



Title	Role of the Atg9a gene in intrauterine growth and survival of fetal mice
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## **Role of the *Atg9a* gene in intrauterine growth and survival of fetal mice**

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**Short title: *Atg9a* gene in murine fetal growth and survival**

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1 **Abstract**

2 Autophagy is activated by environment unfavorable for survival and requires Atg9a protein.  
3 Mice heterozygous for  $p57^{Kip2}$ , devoid of the imprinted paternal allele ( $p57^{Kip2+/-}$ ), are known  
4 to develop hypertension during pregnancy. To determine whether fetal *Atg9a* is involved in  
5 the intrauterine survival and growth of fetal mice, this study was performed on *Atg9a*  
6 heterozygous ( $Atg9a^{+/-}$ ) pregnant mice with and without  $p57^{Kip2+/-}$ . The pregnant mice  
7 heterozygous for both knockout alleles of *Atg9a* and  $p57^{Kip2}$  ( $Atg9a^{+/-}/p57^{Kip2+/-}$ ), but not  
8 those heterozygous for *Atg9a* alone, developed hypertension during pregnancy. Placental  
9 expression of *Atg9a* mRNA was significantly decreased in the  $Atg9a^{-/-}$  mice compared to  
10  $Atg9a^{+/-}$  or  $Atg9a^{+/+}$  mice. The  $Atg9a^{-/-}$  fetal mice exhibited significantly retarded growth  
11 and were more likely to die *in utero* compared to  $Atg9a^{+/+}$  and  $Atg9a^{+/-}$  fetal mice. Growth  
12 retardation was observed in the presence of maternal hypertension in  $Atg9a^{-/-}$  fetal mice.  
13 These results suggest that  $Atg9a^{-/-}$  fetal mice from pregnant dams heterozygous for both  
14 knockout alleles of *Atg9a* and  $p57^{Kip2}$  are more susceptible to hypertensive stress than fetuses  
15 with intact autophagic machinery.

16 **Key words:** Autophagy, Fetal growth restriction, Hypoxia, Intrauterine fetal death,  
17 Hypertension

18

19

## 20 **1. Introduction**

21 Autophagy is a bulk degradation system, which controls the clearance and reuse of  
22 intracellular constituents, and is important for the maintenance of an amino acid pool essential  
23 for survival [1-3]. Autophagy can be activated by nutritional deprivation and intracellular  
24 stress, such as hypoxia. Genetic studies in yeast have identified many autophagy-related (*Atg*)  
25 genes that are required for autophagosome formation essential for autophagy, such as *Atg3*,  
26 *Atg5*, *Atg7*, *Atg9a*, and *Atg16L1* [4]. Most of the *Atg* genes are conserved in higher eukaryotes.  
27 In yeast, autophagy-defective mutants were unable to survive under conditions of nitrogen  
28 starvation [5]. Similarly, the *Atg3*-, *Atg5*-, *Atg7*-, *Atg9a*-, and *Atg16L1*-knockout mice  
29 (genotypes of each *Atg*<sup>-/-</sup>) did not survive, and usually died within **one** day after birth [6-9].  
30 Thus, autophagy was found to be crucial for survival in the neonatal period [6-10], while mice  
31 heterozygous for the knockout allele of the *Atg9a* gene (*Atg9a*<sup>+/-</sup>) grew normally [9]. Even in  
32 the embryonic stages, a shortage of nutrient and oxygen supply from the placenta occurs in  
33 human fetuses in certain clinical conditions, such as hypertensive pregnancies and maternal  
34 malnutrition, leading to fetal growth restriction (FGR) and intrauterine fetal death (IUFD)  
35 [11-13]. Therefore, similar adverse events may occur in response to unfavorable intrauterine  
36 environments in fetal mice devoid of autophagic machinery. Indeed, FGR and IUFD have  
37 been reported in fetal mice deficient in *beclin 1*, which is another gene essential for autophagy  
38 [14].

39 The *Atg9b* gene - expressed in the placenta - functionally complements *Atg9a* [19].

40 Therefore, *Atg9b* protein expression may be higher in the placenta of *Atg9a*<sup>-/-</sup> fetal mice.

41 When the expression of Atg9b protein is increased, autophagic activity may be monitored by  
42 quantifying the expression of LC3-II (truncated protein of LC3-I) in the placenta of *Atg9a*<sup>-/-</sup>  
43 fetal mice.

44 The p57<sup>Kip2</sup>, a potent inhibitor of the cyclin-cyclin dependent kinase (CDK)  
45 complex, is a paternally imprinted gene located within a cluster of imprinted genes in humans  
46 (chromosome 11p15.5) and mice (distal chromosome 7) [15,16]. As p57<sup>Kip2</sup><sup>+/-</sup> (heterozygous,  
47 lacking the paternal allele) pregnant mice develop abnormalities similar to preeclampsia,  
48 including hypertension and proteinuria when carrying a fetus without p57<sup>Kip2</sup> expression [17],  
49 these mice can be used as an animal model for hypertensive disorders in pregnancy. The  
50 present study was performed to determine whether fetal *Atg9a* is involved in survival and *in*  
51 *utero* growth in pregnant mice heterozygous for *Atg9a* (*Atg9a*<sup>+/-</sup>) with and without p57<sup>Kip2</sup><sup>+/-</sup>.  
52 In addition, expressions of *Atg9b* mRNA, Atg9b protein and LC3-II protein were compared  
53 between placentas with *Atg9a*<sup>-/-</sup> and *Atg9a*<sup>+/+</sup>.

