnf12*/*Xist* expression, improved iXCI, and successfully redeemed the skewed sex ratio to nearly 50% in mouse IVF offspring. Thus, our data show that iXCI is one of the major epigenetic barriers for the developmental competence of female embryos during preimplantation stage, and targeting erroneous epigenetic modifications may provide a potential approach for preventing IVF-associated complications.

in vitro fertilization | sex ratio | X chromosome inactivation | Xist | Rnf12

There have been more than 5 million people born from in vitro fertilization (IVF) since the birth of Louise Brown on July 25, 1978 (1); this makes IVF as one of the most effective and successful assisted reproductive technologies that are routinely used for treating infertility that affects ∼15% couples globally (2). Nowadays, IVF contributes to 1–5% of all newborns in developed countries (2). In addition, IVF is one of the most promising technologies for accelerating the intensive propagation or improving intensity of genetic selection from genetically superior individuals in domestic animals (3). Although the great majority of IVF-conceived offspring are in good health, epidemiologic analyses in humans and laboratory studies in animals show that IVF is associated with various short- or long-term consequences, such as miscarriage, preterm birth, lower birth weight, skewed sex ratio, and higher disease risks in later life (4–8).

Dynamic epigenetic reprogramming occurs during preimplantation development (9). Environmental perturbations such as manipulation and culture of embryos in vitro during IVF are expected to influence the epigenetic programming of the developing embryos during this critical period, often leading to nonrandom epigenetic errors such as aberrant DNA methylation and histone modifications (10, 11). Indeed, aberrant DNA methylation of some genes has been linked to certain complications, including vascular dysfunction and imprinting disorders, in IVF offspring (12, 13).

Skewed sex ratio is an issue of great concern among the IVF-associated consequences. The first case of IVF-associated sex skewing was reported in bovine as early as 1991 (14), and this phenomena has been confirmed repeatedly in bovine and porcine IVF embryos (15–17). Recent epidemiologic data from Oceania and United Kingdom also show that IVF can result in sex skewing in humans with a higher male birth rate (5, 6). Sex ratio is an important indicator of reproductive health that tends to be sensitive or even adaptive to many factors (18, 19), and its skewing has a potential effect on long-term social and behavior consequences (20).

IVF-associated sex skewing reflects potential sex-biased embryonic developmental defects (21). The sex-biased embryonic defects are reminiscent of the disrupted sex-specific epigenetic events. X chromosome inactivation (XCI) is a female-specific epigenetic event that occurs during early development for balancing the X-linked gene dosage between males and females. Previous studies show that disturbed XCI caused by mutation or deletion of related genes results in sex skewing in mice (22–24). Thus, we hypothesized that the IVF process may disturb the XCI status, thereby leading to the skewed sex ratio. We report herein that skewed sex ratio in mouse IVF offspring is a result of female-biased developmental defects due to impaired XCI (iXCI) via reduced ring finger protein

Significance

Sex ratio is an important indicator of reproductive health, and its skewing reflects disturbed embryonic development. This study focused on sex skewing, which has recently identified in human in vitro fertilization (IVF) babies. We reported herein that the skewed sex ratio in mouse IVF offspring was due to the impaired imprinted X chromosome inactivation via suppressing the ring finger protein 12 (*Rnf12*)/X-inactive specific transcript (*Xist*) pathway; the sex skewing can be corrected by overexpressing *Rnf12* or by supplementation of retinoic acid in embryo culture medium. Hence, our study not only identified a major epigenetic error responsible for sex skewing in IVF offspring, but also implicated a potential strategy for preventing sex skewing and IVF-associated complications by targeting erroneous epigenetic modifications induced by IVF.

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The authors declare no conflict of interest.

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12 (*Rnf12*)/X-inactive specific transcript (*Xist*) expression. Accordingly, correcting this epigenetic error by overexpression of *Rnf12* to compensate iXCI *via Xist* up-regulation or by incubation of the IVF embryos with an epigenetic modulator retinoic acid (RA) redeemed the skewed sex ratio. Thus, our data show that iXCI is one of the major epigenetic barriers for the developmental competence of female embryos during preimplantation stage and targeting erroneous epigenetic modifications may provide a potential approach for preventing IVF-associated complications.

