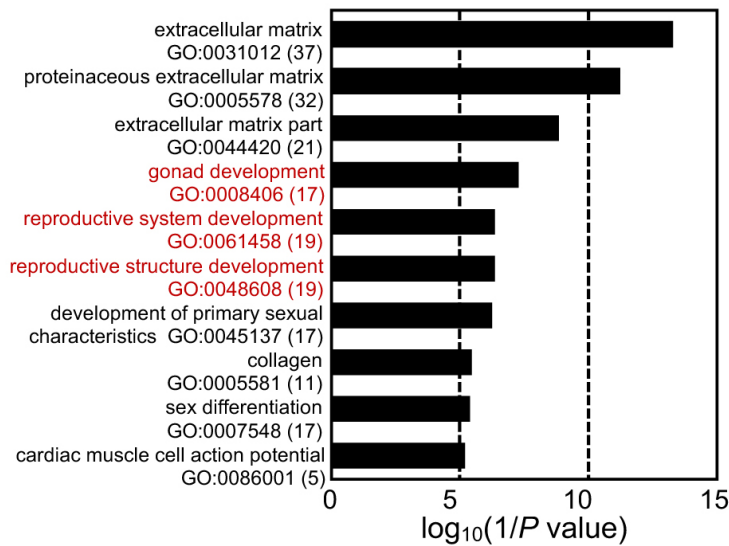


Treatment	days 0–5	days 5–11	days 11–17
Control	basal medium (10% FBS + alpha-MEM)	basal medium + vehicle	basal medium
ICI		basal medium + 10 $\mu$ M ICI	
MPP		basal medium + MPP	
SPS		10% SPS + alpha-MEM	
SPS + AMH		10% SPS + alpha-MEM + AMH	
AFP		basal medium + AFP	

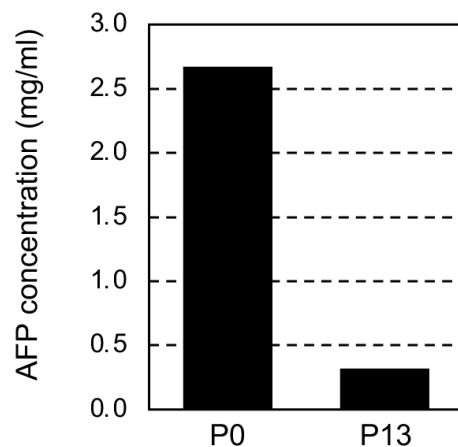
**Fig. S1. Schematic illustration of the experiments.**

(A) Procedure for ovarian culture. Embryonic day (E) 12.5 female mouse ovaries were collected and cultured on Transwell-COL membrane under various culture conditions. (B) Time course of experiments. The whole culture period was 17 days. To identify factors affecting follicle assembly, basal medium ( $\alpha$ -MEM supplemented with 10% FBS) was switched to each experimental medium on day 5. On day 11 of culture, the experimental medium was switched to basal medium. mRNA expression analysis and ChIP-qPCR analysis were conducted on days 7 and 9, respectively. Secondary follicles from the cultured ovaries were isolated on day 17, and secondary follicle formation was assessed.

