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Arp3 is required during preimplantation development of the mouse embryo

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Abstract The role of Arp3 in mouse development was investigated utilizing a gene trap mutation in the Arp3 gene. Heterozygous Arp3$^{WT/GT}$ mice are normal, however, homozygous Arp3$^{GT/GT}$ embryos die at blastocyst stage. Earlier embryonic stages appear unaffected by the mutation, probably due to maternal Arp3 protein. Mutant blastocysts isolated at E3.5 fail to continue development in vitro, lack outgrowth of trophoblast-like cells in culture and express reduced levels of the trophoblast marker Cdx2, while markers for inner cell mass continue to be present. The recessive embryonic lethal phenotype indicates that Arp3 plays a vital role for early mouse development, possibly when trophoblast cells become critical for implantation.

Keywords: Arp3-deficient mouse; Embryonic lethality; Abnormal blastocysts

1. Introduction

Motion of cells in animals and protists, organelle movement and intercellular transport, and cell morphology in plants and fungi are intimately associated with dynamic remodeling of the actin cytoskeleton. In all eukaryotes actin dynamics include de novo nucleation, site-directed branching, and elongation of actin filaments. Activation of the seven-subunit Arp2/3 complex leads to recruitment of actin into an expanding array of filaments branching off one another to form the actin filament network [1–3]. The role of the Arp2/3 complex is to augment the slow spontaneous rate of actin filament nucleation [reviewed in 4,5].

The Arp2/3 complex regulates actin filament morphology in yeast [6,7], *Drosophila* [8], *Caenorhabditis elegans* [9], and in mammalian cells [10]. Formation of lamellipodia and filopodia at the leading edge of migrating animal cells involves the Arp2/3 complex [10] and without this activity cell motility is severely compromised. *Drosophila* cells transfected with siRNA against components of the Arp2/3 complex failed to form the typical motility-driving membrane protrusions [11,12] and RNA interference in *C. elegans* caused cell migration defects in the early worm embryo preventing the establishment of the basic body plan [9]. Lethal phenotypes prior to cell migration in pre-gastrulating *Drosophila* embryos may be related to disruption of endocytic processes, suggesting that Arp2/3-mediated actin nucleation and polymerization is also involved in the propulsion of endocytic vesicles [13,14]. In non-motile yeast cells the Arp2/3 complex plays a role for internalization of endocytic vesicles [15,16] and yeast strains lacking components of the Arp2/3 complex die or exhibit severe growth retardation [17,18]. Loss of function mutations in *Drosophila* demonstrate the role of the Arp2/3 complex in multicellular organisms, for instance to form proper ring canals in oocytes [8].

No loss of function experiments in mouse or other vertebrate organisms have been described. Silencing of Arp3 by RNA interference in immortalized mouse embryonic fibroblasts resulted in reduced intracellular motility of *Listeria monocytogenes* and decreased actin nucleation activity, but interestingly Arp3-deficient fibroblasts were viable and exhibited normal leading-edge actin structures, as well as appropriate surface motility, and locomotion [19]. Previous reports on cell lines depleted for components of the Arp2/3 complex clearly showed alterations of cell shape and spreading [12], as well as defects in cell growth [20].

Here, we utilized an Arp3-deficient gene trap mutant mouse to investigate the role of Arp3 in early embryonic development. We show that heterozygous Arp3$^{WT/GT}$ mice are apparently normal but that homozygous Arp3$^{GT/GT}$ mutants die between E3.5 and E4.5. Our results demonstrate the critical developmental role of Arp3, which is likely to be associated with embryo implantation. We posit that an earlier Arp3 mutant phenotype may be obscured by maternal Arp3 that is still present in blastocysts.

