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The *ADP-ribosylation factor 1* gene is indispensable for mouse embryonic development after implantation

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ABSTRACT

ADP-ribosylation factor (Arf) 1 is thought to affect the morphologies of organelles, such as the Golgi apparatus, and regulate protein trafficking pathways. Mice have six Arf isoforms. In knockdown experiments with HeLa cells, no single Arf isoform among Arf1–5 is required for organelle morphologies or any membrane trafficking step. This suggests that the cooperation of two or more Arfs is a general feature. Although many cell biological and biochemical analyses have proven the importance of Arf1, the physiological roles of Arf1 in mice remain unknown. To investigate the activity of Arf1 *in vivo*, we established *Arf1*-deficient mice. $Arf^{-/-}$ blastocysts were identified at the expected Mendelian ratio. The appearance of these blastocysts was indistinguishable from that of wild-type and $Arf^{+/-}$ blastocysts, and they grew normally in an *in vitro* culture system. However, $Arf^{-/-}$ embryos were degenerated at E5.5, and none survived to E12.5, suggesting that they died soon after implantation. These data establish for the first time that the *Arf1* gene is indispensable for mouse embryonic development after implantation.

1. Introduction

ADP-ribosylation factors (Arfs) are 20-kDa GTP-binding proteins. The activities of Arfs affect the morphologies of organelles, such as the Golgi apparatus and early endosomes, and regulate protein trafficking through the secretory and endocytic pathways [1,2]. Mice have six Arf isoforms (Arf1–6), which are grouped into three classes (I–III). Class I, Arf1–3, are 96% identical. Class II, Arf4 and Arf5, are 90% identical to each other and 81% identical to Arf1. Class III is only Arf6 and is the most divergent of the Arf proteins. Studies have mainly focused on Arf1 at the Golgi apparatus and Arf6 at the plasma membrane; however, Arf3–5 are also present on Golgi membranes.

Arf1 regulates vesicle formation on intracellular membranes. The budding of vesicles from Golgi cisternae is fully reconstituted in the presence of Arf1 and coatomer (COPI) [3–6]. Arf1 recruits coatomers to budding vesicles and is then released from these vesicles to stimulate their uncoating and fusion with target membranes [3,7] and COPI on the endoplasmic reticulum [8–11]. The coupling of Arf1 and Cdc42 activities regulates endocytosis at the plasma membrane. Thus, endocytosis and secretion share the common regulator Arf1, which provides a molecular basis for crosstalk between these two processes [12]. In addition, Arf1 may also function in the control of integrin-mediated cell adhesion [13].

Arf1 plays important roles in many biological functions. For example, Arf1 regulates epidermal growth factor-dependent breast cancer cell invasion, growth, and migration [14,15]. Phosphatase of regenerating liver 3 exerts a pro-migratory role through the activation of Arf1, inducing faster trafficking of integrin molecules in colorectal cancer [16]. Therefore, inhibitors of Arf1 were developed with the expectation that they would be valuable tools to study membrane trafficking and also be anticancer drug candidates [17].

Small interfering RNA can be used to specifically deplete each human Arf and thereby examine the roles of Arf1–5 in live cells [18–20]. Surprisingly, no single Arf, including Arf1, is required for Golgi function or any step of membrane trafficking examined in HeLa cells. Instead, pairs of Arfs cooperate at particular steps. For example, Arf1 and Arf4 act redundantly during transport in the early secretory pathway. These results suggest that the

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cooperation of two or more Arfs at the same site is a general feature of Arf signaling. This is supported by the fact that yeast has two Arfs and that the disruption of both genes is lethal; thus Arfs are essential for mitotic growth in yeast [21]. Although many cell biological and biochemical analyses have proven the importance of Arf1, the physiological role of Arf1 in mice, as well as the cooperation of two or more Arfs at the same site, remains to be elucidated.

We have analyzed the novel Arf GTPase-activating proteins SMAP1 and SMAP2, which seem to activate the GTPase activity of Arf6 and Arf1, respectively [22–27]. We recently showed that single knockout mice of *SMAP1* and *SMAP2* have specific defects, although they develop normally [28,29]. To extend our analysis of SMAP2, we have attempted to elucidate the physiological roles of Arf1 in mice.