Results and Discussion

Sex Skewing in IVF Offspring Is Linked to Female-Biased Developmental Defects During Peri-Implantation Stage. To test the temporal patterns of IVF-associated sex skewing, we generated mouse embryos under standardized IVF conditions (25) and used in vivo fertilized embryos as the control (IVO group; Fig. 1*A*). We found that IVF resulted in a significantly higher male birth rate (57.17% vs. 50.89%; Fig. 1*B* and Fig. S1*A*); further analysis showed that sex skewing did not occur before implantation, because sex ratios of both IVF and IVO blastocysts were near 50% (Fig. 1*B* and Fig. S1*B*). After implantation, IVF embryos had a lower survival rate that could be observed as early as embryonic day (E)7.5 (Fig. S2*A*). At E7.5, IVF female embryos showed significantly greater incidence in morphological abnormalities than their male counterparts (10.01% vs. 2.60%), accompanied by a higher frequency of abnormal extraembryonic tissues (Fig. 1 *C* and *D* and Figs. S1*C* and S2*B*). These results show that IVF embryos have female-biased developmental defects during the peri-implantation stage, which is responsible for the skewed sex ratio in their offspring.

iXCI Is Impaired in IVF Female Embryos. The observed female-biased developmental defects led us to hypothesize that a certain sex-specific epigenetic event was disturbed by IVF. Because the observed female-biased defects are similar to that previously described in mouse embryos with disturbed XCI caused by paternally inherited *Xist* mutations (22, 23), we postulated that the IVF embryos may have impaired XCI. Mice undergo two waves of XCI: iXCI initiates in early preimplantation embryos and persists in extraembryonic tissues, and random XCI (rXCI) occurs in the embryonic cells shortly after implantation (26–28). By detecting the H3K27me3, a classic marker for establishment of XCI by achieving X chromosome wide heterochromatinization of transcriptional depression (28), we first confirmed that rXCI was not impaired in either morphologically normal or abnormal E7.5 IVF female embryos because H3K27me3 enrichment was normal in nearly all epiblast cells (Fig. 2 *A* and *B*). In contrast, iXCI was impaired in abnormal E7.5 IVF female embryos because H3K27me3 domains were significantly decreased in the extraembryonic tissues of these embryos (Fig. 2 *A* and *B*). We then examined whether iXCI was affected by IVF during the preimplantation stage when it establishes (27). As expected, nearly all IVO female blastocysts ($n = 45$) consistently showed a single H3K27me3 domain in each trophoblast cell (Fig. 2 *C* and *D*). In contrast, a substantial proportion of the IVF female blastocysts ($n = 61$) contained trophoblast cells lacking the H3K27me3 domain, with a variable frequency ranging from 4.8% to 100.0% (Fig. 2 *C* and *D*). Both IVO and IVF male blastocysts had no H3K27me3 domain (Fig. S3*A*). These results confirmed that iXCI is impaired in a proportion of preimplantation IVF female embryos, whereas male embryos have no ectopic iXCI. This notion is further supported by the fact that mRNAs of several X-linked nonescaping genes, including ATPase, Cu++ transporting, alpha polypeptide (*Atp7a*), FYVE, RhoGEF and PH domain-containing 1 (*Fgd1*), phosphoglycerate kinase 1 (*Pgk1*), cysteine-rich hydrophobic domain 1 (*Chic1*), and kinesin family member 4 (*Kif4*), were up-regulated in IVF female embryos (Fig. S3*B*). We also found that both the fertilization process (FP) and culture process (CP) of IVF contributed to iXCI impairment (Fig. S4 *A* and *B*). In comparison with FP or CP alone, the IVF process caused the most severe loss of H3K27me3 domain in the embryos, suggesting a synergetic effect of CP and FP on iXCI impairment. Furthermore, we also found that the IVF-induced iXCI impairment was not limited to a specific genotype or culture condition (Fig. S4*C*). In rabbits, H3K27me3 immunostaining revealed impaired XCI in IVF female blastocysts as well (Fig. S5*A*). In cows, H3K27me3 is less useful for determining XCI status in blastocysts (Fig. S5*B*); up-regulation of representative X-linked nonescaping glucose-6-phosphate dehydrogenase (*G6pd*) and *Pgk* genes (16) suggests impaired XCI in IVF female bovine embryos. In humans, although comparisons cannot be made with naturally conceived embryos due to ethical considerations, the observed incomplete initiation of XCI at