54

## 55 **2. Materials and Methods**

### 56 **2.1. Mice**

57 Mice housed in a temperature- and humidity-controlled room were maintained under a  
58 12:12-hour light-dark schedule with free access to food and water. All procedures were  
59 performed in accordance with the Local Ethical Commission for Animal Experiments at  
60 Hokkaido University (Japan). Female and male mice (129Sv × C57BL/6) heterozygous for  
61 *Atg9a* (*Atg9a*<sup>+/-</sup>) [9] were provided by a co-author (TS). Male mice heterozygous for p57<sup>Kip2</sup>,

62 lacking the imprinted paternal allele ( $p57^{Kip2+/-}$ ) [18], were provided by co-authors (KN and  
63 KN). In the current study, female mice of  $Atg9a^{+/-}$  alone were mated with male mice of  
64  $p57^{Kip2+/-}$  to generate female mice heterozygous for knockout alleles of  $Atg9a$  and  $p57^{Kip2}$   
65 ( $Atg9a^{+/-}/p57^{Kip2+/-}$ ). The genotype of generated  $Atg9a^{+/-}/p57^{Kip2+/-}$  female was determined  
66 by PCR of tail DNA. Female mice of  $Atg9a^{+/-}$  and  $Atg9a^{+/-}/p57^{Kip2+/-}$  were mated with male  
67 mice of  $Atg9a^{+/-}$  to generate four embryo groups (groups A1, A2, B1 and B2, Tab. 1) divided  
68 according to differences in mother and fetal  $Atg9a$  genotypes. Pregnancy stage was expressed  
69 as the number of days *post coitum* (dpc). Thirteen pregnant mice with  $Atg9a^{+/-}$  and 13  
70 pregnant mice with  $Atg9a^{+/-}/p57^{Kip2+/-}$  were used in the study. Pregnant mice of both groups  
71 were sacrificed on 13.5, 15.5, and 17.5 dpc for determination of the number of fetuses,  
72 measurement of live fetal body weight and determination of fetal  $Atg9a$  genotypes by PCR of  
73 tail DNA (Fig. 1).

74

## 75 **2.2. Measurement of blood pressure in pregnant mice**

76 Blood pressure was measured with a tail-cuff using a CODA™ monitor (Kent Scientific,  
77 Torrington, CT, USA), 5 – 10 times at 30 s intervals, 15 min after the behavior and heart rate  
78 of the pregnant mice were stabilized in the morning of 5.5, 9.5, 13.5, 15.5, and 17.5 dpc.  
79 Blood pressure values are reported as the means of at least three measurements varying by 5%  
80 obtained in one session.

81

82

83 **2.3. Measurement of fetal body weight**

84 The body weights of pregnant mice and alive fetal mice were measured using Electronic  
85 balance FA-200 (A&D, Tokyo, Japan). Pregnant mice were anesthetized deeply with ether  
86 and dissected in the lower abdomen. The multilocular uterus containing a fetus in each  
87 locus was incised and fetuses with their placentas were separated from the uterus. Some  
88 fetuses, based on their color and size, appeared to be dead at sacrifice. Fetuses that were less  
89 pinkish and smaller than others were judged as dead (Fig. 1; upper panel). These fetuses were  
90 subjected to *Atg9a* genotype determination, but their body weights were not measured.

91

92 **2.4. DNA extraction, PCR amplification and electrophoresis**

93 The *Atg9a* genotypes of the fetuses (Fig. 1; lower panel) and placentas were analyzed using  
94 PCR of fetal tail DNA and placental DNA. The DNA was extracted using a DNA extraction  
95 kit (DNeasy<sup>®</sup> Blood & Tissue Kit; Quiagen, Valencia, CA, USA). PCR was performed in a  
96 final volume of 15  $\mu$ L consisting of 1  $\mu$ L of genomic DNA, 1.5  $\mu$ L of 10 $\times$  Taq buffer, 1.5  $\mu$ L  
97 of dNTPs (2.5 mM each), 0.6  $\mu$ L each of forward and reverse primers, 9.7  $\mu$ L of nuclease-free  
98 water, and 0.1  $\mu$ L of Taq DNA polymerase, under the following conditions: 3 min at 95 $^{\circ}$ C, 35  
99 cycles at 95 $^{\circ}$ C for 20 s and 68 $^{\circ}$ C for 1 min. The primers for the wild-type allele were:  
100 5'-CCAGAGCCTGTCATGGTACTGGGAACC-3' (primer I) and  
101 5'-CCTCAAGGAGCAGGTGCAGCGAGATGG-3' (primer II). For the knockout allele,  
102 primer I and 5'-CTAAAGCGCATGCTCCAGACTGCCTTG-3' (primer III) were used. The  
103 amplified products were checked by 2.0% agarose gel electrophoresis with ethidium bromide.

104 The DNA bands were visualized with a UV transilluminator to determine the genotypes (Fig.  
105 1; lower panel).

106

## 107 **2.5. Detection of *Atg9b* mRNA expression**

108 The RNA samples were extracted from 20 mg of frozen placental sample using an RNeasy<sup>®</sup>  
109 Plus Mini Kit (Qiagen) after smashing with MicroSmash<sup>™</sup> (Tomy Seiko, Tokyo, Japan), and  
110 the sample RNA concentrations were measured by NanoDrop<sup>®</sup> (ND-1000; Thermo Scientific  
111 Japan, Kanagawa, Japan). After dilution of each sample with RNase-free water and adjusted  
112 to a uniform density (3 µg/µL), reverse-transcription polymerase chain reaction (RT-PCR)  
113 was performed with Super Script<sup>™</sup> III (Life Technologies, Carlsbad, CA, USA). Quantitative  
114 Real-time PCR was used to measure the expression of *Atg9b* mRNA using SYBR<sup>®</sup> Premix Ex  
115 Taq<sup>™</sup> (Takara, Shiga, Japan) and an ABI 7300 Real-Time PCR System (PE Applied  
116 Biosystems, Foster City, CA, USA). *GAPDH* was used as an internal standard (housekeeping  
117 gene). Real-time PCR was performed in a final volume of 15 µL consisting of 0.6 µL of  
118 sample cDNA, 7.5 µL of 2x Ex Taq buffer, 0.3 µL of 50x ROX Reference Dye, 0.6 µL each of  
119 forward and reverse primers, 5.4 µL of nuclease-free water under the following conditions:  
120 for *Atg9a*, 2 min at 50°C, 10 s at 95°C, 40 cycles at 95°C for 15 s and 59°C for 1 min; and for  
121 *Atg9b* and *GAPDH*, 2 min at 50°C, 10 s at 95°C, 40 cycles at 95°C for 15 s and 62°C for 1  
122 min. The sequences of primers were 5'-GAGCAGGTGCAGCGAGATG-3' (forward) and  
123 5'-GCAGGTCTCTGGACAGTGAGG-3' (reverse) for *Atg9a*,



124 5'-GCATCACATCCAGAACCTGGA-3' (forward) and  
125 5'-CCGCTGATGATAGCTGTAGATCTTG-3' (reverse) for *Atg9b*, and  
126 5'-GGCATTGCTCTCAATGACAA-3' (forward) and 5'-TGTGAGGGAGATGCTCAGTG-3'  
127 (reverse) for *GAPDH*.