2. Materials and methods

2.1. Generation and genotyping of the Arp3 gene trap mutant mouse

Generation and identification by RACE-PCR of mouse embryonic stem (ES) cell clones containing the integrated PT1βgeo gene trap vector have been described previously [21,22]. The ES cell clone A009F03 harboring the Arp3 mutation was used to generate chimeric mice by morula aggregation with CD1 wild-type embryos (E2.5). Chimeras were mated to albino CD1 mice to obtain heterozygous Arp3$^{GT/WT}$ progeny. For genotyping DNA was isolated, digested with BamHI, and hybridized on Southern blots to ^{32}P-labelled hybridization probe (796 bp) that was generated by PCR of Arp3 intron1 (nucleotides 7511–8347). Blastocysts were genotyped by PCR [23] using following primers: forward-strand primer wild-type 1 (U-wt1, 5′-TGCGGAGGTGTGTAAACA-3′); reverse-strand primer wild-type (L-wt, 5′-TCCCCTCCTCTTACAACAC-3′); forward-strand primer wild-type

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2 (U-wt2, 5′-AACCCACAACAACAAAAA-3′); reverse-strand primer gene trap (L-gt, 5′-GCCGCTTGTCCTCCTTGT-3′) for 45 cycles with annealing temperature set at 58 °C.

2.2. Northern blot analysis and RT-PCR

RNA was isolated with guanidinium thiocyanate-phenol and purified with RNAeasy kit (QIAGEN). Electrophoresis was performed on 1.0% agarose/formaldehyde gels. Northern blots were carried out on Hybond nylon membrane (Amersham) with the ^{32}P-labeled BglII/PstI fragment (4×10^6 cpm/ml) of Arp3 cDNA (NCBI GenBank accession: BC005557) used as hybridization probe. It corresponds to the sequence spanning part of exon 8 to nearly all of exon 12.

2.3. Cultivation of mouse embryos

Blastocysts at 3.5 dpc were isolated in M2 medium (Sigma) according to standard protocols. Cultivation was done in M16 medium (Sigma) with 5% CO_2 at 37 °C. For the analysis of cell outgrowth embryos were transferred after one day in culture onto gelatinized tissue culture plates (Nunc) in DMEM medium supplemented with high glucose and sodium pyruvate; 15% FCS, 2 mM L-glutamin; 0.1 mM non-essential amino acids; 0.1 mM β-mercaptoethanol; 1000 U LIF/ml for 5 days.

2.4. Immunoblot of Arp3 and immunohistochemistry

Protein extracts for immunoblots were prepared as described previously [24]. Arp3 protein and α-tubulin, used as loading control, were identified with rabbit anti-Arp3 polyclonal antibody (kindly provided by Theresia Stradal, HZI, Braunschweig) and mouse anti-α-tubulin monoclonal antibody (#T9026 DM 1A, Sigma), respectively. Horseradish peroxidase-conjugated goat anti-rabbit IgG (#A0545, Sigma) and goat anti-mouse IgG (#A9044, Sigma) were used as secondary antibodies and visualized by enhanced chemiluminescence (ECL) on Hyperfilm ECL (Amersham). Proteins were quantified by densitometric scanning of films.

For immunohistochemistry E3.5 or E4.5 embryos were fixed in 4% PFA for 20 min, treated with 0.4% Triton X-100 in PBS for 20 min, and blocked with 0.1% Triton X-100, 1% BSA or 10% donkey serum in PBS for 30 min. After each step embryos were washed five times for 5 min in PBS and antibody staining was performed for 1 h followed by staining of DNA with DAPI for 10 min. Embryos were mounted with Moviol for epifluorescence microscopy using a Leica DMR microscope equipped with a ProgRes C12 camera and software from Jenoptik, Germany. The following antibodies were used: mouse anti-Arp3 IgM monoclonal antibody (49B6, kindly provided by Theresia Stradal) and mouse anti-Arp3 IgG monoclonal antibody (#612134, BD Biosciences) in combination with horse anti-mouse IgG (H+L) coupled to HRP (#PI-2000, Vector Laboratories) in 1:10 dilution as secondary antibody, visualized with DAB (Sigma); rabbit polyclonal anti-Oct4 (Acris), goat polyclonal anti-Nanog (Santa Cruz), goat polyclonal anti-Cadherin (Santa Cruz), mouse monoclonal anti-Cdx2 (Santa Cruz), mouse monoclonal anti-BrdU (Santa Cruz), and rabbit monoclonal anti-active Caspase 3 (Abcam), all applied in dilutions

