1 # Histone demethylase complexes KDM3A and KDM3B cooperate with

2 # OCT4/SOX2 to construct pluripotency gene regulatory network

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26   **Key words:** Pluripotency, H3K9 methylation, Histone demethylase complexes, KDM3A/3B

27   **Summary:** Erasure of H3K9 methylation in porcine pluripotent stem cells depends on the complex

28   of transcription factors OCT4/SOX2 and histone demethylase KDM3A/KDM3B.

29   **Abstract**

30   The pluripotency gene regulatory network of porcine-induced pluripotent stem cells (piPSCs),

31   especially in epigenetics, remains elusive. To determine this biological function of epigenetics, we

32   cultured piPSCs in different culture conditions. We found that activation of pluripotent gene- and

33   pluripotency-related pathways requires the erasure of H3K9 methylation modification which was

34   further influenced by mouse embryonic fibroblast (MEF) served feeder. By dissecting the dynamic

35   change of H3K9 methylation during loss of pluripotency, we demonstrated that the H3K9

36   demethylases KDM3A and KDM3B regulated global H3K9me2/me3 level and that their co-

37   depletion led to the collapse of the pluripotency gene regulatory network. Immunoprecipitation-

38   mass spectrometry (IP-MS) provided evidence that KDM3A and KDM3B formed a complex to

39   perform H3K9 demethylation. The genome-wide regulation analysis revealed that OCT4 (O) and

40   SOX2 (S), the core pluripotency transcriptional activators, maintained the pluripotent state of

41   piPSCs depending on the H3K9 hypomethylation. Further investigation revealed that O/S

42 cooperating with histone demethylase complex containing KDM3A and KDM3B promoted

43 pluripotency genes expression to maintain the pluripotent state of piPSCs. Together, these data offer

44 a unique insight into the epigenetic pluripotency network of piPSCs.

45 **Background**

46 The acquisition of pluripotency depends on the reconstruction of the epigenetic modification

47 during reprogramming. Recent studies have shown that OCT4, SOX2, and KLF4 (OSK)

48 cooperating with epigenetic modifiers mediated the gradually silencing of differentiation genes and

49 activating of pluripotency genes simultaneously during reprogramming [1, 2]. Of note, global H3K9

50 methylation is erased in the later stages of the reprogramming with the activation of pluripotency

51 network [3, 4]. Therefore, the establishment of pluripotent epigenetic regulation network based on

52 histone modification should be the kernel for maintaining the pluripotent state of induced pluripotent

53 stem cells (iPSCs) [4].

54 As an epigenetic modification associated with transcriptionally repressing, H3K9 methylation

55 is a major hindrance during somatic cell reprogramming into iPSCs [3, 4]. Loss of H3K9

56 methylation significantly increases the expression of endogenous pluripotent genes and contributes

57 to the transition of pre-iPSCs into iPSCs [3, 5]. Previous studies also demonstrated that the increase

58 of H3K9 methylation causes the loss of pluripotency in mouse embryonic stem cells (mESCs) [6-

59 8]. However, the molecular mechanism underlying H3K9 hypomethylation in pluripotent gene

60 regions remains unclear in pluripotent stem cells. The KDM3 family, which includes KDM3A and

61 KDM3B, function as epigenetic activators to demethylate intrinsic H3K9 methylation modification

62 [9, 10]. Additionally, KDM3A has been found to play a critical role in a wide range of biological

63 process like embryo development [8], pluripotency maintenance [6], carcinogenesis [11, 12], cell

64   senescence [13], sex differentiation [14], and spermatogenesis [15, 16]. At the same time, KDM3B

65   exhibits similar functions like KDM3A, such as pluripotency maintenance [5, 7], carcinogenesis

66   [17], and spermatogenesis [18]. However, the elaborate mechanism of KDM3A and KDM3B in

67   pluripotency maintenance and their functional redundancy in H3K9 demethylation remain unclear.

68   Pig is an ideal model for human medical research due to its close similarity with human in

69   genetic, anatomical, and physiological condition [19, 20]. Research on porcine PSCs contributes to

70   promising value in the biomedical field [20], though most published porcine PSCs, including ESCs

71   and iPSCs, cannot meet with the stringent criteria for pluripotency identity. We hypothesized the

72   unsound pluripotency gene regulatory network of piPSCs, especially in epigenetics, would be the

73   major obstacle for us to obtain bona fide PSCs *in vitro*. To solve this issue, the present study used

74   piPSCs generated by tetracycline operator (TetO)-inducible system [21] to delineate the mechanism

75   of H3K9 demethylation in piPSCs. Here, we found that KDM3A/KDM3B-meditated H3K9

76   demethylation plays a critical role in pluripotency maintenance of piPSCs. Moreover, the rescue

77   experiment suggested that not all exogenous genes were required and only O/S were essential for

78   the pluripotency maintenance of piPSCs. By performing genome-wide analysis of H3K9me2/me3

79   modification and O/S occupancy enrichment by ChIP-seq, as well as KDM3A and KDM3B

80   interactome analysis by IP-MS, we thus reveal the novel pluripotency regulation pathway, in which

81   SOX2/OCT4 recruit chromatin remodeling proteins KDM3A/KDM3B to form a transcriptional

82   complex to drive the pluripotency gene regulatory network.

83   **Results**

84   **H3K9 demethylation maintains the pluripotency of piPSCs**

85   We applied a combination of cytokines (LIF and bFGF) and signaling inhibitors (2i: chir99021

86   and sb431542) to maintain piPSCs that were reprogrammed by TetO-inducible systems (TetO-

87   FUW-OSKM and FUW-M2rtTA) [21]. The piPSCs depended on the sustained expression of

88   exogenous genes, which were similar to other reported piPSCs [21-24]. The clonal morphology and

89   pluripotency gradually disappeared after the piPSCs cultured in differentiation medium without

90   doxycycline (DOX) and feeder (Fig. S1A). The expression of both exogenous and endogenous

91   pluripotent genes (*OCT4*, *SOX2*, *LIN28A*, and *NANOG*) were down-regulated in this treatment (Fig.

92   S1B, C). However, the expression of endogenous *KLF4* was not impeded, and *c-MYC* was

93   significantly up-regulated (Fig. S1B). Analysis of global histone methylation showed that the level

94   of H3K9me1/2/3 was significantly increased, whereas the level of H3K4me1/3 was decreased (Fig.

95   S1D). The RT-qPCR analysis further showed the expression of *KDM3A/3B/3C/4B/4C*, which are

96   H3K9 demethylase, was down-regulated significantly (Fig. S1E). These results indicated that the

97   H3K9 hypomethylation and pluripotent factors *OCT4* and *SOX2* were strongly associated with

98   maintaining the pluripotency of piPSCs. Additionally, the downregulation of H3K9 demethylase

99   KDM3A/3B/ 3C/4B/4C might be caused by the loss of pluripotent factors, cytokines, and small

100   molecules during differentiation.

101   To investigate the mechanism in detail, we screened a series of components in the culture

102   medium (Fig. S1F). The results showed that the pluripotency of piPSCs mainly depends on two

103   small molecule inhibitors, DOX, and feeder (Fig. 1A, S1F). Given that the expression of *KDM3A*

104   was significantly down-regulated during differentiation (Fig. S1E), we further tested the expression

105   of *KDM3A* in different treatments. The results showed that the expression of *KDM3A* was affected

106   by feeder. Interestingly, the expression of *KDM3A* was not impeded when DOX was removed, but

107   it was further down-regulated when DOX was removed under F- treatment (Fig. S1G). These results

108   suggested the possible pivotal role of feeder and core pluripotent factors in reducing H3K9

109   methylation.

110   To investigate the mechanisms of increased H3K9 methylation under the feeder-free condition,

111   we focused on the F- and FD- treatments. With the disappearance of typical clone morphology,

112   lower proliferation rate, and high rate of apoptosis under both conditions were observed (Fig. 1A,

113   S2A, B, C). The expression levels of *OCT4* and *SOX2* were gradually down-regulated, whereas

114   those of *KLF4* and *c-MYC* were significantly up-regulated (Fig. 1B, C). The expression of *LIN28A*

115   increased significantly in F- treatment but decreased significantly in FD- treatment (Fig.1B, C).

116   Immunofluorescence also confirmed that the total expressions of OCT4 and SOX2 were

117   significantly decreased and that the piPSCs gradually differentiated into the ectoderm and

118   mesoderm (Fig. S2D, E). These results indicated the pluripotency of piPSCs gradually vanished

119   under F- and FD- treatments. The loss of pluripotency gene expression prompts us to check

120   repression related epigenetic modification H3K9 methylation in these cells. The results revealed

121   that the expression of *KDM3A/3B/3C/4C* genes was remarkably decreased and H3K9 methylation

122   was gradually increased in F- and FD- treatments (Fig. 1C, D, F). Correspondingly,

123   immunofluorescence showed that the level of H3K9me2/3 increased gradually, whereas the level of

124   H3K4me3 which involved in the regulation of gene activation decreased (Fig. 1E, S2F). Further

125   analysis demonstrated that the fluorescence intensity of H3K9me2/me3 at the marginal zone of

126   clone was higher than that inside in the F- treatments (Fig. 1E), whereas the fluorescence intensity

127   of H3K4me3 had an opposite trendency (Fig. S2G). This result was closely associated with the

128   phenotype of cell marginal differentiation. These observations suggested that the increased H3K9

129   methylation in the F- and FD- treatments lead to cell differentiation. Moreover, the function of

130   pluripotent factors may depend on the demethylation of H3K9me2/3 mediated by feeder.