128

## 129 **2.6. Immunodetection of Atg9b and LC3-II**

130 Western blotting was performed to analyze the expression levels of Atg9b protein and  
131 microtubule-associated protein light chain 3 (LC3)-II (truncated protein of LC3-I). The  
132 sample proteins were extracted from 20 mg of frozen placental tissues after mixing with 100  
133  $\mu$ L of 2% Triton X-100 lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 2% Triton X-100, 2  
134 mM PMSF, and complete protease inhibitor cocktail), and then sonicated (Handy Sonic<sup>®</sup>;  
135 Tomy Seiko, Tokyo, Japan), extracted, and the protein concentration was measured by BCA  
136 Protein Assay (Thermo Fisher Scientific, Rockford, IL, USA).

137 Aliquots of 12  $\mu$ g of protein samples were diluted with 1:2 volumes of Laemmli  
138 sample buffer (10% SDS, 1 M DTT, 0.5 M Tris-HCl, pH 6.8, 0.001% bromophenol blue, and  
139 5% glycerol), boiled at 95°C for 5 min, separated by SDS-polyacrylamide gel electrophoresis  
140 (SDS-PAGE) using 8% gels for Atg9b and 13% for LC3-II, electrotransferred onto PDVF  
141 membranes (Amersham Biosciences, Little Chalfont, UK), and blocked with 1.5% skim milk  
142 in TBS-T (1 $\times$  TBS with 0.08% Tween) at room temperature for 60 min. The membranes were  
143 incubated with the following primary antibodies at 4°C overnight: Atg9b (anti-ATG9B

144 antibody, 1:1000; NB110-74833; NOVUS, Littleton, CO, USA), LC3-II (anti-LC3, 1:800;  
145 MBL, Nagoya, Japan). GAPDH was also detected as a loading control in this study using an  
146 anti-GAPDH mouse IgG antibody at a dilution of  $1:4 \times 10^6$ .

147           After washing with TBS-T, the membranes were incubated with secondary antibody  
148 at room temperature for 60 min. ECL™ anti-rabbit IgG, horseradish peroxidase-conjugated  
149 whole antibody (1:10000; GE Healthcare, Little Chalfont, UK) was used for Atg9b and  
150 LC3-II, and ECL™ anti-mouse IgG, horseradish peroxidase-conjugated whole antibody  
151 (1:10000; GE Healthcare) for GAPDH. The protein bands were visualized with ECL solution  
152 (Immobilon™ Western, Chemiluminescent HRP Substrate; Millipore, Little Chalfont, UK)  
153 and quantified with ImageQuant LAS4000 (GE Healthcare Japan, Tokyo, Japan).

154

## 155 **2.7. Statistical analysis**

156 Data are presented as mean±standard deviation. Statistical analysis was performed using the  
157 JMP® Pro 11 statistical software package (SAS, Cary, NC, USA). Differences between two  
158 groups were tested using the Mann-Whitney U test (Figs. 2, 5, and 6). Differences between  
159 more groups were tested using the Steel-Dwass test after confirmation of significant  
160 difference with Kruskal-Wallis test (Figs. 3, 4, and 7). Differences between frequencies were  
161 tested using Fisher's exact test with Bonferroni correction (Table 2). In all analyses,  $P < 0.05$   
162 was taken to indicate statistical significance.

163

## 164 **3. Results**

165 *Atg9a*<sup>+/-</sup> mice (used to obtain A1 and A2 groups, Tab. 1) did not exhibit significant changes in  
166 systolic blood pressure (SBP) during pregnancy (Fig. 2), while *Atg9a*<sup>+/-</sup>/*p57*<sup>Kip2 +/-</sup> mice (used  
167 to obtain B1 and B2 groups, Tab. 1) exhibited a significant increase in SBP at 9.5 dpc (113 ±  
168 11.6 mm Hg) and thereafter (116 ± 12.6, 125 ± 16.2, and 121 ± 12.8 mm Hg for 13.5, 15.5,  
169 and 17.5 dpc, respectively) compared to SBP baseline (102 ± 12.0 mm Hg) determined before  
170 pregnancy. The SBP differed significantly between the two groups (A vs. B) at 9.5 dpc (113 ±  
171 11.6 vs. 99 ± 9.5 mm Hg), 15.5 dpc (125 ± 16.2 vs. 95 ± 10.5 mm Hg), and 17.5 dpc (121 ±  
172 12.8 vs. 102 ± 12.1 mm Hg). Pre-pregnancy body weight did not differ significantly between  
173 female mice of *Atg9a*<sup>+/-</sup> (normotensive pregnancy [A] group; n=13; Tab. 1) and *Atg9a*  
174 <sup>+/-</sup>/*p57*<sup>Kip2 +/-</sup> (hypertensive pregnancy group [B]; n=13) groups (22.8 ± 2.7 vs. 21.7 ± 2.5 g,  
175 respectively).

176 The expression of *Atg9a* mRNA was significantly reduced in *Atg9a*<sup>-/-</sup> placentas  
177 compared to *Atg9a*<sup>+/-</sup> and *Atg9a*<sup>+/+</sup> placentas (Fig. 3). Then, placental expression of *Atg9b*  
178 mRNA, Atg9b protein and LC-3-II protein (Fig. 4) were analyzed in relation to placental  
179 *Atg9a* genotype (*Atg9a*<sup>+/+</sup> vs. *Atg9a*<sup>-/-</sup>) and the presence or absence of gene *p57*<sup>Kip2 +/-</sup>  
180 (indicative of hypertension). The *Atg9b* mRNA expression in *Atg9a*<sup>-/-</sup> placentas was  
181 comparable to that of *Atg9a*<sup>+/+</sup> placentas. However, in the presence of hypertension, the *Atg9b*  
182 mRNA level was significantly higher in *Atg9a*<sup>+/+</sup> placentas than that in *Atg9a*<sup>-/-</sup> placentas. The  
183 expression of Atg9b and LC3-II proteins was significantly higher in *Atg9a*<sup>-/-</sup> placentas than in  
184 *Atg9a*<sup>+/+</sup> placentas in the presence of hypertension (Fig. 4).