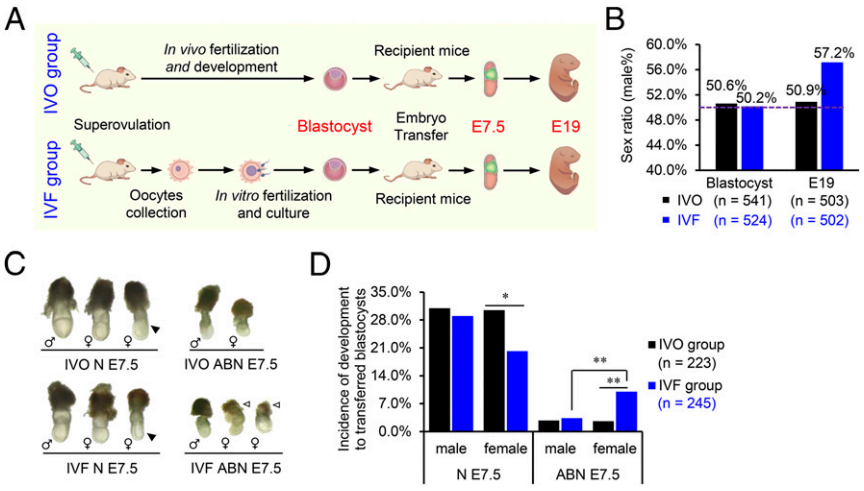

Fig. 1. Sex ratio is skewed in IVF embryos due to female-biased abnormal embryonic development. (*A*) Schematic diagram of the experimental design. Embryos after IVF and development (IVO group, control) or IVF and culture (IVF group) were sampled at the blastocyst stage, E7.5, and at birth (E19), followed by detailed examinations. (*B*) Sex ratio (proportion of males) of blastocysts and E19 fetuses in IVO and IVF groups. The dotted line indicates the expected natural sex ratio of 50%. (*C*) Representative images of IVO and IVF E7.5 embryos with normal (N) or abnormal (ABN) morphologies. Solid arrowhead indicates developmentally delayed embryos with normal morphology. A large proportion of IVF female ABN E7.5 embryos displayed severe disorganized extraembryonic tissues (open arrowhead). (*D*) Incidence of development of male and female embryos with different morphologies at E7.5. The number (*n*) of embryos examined in each group is indicated. $*P < 0.05$, $**P < 0.01$.