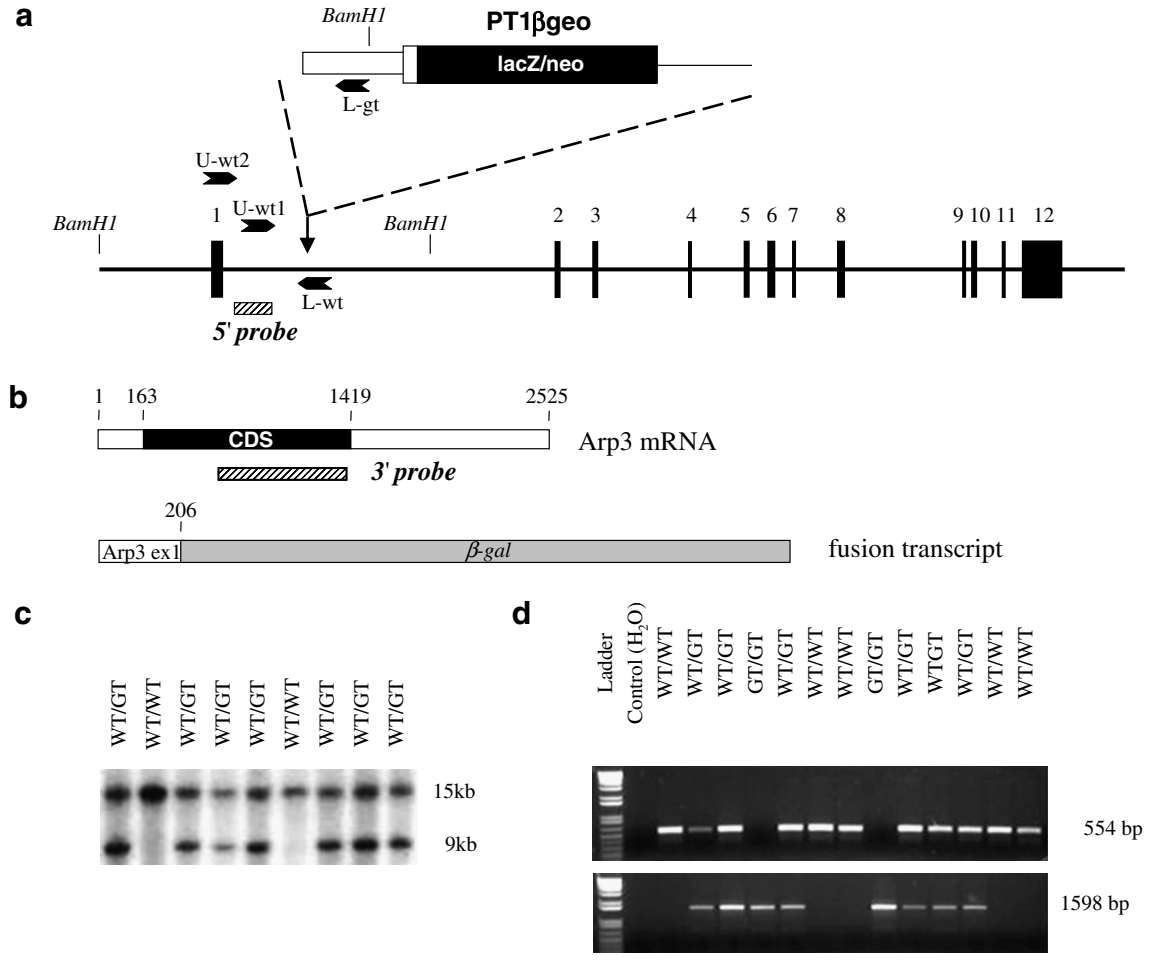


Fig. 1. The gene trap mutation in the mouse Arp3 gene. (a) The PT1βgeo vector has integrated into the first intron of the Arp3 gene that consists of 12 exons. BamH1 sites, 5′ hybridization probe, and primers used for genotyping are indicated. (b) Wild-type Arp3 mRNA is compared to mutant Arp3/βgeo fusion transcript containing only 206 nucleotides of Arp3 exon 1 that codes for 14 N-terminal amino acids. (c) Southern blot genotyping of a typical litter from heterozygous parents. BamHI digested DNA was hybridized with 5′ probe. Wild-type and Arp3 mutant alleles are represented by 15 kb and 9 kb fragments, respectively. (d) PCR-based genotyping of early embryos using Arp3-specific and vector-specific primers. Wild-type and mutant alleles are recognized by products of 554 and 1598 nucleotides, respectively.

between 1:50 and 1:100. Secondary antibodies (IgG) were raised in donkey and obtained from Jackson Laboratories: anti-goat Cy3, anti-mouse Cy2, anti-mouse Cy3, anti-rabbit Cy2. These were used in a dilution of 1:400.

2.5. BrdU labeling

E3.5 blastocysts were isolated, cultured in M16 medium (Sigma) supplemented with 10 μM BrdU (Sigma) for 16 h, and fixed in 4% paraformaldehyde (PFA) for 20 min. For immunofluorescence cells were washed in PBS and treated with 0.5 M HCl for 30 min.

3. Results

3.1. Generation of the Arp3 mouse mutant by gene trap vector insertion