In this study, to investigate the activity of Arf1 *in vivo*, we established *Arf1*-deficient ($Arf^{-/-}$) mice, which lacked the entire open reading frame of *Arf1*. $Arf1^{-/-}$ blastocysts were indistinguishable from wild-type and heterozygous ($Arf1^{+/-}$) blastocysts and grew normally in an *in vitro* culture system. $Arf1^{-/-}$ embryos were degenerated at E5.5, and no viable $Arf1^{-/-}$ pups were born, suggesting that $Arf1^{-/-}$ embryos died soon after implantation. Our results show that *Arf1* is indispensable for mouse embryonic development after implantation.

2. Materials and methods

2.1. Animals

This study was approved by the Committee of Animal Experiments, Nara Women's University (approval ID: 06-13).

2.2. Generation of Arf1-deficient mice

A genomic clone encompassing the four exons containing the entire coding sequence of *Arf1* was isolated from a C57BL/6J BAC library (RP23-316M1, BACPAC) using Red/ET methods (Gene Bridge). A targeting vector was constructed in which the marker gene PGK-neo-pA, loxP-flanked neomycin resistance (neo) cassette, replaced the entire coding sequence of *Arf1*. A DT-A fragment was ligated to the 5′ end of the targeting vector for negative selection. The targeting vector was linearized by *Sal I* digestion and introduced into TT2 ES cells by electroporation [31]. Eight clones were positive for homologous recombination, as determined by PCR, and five clones had undergone homologous recombination, as determined by Southern blot analysis. Three clones were injected into 8-cell-stage embryos. Chimeras were mated with C57BL/6J female mice and two germline transmissions of the disrupted *Arf1* allele were confirmed (Accession No. CDB0702K: http://www.cdb.riken.jp/arg/mutant%20mice%20list.html). Later mice were crossed to a Cre deleter strains (CAG-Cre-TG mice) to remove neo cassette.

2.3. Southern blot analysis

Genomic DNA (10 μg) was digested with the appropriate restriction enzymes (*Sma I*, *Spe I*, or *EcoRI*), electrophoresed through 0.8% agarose gels, and blotted onto Hybond N+ membranes (GE Healthcare Life Science) using standard procedures. 5′ and 3′ probes were amplified from BAC DNA using the following primers sets: Arf1-LP-2-5′: 5′-AGGCTCATGGCAATCTTCAATCTTGT GC-3′ and Arf1-LP-2-3′: 5′-GCTGGAGTGAGGCAGACACACAGGACA AAG-3′ for the 5′ probe, and Arf1-SP-2-5′: 5′-GGAGCAGCTTCTGGG CCTTCACCTTTAACC-3′ and Arf1-SP-2-3′: 5′-CACCCTTAATAGGGCC TTTCCTAAAAGAC-3′ for the 3′ probe. The following PCR program was used to produce the 3′ probe using standard Taq polymerase (Greiner Bio One): 94 °C for 3 min, 35 cycles of 94 °C for 1 min, 55 °C for 2 min, and 72 °C for 3 min, and finally 72 °C for 7 min. The following PCR program was used to produce the 5′ probe using KOD-FX polymerase (TOYOBO): 94 °C for 2 min, 40 cycles of 94 °C for 10 s and 68 °C for 1 min, and finally 68 °C for 15 min. A neo probe of 620 bp was prepared from the pKJ2-neo plasmid by double digestion with *Bam HI* and *Pst I*. Each probe fragment was recovered from an agarose gel with QIAX II (Qiagen). Radiolabeled probes were prepared using random priming kits (TAKARA). Southern blots were hybridized and washed at 65 °C using previously described conditions (http://www.cdb.riken.jp/arg/download_file/06.protocol.pdf). Bands were visualized by autoradiography on Kodak X-Omat AR film.

2.4. Genotyping of mice by PCR

Genomic DNA from mouse tails and embryos was isolated by standard methods.