131   **Feeder and pluripotent factors synergize to construct the pluripotency network.**

132   We attempted to gain insights into the molecular consequences that were triggered in response

133   to the action of these treatments. We performed RNA sequencing to provide a snapshot of the

134   transcriptional dynamics after the pluripotency changed. Transcriptional changes in both directions

135   were observed in F- (272 up versus 443 down) and FD- treatments (1786 up versus 1144 down; Fig.

136   2A). Notably, down-regulated genes accounted for the majority in the differentially expressed genes

137   (DEGs) in F- treatment (Fig. 2A). Given the significant increase of H3K9me2/3 in this treatment,

138   feeders might promote the gene expression by facilitating the erasure of H3K9me2/3. However,

139   more up-regulated transcription was observed in FD- treatment (Fig. 2A), suggesting that core

140   pluripotent factors mainly inhibited transcriptional output to maintain pluripotency. Further analysis

141   showed 200 of 272 up-regulated genes in the F- treatment were also up-regulated in FD- treatment,

142   and 226 of 443 down-regulated genes in the F- treatment were also down-regulated in FD- treatment

143   (Fig. 2B), indicating that these two treatments went through a consecutive process of differentiation.

144   Additionally, the pluripotency-related genes and *KDM3A/3B/4C* showed a gradient descending

145   trendency under F- and FD- treatments (Fig. 2C). This indicated feeder and exogenous pluripotency

146   genes processed a complementary regulation during piPSCs maintenance.

147   To further investigate this process, we applied cluster analysis for the DEGs. Gene ontology

148   analysis of DEGs revealed significant enrichment in GO terms "positive regulation of

149   developmental process", "MAPK cascade", "positive regulation of fibroblast proliferation", and

150   "cell cycle arrest" in response to F- and FD- treatments (Clusters 1 and 2; Fig. 2D). FD- responsive

151   down-regulated genes were enriched for "neuronal differentiation", "chromosome segregation", and

152   "DNA repair" (Clusters 3 and 4; Fig. 2D). These results indicated the DOX (exogenous genes)

153   mainly inhibited the expression of differentiation-related genes and activated DNA repair-related

154   genes to maintain the pluripotency of piPSCs. The cluster analysis also revealed that the feeder

155   mainly promoted the expression of the SRY-related HMG-box (SOX) family of transcription factors

156   (Clusters 1 and 4) to maintain the pluripotency of piPSCs which is consistent with feeder's function

157   reported before [25, 26]. Some transcription factors in SOX family (Cluster 4) were co-activated by

158   feeder and exogenous genes, such as *SOX3* [27] (Fig. 2D). The result suggested that reprogramming

159   factors cooperated with feeder to drive the pluripotency gene regulatory network.

160   **H3K9me2/3 prevents pluripotent transcription factors regulating on the target genes**

161   To further investigate the role of H3K9 methylation on the maintenance of pluripotency, ChIP-

162   seq of H3K9me2/3 was performed in MOCK and FD- groups. The ChIP-seq showed that the

163   genome-wide level of H3K9me2/3 was significantly increased in FD- treatment (Fig. 3A, B). The

164   binding sites of H3K9me2/3 were located mainly in the intergenic region and intron rather than

165   promoter, and no significant difference was noted between the MOCK and FD- near the

166   transcription start sites (Fig. S3A, B). These results suggested that H3K9me2/3 inhibited gene

167   expression by acting on the other transcriptional regulatory regions other than promoters. Further

168   analysis of de novo peaks revealed H3K9me2 and H3K9me3 only shared 2211 peaks in FD-

169   treatment which account for 11% of the newly added peaks of H3K9me3. However, the analysis of

170   the peak-associated genes showed that there were 226+474+2272 genes affected by H3K9me2 and

171   H3K9me3 in FD- treatment in which only 226 genes were co-regulated, accounting for 19.4% of

172   the H3K9me3 genes in this treatment (Fig. S3C). This result suggested that H3K9me3 cooperates

173   with H3K9me2 to regulate the partial target gene under FD- treatment, while the binding sites are

174   different. Combined analysis of ChIP-seq and RNA-seq of MOCK and FD- groups were performed

175   to clarify the relationship between H3K9me2/3 and pluripotency maintenance. Considering that

176   H3K9 methylation will silence gene expression, the associated genes were divided into four parts:

177   the downregulated genes marked by FD- specified H3K9me2/3; the upregulated genes marked by

178   MOCK specified H3K9me2/3. Further GO analysis of genes in Parts 1 and 3 reveal their enrichment

179   in "positive regulation of fibroblast proliferation", "positive regulation of apoptotic process",

180   "positive regulation of ERK1 and ERK2 cascade" and "positive regulation of epithelial to

181   mesenchymal transition" (Fig. S3D). The genes in Parts 2 and 4 included several potential

182   pluripotent genes, such as *NR6A1*(Fig. S3D) [28]. The result suggested that H3K9me2/3 participated

183   not only in the inhibition of pluripotent genes during differentiation but also in the inhibition of

184   somatic genes in the pluripotent state.

185   To further clarify the role of H3K9me2/3 in the maintenance of pluripotent state, motif analysis

186   of H3K9me2/3 peaks was performed. Results revealed that H3K9me2 peaks only bound sites in

187   FD- group contained pluripotent transcription factor binding sites, such as OCT4 and SOX2 (Fig.

188   3C). Although this finding is not clear on H3K9me3, more peaks have been found to contain

189   pluripotency factors binding sites in FD- group compared to the MOCK group. This result implied

190   that H3K9me2/3 prevented interaction between pluripotent transcription factors and their target

191   genes, especially H3K9me2. To confirm this, we found a significant enrichment of H3K9me2

192   modification occurred in the upstream or downstream region of pluripotent genes *OCT4, LIN28A,*

193   *TAF4B,* and *SALL4* in the FD- group (Fig. 3D), suggesting that these genes expression was

194   suppressed (Fig. S3E, F). These results suggested that H3K9me2/3 represses gene expression by

195   preventing the interaction between pluripotent transcription factors and target genes.

196   **Combined loss of KDM3A and KDM3B reveals critical roles of H3K9 demethylation in**

197   **piPSCs maintenance**

198   H3K9 methylation was unevenly distributed in chromosomes and regulated by KDM3A/B.

199   However, the molecular mechanism remained unclear. To further directly investigate the role of

200   H3K9 hypermethylation in the maintenance of piPSCs, we down-regulate the expressions of

201   *KDM3A* and *KDM3B* by shRNA. Unexpectedly, the depletion of KDM3A only resulted in a slight

202   decrease in the proliferation of piPSCs (Fig. S4A, B), and the global level of H3K9me2/3 was only

203   slightly up-regulated (Fig. 4A, B). KDM3B loss not only significantly reduced the size of clones

204   and proliferation rates but also decreased the degree of AP staining. However, the level of

205   H3K9me1/2/3 remained unchanged (Fig. 4A, B). The results varied from previous studies on

206   mESCs [6, 7]. KDM3A and KDM3B possess intrinsic H3K9 demethylating activity, so their

207   functions may be complementary in the H3K9 demethylation. As expected, the co-depletion of

208   KDM3A and KDM3B by shRNA induced a significant growth arrest and pluripotency loss in this

209   group (Fig. 4A, B, S4A, B). Consistent to this, H3K9me2/3 was increased significantly (Fig. 4B).

210   These results suggested that KDM3B could rescue the depletion of KDM3A, but KDM3A could not

211   rescue that of KDM3B, suggesting that KDM3B might be involved in other biological processes.

212   To molecularly characterize KDM3A/B function, the profiles of the transcriptomes of shRNA

213   treated samples (shKDM3A, shKDM3B, and shKDM3A/B groups) were analyzed as well as empty

214   vector control (NC) by RNA-sequencing. Although transcriptome did not change much in

215   shKDM3A group (11 up versus 27 down), the gene expression in shKDM3B and shKDM3A/B was

216   observed significantly changing with 1099 and 918 up-regulation; 1475 and 1727 down-regulation

217   respectively (Fig. S4C). This finding indicates the depletion of KDM3A alone had no significant

218 effect on the pluripotency of piPSCs. Further analysis revealed shKDM3B shared 50% (upregulated)

219 and 63% (downregulated) dysregulated genes with shKDM3A/B group, explaining why KDM3B

220 could rescue the lack of KDM3A (Fig. 4C). Of note, down-regulated genes (925 downregulated vs

221 502 upregulated) accounted for the majority in the DEGs of shKDM3B and shKDM3A/B treatments,

222 which suggested KDM3A and KDM3B played transcriptional activation roles in the transcription

223 regulation. The DEGs were then classified into five clusters (Fig. 4D). KEGG analysis was

224 performed on each cluster to clarify the effects of KDM3A and KDM3B on the maintenance of

225 piPSCs. Genes with functions in pathways of "signaling pathways regulating pluripotency of stem

226 cells", "Wnt signaling pathway", and "Pathways in cancer" were significantly down-regulated in

227 shKDM3B and shKDM3A/B groups (cluster A), whereas PI3K-Akt signaling pathway-associated

228 genes were expressed at lower levels only in the shKDM3A/B group (cluster C). The regulation of

229 associated genes on "oxidative phosphorylation" was up-regulated in the shKDM3B and

230 shKDM3A/B groups (cluster E). Genes representing "TGF-beta signaling" and "metabolic

231 pathways" were gradually up-regulated in the shKDM3B and shKDM3A/B groups, and these genes

232 up-regulated more in shKDM3A/B group compared to shKDM3B only (cluster D). Unexpectedly,

233 268 genes representing MAPK signaling pathway were expressed at higher levels in the shKDM3B

234 group, whereas the expression of these genes decreased after interfering with KDM3A expression

235 on the basis of shKDM3B (cluster B; Fig. 4D). The transcriptome analysis results suggested that the

236 depletion of KDM3A and KDM3B destroyed the pluripotency of piPSCs by decreasing expression

237 of genes related to "Signaling pathways regulating pluripotency of stem cells", "Wnt signaling

238 pathway", and "PI3K-Akt signaling pathway". In addition, the piPSCs tended to differentiate and

239 respond to oxidative stress, such as TGF-beta signaling pathway and oxidative phosphorylation,

240   when depleted both KDM3A and KDM3B by shRNA (cluster D and E). These results suggested

241   that erasure of H3K9me2/3 by KDM3A and KDM3B played a critical role in activating pluripotency

242   related signaling pathways, such as Wnt signaling pathway, and PI3K-Akt signaling pathway.