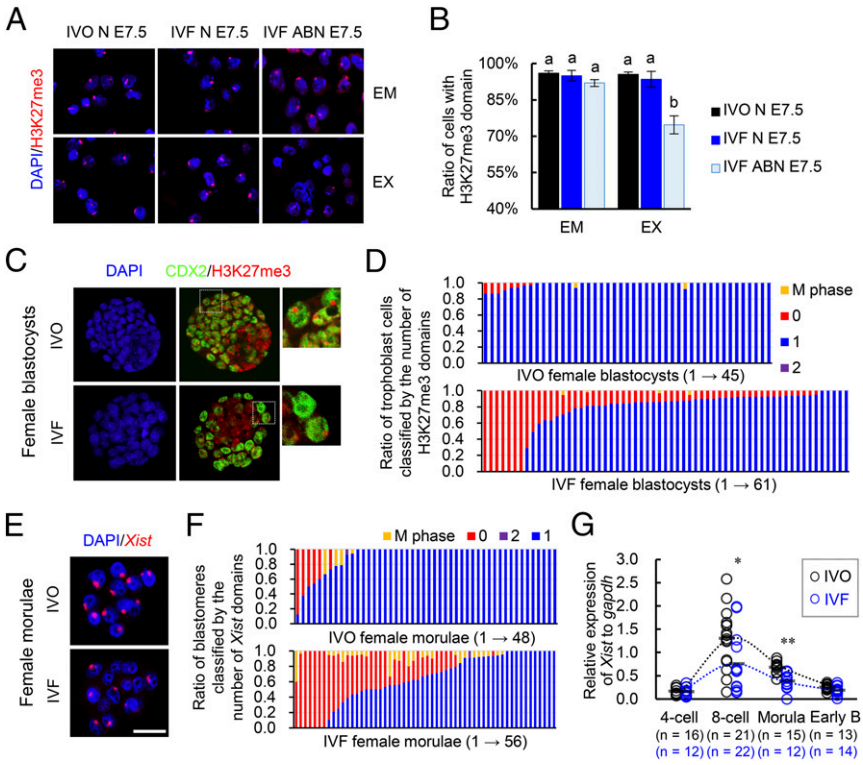

Fig. 2. iXCI is impaired in IVF female embryos. (*A*) Representative H3K27me3 immunostaining in cells isolated from embryonic (EM) and extraembryonic (EX) tissues of normal IVO (IVO N) and IVF (IVF N), as well as abnormal IVF (IVF ABN) female embryos at E7.5. (*B*) Percentage of H3K27me3-positive cells. Values with different letters are significantly different ($P < 0.05$). (*C*) Representative immunostaining for H3K27me3 (red) in the nuclei (DAPI) of IVO and IVF female blastocysts colabeled with CDX2 (green)-positive trophoblast cells. (*Rightmost panels*) Higher magnification of boxed regions. (*D*) The ratio of trophoblast cells classified by the number (0–2) of H3K27me3 domains. (*E*) Representative localization of *Xist* expression in the nuclei (DAPI) of IVO and IVF female morulae. (*F*) The ratio of blastomeres classified by the number (0–2) of *Xist* domains. (*D* and *F*) Each bar represents one female embryo. (*G*) Expression levels of *Xist* in female IVO and IVF embryos during preimplantation stage. Circles represent the relative expression value in each embryo. Horizontal gray lines represent the mean values. The number of embryos in each group is indicated. Early B, early blastocyst. $*P < 0.05$, $**P < 0.01$. (Scale bar, 50 μm.)

the blastocyst stage in IVF embryos may be a reflection of impaired XCI (29, 30). Thus, despite different mechanisms of XCI among species, these data suggest that IVF-induced XCI impairment seems to be common in these species.

iXCI via Reduced *Rnf12*/*Xist* Expression in IVF Female Embryos Is Responsible for the Skewed Sex Ratio at Birth. Next, we asked whether the impaired iXCI is responsible for the female-biased developmental defects and the sex skewing in IVF embryos. Because XCI is initiated by *Xist* RNA coating (31), we first examined *Xist* mRNA expression during preimplantation development. A substantial proportion of IVF female morulae showed degrees of absence of *Xist* domains within the nucleus of each blastomere (Fig. 2 *E* and *F*). Both IVO and IVF males had no *Xist* expression (Fig. S3C). Single embryo quantitative RT-PCR (qRT-PCR) analysis also confirmed that *Xist* expression was significantly inhibited in a proportion of preimplantation IVF female embryos from eight-cell to morula stages (Fig. 2*G*), suggesting insufficient initiation and establishment of iXCI because *Xist* expression is essential for initiating iXCI (31). Because *Xist* overexpression would induce transcriptional repression not only in the X chromosome but also in autosomes (32), we assessed whether expression of other upstream regulators of *Xist* could be used to compensate for impaired iXCI (Fig. S6). Among them, *Rnf12* was down-regulated in preimplantation IVF female embryos (Fig. 3*A*) in association with decreased *Xist* mRNA expression (Fig. 2*G*). We also confirmed that RNF12 protein was decreased in IVF preimplantation embryos by both immunofluorescence microscopy and immunoblotting (Fig. S7 *A* and *B*). Moreover, *Rnf12* knockdown by siRNA (Fig. S7 *C* and *D*) led to *Xist* down-regulation and loss of *Xist* domain within the nucleus of female embryos (Fig. S7 *C* and *E*), suggesting a crucial role of *Rnf12* transcription in the expression of *Xist* during preimplantation stage. In addition, RNF12 can specifically activate *Xist* expression in the paternal X chromosome during iXCI and also prevent excessive up-regulation of *Xist* with a rapid feedback mechanism (24, 33). Together, these data show that *Rnf12* overexpression can be used to compensate impaired iXCI by fine-tuning *Xist* expression in IVF female embryos. As expected, overexpression of *Rnf12* (Fig. 3*B*) resulted in increased *Xist* expression (Fig. 3*C*) to the levels comparable to that in IVO embryos (Fig. 2*G*) during XCI initiation. *Rnf12* overexpression also significantly improved iXCI status in IVF female embryos as revealed by *Xist* RNA-FISH and H3K27me3 immunostaining, without inducing ectopic iXCI in both females and males (Fig. 3 *D* and *E* and Fig. S8 *A*–*C*).