The embryonic stem cell clone A009F03, which we generated as part of a large-scale gene trap approach to study gene functions in mouse [21,22], contains the PT1βgeo vector inserted into the first intron of the Arp3 gene (Fig. 1a). The mouse Arp3 gene extends over 12 exons on chromosome 1. The mRNA is 2525 nucleotides in length (GenBank accession number NM_023735) and codes for a protein of 418 amino acids. The gene trap event was identified in the ES cell clone by 5′RACE-PCR, detecting a fusion transcript encoding fourteen N-terminal amino acids of Arp3 and β-galactosidase/neo (β-geo; Fig. 1b). The annotated ES cells were aggregated to wild-type embryos to derive mouse chimeras and subsequently a mouse line carrying the gene trap mutation. A BamHI RFLP was established to distinguish Arp3 wild-type (15 kb) and mutant (9 kb) alleles (Fig. 1c). Early embryos were genotyped by PCR using suitable primers derived from gene and vector sequences (Fig. 1a,d). Significantly, no $Arp3^{GT/GT}$ offspring were detected in litters from heterozygous $Arp3^{WT/GT}$ parents 4 weeks after birth, indicating that the homozygous mutant was not viable (see Table 1).

Table 1
Genotypes of progeny from heterozygous Arp3 parents

Age	(*n*)	WT/WT	WT/GT	GT/GT
P 30	153	54	99	0
E13.5	7	2	5	0
E12.5	9	4	5	0
E10.5	34	13	21	0
E 8.5	34	12	22	0
E 4.5	49	13	36	0
E 3.5	111	39	56	16
E 2.5	32	9	17	6

Genotyping of embryos was performed with PCR.