243   **Histone demethylase KDM3A play a dominant role in maintaining pluripotency**

244   Previous studies have demonstrated that the depletion of either KDM3A or KDM3B causes

245   the increase of H3K9 methylation and loss of pluripotency in mESCs [6, 7]. However, a similar

246   phenomenon only appeared in piPSCs when both KDM3A and KDM3B were down-regulated,

247   implying a novel and complex regulatory mechanism in piPSCs. To further reveal the demethylation

248   mechanism regulated by KDM3A/B, they were respectively overexpressed in piPSCs (Fig. 4E, F).

249   The results showed that global H3K9me2/3 of piPSCs was remarkably decreased after

250   overexpression of KDM3A or KDM3B (Fig. 4F, S4D). Differences in clone phenotypes were

251   observed between T3F-KDM3A and T3F-KDM3B groups (Fig. 4E). Meanwhile, higher cell

252   proliferation rate has been detected after KDM3A overexpression while KDM3B overexpression

253   inhibits cell proliferation (Fig. S4E), suggesting that KDM3A and KDM3B were involved in

254   different biological processes.

255   To understand the molecular mechanism of KDM3A and KDM3B in piPSCs regulation, we

256   profiled the transcriptomes of T3F-KDM3A and T3F-KDM3B by RNA-seq. We found KDM3A

257   (460 up versus 283 down) and KDM3B (378 up versus 258 down) had divergent transcriptional

258   output (Fig. S4C). Only 128 up-regulated genes and 76 down-regulated overlapped genes shared by

259   the two groups, accounting for 18% and 16.4% of the total number of differently expressed genes,

260   respectively (Fig. S4F). The divergent transcriptional response again confirmed that KDM3A and

261   KDM3B were involved in different biological processes. This finding explained why the depletion

262   of KDM3B alone impairs the piPSCs pluripotency without altering global H3K9me2/3 modification.

263   KEGG analysis was performed on the genes to clarify the effects of the overexpression of KDM3A

264   and KDM3B on the maintenance of piPSCs. Genes with functions in pathways of "Signaling

265   pathways regulating pluripotency of stem cells", "glutathione metabolism", and

266   "glycerophospholipid metabolism" were significantly upregulated in T3F-3A group, whereas genes

267   associated with MAPK, apoptosis, and TGF-beta signaling pathways were downregulated only in

268   T3F-3A group (Fig. 4G). Nonetheless, overexpression of KDM3B enriched pathways unrelated to

269   pluripotency (Fig. S4G). We also found naive pluripotent gene *TBX3* [29, 30] and *WNT* ligands

270   upregulated while the expression of *JUN* which inhibiting reprogramming downregulated after

271   overexpression of KDM3A [2] (Fig. S4H). However, the overexpression of KDM3B does not have

272   the same effect. Together, our results suggested that the KDM3A maintained the pluripotent network

273   of piPSCs by activating the expression of genes of signaling pathways regulating pluripotency as

274   well as inhibiting genes associated with MAPK, apoptosis, and TGF-beta signaling pathways. The

275   co-depletion of KDM3A and KDM3B influenced the pluripotency of piPSCs, but only

276   overexpression of KDM3A could promote the pluripotency, suggesting KDM3A play a dominant

277   role in maintaining pluripotency by H3K9 demethylation.

278   **OCT4 and SOX2 are the core factors for the maintenance of piPSCs**

279   Considering that the downregulation of OSKM also leads to the increase of H3K9 methylation

280   under F- condition, it is necessary to further investigate the role of core pluripotent genes in the

281   maintenance of pluripotency. After removing DOX, the proliferation of piPSCs was significantly

282   decreased, and the classical ESC like clonal morphology gradually destroyed (Fig. S1F, 5A). The

283   expressions of endogenous *OCT4* and *SOX2* were significantly down-regulated, whereas those of

284   endogenous *KLF4* and *c-MYC* were significantly up-regulated in FD- treatment (Fig. 1B). Therefore,

285   four pluripotency genes OSKM may have different roles in piPSCs maintenance. Next, we try to

286   determine single pluripotency gene's function by using a lentivirus overexpression system carried

287   EF1 promoter (Fig. S5A, B, C). The results showed that piPSCs could maintain clonal morphology

288   under DOX- condition when OCT4 or c-MYC was complemented (Fig. S5A). However, the AP

289   staining of c-MYC group gradually presented negatively as the passage times increased under the

290   DOX- condition (Fig. S5D). Given the role of SOX2 may depend on OCT4, we then try to

291   overexpress both factors in piPSCs to explore whether O/S can recapitulate exogenous OSKM

292   function to maintain pluripotency. After overexpressed O/S (EF1α-OCT4-P2A-SOX2) or OCT4

293   (EF1α-3×FLAG-OCT4) alone, cell lines can be expanded more than 20 passages, and still maintain

294   the phenotype without differentiation (Fig. 5A). The proliferation rate is similar when compared OS

295   co-overexpression cell line with the control, however, the OCT4 overexpressing line is lower than

296   the control (Fig. S5E). Further analysis showed that the expression of total *SOX2* was positively

297   correlated with *LIN28A* expression level and negatively correlated with *NANOG* and *c-MYC* (Fig.

298   5B). These results indicated that SOX2 was also a core transcription factor that regulated the

299   proliferation of piPSCs, and its function depended on OCT4.

300   ChIP-seq of OCT4 and SOX2 were performed to analyze their mechanism of transcriptional

301   regulation. The results showed that the binding sites of OCT4 and SOX2 were mainly located in the

302   intergenic region and intron rather than promoter, suggesting that they activated gene expression by

303   acting on the distal enhancer region (Fig. S5F). It is noteworthy that the OCT4 shares about 50%

304   (5554) peaks with SOX2, which accounts for 74.6% (3376/4523) of the genes OCT4 target (Fig.

305   5C), supporting interaction between OCT4 and SOX2 during binding to maintain gene expression.

306   These findings were similar to the results reported in mouse [1, 31, 32]. Motif analysis indicating

307   OCT4 and SOX2 were of high similarity (Fig. S5G), which is consistent with the peak binding

308   analysis between the two proteins. Therefore, we performed a bimolecular fluorescence

309   complementary test to directly investigate the proteins interacting with OCT4 and SOX2 (Fig. S7B).

310   To reveal why OCT4 and SOX2 were necessary for piPSCs, we further analyzed their peaks

311   in pluripotency genes *OCT4*, *SOX2*, *LIN28A*, and *NANOG*. ChIP-seq showed that all the genes were

312   co-occupied by OCT4 and SOX2 (Fig. 5D). We further validated the regulation by double luciferase

313   test and found all the four genes can be activated by O/S, and *LIN28A* showing significantly higher

314   luciferase activity (13-fold change) than other genes (Fig. S5H). KEGG analysis also revealed

315   OCT4 and SOX2 targets are mainly enriched in the pathway related to "Wnt/β-catenin signaling

316   pathway", "TGF-β signaling pathway", and "signaling pathway regulating pluripotency of stem

317   cells" (Fig. 5E). These results indicated OCT4 and SOX2 formed heteromeric dimers to maintain

318   the porcine pluripotent regulatory network.