We further determined whether improvement of iXCI could rescue the developmental defects in IVF female embryos. *Rnf12* overexpression had no evident side effects on blastocyst development as it did not affect the blastocyst formation rate (83.5% vs. 84.2%) and their sex ratio (Fig. 3*H*). However, the proportion of morphologically abnormal female embryos was significantly decreased at E7.5 after overexpressing *Rnf12* (4.09% vs. 9.89%; Fig. 3*F*), although the iXCI status remained impaired in abnormal embryos (Fig. 3*G*). These results showed that improved iXCI status due to *Rnf12*/*Xist* up-regulation can rescue the developmental defects in IVF female embryos. Furthermore, *Rnf12* overexpression resulted in correction of the skewed sex ratio (58.16%) of the nontreated IVF offspring to 51.92% at birth (Fig. 3*H*), comparable

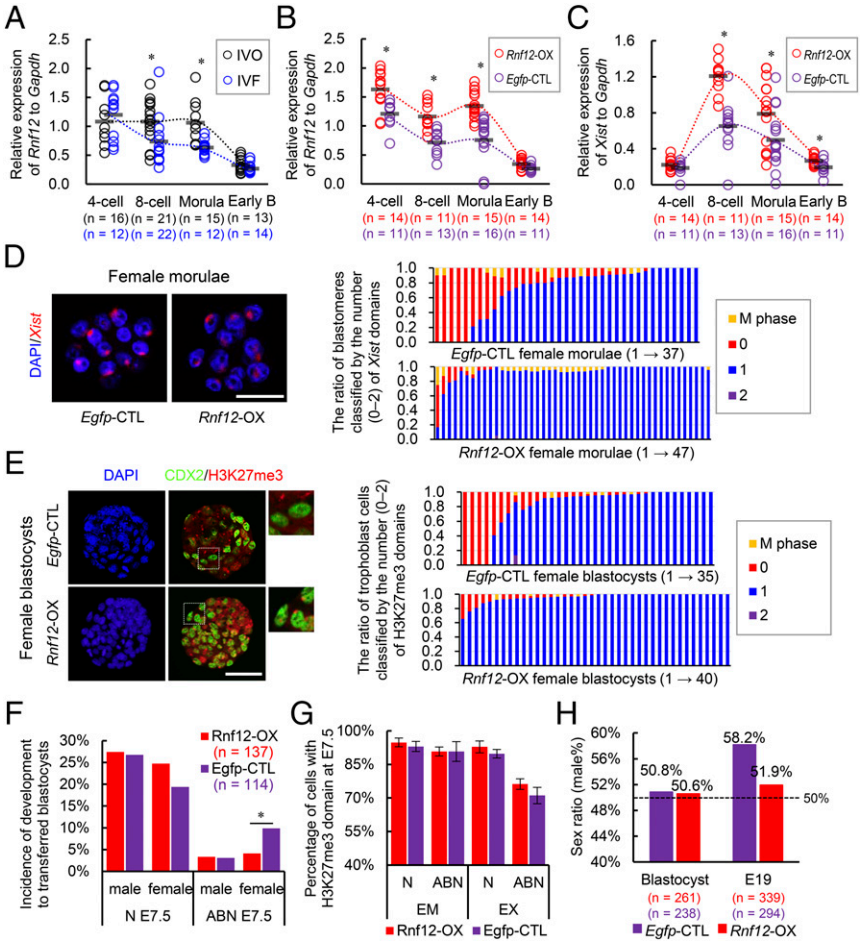

Fig. 3. iXCI impairment in IVF female embryos is responsible for the skewed sex ratio at birth. (*A*) *Rnf12* expression levels in female IVO and IVF embryos during the preimplantation stage. (*B*) *Rnf12* and (*C*) *Xist* expression levels in female embryos overexpressing *Rnf12* (*Rnf12*-OX) and control (*Egfp*-CTL) during the preimplantation stage. (*A–C*) Circles represent the relative expression level in each embryo. Horizontal gray lines represent the mean values. Early B, early blastocyst. (*D*) RNA-FISH of *Xist* expression in female morulae. The ratio of blastomeres classified by the number of *Xist* domains is presented on the right. (*E*) H3K27me3 immunostaining in female blastocysts. The ratio of trophoblast cells classified by the number of H3K27me3 domains is presented on the right. (*D* and *E*) Each bar represents one female embryo. (*F*) Incidence of development of *Rnf12*-OX and *Egfp*-CTL embryos with normal (N) or abnormal (ABN) morphology at E7.5. (*G*) Percentage of H3K27me3-positive cells from the embryonic (EM) and extraembryonic (EX) tissues of E7.5 female embryos. (*H*) Sex ratios in *Rnf12*-OX and *Egfp*-CTL groups. The dotted line indicates the expected proportion of 50%. The number of embryos/fetuses in each group is indicated. *$P < 0.05$. (Scale bar, 50 μm.)