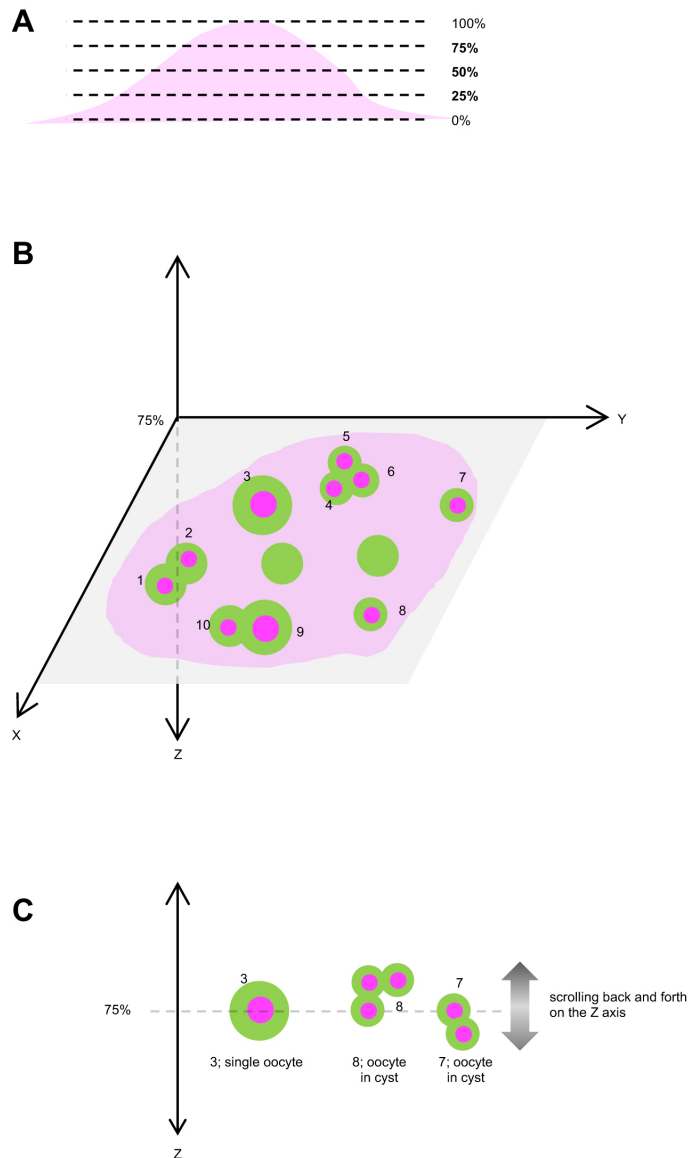


**Fig. S2. Enriched GO terms (top 10) in differentially expressed genes in ovaries cultured with basal medium compared with those from P0 mice.**

RNA-seq data were obtained from DRA010141. Significantly enriched GO terms consisted of gonad development, reproductive system, reproductive structure development, and others. The number in parentheses represents the number of genes annotated to each GO term. GO, gene ontology.



**Fig. S3. AFP levels in the serum from P0 and P13 mice.**



**Fig. S4. Method for counting the number of single oocytes or oocytes in cysts in the ovaries.**

Z-stack images were obtained at 1- $\mu$ m thickness. (A) The number of oocytes was counted on a plane with 25%, 50%, and 75% Z-positions of the full height, respectively. (B) DDX4-labeled cells (green) with visible nuclei (magenta) were defined as oocytes. An oocyte connected with any neighboring oocyte(s) was defined as an oocyte in a cyst, whereas an oocyte that was not connected with any neighboring oocyte(s) was defined as a single oocyte in the three-dimensional images. (C) Oocyte cysts were assessed by scrolling back and forth along the Z-axis until the end of the oocyte was observed. In the example shown in this figure, a total of 10 oocytes were counted on a plane with 75% of the height. Among them, there was single oocyte identified. The percentage of single oocytes in an ovary was calculated as (total number of single oocytes on planes with 25%, 50%, and 75%)/(total number of oocytes on planes with 25%, 50%, and 75%).

**Table S1. Biological components in FBS used in this study.**

Compound	Concentration
Estradiol	8.5 pg/ml
alpha-fetoprotein	130.5 ng/ml

**Table S2. The percentage of single oocytes per total oocytes in ovaries cultured in basal medium (control).**

Sample No.	day of culture	No. of oocytes			% of single oocytes
		single	in cyst	total	
1		25	2553	2578	1.0%
2		17	1617	1634	1.0%
3		16	1858	1874	0.9%
4	7	42	1272	1314	3.2%
5		68	792	860	7.9%
6		40	1503	1543	2.6%
	<b>mean</b>	<b>34.7</b>	<b>1599.2</b>	<b>1633.8</b>	<b>2.8%</b>
7		34	1660	1694	2.0%
8		62	1310	1372	4.5%
9		29	1152	1181	2.5%
10	8	104	1001	1105	9.4%
11		78	1166	1244	6.3%
12		74	266	340	21.8%
	<b>mean</b>	<b>63.5</b>	<b>1092.5</b>	<b>1156.0</b>	<b>7.7%</b>
13		172	985	1157	14.9%
14		17	1785	1802	0.9%
15		60	1508	1568	3.8%
16	9	166	830	996	16.7%
17		41	811	852	4.8%
18		42	1176	1218	3.4%
	<b>mean</b>	<b>83.0</b>	<b>1182.5</b>	<b>1265.5</b>	<b>7.4%</b>
19		143	866	1009	14.2%
20		90	1329	1419	6.3%
21		147	980	1127	13.0%
22	10	43	464	507	8.5%
23		80	485	565	14.2%
24		14	490	504	2.8%
	<b>mean</b>	<b>86.2</b>	<b>769.0</b>	<b>855.2</b>	<b>9.8%</b>
25		58	840	898	6.5%
26		66	418	484	13.6%
27		81	318	399	20.3%
28	11	87	322	409	21.3%
29		89	411	500	17.8%
30		69	719	788	8.8%
	<b>mean</b>	<b>75.0</b>	<b>504.7</b>	<b>579.7</b>	<b>14.7%</b>
31		134	449	583	23.0%
32		165	409	574	28.7%
33		164	303	467	35.1%
34	12	184	318	502	36.7%
35		130	249	379	34.3%
36		148	240	388	38.1%
	<b>mean</b>	<b>154.2</b>	<b>328.0</b>	<b>482.2</b>	<b>32.7%</b>
37		256	230	486	52.7%
38		223	329	552	40.4%
39		214	213	427	50.1%
40	13	169	328	497	34.0%
41		113	261	374	30.2%
42		127	33	160	79.4%
	<b>mean</b>	<b>183.7</b>	<b>232.3</b>	<b>416.0</b>	<b>47.8%</b>