319   **O/S and KDM3A/3B synergize to construct the porcine pluripotent networks**

320   Previous studies in mES have demonstrated the direct activation of KDM3A/3B by OCT4 [6,

321   8]. To determine if this regulation link still exists in piPSCs, we overexpressed OCT4 and SOX2 in

322   piPSCs (Fig. S6A). The results showed that the expression of *KDM3A* and *KDM3B* were not

323   affected, and the promoter activity of *KDM3A* and *KDM3B* cannot be activated by OCT4 and SOX2

324   (Fig. S6B, C). In addition, the expression of endogenous *OCT4* and *SOX2* did not further increase

325   significantly when KDM3A or KDM3B were overexpressed (Fig. S6D). Given the dependence

326   between transcription factors O/S and hypomethylation of H3K9 in this study (Fig. 3C), the

327   relationship between them might be a cooperator. To further explore the physiological relevance of

328   our findings that OCT4 and SOX2 maintain piPSCs pluripotency is dependent on H3K9

329   hypomethylation, we ask how many OCT4 and SOX2 peaks co-occupied by H3K9me2/3. Finally,

330   we found about 20% OCT4 (18%, 1984/10817) and SOX2 (22%, 2346/10870) peaks modified by

331   H3K9me2 while fewer peaks have been found modified by H3K9me3 in FD- condition (Fig. 6A).

332   Furthermore, we analyzed the change of H3K9me2/3 in OCT4 and SOX2 binding sites. Previous

333   results showed that there was no significant difference near the transcription start sites (Fig. S3B),

334   but the level of H3K9me2 at the OCT4 and SOX2 binding sites increased significantly in FD- group,

335   indicating that H3K9 methylation directly prevented the function of OCT4/SOX2 (Fig. 6B, C). The

336   KEGG enrichment of peak-associated genes shared by OCT4/SOX2 and H3K9me2 showed that the

337   genes were enriched in pathways like Wnt/β-catenin signaling pathway which is important for

338   pluripotency of stem cells regulation (Fig. 6D). This result indicated that OCT4 and SOX2 drove

339   critical pluripotent pathway depending on the erasure of H3K9me2. In addition, ATAC-seq of

340   MOCK and FD- groups also showed that with the increase of H3K9me2 in the O/S binding sites,

341   the accessibility of these sites in the FD-group decreased significantly (Fig. 6E). Motif analysis

342   further showed that the proportion of pluripotency related motifs (OCT4/SOX2) was decreased,

343   while the proportion of somatic transcription factors (Fra1/Jun-AP1/AP1) were increased in FD-

344   treatment, indicating the OCT4/SOX2 binding patterns change in this treatment (Fig. S6E).

345   Interestingly, conjoint analysis between the RNA-seq of T3F-3A and ChIP-seq of O/S not only

346   reconfirmed the above results but also found KDM3A was involved in the inhibition of O/S-

347   mediated TGF-β and MAPK signaling pathways (Fig. S6F, G). ChIP-seq analysis further validates

348   OCT4 and SOX2 binding to the enhancer and promoter regions of pluripotent genes *OCT4*, *LIN28A*,

349   *NANOG,* and Wnt receptor proteins *FZD8* in piPSCs, but these binding regions showed increased

350   H3K9me2 in the FD- treatment, that is, the binding sites of OCT4 and SOX2 were closed (Fig. 6F).

351   These results suggested that OCT4 and SOX2 activated key pluripotent genes depending on the

352   erasure of H3K9me2.

353   To further dissect the demethylation mechanism underlying KDM3A and KDM3B in the O/S

354   binding sites, we applied IP-MS to directly investigate the proteins interacted to KDM3A and

355   KDM3B under normal condition (Fig. 7A, B, S7A). The results showed an interaction between

356   KDM3A and KDM3B. Additional studies were conducted to verify the direct binding between

357   KDM3A and KDM3B using a bimolecular fluorescence complementary (BiFC) technique (Fig.

358   S7B). GO analysis was performed on the proteins belonging to each part to clarify the different

359   effects of KDM3A and KDM3B on the maintenance of piPSCs. Partners shared by KDM3A and

360   KDM3B were enriched with functions in "DNA ligation involved in DNA repair", "histone H3-K9

361   demethylation", and "DNA topological change". KDM3A specific interacting proteins were

362   enriched with functions in "positive regulation of telomerase activity". The specific KDM3B-

363   interacting proteins were enriched with functions in "positive regulation of Wnt protein secretion"

364   and "glycolytic process" (Fig. 7C). These results suggested that KDM3A and KDM3B co-regulated

365   gene activation processes in the chromatin level but also individually involved in different biological

366   processes.

367   Further analysis of the IP-MS revealed that KDM3A interacted with SOX2, whereas KDM3B

368   interacted with OCT4, which were further confirmed using IP-WB (Immunoprecipition combined

369   with western blot) (Fig. 7B, D). A large number of high mobility group (HMG) proteins involved in

370   chromatin remodeling [33, 34] were significantly enriched in the intersection of the IP-MS of

371   KDM3A and KDM3B (Fig. 7B). BiFC test again demonstrated the direct interaction between OCT4,

372   SOX2, KDM3A, and KDM3B (Fig. S7B). These results suggested that OCT4/SOX2,

373   KDM3A/KDM3B, and HMG proteins formed a transcription complex to activate downstream genes

374   (Fig. 7E). This new mechanism of pluripotency maintenance is important for the establishment of

375   porcine naïve-state PSCs.

376   **Discussion**

377   The deconstruction of pluripotency gene regulatory network is a challenging and urgent issue

378   need solving to obtain bona fide porcine pluripotent cells across the globe. In this study, we used

379   the piPSCs generated by TetO-inducible system to gain a clear description of how the porcine

380   epigenetic pluripotency network was maintained. Our results demonstrated that OCT4 and SOX2

381   cooperate with KDM3A/KDM3B to support the complex formation of super-transcription drivers

382   to porcine pluripotency network (Fig. 7E).

383   KDM3A/KDM3B-meditated H3K9 demethylation plays a critical role in early mouse

384   embryogenesis and mES maintenance [6-8]. However, no detailed information can be found on

385   H3K9 methylation regulating porcine embryogenesis and ESC maintenance. In addition, the rules

386   of KDM3A and KDM3B in H3K9 demethylation remained unclear. Our results demonstrate that

387   KDM3A and KDM3B form a synergistic complex to perform demethylation, and only suppressing

388   both of them will achieve global H3K9 hypermethylation. The conventional view is that KDM3A

389   and KDM3B may function in a redundant manner [8-10]. However, our study also suggests that

390   KDM3A and KDM3B are novel cooperative mechanisms in the erasure of H3K9 methylation. The

391   single-cell sequencing of porcine embryo shows that KDM3A is highly expressed in Morula, ICM,

392   and Epiblast, but KDM3B is only specifically expressed in Epiblast (Fig. S7C). The expression

393   pattern was similar to *SOX2*, suggesting that a similar interaction mechanism might exist in Epiblast

394   [35]. Our results also reveal that the overexpression of KDM3A can significantly improve the

395   proliferation and anti-apoptosis ability of pluripotent stem cells, but that of KDM3B leads to a

396   decline in cell proliferation. The results suggest that KDM3A is the preferred choice for the H3K9

397   demethylation and excessive KDM3B affects pluripotency by promoting other biological processes

398   under normal condition. Our study provides a novel mechanism in which histone demethylase

399   performs H3K9 demethylation in porcine pluripotent stem cells.

400   Porcine pluripotent stem cells, including embryonic stem cells (ESCs) and iPSCs, are facing a

401   very serious problem [36]. At present, piPSCs can be obtained by ectopic expression of defined

402   pluripotent transcription factors [24, 37, 38]. However, the maintenance of pluripotent state depends

403   on the continuous expression of exogenous genes, and endogenous pluripotent genes are not fully

404   activated [22-24, 37-41]. Therefore, most of piPSCs also fail to contribute to chimeras with

405   exceptions that are only detected by genomic PCR analysis [22, 23]. Porcine embryonic stem cell-

406   like cells (pESLCs) exhibited some features of pluripotency, but these cells could not maintain self-

407   renewal for a long time [42-44] [45]. The generation of pESCs and acquisition of imperfect piPSCs

408   always fail because the porcine endogenous pluripotent network cannot be maintained stably.

409   OCT4, which is a core factor during reprogramming, is often used as a marker for

410   reprogramming cells to reach iPSC state [37, 46, 47]. Numerous studies have shown that the

411   depletion of OCT4 causes pluripotent stem cells to gradually lose their pluripotency [32, 48]. Our

412   study demonstrates that the deficiency of endogenous OCT4 expression is the main reason porcine

413   pluripotent stem cells cannot be maintained for a long time. Several studies on pig early embryos

414   have indicated that OCT4 is generic expressed in ICM and TE, which differs from that in humans

415   and mice (Fig. S7C) [35]. The regulatory mechanism of endogenous OCT4 in pigs may be unique.

416   Although our study did not further increase endogenous OCT4 by changing the culture system, we

417   demonstrated that low expression of OCT4 is associated with high levels of H3K9me2 in its

418   promoter and enhancer region (Fig. 3D). We speculate that H3K9me2 level must be decreased in

419   OCT4 promoter region during the establishment of porcine ESCs.

420   SOX2, a molecular marker specifically expressed in pig ICM [49], cooperates with OCT4 to

421   activate downstream pluripotent genes [31, 32]. According to our results, OCT4 and SOX2 together

422   directly activate many pluripotency related pathways and regulate each other, and this finding is

423   similar to that in other reports [1, 31, 32]. However, differences are noted in downstream pathways

424   and genes, especially the TGF-β signaling pathway, which must be directly inhibited by O/S in

425   piPSCs. The IP-MS of KDM3A and KDM3B shows that both bind a large number of proteins

426   associated with chromatin remodeling, such as HMGB2. Our further study demonstrates that SOX2

427   and OCT4 directly bind to KDM3A and KDM3B, respectively, suggesting that the SOX2/OCT4

428   recruit KDM3A/KDM3B and chromatin remodeling proteins form a transcriptional complex to

429   drive the pluripotency gene regulatory network. This novel mechanism is similar to the previously

430   reported super enhancer. Understanding the mechanistic basis for forming the complex between

431   pluripotent and non-pluripotent cells reveals further insights into the nature of the pluripotent state.