to that of IVO group (50.89%; Fig. 1*B*). Although the exact causes of reduced *Rnf12* expression in IVF female embryos remain to be determined, we found that negative *Rnf12* regulators (34), i.e., Nanog homeobox (*Nanog*) and POU domain, class 5, transcription factor 1 (*Oct4*), were dysregulated in IVF female embryos (Fig. S8*D*). We found *Nanog* expression to be higher in IVF female embryos (Fig. S6); however, immunofluorescence microscopy analysis showed evident mislocalization of NANOG protein in some individual trophoblast cells of IVF female blastocysts (Fig. S8*E*). These findings show that *Nanog* expression in IVF embryos is dysregulated in a spatiotemporal manner, which may partially explain the inhibited *Rnf12*/*Xist* expression in IVF embryos. Nonetheless, our findings show that iXCI impairment is a major epigenetic error responsible for the female-biased developmental defects and skewed sex ratio at birth in mouse IVF offspring.

Supplementation of RA in Embryo Culture Medium Improved iXCI Status and Corrected Skewed Sex Ratio of IVF Offspring. Having determined a causal role of impaired iXCI in the skewed sex ratio at birth, we then asked whether improving iXCI status via supplementation of epigenetic modulators in culture medium could prevent sex skewing in mouse IVF offspring. Because establishment and maintenance of XCI involves *Xist* coating, histone modifications, and DNA methylation (35), we screened several well-known related modulators as potential candidates (Fig. 4*A* and Fig. S9*A*). We found that supplementation of RA in the embryo culture medium significantly up-regulated *Rnf12*/*Xist* expression and improved the iXCI status in IVF female embryos (Fig. 4 *A–C* and *E*). Moreover, RA supplementation effectively corrected the skewed sex ratio (57.17%) in nontreated mouse IVF offspring to 52.03% at birth (Fig. 4*D*). In mouse ES cells, RA up-regulates *Xist* expression by inhibiting the expression of pluripotency factors (36). Similarly, we found that RA suppressed *Nanog* and *Oct4* expression in preimplantation mouse IVF female embryos (Fig. 4*E*), and these effects were reversed by RA withdrawal from the culture medium (Fig. 4*E*). RA had no significant adverse effect on the developmental potential as the cell number ratio of trophoblast to inner cell mass in treated IVF embryos did not differ from that of untreated ones (2.54 ± 0.61 vs. 2.50 ± 0.58). Interestingly, we found that the expression of key genes involved in the synthesis and metabolism of RA can be detected in preimplantation embryos and fallopian tube (Fig. S9*B*). Thus, it is possible that dysregulation of fallopian tube-derived RA and other factors such as cytokines and hormones