3.2. Reduced expression of Arp3 in heterozygous mutants causes no phenotype

By Northern blot analysis we detected the 2.5 kb Arp3 mRNA in all examined tissues of the adult mouse, albeit at quite different levels (Fig. 2a). This observation essentially confirmed that Arp3 is expressed ubiquitously. Heterozygous Arp3 mouse mutants displayed no apparent phenotype, despite the fact that the relative abundance of Arp3 message was markedly reduced in organs of heterozygous compared to wild-type mice (Fig. 2b). Western blot analysis of protein extracts from heart, liver, and muscle revealed about 50% less Arp3 protein in heterozygous mutant than in wild-type animals (Fig. 2c,d). These results indicate that the gene trap

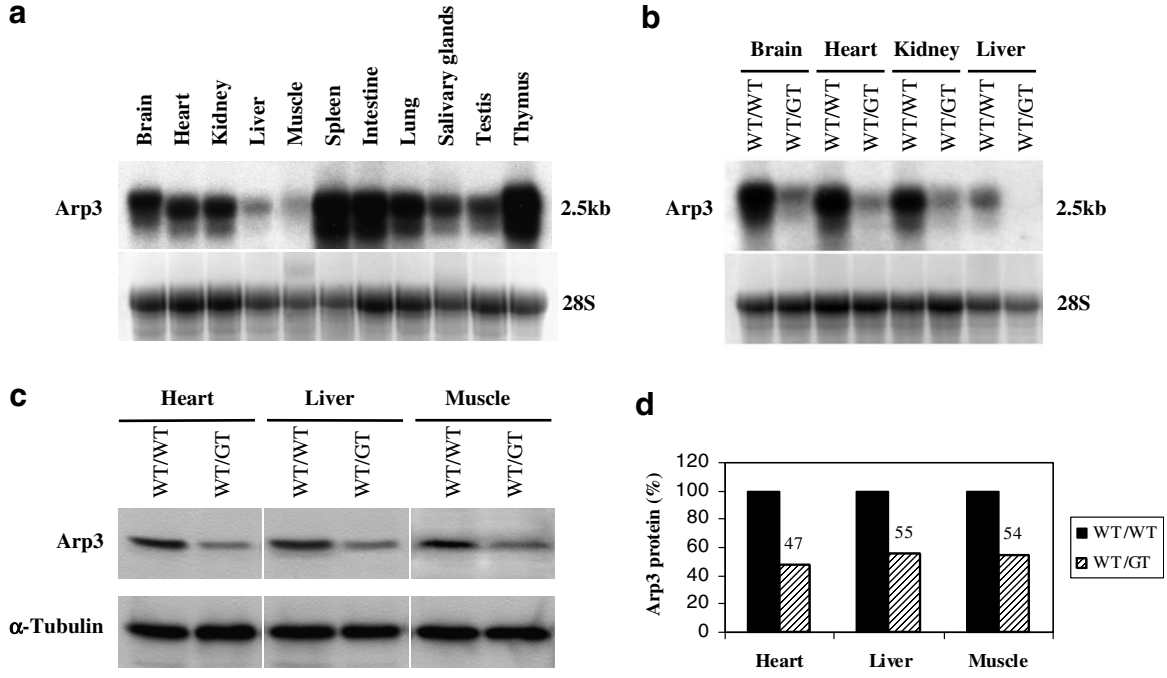


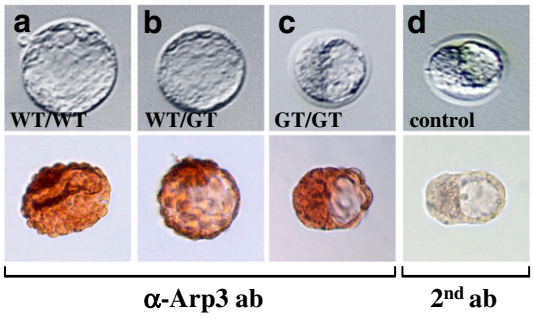
Fig. 2. Expression of Arp3 in wild-type and mutant mice. (a) RNA blot from tissues of the adult mouse indicates the ubiquitously expressed 2.5 kb Arp3 mRNA. 28S rRNA served as a gel loading control. (b) The Northern blot illustrates that Arp3 mRNA is significantly reduced in tissues from heterozygous mutant mice compared to wild-type. (c) Immunoblot of Arp3 in protein extracts (40 μg) from organs of wild-type and heterozygous mutant mice indicates that Arp3 protein is markedly diminished in heterozygous mice. α-tubulin serves as a loading control. (d) Quantification of relative Arp3 levels by densitometric scanning of the film shown in panel c.

integration indeed eliminated the production of Arp3 protein. We therefore imply that homozygous mutants totally lack Arp3 protein, although their early embryonic lethality precludes the experimental determination of protein levels (see below). These data indicate that approximately 50% of normal Arp3 levels are sufficient to form functional Arp2/3 complexes and support normal mouse development.