185 The *Atg9a*<sup>+/+</sup>, *Atg9a*<sup>+/-</sup>, and *Atg9a*<sup>-/-</sup> fetal mice accounted for 30% (69/231), 53%

186 (123/231), and 17% (39/231) of the total number of fetuses, respectively, with the number of  
187 *Atg9a*<sup>-/-</sup> fetal mice lower than the expected Mendelian frequency (Tab. 2). The fraction of  
188 dead fetal mice appeared to be greater with decreasing number of fetal *Atg9a* alleles: 1.4%  
189 (1/69), 14% (17/123), and 26% (10/39) for *Atg9a*<sup>+/+</sup>, *Atg9a*<sup>+/-</sup>, and *Atg9a*<sup>-/-</sup> fetuses,  
190 respectively. The prevalence rate of IUFD was significantly higher in *Atg9a*<sup>-/-</sup> fetal mice than  
191 in *Atg9a*<sup>+/+</sup> fetal mice (26% [10/39] vs. 1.4% [1/69]) and in fetal mice with at least one *Atg9a*  
192 allele (*Atg9a*<sup>+/+</sup> + *Atg9a*<sup>+/-</sup>) (26% [10/39] vs. 9.4% [18/192]).

193           The fetal body weight was significantly lower in fetuses carried by hypertensive  
194 dams than those of normotensive dams (B1 and B2 < A1 and A2) at all stages of pregnancy  
195 (Fig. 5). The fetal body weight was also significantly lower in *Atg9a*<sup>-/-</sup> fetal mice (groups A2+  
196 B2) than in *Atg9a*<sup>+/+</sup> and *Atg9a*<sup>+/-</sup> fetal mice (groups A1 +B1) on 13.5 and 17.5 dpc (Fig. 6).  
197 Finally, the body weight of fetuses carried by hypertensive dams was usually lower than that  
198 fetuses of normotensive dams (Fig. 7). At any stage of pregnancy, fetal body weight did not  
199 differ between the groups carried by normotensive dams (A1 vs. A2). In contrast, body weight  
200 of fetuses carried by hypertensive dams was significantly lower in group B2 than in group B1  
201 on 15.5 and 17.5 dpc (Fig. 7).

202

#### 203 **4. Discussion**

204 The current study demonstrated that the growth of *Atg9a*<sup>-/-</sup> fetal mice was retarded compared  
205 with *Atg9a*<sup>+/+</sup> and *Atg9a*<sup>+/-</sup> fetal mice. This confirmed the results of previous studies in which  
206 autophagy-defective fetal mice devoid of *Atg* genes other than *Atg9a* were born with lower

207 birth weight than those with normal (in relation to autophagy) phenotype [6,7,10]. In addition,  
208 it was suggested that *Atg9a*-knockout fetal mice are characterized by an increased likelihood  
209 of dying *in utero*, as it was reported for *beclin 1*-knockout fetal mice [14].

210           Since in mice autophagy remains at a low level throughout the embryonic period  
211 and massive autophagy occurs transiently soon after birth in normal neonatal mice, autophagy  
212 was suggested to play minor role in fetal mouse development [6]. Therefore, fetal growth  
213 restriction of *Atg9a*-knockout fetal mice has not been emphasized in previous reports [6,7,10].  
214 The body weights of *Atg3*<sup>-/-</sup>, *Atg5*<sup>-/-</sup>, and *Atg7*<sup>-/-</sup> neonatal mice were significantly lower  
215 than those of wild-type and heterozygous mice [6,7,10]. As all *Atg3*<sup>-/-</sup>, *Atg5*<sup>-/-</sup>, *Atg7*<sup>-/-</sup>, and  
216 *Atg9a*<sup>-/-</sup> mice are indeed defective in autophagy [6,7,9,10], these results suggest that the lack  
217 of functional autophagic machinery in fetuses resulted in a lower birth weight. Thus, the fetal  
218 autophagic machinery has a protective function against FGR. This notion was further  
219 supported by our findings that FGR was observed in *Atg9a*<sup>-/-</sup> fetal mice carried by  
220 hypertensive dams.

221           Although in the current study, the fetus size in normotensive dams did not differ  
222 significantly among different fetal *Atg9a* genotype, the Atg9b and LC3-II proteins were  
223 present in the placentas of *Atg9a*<sup>-/-</sup> fetal mice. The placental autophagy may have prevented  
224 FGR in *Atg9a*<sup>-/-</sup> fetal mice carried by normotensive dams. The deleterious effect of  
225 hypertension in addition to the lack of fetal autophagy, in turn, may have outweighed the  
226 protective effect of placental autophagy on FGR in fetuses carried by hypertensive dams.

227           Maternal hypertension is a well-known detrimental factor for pregnancy outcome,

228 causing FGR and IUFD in humans [12]. Placental insufficiency due to poor blood supply *via*  
229 the uterine arteries is the most common cause of fetal starvation, and the increased impedance  
230 to flow within uterine arteries occurs frequently in hypertensive disorders in pregnancy  
231 [11,13]. In such an unfavorable intrauterine environment, fetal autophagy may be activated,  
232 and placental autophagy was indeed increased in women with hypertensive disorders and/or  
233 FGR [20-24]. In addition, the expression of LC3-II protein in the human placenta tended to  
234 linearly increase with a decreasing normalized infant birth weight [24]. The findings in  
235 humans and mice suggest that dysfunction of the fetal autophagic machinery is causally  
236 associated with FGR, and an unfavorable intrauterine environment such as maternal  
237 hypertension outweighs the protective role of fetal autophagy in FGR, leading to FGR even  
238 after the activation of autophagy.