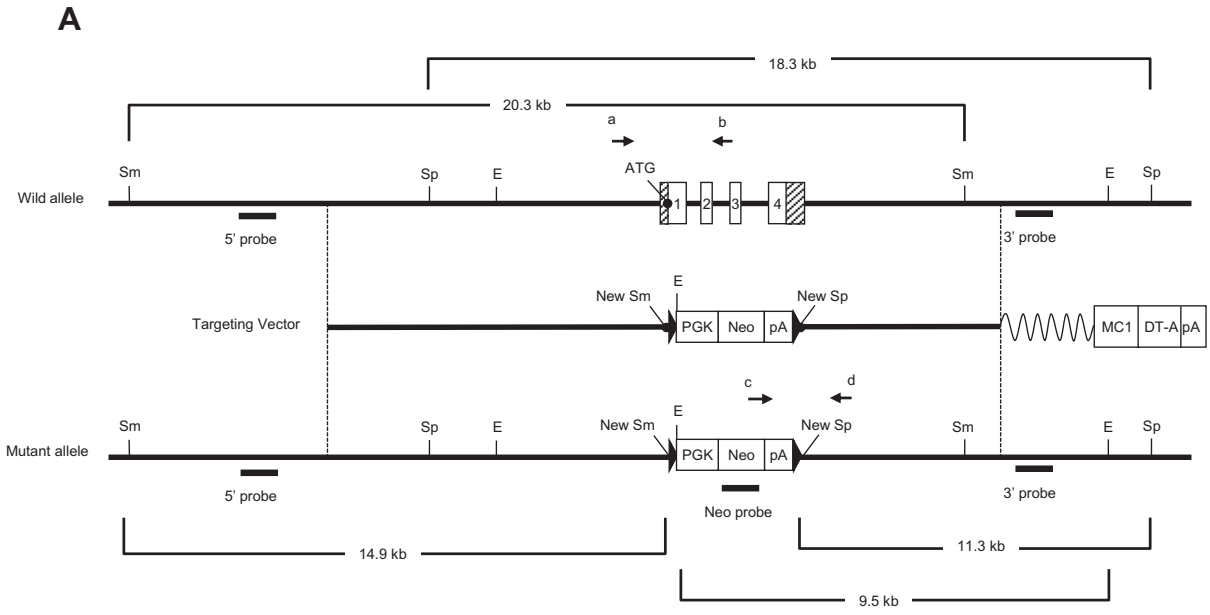
Genotyping of mouse tails and embryos was performed using PCR with standard Taq polymerase (Greiner Bio One) and specific primers designed to amplify the mutant or wild-type allele. The primers used for PCR analysis were as follows: Arf1-F2: 5′-GCAG GGGACTCCTACATAGGTGTCTCTGAC-3′ and Arf1-R2: 5′-GAAACAT GGCCACATTAGTGCAC-3′ for the wild-type allele, neo 2: 5′-CAGTCG CCTTCTATCGCCTTCTTGACG-3′ and Arf1 C: 5′- AGGGCTCTCAATCTC AGGAAGAAGCCTGGC-3′ for the knockout allele, and Arf1-F2 and Arf1 C for the neo deleted knockout allele. The following PCR program was used: 94 °C for 3 min, 32 cycles of 94 °C for 15 s, 60 °C for 30 s, and 72 °C for 1 min, and finally 72 °C for 7 min. For genotyping, one blastocyst was lysed in 10 μl of lysis buffer (330 μg/ml proteinase K) for 1 h at 55 °C, and then proteinase K was heat-inactivated for 10 min at 95 °C. Genomic DNA was isolated from cultured blastocysts by standard methods. Genotyping was performed as described above using KOD-FX polymerase (TOYOBO). The PCR program was as follows: 94 °C for 2 min, 40 cycles of 98 °C for 10 s, 60 °C for 30 s, and 68 °C for 1 min, and finally 68 °C for 15 min.

2.5. Histological examination

Histological examinations were performed as described previously [32]. In brief, uteri at E5.5 were fixed in Bouin's solution, dehydrated with ethanol, and embedded in paraffin. Sections were stained with hematoxylin and eosin.