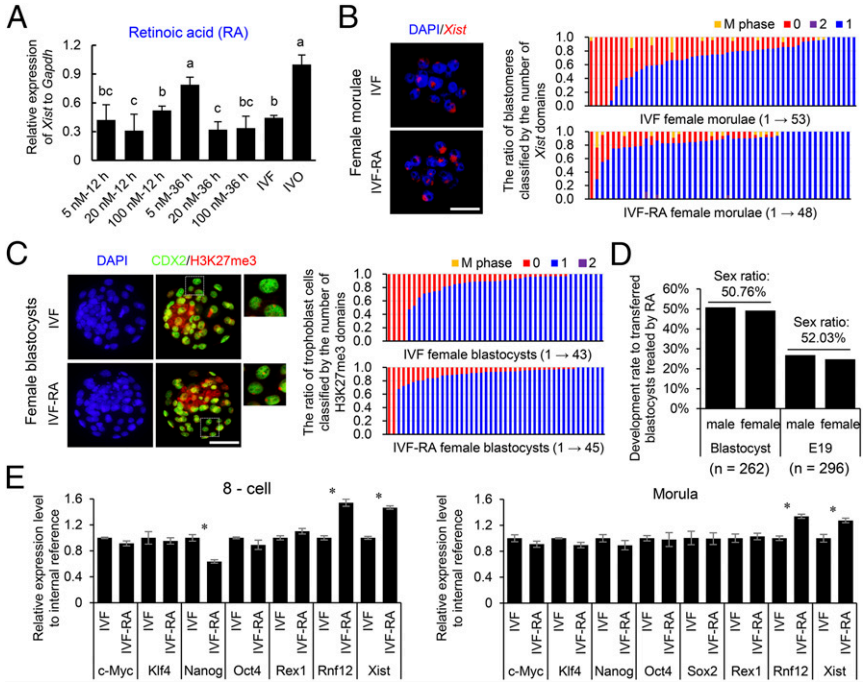

Fig. 4. Supplementation of RA in the culture medium improves the iXCI status and corrects the skewed sex ratio in IVF offspring. (*A*) The relative *Xist* expression in RA-treated IVF female morulae. Values with different letters are significantly different ($P < 0.05$). At least 50 embryos were evaluated in each group for each replicate. (*B*) Representative nuclear *Xist* noncoding RNA localization in RA-treated female morulae. The ratio of blastomeres classified by the number (0–2) of *Xist* domains is presented on the right. (*C*) Representative nuclear H3K27me3 immunostaining in RA-treated female blastocysts. The ratio of trophoblast cells classified by the number (0–2) of H3K27me3 domains is presented on the right. (*Rightmost panels*) Higher magnification of boxed regions. (*B* and *C*) Each bar represents one female embryo. (*D*) Development rates of RA-treated male and female embryos at the blastocyst stage and E19. The number of embryos or fetuses in each group is indicated. (*E*) The effect of RA on the expression levels of the pluripotency factors, *Rnf12* and *Xist*, in pooled IVF female embryos at the eight-cell and morula stages. *Sox2* was not detected at the eight-cell stage. $*P < 0.05$. (Scale bar, 50 μm.)

contribute to the compromised development of IVF embryos, probably via epigenetic involved mechanisms such as XCI.

Conclusions

Sex ratio, as an important indicator of reproduction health, tends to be skewed by many endogenous or exogenous factors, including altered nutrition [e.g., high-fat diet (37, 38) and glucose concentration (39)], environmental conditions [e.g., crowded conditions (40)], and other factors (e.g., IVF processes). However, the underlying mechanisms are likely to be complex and remain largely unknown.

Our current study, focusing on the sex skewing induced by IVF conditions, identified that this skewed sex ratio is due to impaired iXCI via reduced *Rnf12/Xist* expression during early embryonic development; supplementation of an epigenetic modulator RA in embryo culture medium can correct the skewed sex ratio in mouse IVF offspring (Fig. 5). Our current findings have suggested a potential strategy for preventing sex skewing and IVF-associated complications by targeting erroneous epigenetic modifications induced by IVF. Moreover, considering the crucial role of epigenetic reprogramming in early embryonic development, the changed epigenetic modifications may also involve in sex skewing induced by other factors.

Materials and Methods

Details are in the *SI Materials and Methods*, including experimental procedures and reagents.

Animals. ICR female mice aged 7–8 wk, ICR male mice aged 10–12 wk, and (C57BL/6 × DBA/2) F1 (BDF1) male mice aged 10–12 wk were fed ad libitum and housed under controlled lighting conditions (12 light:12 dark). They were maintained under specific pathogen-free conditions. All animal experiments were approved by and performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of China Agricultural University.