239           The *Atg9a*<sup>-/-</sup> fetal mice had a high likelihood of *in utero* death in the present study.  
240 In addition, the frequency of *Atg9a*<sup>-/-</sup> fetal mice was somewhat lower than the expected  
241 Mendelian frequency, even after including dead fetuses. Similar findings, but with a more  
242 severe phenotype, were reported in *beclin 1*-knockout fetal mice. No homozygous mutant  
243 offspring (*beclin 1*<sup>-/-</sup>) were born to dams heterozygous for *beclin 1* because all *beclin 1*<sup>-/-</sup>  
244 fetal mice had severely retarded growth and died in the early embryonic stages [14]. Such  
245 early-dead fetuses become conceptus traces and are not recognized as fetuses [14]. These  
246 early embryonic deaths may help to explain why less *Atg9a*<sup>-/-</sup> fetal mice than expected were  
247 observed in the current study. In addition, strict classification of fetal mice as dead or alive  
248 was not easy and was based on color and size. Therefore, some fetal mice classified as dead

249 might have been alive.

250           In conclusion, in the current study we have focused on the examination of the  
251 effects of *Atg9a* deficiency in fetal mice on fetal growth and survival. We demonstrated that  
252 *Atg9a*-knockout (*Atg9a*<sup>-/-</sup>) fetal mice were likely to show growth restriction, especially in the  
253 presence of maternal hypertension. This suggests that dysfunction of fetal autophagic  
254 machinery causes lower fetal body weight. In addition, the results of the study indicated that  
255 fetal mice devoid of *Atg9a* had an increased likelihood of dying *in utero*, similar to the  
256 observations reported previously for autophagy-defective *beclin 1*<sup>-/-</sup> mice [14].

257

#### 258 **Conflict of Interest**

259 None declared.

260

261

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331

332

333 **Figure captions**

334 Fig. 1. Exemplary images of fetuses at 17.5 day post coitum. A/ *Atg9a*<sup>-/-</sup> fetuses judged as  
335 dead, B/ *Atg9a*<sup>-/-</sup> fetuses with growth retardation, C/ *Atg9a*<sup>+/+</sup> fetuses with normal growth.

336 The *Atg9a* genotypes of the fetuses were determined by PCR of mouse tail DNA according to  
337 the presence or absence of wild type (WT) and knockout (KO) alleles.

338

339 Fig. 2. Systolic blood pressure (mean ± SD) in *Atg9a*<sup>+/-</sup>/*p57<sup>Kip</sup>*<sup>+/-</sup> and *Atg9a*<sup>+/-</sup> pregnant mice.

340 The numbers of pregnant mice are indicated in parentheses. \**P* < 0.05 between the two  
341 examined groups of mice; †*P* < 0.05 between a particular day post coitum (dpc) and a  
342 pre-pregnancy day (SBP baseline).

343

344 Fig. 3. Expression of *Atg9a* mRNA in the placentas of *Atg9a*<sup>+/+</sup>, *Atg9a*<sup>+/-</sup> and *Atg9a*<sup>-/-</sup> mice.

345 The mice were sacrificed at 15.5 day post coitum; the numbers of pregnant mice are indicated  
346 in parentheses. Different superscripts mean significant differences (*P*<0.05).

347

348 Fig. 4. Expression of *Atg9b* mRNA, Atg9b protein and LC3-II protein in placentas with

349 *Atg9a*<sup>+/+</sup> and *Atg9a*<sup>-/-</sup> genotypes. The pregnant mice were sacrificed at 17.5 day post coitum.

350 Please refer to Table 1 for group (A1, A2, B1, and B2) definition. Different superscripts mean  
351 significant differences (*P*<0.05).

352

353 Fig. 5. Fetal body weight in the A1+A2 (normotensive) and B1+B2 (hypertensive) mice on  
354 13.5, 15.5, and 17.5 days post coitum (dpc). Numbers of fetuses are presented in parentheses.  
355 Upper and lower ends of the box and horizontal line inside the box indicate 75th percentile,  
356 25th percentile, and median values, respectively. \* $P < 0.05$  between the two compared groups.  
357 Please refer to Table 1 for group (A1, A2, B1, and B2) definition.

358

359 Fig. 6. Fetal body weight in the A1+B1 and A2+B2 mice on 13.5, 15.5, and 17.5 days post  
360 coitum (dpc). Numbers of fetuses are presented in parentheses. Upper and lower ends of the  
361 box and horizontal line inside the box indicate 75th percentile, 25th percentile, and median  
362 values, respectively. \* $P < 0.05$  between the two compared groups. Please refer to Table 1 for  
363 group (A1, A2, B1, and B2) definition.