2.6. In vitro culture of blastocysts

Blastocysts were analyzed as described previously [33,30], with some modifications. In brief, recovered blastocysts were first imaged using an Olympus microscope (IX70) equipped with a digital camera (SHIMAZ MOTICAM) and then cultured in high-glucose DMEM (Sigma) containing pyruvate, non-essential amino acids, and 15% fetal calf serum in 24-well plates. On day 7, each embryo was imaged using an Olympus microscope (IX70) equipped with a digital camera (SHIMAZ MOTICAM) and genotyped by PCR as described above. Some cultures were stained with Hoechst 33342 (TAKARA), observed using a LEICA fluorescence microscope (DMI 3000B), and genotyped by PCR as described above. Areas of ICM and TE were calculated from the images using Motic Images Plus 2.3S. Thereafter, the ICM ratio (ICM area/TE area) was calculated using Excel.



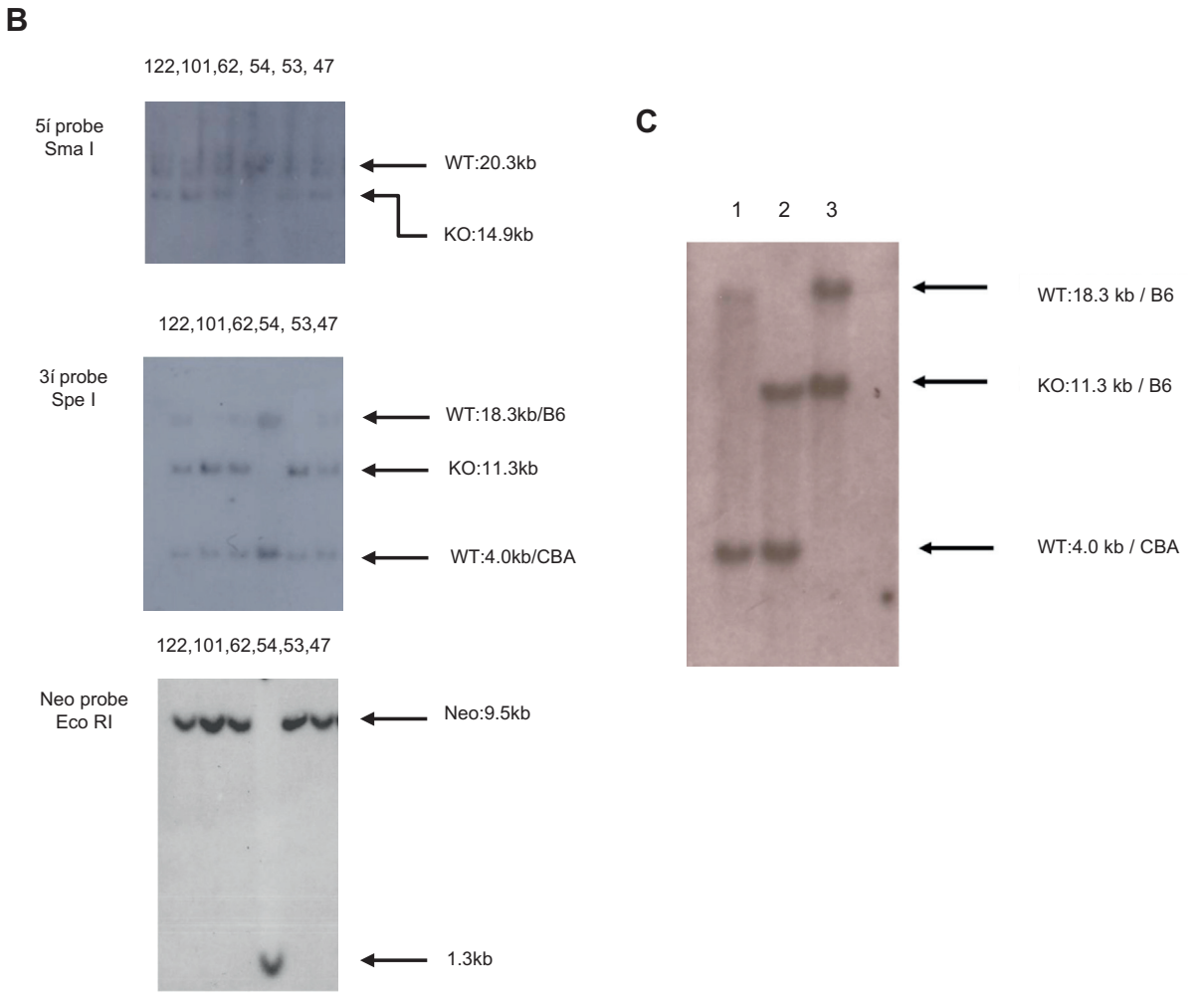


Fig. 1. Generation of *ADP-ribosylation factor 1* (*Arf1*)-deficient mice. (A) Schematic of the targeting strategy used to disrupt *Arf1*. The open reading frame sequence is replaced by the loxP (⏵)-flanked neo-expression cassette. Sp, Sm, and E represent *Spe I*, *Sma I*, and *Eco RI*, respectively. Primers a, b, c, and d are Arf1-F2, Arf1-R2, neo2, and Arf1 C (see Section 2 for descriptions), respectively. (B) Southern blot analysis of embryonic stem (ES) cells using the 5′, 3′, and neo probes described in (A). Numbers above the lanes are the ES cell clone number. Clones 47, 53, 62, 101, and 122 are expected homologous recombinants, whereas clone 54 is not. The short band in clone 54 detected by the neo probe was derived from a non-homologous integration site. It should be noted that the 3′ probe has a polymorphism in the *Spe I* restriction enzyme digestion