432   **Material and Methods**

433   **Statement of Ethics**

434   All animal experiments were performed in strict accordance with the Guide for the Care and

435   Use of Laboratory Animals (Ministry of Science and Technology of the People's Republic of China,

436   Policy No. 2006398) and were approved by the Animal Care and Use Center of the Northwest A &

437   F University.

438 **Cell culture**

439   Porcine iPSCs was cultured on feeder (MEF, mouse embryonic fibroblasts) and maintained in

440 LB2i medium, consisting of DMEM (Hyclone, USA) supplemented with 15% FBS (VISTECH,

441 New Zealand), 0.1 mM NEAA (Gibco, USA), 1 mM L-glutaMAX (Gibco, USA), 10 ng/ml LIF

442 (Sino Biological, 14890 -HNAE), 10 ng/ml bFGF (Sino Biological,10014-HNAE), 0.1 mM β-

443 mercaptoethanol (Sigma Aldrich, M3148, USA), 3 μM CHIR99021 (MCE, HY-10182), 2 μM

444 SB431542 (Selleck, S1067), 4 μg/ml doxycycline (Sigma Aldrich, D9891), 100 units/ml penicillin

445 and 100 μg/ml streptomycin. piPSCs were passaged using TrypLE™ Select (Invitrogen, USA) into

446 a single cell at 2×10^4 cells per 12-well plate every 5–6 days. Differentiation was performed in

447 differentiation medium: DMEM (Hyclone, USA) supplemented with 15% FBS, 0.1mM NEAA, 1

448 mM L-glutaMAX, 0.1 mM β-mercaptoethanol, 100 units/ml penicillin and 100 μg/ml streptomycin.

449 HEK293T and MEFs were cultured in DMEM supplemented with 10% FBS and 100 units/ml

450 penicillin, 100 μg/ml streptomycin [21].

451 **Plasmids and cloning**

452   All lentivirus backbones vectors were derived from pCDH-CMV-MCS-EF1-GreenPuro

453 (CD513B-1, SBI, Mountain View, CA, USA) by Seamless Cloning and Assembly Kit (Novoprotein,

454 China). The following vectors used in this study were constructed using the same strategy and its

455 information is shown in Table S1.

456   **shRNA vectors**

457   shRNAs were designed using the online shRNA design tool from Invitrogen

458 (http://rnaidesigner.thermofisher.com/rnaiexpress/setOption.do?designOption=shrna&pid=523099

459 9276329139407/) with default parameters. And the potential off-target effects were filtered out

460 using BLAST against the porcine genome.

461 Sense and antisense oligonucleotides of the shRNA duplex were synthesized and cloned into

462 an in-house designed pCDH-U6-MCS-EF1-GFP-T2A-PURO or pCDH-U6-MCS-EF1-GFP-T2A-

463 mCherry Lentivector. All shRNAs were tested for each gene in piPSCs and knockdown efficiencies

464 of 70% or more were used in this study. The vector and sequence information of shRNAs used in

465 this study is shown in Table S1 and S2.

466 **Overexpression vectors**

467 The p-O/S/K/M/L(*OCT4/SOX2/KLF4/c-MYC/LIN28A*) gene were PCR-amplified from

468 porcine blastocysts and subcloned into an in-house pCDH-EF1-MCS-T2A-PURO vectors. The

469 3×FLAG-OCT4, 3×FLAG-SOX2, 3×FLAG-KDM3A and 3×FLAG-KDM3B were generated by

470 PCR and subcloned in pCDH-TetO-3XFLAG-MCS-T2A-PURO. The Rosa26-EF1α-3×FLAG-

471 OCT4, and Rosa26-EF1α-OCT4-P2A-SOX2 were generated by PCR and subcloned in Rosa26-

472 EF1α-MCS-T2A-PURO. Coding regions of all overexpression vectors were verified by sequencing.

473 Subsequently, these overexpression vectors were transfected into HEK293T cell and identified by

474 Western blot. The vector information of these genes used in this study is shown in Table S1.

475 **BiFC vectors**

476 The *OCT4/SOX2/KDM3A/KDM3B/WNT2* gene were generated by PCR and subcloned in

477 pBiFC-VC155 and pBiFC-VN173. Coding regions of all overexpression vectors were verified by

478 sequencing. The vector information of these genes used in this study is shown in Table S1.

479 **Luciferase vectors**

480 To verify the ChIP-seq of OCT4 and SOX2, the porcine target gene promoter and enhancer

481 were amplified by PCR from total genomic DNA extracted from piPSCs and subcloned into an in-

482   house PGL3-basic vector (Promega, E1751) by Seamless Cloning. The detailed information of these

483   Luciferase vectors is shown in Table S1.

484   **Lentivirus packaging and transduction**

485   HEK293T cells were seeded onto a 6-well plate and grown to 70–80% confluence. Then the

486   lentivirus backbone and package vector (pVSV-G and psPAX2) were transfected into HEK293T

487   cells using PEI (polyethyleneimine, sigma). For transfection of per well, 1 µg pVSV-G, 1µg psPAX2

488   and 2 µg lentivirus backbone vector were diluted in 200 µl optiMEM and vortexed. 12 µl PEI

489   (1mg/ml) was added to the plasmid mix, vortexed, incubated for 15 min at room temperature then

490   added to cells. After 12 h, the medium was replaced with fresh medium added lipid and cells were

491   maintained for 48 -72 h. Lentivirus (culture supernate) was collected, filtered through 0.45 µm filter

492   to remove debris. For the lentiviral transduction, 2×10^4 cells were plated on MEF-coated 12 well

493   plate per well and allowed to attach overnight. Virus and fresh media were added at a ratio of 1:1

494   supplemented with 4 µg/ml Polybrene into this well. The Cells were incubated with mixed media

495   overnight, washed with PBS and replaced with fresh medium. After 1 week of culture, stably

496   infected colonies were selected with puromycin (10 µg/ml) for 24 h, and viral titer was calculated

497   by counting the GFP-positive colonies.

498   **AP staining**

499   Cells were fixed with 4% paraformaldehyde in PBS (pH 7.4) for 15 min at room temperature,

500   washed twice using ice-cold PBS and developed with AST Fast Red TR and α-Naphthol AS-MX

501   Phosphate (Sigma Aldrich) according to the manufacturer's instructions. Then the cells were

502   incubated with the mixture (1.0 mg/ml Fast Red TR, 0.4 mg/ml Naphthol AS-MX in 0.1 M Tris-

503   HCL Buffer) at room temperature. After 20 min, the AP-positive iPS colonies showed in red color.

504   The images were collected by a Nikon phase contrast microscope.

505   **RNA extraction, Reverse transcription, and Quantitative real-time PCR**

506   Total RNA was extracted by RNAiso Plus (Takara, 9109) and purified with the guanidine

507   isothiocyanate - phenol chloroform. Reverse transcription was performed using Fast Quant RT Kit

508   (TIANGEN, KR106). Fluorescence quantitative PCR analyses of all samples were performed using

509   a Bio-Rad CFX96 and SYBR green master mix (TIANGEN, FP215). Semi-quantitative PCR

510   reactions were performed for 30 cycles by 2×TSINGKE Master Mix (TSINGKE, TSE004). The

511   primer information used in this study is provided in Table S3. The expression of target gene was

512   normalized against transfection of control vector. Data of Q-PCR are calculated by Bio-Rad

513   software CFX3.1 and derived from three independent experiments.

514   **Western blot**

515   The cells were digested by TrypLE™ Select and then transferred immediately to a 1.5 ml tube

516   on ice. The cell suspension was then centrifuged at 5000g for 3 min and supernatant was discarded.

517   The cell sediment was lysed by RIPA buffer (Beyotime, P0013B) for 30 min on ice, added to 5×

518   SDS-PAGE loading buffer (GENSHARE G, JC-PE007), and heated at 100 °C for 5 min, then loaded

519   onto 8-12% SDS-PAGE gel. The SDS-PAGE gels were run at 100V for 1.5 h and transferred to a

520   PVDF membrane by semidry electrophoretic transfer (Bio-Rad) for 45 min at 15 V. The transferred

521   membrane was blocked with 8% skim milk) at room temperature for 2 h, and then incubated with

522   the primary antibody in TBS-T buffer (20 mM Tris/HCl pH 8.0, 150 mM NaCl, 0.05% Tween 20)

523   at 4 °C overnight. After washing three times with TBS-T buffer, the blot was incubated with

524   secondary antibody at 37°C for 1h, then washed three times. Enhanced chemiluminescent substrate

525   (Biodragon, BF06053-500) was used to detect the HRP signal and the western blot images were

526   collected using the Chemiluminescent Imaging System (ZY058176, Tanon-4200, China). The

527   information of antibodies used in this study was listed in Table S4.

528   **Immunofluorescence microscopy**

529   The cells were fixed with 4% paraformaldehyde in PBS (pH 7.4) for 15 min at room

530   temperature. Fixed cells were washed twice using ice-cold PBS, permeabilized with 0.1% Triton X-

531   100 in PBS for 10 min, and subsequently blocked for 2 h at room temperature in PBS containing 5%

532   FBS. The cells were incubated with blocking buffer containing primary antibodies at 4°C overnight.

533   The secondary antibodies were diluted in a blocking buffer and incubated at 37°C for 1h. After

534   washing with PBS for three times, the nuclei were stained by 10 μg/ml Hoechst 33342 for 8 min.

535   Finally, the images were collected by an EVOS fluorescence microscope. The information of

536   antibodies used in this study was listed in Table S4.