Preparation of Mouse Embryos. All experiments involved in embryo preparation were performed as previously described (25), with minor modifications. ICR female mice were superovulated by an i.p. injection of 5 IU

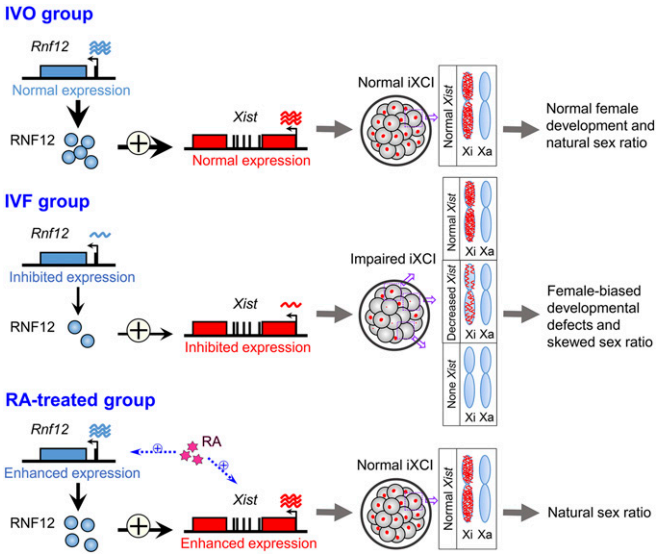

Fig. 5. A model illustrating the causal role of iXCI in sex skewing in mouse IVF offspring. IVF induces impaired iXCI via reduced *Rnf12/Xist* expression during early mouse embryonic development, resulting in female-biased developmental defects at the peri-implantation stage and skewed sex ratio at birth. Supplementation of an epigenetic modulator RA can correct the skewed sex ratio by improving the iXCI status through up-regulating the *Rnf12/Xist* expression in IVF female embryos.

pregnant mare serum gonadotropin and a further i.p. injection 48 h later of 5 IU human chorionic gonadotropin (hCG). In the IVO group, superovulated ICR females were cocaged individually with ICR males after the hCG injection. The following morning, successful mating was confirmed by the presence of a vaginal plug. In some experiments, superovulated ICR females were mated individually with BDF1 males after the hCG injection. In the IVF group, sperm were obtained from the cauda epididymis and capacitated for 1 h in human tubal fluid (HTF; SAGE) medium at 37 °C in 5% CO_2. Oocytes were collected from the ampullae at 14 h after hCG treatment. Gametes were then coincubated in HTF medium for 4 h at 37 °C in 5% CO_2. After 4 h in the incubator, zygotes were washed and cultured to the blastocyst stage in potassium simplex optimization medium containing amino acids (KSOM+AA; Millipore) under mineral oil at 37 °C in 5% CO_2.

Pseudo-pregnant female mice (recipients) were cocaged individually with vasectomized males 3.5 d before embryo transfer. The morning after mating, the recipients were checked for the presence of a vaginal plug. The day of plugging was considered to be day 0.5 of the pseudo-pregnancy. Well-developed blastocysts with similar morphologies were selected for embryo transfer. Twelve blastocysts were transferred to each recipient, with six embryos in each uterine horn. For some experiments, pronuclear stage embryos were transferred into the oviducts of day 0.5 recipients.

Collection of Mouse Embryos. IVO embryos at the four-cell, eight-cell, and morula stages (54–56, 68–70, and 78–80 h after hCG, respectively) were recovered from donors by flushing the fallopian tubes with M2 medium. Embryos at the blastocyst stage (92–94 h after hCG) were recovered from donors by flushing the uterus with M2 medium (25). IVF embryos at different preimplantation stages were collected based on their developmental progress and morphology. Before the final collection, samples were washed with acidic Tyrode's solution (Sigma) to eliminate the zona pellucida. Any residual contaminants were depleted via a short series of additional washes in PBS/0.1% polyvinyl alcohol (PVA).

At E7.5, the conceptuses covered with the decidual mass were gently teased away from the uterus. The decidua in which the conceptus embedded was peeled off, and the parietal yolk sac was opened to expose the visceral yolk sac endoderm layer. Embryonic and extraembryonic parts of the E7.5 embryos were separated and washed in PBS before further analyses.

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