**Table S3. The percentage of single oocytes per total oocytes in ovaries cultured in the ICI-containing medium.**

sample No.	day of culture	No. of oocytes			% of single oocytes	P-value (v.s. Control)
		single	incyst	total		
43		20	1792	1812	1.1%	
44		39	1765	1804	2.2%	
45		36	1921	1957	1.8%	
46	7	115	1508	1623	7.1%	
47		71	2092	2163	3.3%	
48		68	1887	1955	3.5%	
	<b>mean</b>	<b>58.2</b>	<b>1827.5</b>	<b>1885.7</b>	<b>3.2%</b>	0.782
49		80	809	889	9.0%	
50		27	1050	1077	2.5%	
51	8	11	1100	1111	1.0%	
52		72	900	972	7.4%	
53		131	812	943	13.9%	
54		135	1020	1155	11.7%	
	<b>mean</b>	<b>76.0</b>	<b>948.5</b>	<b>1024.5</b>	<b>7.6%</b>	0.967
55		65	985	1050	6.2%	
56		27	925	952	2.8%	
57	9	60	945	1005	6.0%	
58		93	608	701	13.3%	
59		73	1091	1164	6.3%	
	<b>mean</b>	<b>65.7</b>	<b>917.1</b>	<b>982.8</b>	<b>7.0%</b>	0.875
60		281	947	1228	22.9%	
61		238	850	1088	21.9%	
62	10	147	1051	1198	12.3%	
63		148	483	631	23.5%	
64		149	712	861	17.3%	
65		102	1150	1252	8.1%	
	<b>mean</b>	<b>177.5</b>	<b>865.5</b>	<b>1043.0</b>	<b>17.7%</b>	0.037
66		111	611	722	15.4%	
67		121	662	783	15.5%	
68	11	114	697	811	14.1%	
69		240	188	428	56.1%	
70		259	595	854	30.3%	
	<b>mean</b>	<b>170.4</b>	<b>603.1</b>	<b>773.5</b>	<b>24.8%</b>	0.273
71		165	361	526	31.4%	
72		192	609	801	24.0%	
73	12	191	494	685	27.9%	
74		283	266	549	51.5%	
75		350	204	554	63.2%	
76		180	197	377	47.7%	
	<b>mean</b>	<b>226.8</b>	<b>355.2</b>	<b>582.0</b>	<b>40.9%</b>	0.322
77		200	80	280	71.4%	
78		153	108	261	58.6%	
79	13	314	90	404	77.7%	
80		346	80	426	81.2%	
81		273	38	311	87.8%	
82		288	71	359	80.2%	
	<b>mean</b>	<b>262.3</b>	<b>77.8</b>	<b>340.2</b>	<b>76.2%</b>	0.025

**Table. S4.** Genes included in three GO terms “gonad development”, “reproductive system development”, and “reproductive structure development” using annotations for the differentially expressed gene set in *in vitro*-derived ovaries.

Symbol	Fold Change	log <sub>2</sub> RPKM	
		<i>in vitro</i> (Control)	<i>in vivo</i>
<i>Amh</i>	11.57	11.4	7.9
<i>Foxa1</i>	6.19	5.3	2.7
<i>Nos2</i>	5.21	9.9	7.5
<i>Frzb</i>	4.23	6.3	4.2
<i>Inha</i>	3.17	14.4	12.7
<i>Sfrp1</i>	-3.04	12.2	13.8
<i>Kdr</i>	-3.14	10.7	12.3
<i>Gata3</i>	-3.34	6.8	8.5
<i>Lfng</i>	-3.64	6.9	8.7
<i>Ccnd1</i>	-4.28	9.3	11.4
<i>Srd5a2</i>	-4.91	4.5	6.8
<i>Rbp4</i>	-4.54	5.9	8.1
<i>Gata1</i>	-4.01	6.4	8.4
<i>Sfrp2</i>	-4.21	7.4	9.5
<i>Cga</i>	-4.67	4.0	6.2
<i>Osr1</i>	-6.57	6.2	8.9
<i>Myocd</i>	-6.95	4.0	6.8
<i>Sfrp5</i>	-22.09	1.9	6.3
<i>Wnt10b</i>	-25.56	1.7	6.3