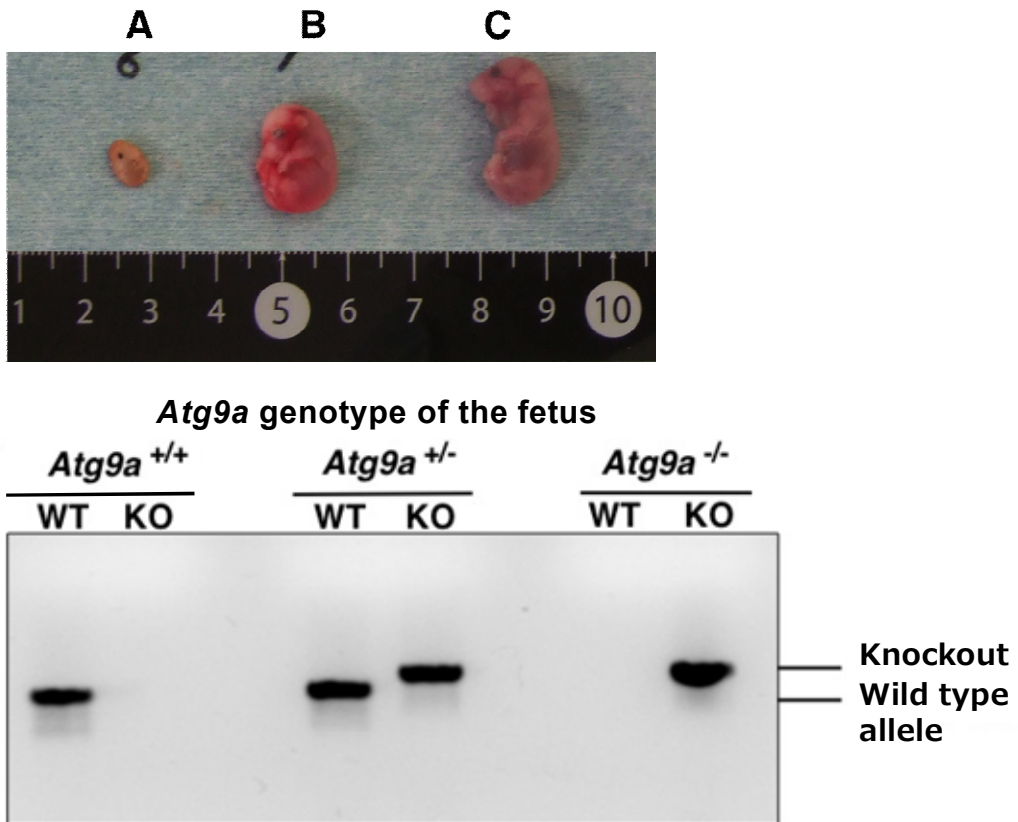
364

365 Fig. 7. Fetal body weight in the A1, A2, B1 and B2 mice on 13.5, 15.5, and 17.5 days post  
366 coitum (dpc). Numbers of fetuses are presented in parentheses. Upper and lower ends of the  
367 box and horizontal line inside the box indicate 75th percentile, 25th percentile, and median  
368 values, respectively. \* $P < 0.05$  between the two compared groups. Please refer to Table 1 for  
369 group (A1, A2, B1, and B2) definition.

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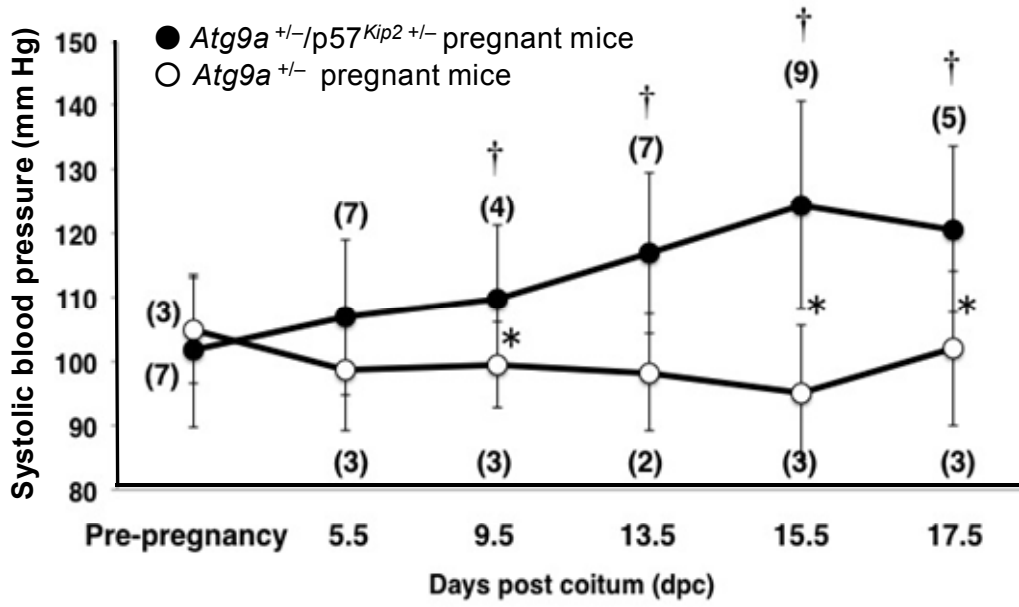
372 Fig. 1.



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375 Fig. 2.

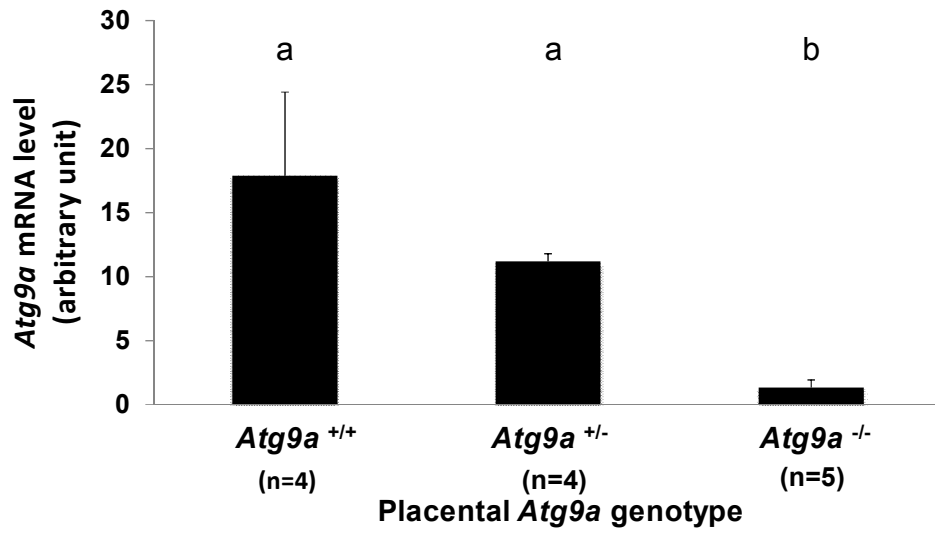


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379 Fig. 3.