3.3. Arp3 function is essential in preimplantation embryos

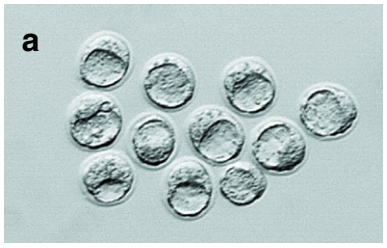
Genotyping of 153 mice from heterozygous parents at P30 indicated that no homozygous Arp3 mutants survived this postnatal period (Table 1). Likewise, no homozygous Arp3$^{GT/GT}$ embryos were found between E4.5 and E13.5. In contrast, E2.5 morulae were obtained at the Mendelian ratio of genotypes, whereas homozygous E3.5 blastocysts were present only at about 50% of the expected value. These results suggest that loss of Arp3 causes embryonic lethality between E3.5 and E4.5. We also compared the developmental potential of E3.5 wild-type and mutant blastocysts in vitro over a 24-h culture period. Of 85 randomly chosen E3.5 blastocysts (Fig. 3a) 49 embryos developed properly into expanded blastocysts (Fig. 3b), while 36 embryos appeared developmentally arrested (Fig. 3c). Genotyping of all embryos after cultivation revealed that no homozygous Arp3$^{GT/GT}$ embryo progressed in development, whereas two-thirds of wild-type and heterozygous blastocysts formed fully expanded blastocysts in vitro. In keeping with this observation, all homozygous but only about 30% of wild-type and heterozygous embryos failed to further develop in culture, indicating that Arp3 is essential during the blastocyst stage.

Interestingly, using two Arp3-specific antibodies that do not recognize epitopes within the N-terminal 14 amino acids (T. Stradal, personal communication) we detected significant and similar amounts of Arp3 protein in E3.5 blastocysts of all three genotypes including 10 homozygous Arp3$^{GT/GT}$ embryos (Fig. 4). We interpret this finding as an indication that mater-

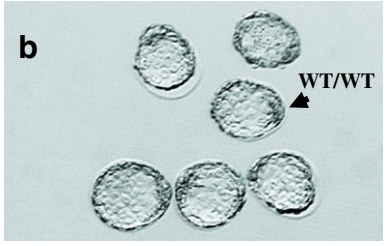


Stage	Total	WT/WT	WT/GT	GT/GT
E3.5	49	14	25	10

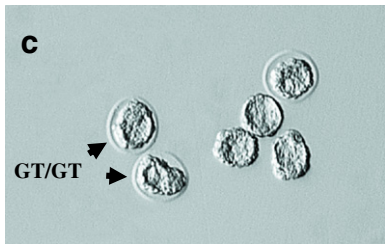
Fig. 4. Immunohistochemical staining of Arp3 protein in E3.5 embryos. 49 blastocysts were first stained with Arp3-specific antibody (a–c) and then genotyped by PCR. Embryos of all genotypes contain Arp3 protein. (d) Control staining with secondary antibody only.