537   **Chromatin Immunoprecipitation and ChIP-seq**

538   **ChIP Assay**

539   The 1×10^7 cells were crosslinked with 1% formaldehyde for 10 min at room temperature, then

540   added to 125 mM Glycine to neutralize the formaldehyde. The crosslinked cells were washed three

541   times using ice-cold PBS, subsequently scraped off and transferred to a 15ml centrifuge tube. The

542   cell suspension was spun down for 5 min at 2000g and the supernatant was completely removed.

543   The sediments were lysed by Nuclear Lysis Buffer to obtain chromatin extracts, that were treated

544   with ultrasound to obtain DNA fragments with an average size of 200-500 bp. The complexes of

545   target proteins and DNA fragments are immunoprecipitated by specific antibodies and protein A/G

546   magnetic beads. The information of antibodies used in this study were listed in Table S4.

547   **ChIP-seq and data analysis**

548   The DNA fragments of IP and Input were used for stranded DNA library preparation. Then the

549 library was detected by agarose electrophoresis, quantified using Qubit 2.0 and sequenced on

550 Illumina HiSeq2500 PE150. Raw sequencing data was first filtered by Trimmomatic [50] (version

551 0.36), low-quality reads were discarded and the reads contaminated with adaptor sequences were

552 trimmed. The clean reads were mapped to the reference genome of *Sus scrofa*

553 fromGCF_000003025.6(ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCF/000/003/025/GCF_00000302

554 5.6_Sscrofa11.1/GCF_000003025.6_Sscrofa11.1_genomic.fna.gz) using STAR software (version

555 2.5.3a) with default parameters [51]. Then the distribution, coverage, homogeneity and chain

556 specificity of reads were evaluated by RSeQC (version 2.6) [52]. Peaks in the ChIP-Seq datasets

557 were called with MACS2 (version 2.1.1) [53] and associated with genes using Homer (version v4.10)

558 [53, 54]. The distribution of call peak on the chromosomes and functional elements was performed

559 by deepTools（version 2.4.1）[55]and ChIPseeker (version 1.5.1) [56]. Peak maps of reads across

560 the genome are described using IGV (version 2.4.16) [57]. The motif analysis was performed using

561 Homer (version v4.10) [54]. Gene ontology (GO) analysis and Kyoto encyclopedia of genes and

562 genomes (KEGG) enrichment analysis for the peak related genes were both implemented by

563 KOBAS software (version: 2.1.1) with a corrected P-value cutoff of 0.05 to judge statistically

564 significant enrichment [58]. Signal tracks of features (H3K9me2 and H3K9me3) were calculated

565 by using Deeptools with parameter "ComputeMatrix". We remove the noise by subtracting the

566 INPUT matrix from the IP matrix and showed the features at peaks regions and up- and downstream

567 regions within ±2kb.

568 **Digital RNA-seq**

569 **RNA extraction, library preparation and sequencing**

570   Total RNAs were extracted from samples using TRIzol (Invitrogen) following the methods

571   [59]. DNA digestion was carried out after RNA extraction by DNase I. RNA quality was determined

572   by examining A260/A280 with NanodropTM One spectrophotometer (Thermo Fisher Scientific

573   Inc). RNA Integrity was confirmed by 1.5% agarose gel electrophoresis. Qualified RNAs were

574   finally quantified by Qubit3.0 with QubitTM RNA Broad Range Assay kit (Life Technologies).

575   2 µg total RNAs were used for stranded RNA sequencing library preparation using KC-

576   DigitalTM Stranded mRNA Library Prep Kit for Illumina® (Catalog NO. DR08502, Wuhan

577   Seqhealth Co., Ltd. China) following the manufacturer's instruction. The kit eliminates duplication

578   bias in PCR and sequencing steps, by using unique molecular identifier (UMI) of 8 random bases

579   to label the pre-amplified cDNA molecules. The library products corresponding to 200-500 bps were

580   enriched, quantified and finally sequenced on Hiseq X 10 sequencer (Illumina).

581   **RNA-Seq data analysis**

582   Raw sequencing data was first filtered by Trimmomatic (version 0.36) [50], low-quality reads

583   were discarded and the reads contaminated with adaptor sequences were trimmed. Clean Reads were

584   further treated with in-house scripts to eliminate duplication bias introduced in library preparation

585   and sequencing. Briefly, clean reads were first clustered according to the UMI sequences, in which

586   reads with the same UMI sequence were grouped into the same cluster, resulting in 65,536 clusters.

587   Reads in the same cluster were compared to each other by pairwise alignment, and then reads with

588   sequence identity over 95% were extracted to a new sub-cluster. After all sub-clusters were

589   generated, multiple sequence alignment was performed to get one consensus sequence for each sub-

590   cluster. After these steps, any errors and biases introduced by PCR amplification or sequencing were

591   eliminated [60, 61].

592   The de-duplicated consensus sequences were used for standard RNA-seq analysis. They were

593   mapped to the reference genome of *Sus scrofa* from GCF_000003025.6(ftp://ftp.ncbi.nlm.nih.gov

594   /genomes/all/GCF/000/003/025/GCF_000003025.6_Sscrofa11.1/GCF_000003025.6_Sscrofa11.1_

595   genomic.fna.gz) using STAR software (version 2.5.3a) [51] with default parameters. Reads mapped

596   to the exon regions of each gene were counted by feature Counts (Version 1.5.1) [62] and then

597   FPKMs were calculated. Genes differentially expressed between groups were identified using the

598   edgeR package (version 3.12.1) [63]. An FDR corrected p-value cutoff of 0.05 and Fold-change

599   cutoff of 2 were used to judge the statistical significance of gene expression differences. Gene

600   ontology (GO) analysis and Kyoto encyclopedia of genes and genomes (KEGG) enrichment

601   analysis for differentially expressed genes were both implemented by KOBAS software (version:

602   2.1.1) [58] with a corrected P-value cutoff of 0.05 to judge statistically significant enrichment. We

603   clustered and showed our data through hierarchical clustering of R package "pheatmap" with

604   parameter "cutree_rows" based on cluster results. Hierarchical clustering was performed with a

605   distance matrix between two parts. Distance measure used in clustering rows by calculating Pearson

606   correlation.

607   **ATAC-seq**

608   5×10^4 cells were treated with cell lysis buffer and nucleus was collected by density gradient

609   centrifuging for 30min at 120000g in 60% Percoll and 2.5M sucrose solution. Transposition and

610   high-throughput DNA sequencing library were carried out by TruePrep DNA Library Prep Kit V2

611   for Illumina kit (Catalog NO. TD501, Vazyme). The library was sequenced on Novaseq 6000

612   sequencer (Illumina).

613   Raw sequencing data was first filtered by Trimmomatic (version 0.36). Clean Reads were

614 further treated with FastUniq (version 1.1) to eliminate duplication. Reads were mapped to the

615 reference genome of Sus scrofa from GCF_000003025.6 using bowtie2 software (version 2.2.6)

616 with default parameters. The MACS2 software (version 2.1.1) was used for peak calling. The Homer

617 (version 4.10) was used for motifs analysis. We got OS binding sites from ChIP-seq of O/S in this

618 study. Then, genome signal tracks matrix of ATAC-seq nearby O/S sites were calculated by

619 computeMatrix of deeptools. Signal of ATAC-seq nearby O/S sites were visualized by ggplot2.

620 **IP-MS**

621 **Immunoprecipitation**

622 1×10^7 cells were digested by TrypLE™ Select and then transferred immediately to a 1.5 ml

623 tube on ice. The cell suspension was then centrifuged at 5000g for 3 min and supernatant was

624 discarded. The cell sediment was lysed by IP buffer (Beyotime, P0013) for 30 min on ice and then

625 centrifuged at 12000g for 10 min and supernatant was collected. The supernatant was incubated

626 with anti-FLAG magnetic beads (Sigma, F2426) overnight at 4°C, washed five times with IP buffer,

627 added to 5× SDS-PAGE loading buffer (GENSHARE G, JC-PE007), and heated at 100 °C for 5

628 min. Immunoprecipitates were subjected to SDS-PAGE and probed with indicated antibodies.

629 **Mass Spectrometry**

630 **In-gel Digestion**

631 For in-gel tryptic digestion, gel pieces were destained in 50 mM NH4HCO3 in 50% acetonitrile

632 (v/v) until clear. Gel pieces were dehydrated with 100 μl of 100% acetonitrile for 5 min, the liquid

633 was removed, and the gel pieces rehydrated in 10 mM dithiothreitol and incubated at 56 °C for 60

634 min. Gel pieces were again dehydrated in 100% acetonitrile, the liquid was removed and gel pieces

635 were rehydrated with 55 mM iodoacetamide. Samples were incubated at room temperature in the

636   dark for 45 min. Gel pieces were washed with 50 mM NH4HCO3 and dehydrated with 100%

637   acetonitrile. Gel pieces were rehydrated with 10 ng/μl trypsin resuspended in 50 mM NH4HCO3

638   on ice for 1 h. Excess liquid was removed and gel pieces were digested with trypsin at 37 °C

639   overnight. Peptides were extracted with 50% acetonitrile/5% formic acid, followed by 100%

640   acetonitrile. Peptides were dried to completion and resuspended in 2% acetonitrile/0.1% formic acid.