site between B6 and CBA. (C) Southern blot analysis of mice using the 3′ probe described in (A). Genomic DNA from TT2 ES cells (lane 1), a chimeric mouse produced using targeted ES cells (lane 2), and an F1 mouse produced from a cross between a chimeric mouse and a C57BL/6J female mouse (lane 3). It should be noted that there was a restriction enzyme polymorphism as described in (B).

Table 1
Genotypes of offspring generated from intercrosses of *Arf1*$^{-/+}$ mice.

	+/+	+/−	−/−	Empty	Total
P21	67	80	0	0	147
E12.5	2	5	0	3	10
E3.5	11	22	12	0	45

Arf1, ADP-ribosylation factor 1; P, postnatal day.

3. Results and discussion

3.1. Generation of Arf1-deficient mice

We generated an *Arf1*-defcient allele (*Arf1*$^{-/-}$) by gene-targeting in mouse embryonic stem (ES) cells. The *Arf1*-targeting construct comprised an expression cassette in which the neomycin-resistance gene replaced genomic DNA encoding the entire open reading frame of *Arf1* (Fig. 1A). Five clonal ES cell lines bearing the targeted allele were identified by PCR and Southern blot analysis (Fig. 1B). Three of these were used to generate chimeric mice. F1 heterozygous mice were identified by Southern blot analysis (Fig. 1C), and their offspring were used for all subsequent experiments. Heterozygous mice exhibited no obvious abnormalities. Later mice were crossed to a Cre deleter strains (CAG-Cre-TG mice) to remove neo cassette.

3.2. Arf1$^{-/-}$ mice exhibit embryonic lethality

Of the examined 147 postnatal day (P) 21 offspring generated from intercrosses between *Arf1*$^{+/-}$ mice, 80 were *Arf1*$^{+/-}$ and 67 were wild-type (Table 1). Surprisingly, no viable *Arf1*$^{-/-}$ pups were identified, indicating that homozygous *Arf1* mutation kills mice prior to weaning (Table 1).