	Total	WT/WT	WT/GT	GT/GT
E3.5 Blastocysts freshly prepared	85	--	--	--



Cultivation (24h)	Total	WT/WT	WT/GT	GT/GT
E4.5 Blastocysts expanded	49	13	36	0



Cultivation (24h)	Total	WT/WT	WT/GT	GT/GT
E4.5 Blastocysts undeveloped	36	7	15	14

Fig. 3. Phase-contrast images of isolated blastocysts cultivated for 24 h in vitro. (a) Representative examples of E3.5 blastocysts at the time of isolation. (b) Embryos that have developed to expanded blastocysts after 24 h of cultivation in vitro. The table lists numbers of embryos for the indicated genotype within this subgroup. (c) Representative pictures of embryos that failed to develop over 24 h of cultivation in vitro. Note the smaller blastocysts of irregular shape with scattered inner cell mass. Arrowheads mark homozygous mutant embryos. The table indicates the numbers of embryos with corresponding genotypes.

nal Arp3 is still present in embryos at E3.5, and this may be sufficient to rescue the early stages of mouse development.

Prolonged cultivation of 20 blastocysts for five days demonstrated that six Arp3-deficient blastocysts failed to attach to

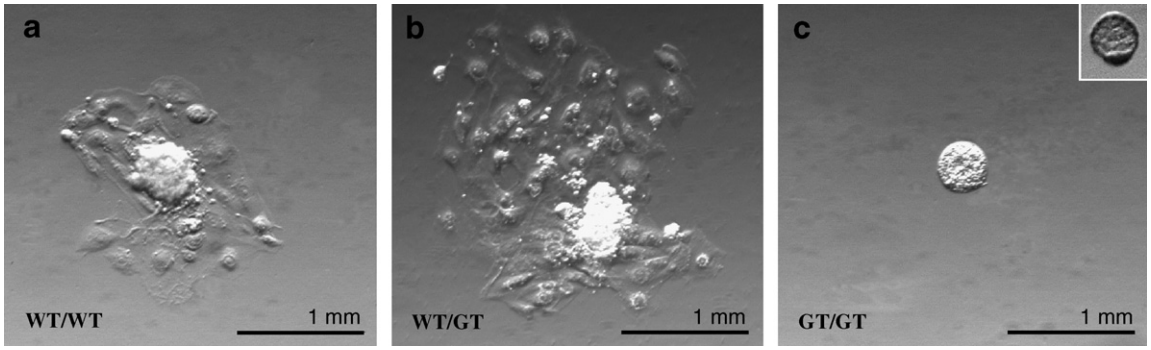


Fig. 5. Microscopic images of blastocysts from wild-type (a), heterozygous (b), and homozygous (c) Arp3 mutant mice after 6 days in culture. Note the extensive outgrowth of cells that normally spreads around the aggregate of the inner cell mass. Arp3-deficient embryos are unable to attach to the substratum with no cells growing out of the blastocyst (c). Inset in panel c shows a wild-type E3.5 blastocyst for comparison.