641   **LC-MS/MS Analysis**

642   The tryptic peptides were dissolved in 0.1% formic acid (solvent A), directly loaded onto a

643   home-made reversed-phase analytical column (15 cm length, 75 μm i.d.). The gradient was

644   comprised of an increase from 6% to 23% solvent B (0.1% formic acid in 98% acetonitrile) over 16

645   min, 23% to 35% in 8 min and climbing to 80% in 3 min then holding at 80% for the last 3 min, all

646   at a constant flow rate of 400 nl/min on an EASY-nLC 1000 UPLC system.

647   The peptides were subjected to NSI source followed by tandem mass spectrometry (MS/MS)

648   in Q ExactiveTM Plus (Thermo) coupled online to the UPLC. The electrospray voltage applied was

649   2.0 kV. The m/z scan range was 350 to 1800 for full scan, and intact peptides were detected in the

650   Orbitrap at a resolution of 70,000. Peptides were then selected for MS/MS using NCE setting as 28

651   and the fragments were detected in the Orbitrap at a resolution of 17,500. A data-dependent

652   procedure that alternated between one MS scan followed by 20 MS/MS scans with 15.0s dynamic

653   exclusion. Automatic gain control (AGC) was set at 5E4.

654   **Data Processing**

655   The resulting MS/MS data were processed using Proteome Discoverer 1.3. Tandem mass

656   spectra were searched against UniPort database. Trypsin/P (or other enzymes if any) was specified

657   as cleavage enzyme allowing up to 2 missing cleavages. The mass error was set to 10 ppm for

658   precursor ions and 0.02 Da for-fragment ions. Carbamidomethyl on Cys were specified as fixed

659   modification and oxidation on Met was specified as variable modification. Peptide confidence was

660   set at high, and peptide ion score was set > 20.

661   **Luciferase assay for enhancer activity**

662   All Luciferase vectors were derived from PGL3-basic vector (Promega, E1751) by Seamless

663   Cloning. Luciferase activity was measured using dual-luciferase detection kit (Beyotime, RG027)

664   as described in the manufacturer's procedure.

665   **Statistical analysis**

666   All the experiments had three independent biological replicates, except RNA-seq which were

667   replicated twice. The ChIP samples were collected from three independent biological experiments,

668   then mixed for ChIP-seq. Statistical significance was accepted at $P < 0.05$ and calculated using one-

669   way ANOVA in Excel 2016.

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673   **Competing interests**

674   All authors read and approved the final manuscript and declare no competing financial interests.

675   **Author contributions**

676   Zhenshuo Zhu and Jinlian Hua designed the study and wrote the manuscript. Zhenshuo Zhu,

677   Xiaolong Wu, Juqing Zhang, Shuai Yu, Qiaoyan Shen, Zhe Zhou, Qin Pan, Wei Yue, Dezhe Qin,

678   Ying Zhang, Wenxu Zhao, Rui Zhang performed the experiments. Zhenshuo Zhu, Qun Li, Sha Peng,

679   Na Li, Shiqiang Zhang, Anmin Lei, Yiliang Miao, Zhonghua Liu, Huayan Wang, Mingzhi Liao,

680   Jinlian Hua analyzed the data.

686   **Data availability**

687   ChIP-seq and RNA-Seq data have been uploaded to National Center for Biotechnology

688   Information Sequence Read Archive (SRA) database under the accession number PRJNA633419.

689   The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium

690   (http://proteomecentral.proteomexchange.org) via the iProX partner repository [64] with the dataset

691   identifier PXD016937.

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874

875 **Figure legends**

876 **Fig. 1. Feeder and exogenous genes affect the global H3K9 methylation in piPSCs. (A)**

877 Representative image of Alkaline Phosphatase (AP) stained colonies after 5 days of clonal growth

878 in the F- (feeder free) and FD- (feeder free and absence of Dox) treatments. The MOCK represents

879 piPSCs grown under normal condition as a blank control. The experiments were performed three

880 times. The scale bar represents 100 μm. **(B)** RT–qPCR analysis of the exogenous reprogramming

881 factors and endogenous pluripotent genes in the F- and FD- treatments. The relative expression

882 levels were normalized to β-actin. Data represent the mean ± s.d.; n = 3 independent experiments.

883 **(C)** Representative Western-Blot of OCT4, Sox2, LIN28A, KDM3A and KDM3B after 5 days of

884 culture in the indicated conditions. The quantitative analysis is shown by histogram. Data represent

885 the mean ± s.d.; n = 3 independent experiments. **(D)** Representative Western-Blot of H3K9me1/2/3

886 after 5 days of culture in the indicated conditions. The quantitative analysis is shown by histogram.

887 Data represent the mean ± s.d.; n = 3 independent experiments. **(E)** Immunofluorescence analysis

888 of H3K9me1/2/3 in the indicated conditions. Intensity values along the indicated path were obtained

889 using ImageJ and shown by line chart. The nuclei were DAPI stained. The scale bar represents 200

890 μm. The experiments were performed three times. **(F)** RT–qPCR analysis of *KDM3A/3B/3C* and

891 *KDM4A/4B/4C* in the F- and FD- treatments. The relative expression levels were normalized to β-

892 actin. Data represent the mean ± s.d.; n = 3 independent experiments.

893 **Fig. 2. Feeder and exogenous genes cooperatively facilitate pluripotent transcriptional**

894 **responses and inhibit the expression of differentiated related genes. (A)** The volcano plot

895 showing the number of up- or downregulated differentially expressed (DE) genes between F- and

896 FD- conditions on day 5 determined by RNA-Seq. n = 2 independent experiments. **(B)** Venn

897 diagrams showing the overlap of up- or downregulated differentially expressed (DE) transcripts

898 between MOCK, F- and FD- conditions. **(C)** Heat map showing changes in the expression of

899 pluripotency, fibroblast markers and epigenetic modification related genes in differentiating

900 treatments obtained from RNA-Seq analysis. **(D)** Four clusters of differential expression highlight

901 the key differences between MOCK, F- and FD- conditions. Gene ontology enrichment for each

902 cluster is presented on the right. Represented genes of each cluster are listed on the left.

903 **Fig. 3. H3K9me2/3 prevents interaction between pluripotent transcription factors and target**

904 **genes. (A)** Metaplots of signal densities for H3K9me2/3 in MOCK and FD- treatments at all

905 H3K9me2/3 bound sites. **(B)** Boxplots of global H3K9 methylation levels in MOCK and FD-

906 treatments. **(C)** The motifs of H3K9me2/3 were identified at MOCK and FD- conditions. Last

907 column: observed and expected motif frequencies (in parentheses). **(D)** Chromatin

908 immunoprecipitation-sequencing (ChIP-seq) analysis of H3K9me2/3 marks at pluripotency gene

909 loci in MOCK and FD- conditions using IGV. Gray bars represent the H3K9 methylation difference

910 area in MOCK and FD- conditions. The size of the peak represents the degree of enrichment. The

911 bold black line indicates the detection regions of ChIP-qPCR.

912 **Fig. 4. KDM3A and KDM3B synergize to maintain the pluripotency network in piPSCs. (A)**

913   Representative image of AP stained colonies after 5 days of clonal growth in the NC (Negative

914   Control), sh3A(shKDM3A), sh3B(shKDM3B) and sh3AB (shKDM3A/B) cell lines. The

915   experiments were performed three times. The scale bar represents 100 μm. **(B)** Representative

916   Western-Blot of KDM3A, KDM3B and H3K9me1/2/3 after 5 days of culture in the normal

917   condition. n = 3 independent experiments. **(C)** Venn diagrams showing the overlap of up- or

918   downregulated differentially expressed (DE) transcripts between sh3B and sh3AB cell lines on day

919   5 determined by RNA-Seq. **(D)** five clusters of differential expression highlight the key differences

920   between NC, sh3B and sh3AB cell lines. KEGG enrichment for each cluster is presented on the

921   right. **(E)** Representative image of AP stained colonies after 5 days of clonal growth in the T3F

922   (Empty vector), T3F-3A (overexpression of KDM3A) and T3F-3B (overexpression of KDM3A)

923   cell lines. The experiments were performed three times. The scale bar represents 100 μm. **(F)**

924   Representative Western-Blot of FLAG and H3K9me1/2/3 after 5 days of culture in the normal

925   condition. n = 3 independent experiments. **(G)** KEGG enrichment of up- or downregulated

926   differentially expressed (DE) genes in T3F-3A cell lines.