To identify the timing of lethality, embryos at the E12.5 stage were collected and genotyped by PCR. Implantation sites without embryos were observed; however, no *Arf1*$^{-/-}$ embryos were obtained at or after E12.5 (Table 1). Since same results were obtained using neo cassette deleted mice (data not shown), observed phenotypes were not due to inserted neo cassette. Instead, abnormal (degenerated) embryos were observed at E5.5 (Fig. 2A). Although wild-type embryos developed an egg cylinder, the structure of four of 14 embryos (28.6%) barely remained intact. The abnormalities of *Arf1*$^{-/-}$ embryos hindered further examinations at E5.5; therefore, blastocysts (at E3.5) were examined (Fig. 2B). Of the 45 blastocysts, 12 (26.7%) were *Arf1*$^{-/-}$. The appearance of *Arf1*$^{-/-}$ blastocysts was indistinguishable from that of wild-type and *Arf*$^{+/-}$ blastocysts at this stage (Fig. 2B and C). Same results were also obtained using neo cassette deleted mice (data not shown). These results indicate that *Arf1* is not necessary for development up to the blastocyst stage but is required for embryogenesis after implantation.

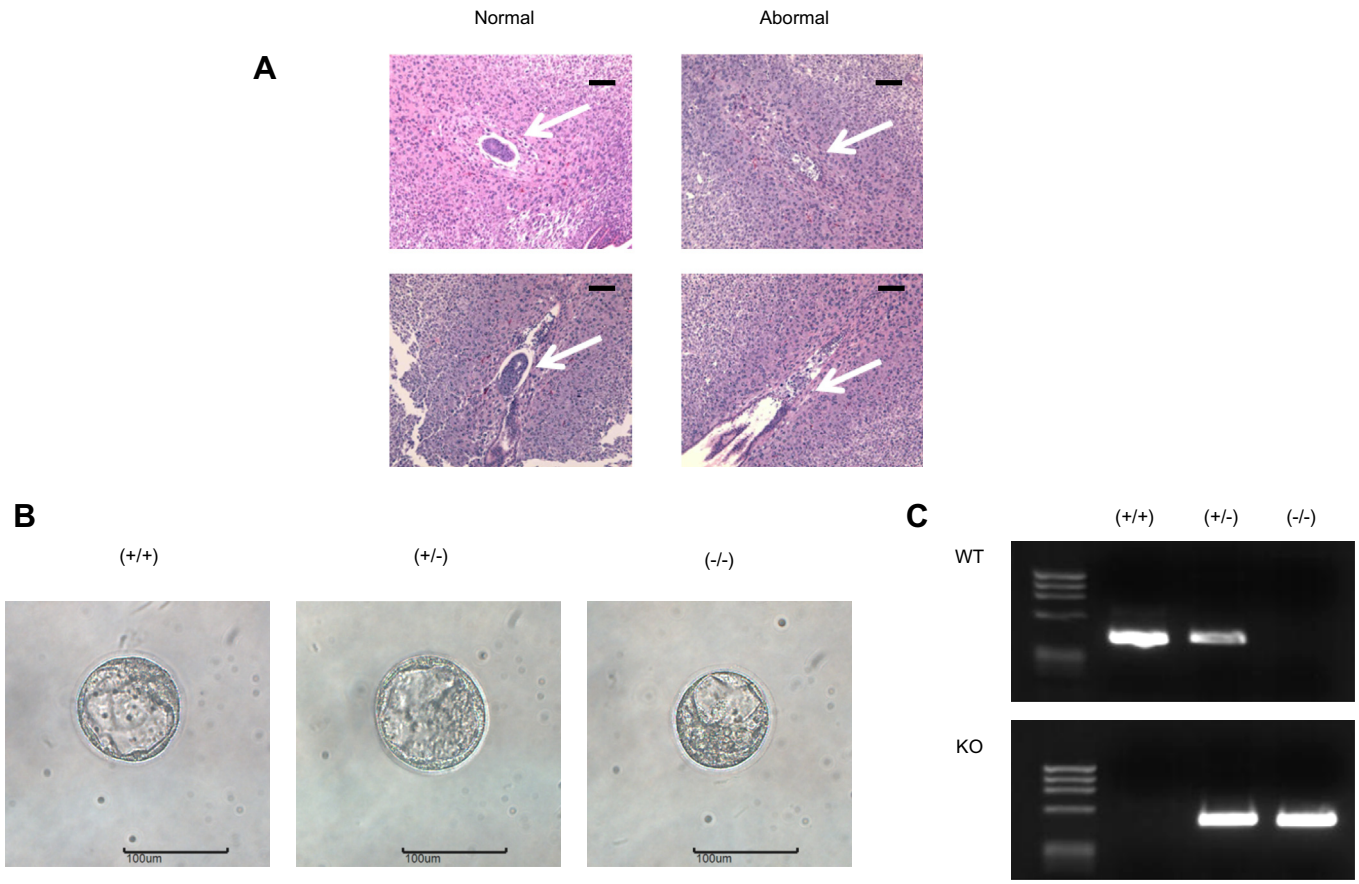


Fig. 2. Embryonic lethality of *ADP-ribosylation factor 1* (*Arf1*)$^{-/-}$ mice. (A) Hematoxylin and eosin staining of normal (wild-type) and abnormal embryos at E5.5. Arrows indicate embryos. Scale bar, 100 μm. (B) Microscopic images of isolated blastocysts. Wild-type (+/+), heterozygous *Arf1*-deficient (+/−), and homozygous *Arf1*-deficient (−/−) blastocysts. Scale bar, 100 μm. (C) PCR genotyping of isolated blastocysts. The upper panel shows PCR detection of the wild-type allele (WT). The lower panel shows PCR detection of the *Arf1*-deficient allele (KO). From left to right, the lanes are ΦX 174/*Hae III* digests, wild-type blastocysts (+/+), heterozygous blastocysts (+/−), and homozygous blastocysts (−/−).