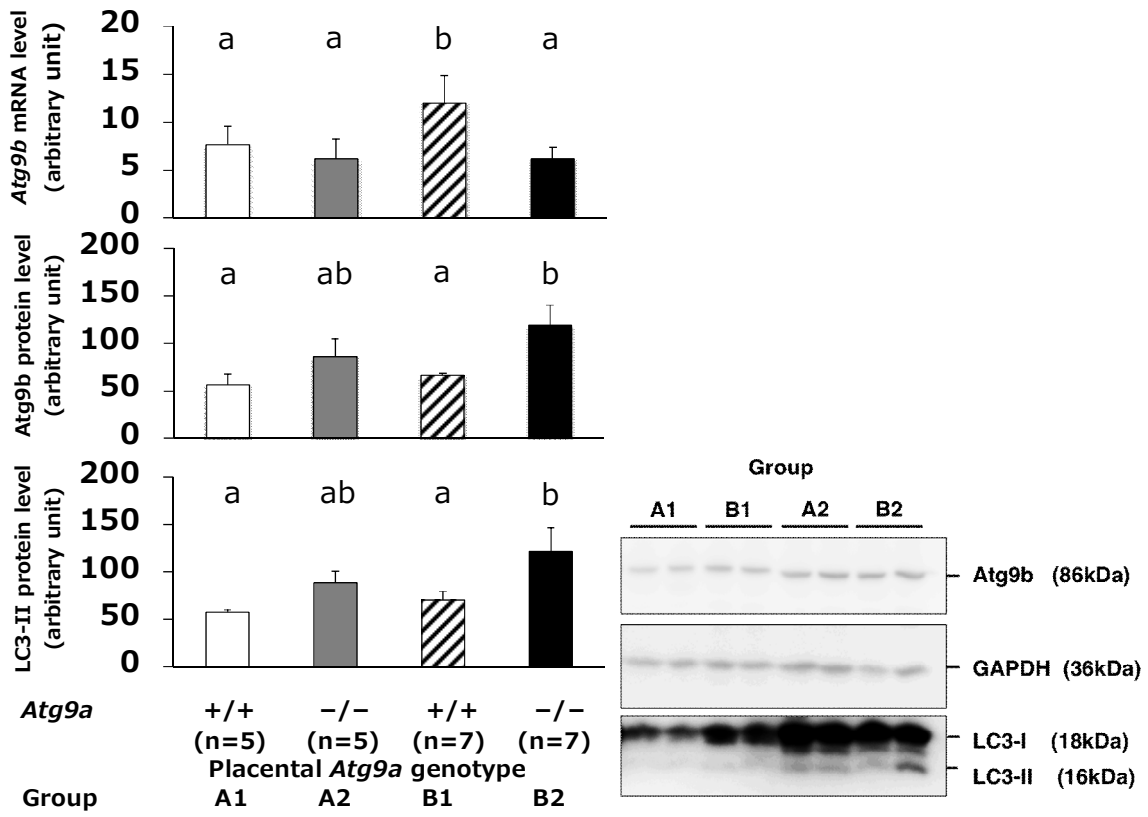
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383 Fig. 4.

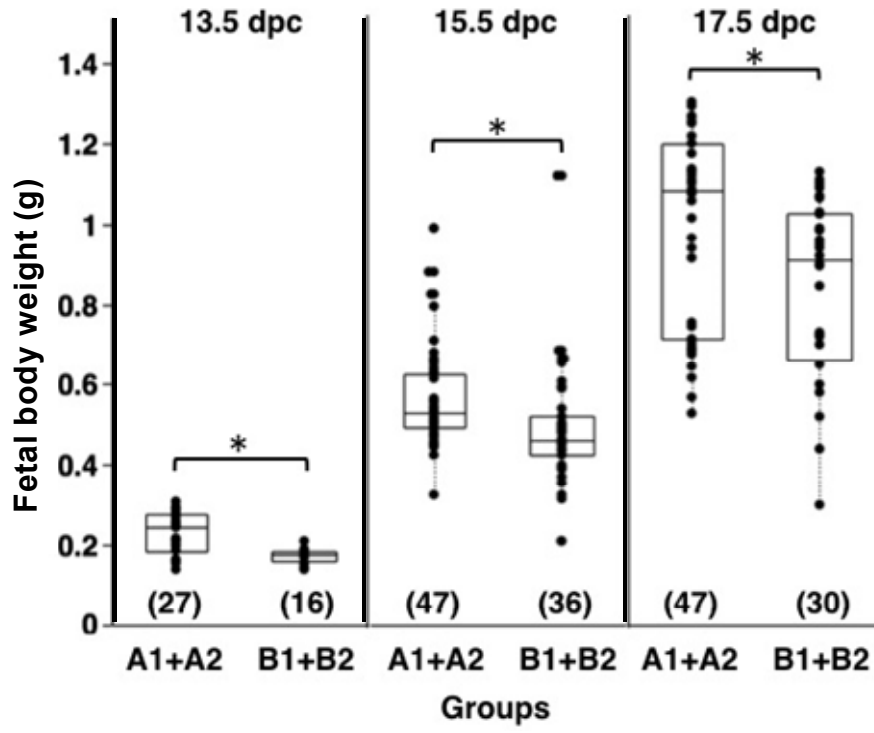


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387 Fig. 5.

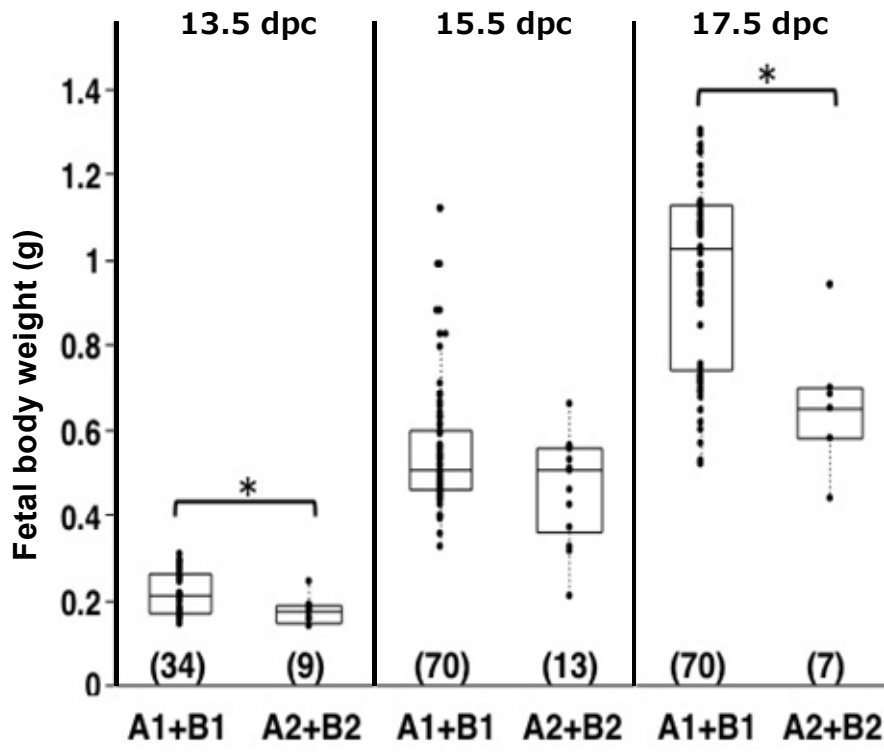


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391 Fig. 6.