927   **Fig. 5. OCT4 and SOX2 was the core factors for the Maintenance of piPSCs. (A)** Representative

928   image of AP stained colonies after 5 days of clonal growth in the R-G (Rosa26-EF1α-GFP, Negative

929   Control), R-FO (Rosa26-EF1α-3×flag-OCT4) and R-OS(Rosa26-EF1α-OCT4-P2A-SOX2) cell

930   lines. The DOX+/- represents the presence/absence of Dox in medium. The scale bar represents 100

931   μm. **(B)** RT–qPCR analysis of the exogenous reprogramming factors and endogenous pluripotent

932   genes in the R-G, R-FO and R-OS treatments. The relative expression levels were normalized to β-

933   actin. Data represent the mean ± s.d.; n = 3 independent experiments. **(C)** Venn diagrams showing

934   the overlap of OCT4 and SOX2 peak and peak associated gene determined by ChIP-Seq. **(D)**

935   Chromatin immunoprecipitation-sequencing (ChIP-seq) analysis of OCT4 and SOX2 at

936   pluripotency gene loci in normal condition using IGV. Gray bars represent the OCT4 and SOX2

937   binding area. The size of the peak represents the degree of enrichment. **(E)** KEGG enrichment of

938   OCT4 and SOX2 peak associated gene.

939   **Fig. 6. The demethylation of H3K9me2 is essential for O/S to activate pluripotent networks.**

940   **(A)** Intersection of OCT4 or SOX2 binding sites and the specific H3K9me2/3 peaks of MOCK and

941   FD-. The number of peaks for each part is given in the Venn diagram. Blue for H3K9me2 peaks,

942   yellow for H3K9me3 peaks, green for oct4 peaks and red for SOX2 peaks. **(B)** Metaplots of signal

943   densities for H3K9me2 in MOCK and FD- treatments at all OCT4 and SOX2 bound sites. **(C)**

944   Boxplots of H3K9me2 levels in MOCK and FD- treatments at all OCT4 and SOX2 bound sites. **(D)**

945   KEGG enrichment of the intersection of OCT4 or SOX2 binding sites and the specific H3K9me2

946   peaks in FD- associated gene. The red marks represent major pluripotent regulatory pathways. **(E)**

947   Metaplots of signal densities for ATAC-seq in MOCK and FD- treatments at all OCT4 and SOX2

948   bound sites. **(F)** The chromatin immunoprecipitation-sequencing (ChIP-seq) analysis of OCT4 and

949   SOX2 at pluripotency gene loci and H3K9me2/3 marks at pluripotency gene loci in MOCK and

950   FD- conditions using IGV. The size of the peak represents the degree of enrichment.

951   **Fig. 7. Cooperative binding of core transcription factors and KDM3A/B to maintain**

952   **pluripotency. (A)** Immunoprecipitation of KDM3A and KDM3B were validated using western blot.

953   piPSCs expressing 3×FLAG-KDM3A or 3×FLAG-KDM3B were used to purify KDM3A or

954   KDM3B-associated proteins. The KDM3A or KDM3B-associated proteins were also detected and

955   identified by mass spectrometry. **(B)** Venn diagrams showing the overlap of KDM3A and KDM3B

956   associated proteins determined by mass spectrometry. The table lists some representative proteins

957   of each section. **(C)** Gene ontology enrichment of KDM3A or KDM3B-associated proteins. **(D)** The

958   correlation between KDM3A, KDM3B, SOX2 and OCT4 were validated by western blotting. **(E)**

959   Model for action of core transcription factors and KDM3A/B during pluripotency maintenance.

960

Figure 1

Figure 2

Figure 3

Figure 4

A

Phase / AP

R-G DOX+   R-FO DOX- P20   R-OS DOX- P20

B

Relative mRNA expression

R-G / R-FO / R-OS

ex-OSKM, total OCT4, total SOX2, endo-OCT4, endo-SOX2, endo-KLF4, endo-c-MYC, LIN28A, NANOG

C

Peak

OCT4 5360 | 5554 | 5436 SOX2

Peak associated gene

OCT4 1147 | 3376 | 1151 SOX2

D

7kb

119668kb 119670kb 119672kb

[1-150]

SOX2

15kb

83800kb 83810kb

[1-120]

LIN28A

10kb

23565kb 23570kb

[1-80]

OCT4

8kb

62932kb 62934kb 62936kb 62938kb

[1-120]

NANOG

OCT4   SOX2

E

KEGG of OCT4 peak associated gene

★ Signaling pathways regulating pluripotency of stem cells
Hippo signaling pathway
★ TGF-beta signaling pathway
Neuroactive ligand-receptor interaction
Basal cell carcinoma
★ Wnt signaling pathway
Renin secretion
Pathways in cancer
Arrhythmogenic right ventricular cardiomyopathy (ARVC)
Melanogenesis
Rap1 signaling pathway
Nicotine addiction
Axon guidance
Long-term depression
Hedgehog signaling pathway
Glycosaminoglycan biosynthesis - heparan sulfate / heparin
Propanoate metabolism
Cocaine addiction
cAMP signaling pathway
Retrograde endocannabinoid signaling

-log10 P-value

KEGG of SOX2 peak associated gene

★ Signaling pathways regulating pluripotency of stem cells
★ Wnt signaling pathway
Melanogenesis
Hippo signaling pathway
Basal cell carcinoma
Axon guidance
Renin secretion
Proteoglycans in cancer
Arrhythmogenic right ventricular cardiomyopathy (ARVC)
Adherens junction
Pathways in cancer
★ TGF-beta signaling pathway
Rap1 signaling pathway
Gap junction
Cocaine addiction
Retrograde endocannabinoid signaling
Dopaminergic synapse
Dilated cardiomyopathy
Hedgehog signaling pathway
Neuroactive ligand-receptor interaction
Focal adhesion
Circadian entrainment
GABAergic synapse
Glycosaminoglycan biosynthesis - heparan sulfate / heparin
Hypertrophic cardiomyopathy (HCM)
Transcriptional misregulation in cancer

-log10 P-value

Figure 6

A

the specific peaks of FD-

H3K9me2: 51577 | 1974 | 8843 (OCT4)
H3K9me2: 51205 | 2346 | 8524 (SOX2)

the specific peaks of MOCK

H3K9me2: 25214 | 329 | 10540 (OCT4)
H3K9me2: 25292 | 251 | 10704 (SOX2)

H3K9me3: 19308 | 172 | 10645 (OCT4)
H3K9me3: 19289 | 191 | 10679 (SOX2)

H3K9me3: 16448 | 531 | 10338 (OCT4)
H3K9me3: 16427 | 552 | 10403 (SOX2)

Legend:
- H3K9me2
- H3K9me3
- OCT4
- SOX2

B H3K9me2 — Signal vs OCT4 bound sites (-2kb to 2kb); H3K9me2 — Signal vs SOX2 bound sites (-2kb to 2kb). FD-, MOCK.

C H3K9me2 — Signal, MOCK vs FD- at OCT4; H3K9me2 — Signal, MOCK vs FD- at SOX2.

E ATAC — Signal vs OCT4 bound sites (-2kb to 2kb); Signal vs SOX2 bound sites (-2kb to 2kb). FD-, MOCK.

D

H3K9 me2 FD- OCT4

KEGG:
- Arrhythmogenic right ventricular cardiomyopathy (ARVC)
- Insulin secretion
- ★ Signaling pathways regulating pluripotency of stem cells
- Rap1 signaling pathway
- Axon guidance
- Nicotine addiction
- Bacterial invasion of epithelial cells
- MAPK signaling pathway
- Retrograde endocannabinoid signaling
- ★ Wnt signaling pathway
- Melanogenesis
- Primary bile acid biosynthesis
- Neuroactive ligand-receptor interaction
- Melanoma

-log10 P-value

H3K9 me2 FD- SOX2

- Axon guidance
- ★ Wnt signaling pathway
- ★ Signaling pathways regulating pluripotency of stem cells
- Insulin secretion
- Nicotine addiction
- Melanogenesis
- Basal cell carcinoma
- Glutamatergic synapse
- Amphetamine addiction
- African trypanosomiasis
- Arrhythmogenic right ventricular cardiomyopathy (ARVC)
- Retrograde endocannabinoid signaling
- Proteoglycans in cancer
- Circadian entrainment
- Hypertrophic cardiomyopathy (HCM)
- Renin secretion

-log10 P-value

F

OCT4; NANOG; LIN28A; FZD8

SOX2, OCT4, H3K9me2 (FD-, MOCK)

Figure 7

A

T3F T3F-3A T3F-3B

FLAG

192 kDa
150 kDa

B

All IP Proteins

312   74   355

T3F-3A   T3F-3B

Groups	Proteins
T3F-3A(only)	HMGB3 HNRNPR SOX2 YTHDF2
intersection of T3F-3A and T3F-3B	HMGN1 HMGN2 BAG2 SMARCA5 CBX5 HMGA1 KDM3A HMGB2 SNW1 KDM3B HMGA2 HMGB1
T3F-3B(only)	CBX3 OCT4

D

Input   IP

T3F T3F-3A T3F-3B   T3F T3F-3A T3F-3B

OCT4

SOX2

C

GO terms

T3F-3A(only)

positive regulation of establishment of -protein localization to telomere
positive regulation of telomere maintenance via telomerase
regulation of translation
RNA splicing
positive regulation of RNA polymerase II -transcriptional preinitiation complex assembly
mRNA splicing, via spliceosome
positive regulation of telomerase activity
ubiquitin-dependent protein catabolic process

0   2   4

intersection of T3F-3A and T3F-3B

nucleosome assembly
DNA topological change
sister chromatid segregation
histone H3-K9 demethylation
DNA unwinding involved in DNA replication
DNA ligation involved in DNA repair
positive regulation of transcription from -RNA polymerase II promoter
positive regulation of DNA binding

0   1   2   3   4

T3F-3B(only)

glycolytic process
mRNA splicing, via spliceosome
tricarboxylic acid cycle
positive regulation of Wnt protein secretion
positive regulation of canonical Wnt signaling pathway
cell-substrate junction assembly
mesodermal cell fate commitment
endodermal cell fate commitment

0   1   2   3

E

Sox2
Oct4
Differentiated state

Feeder
Pluripotent state

KDM3B
KDM3A

Sox2
Oct4

HP1α/HP1γ
H3K9me2
HMGB2
enhancer
promoter

KDM3B
KDM3A
Sox2 Oct4
Pol II