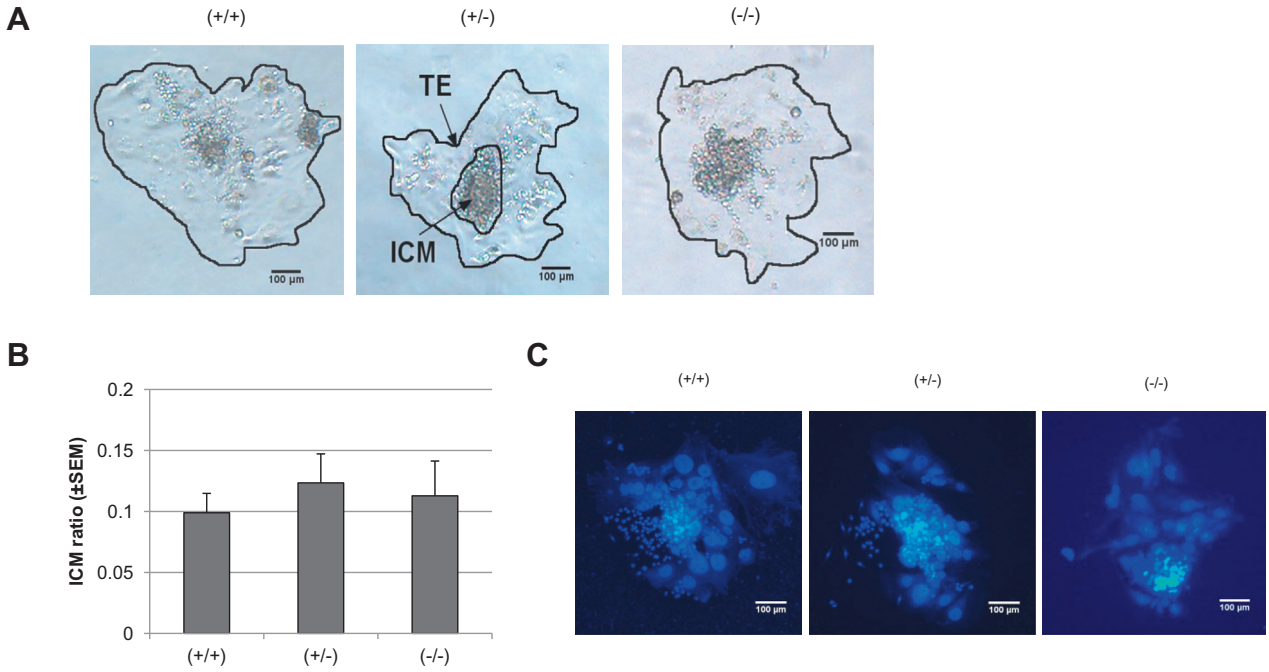


Fig. 3. *ADP-ribosylation factor 1 (Arf1*$^{-/-}$*)* blastocysts grow normally *in vitro*. (A) Images of blastocysts of the indicated genotypes after *in vitro* culture for 8 days. The inner cell mass (ICM) is surrounded by trophectoderm (TE) giant cells. Wild-type blastocysts (+/+), heterozygous *Arf1*-deficient blastocysts (+/−), and homozygous *Arf1*-deficient blastocysts (−/−). Scale bar, 100 μm. (B) Mean ICM ratio (ICM area/TE area) ± standard error of the mean (SEM) for blastocysts of the indicated genotypes. $n = 6$ for +/+, $n = 11$ for +/−, and $n = 6$ for −/−. (C) Hoechst 33342 staining of blastocysts of the indicated genotypes. Scale bar, 100 μm.

3.3. *Arf1*$^{-/-}$ blastocysts grow normally in an in vitro culture system

The death of *Arf1*$^{-/-}$ embryos at early post-implantation stages could be explained by a generalized cellular defect resulting in growth failure or by lineage-specific defects. Arfs are essential for mitotic growth in yeast [21], and *Arf1*$^{-/-}$ blastocysts were indistinguishable from wild-type and *Arf*$^{+/-}$ blastocysts. Therefore, we analyzed the *in vitro* growth capacity of *Arf1*$^{-/-}$ blastocysts (Fig. 3). To this end, blastocysts were collected and cultured individually *in vitro*. After 7 days of culture, both *Arf1*$^{-/-}$ and wild-type blastocysts produced the same trophectoderm (TE) outgrowths and inner cell mass (ICM). There were no differences between wild-type, *Arf1*$^{-/-}$, and *Arf1*$^{+/-}$ blastocysts in terms of the mean ICM ratio (ICM area/TE area) (Fig. 3B) [30]. No nuclear abnormalities were detected by Hoechst 33342 staining (Fig. 3C). Same results were obtained using neo cassette deleted mice (data not shown). Thus, we conclude that the growth of *Arf1*$^{-/-}$ blastocysts is indistinguishable from that of wild-type and *Arf1*$^{+/-}$ blastocysts *in vitro*. These data suggest that *Arf1* is dispensable for the appearance and *in vitro* growth capacity of mouse blastocysts.