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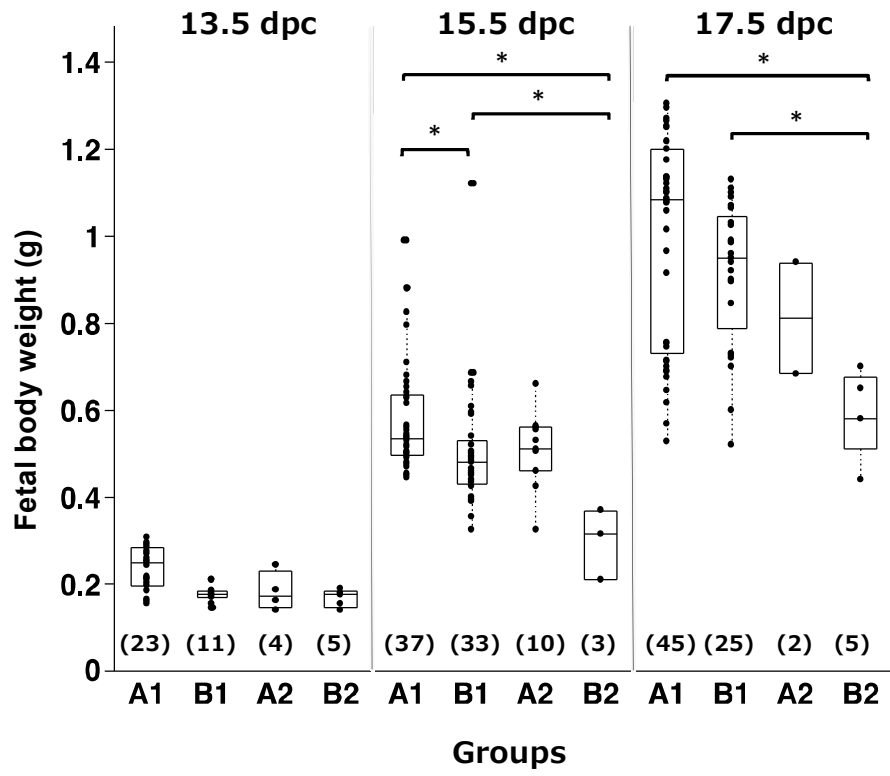


Table 1. Mating patterns and fetal *Atg9a* genotype

	Mating (male × female)	Fetal genotype
Group A1	<i>Atg9a</i> <sup>+/-</sup> × <i>Atg9a</i> <sup>+/-</sup>	<i>Atg9a</i> <sup>+/+</sup> , <i>Atg9a</i> <sup>+/-</sup>
Group A2	<i>Atg9a</i> <sup>+/-</sup> × <i>Atg9a</i> <sup>+/-</sup>	<i>Atg9a</i> <sup>-/-</sup>
Group B1	<i>Atg9a</i> <sup>+/-</sup> × <i>Atg9a</i> <sup>+/-</sup> /p57 <sup>Kip2</sup> <sup>+/-</sup> *	<i>Atg9a</i> <sup>+/+</sup> , <i>Atg9a</i> <sup>+/-</sup>
Group B2	<i>Atg9a</i> <sup>+/-</sup> × <i>Atg9a</i> <sup>+/-</sup> /p57 <sup>Kip2</sup> <sup>+/-</sup> *	<i>Atg9a</i> <sup>-/-</sup>

\*p57<sup>Kip2</sup><sup>+/-</sup> : heterozygous, devoid of the imprinted paternal allele

Table 2. Effects of fetal *Atg9a* genotype on the number of live or dead fetal mice and **the** intrauterine fetal death (IUFD) rate

Dams: <i>Atg9a</i> <sup>+/-</sup> (normotensive group)				
Fetal genotype	n	<i>Atg9a</i> <sup>+/+</sup> (A1)	<i>Atg9a</i> <sup>+/-</sup> (A1)	<i>Atg9a</i> <sup>-/-</sup> (A2)
13.5 dpc	4	12/0	11/3	4/0
15.5 dpc	4	21/0	16/3	10/1
17.5 dpc	5	14/1	31/2	2/6
IUFD rate		2.1% (1/48)	12% (8/66)	30% (7/23)
Dams: <i>Atg9a</i> <sup>+/-</sup> / <i>p57<sup>Kip2</sup></i> <sup>+/-</sup> (hypertensive group)				
Fetal genotype	n	<i>Atg9a</i> <sup>+/+</sup> (B1)	<i>Atg9a</i> <sup>+/-</sup> (B1)	<i>Atg9a</i> <sup>-/-</sup> (B2)
13.5 dpc	2	3/0	8/0	5/0
15.5 dpc	6	9/0	24/4	3/1
17.5 dpc	5	9/0	16/5	5/2
IUFD rate		0.0% (0/21)	16% (9/57)	19% (3/16)
Overall number of fetuses		69 (68/1)	123 (106/17)	39 (29/10)
Overall IUFD rate		1.4% (1/69) <sup>a</sup>	14% (17/123) <sup>b</sup>	26% (10/39) <sup>c</sup>

dpc - day *post coitum*; **n** - number of dams sacrificed.

*P* < 0.05 for a vs. b, a vs. c; and a + b (18/192) vs. c; Fisher's exact test with Bonferroni correction.