As mentioned above, *Arf1*$^{-/-}$ embryos appeared normal at E3.5 and abnormal (degenerated) at E5.5, and no *Arf1*$^{-/-}$ embryos or pups were identified at later time points. Thus, our results established that *Arf1* is indispensable for mouse embryonic development after implantation. At present, we do not know why *Arf1* is dispensable at the blastocyst stage but indispensable after the implantation stage. Taking previously reported knockdown experiments into consideration, we propose two possible explanations for our results. First, Arf1 is dispensable in HeLa cells but indispensable in specific and important cells during mouse embryogenesis after implantation. Alternatively, a residual small amount of Arf1 is sufficient for its normal function in HeLa cells. Unfortunately, *Arf1*$^{-/-}$ mice died at an early stage, which hinders more detailed analysis.

In summary, we established that *Arf1* is indispensable for mouse embryonic development after implantation. In light of our results, the previously proposed concept that the cooperation of two or more Arfs at the same site may be a general feature of Arf signaling should be re-examined.

Author contributions

N.H., H.O., M.S., M.S., and T.W. planned the experiments. N.H., H.O., M.S., K.M., Y.M., S.N., Y.M., H.K., M.S., and T.W. performed the experiments. N.H., H.O., M.S., K.M., and T.W. analyzed the data. N.H., H.O., M.S., Y.M., M.S., and T.W. wrote the manuscript.

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