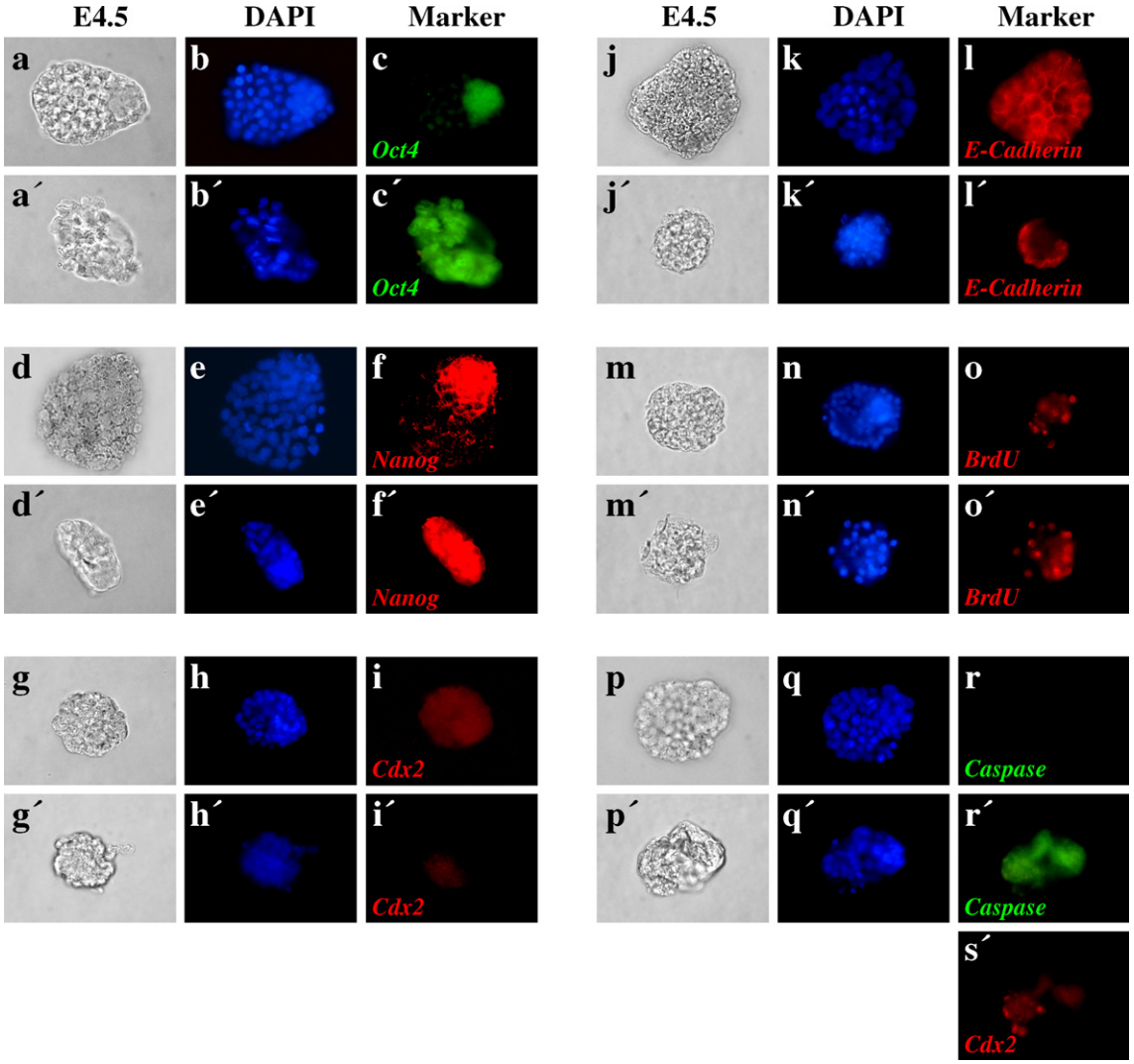


Fig. 6. Expression of marker genes in cultured blastocysts of wild-type (a–r) and mutant (a′–s′) phenotypes. Blastocysts (E3.5) from Arp3$^{WT/GT}$ intercrosses were cultivated in vitro for 24 h and subjected to immunofluorescence staining with indicated antibodies. Brightfield micrographs and DAPI stainings of nuclei are also shown. Note that Oct4 (c), Nanog (f), Cdx2 (i) and E-cadherin (l) are differentially expressed in blastocysts of normal appearance and those that are collapsed (c′, f′, i′, l′). Cell proliferation was determined by BrdU incorporation and appears similar in blastocysts of wild-type (o) and mutant phenotype (o′). Apoptotic cells are not detected in wild-type embryos (r), but are present in mutant blastocysts (r′).

gelatinized tissue culture dishes and formed no outgrowth of trophoblast-like cells, while 4 wild-type and 10 heterozygous embryos readily adopted the typical morphology of aggregated inner cell mass and trophoblast outgrowth (Fig. 5). This observation suggested that Arp3 may play a particular role in trophoblast cells, although it does not rule out important Arp3 functions in other cells or at earlier stages of development.

To analyze the Arp3 mutant phenotype in more detail, we investigated cell proliferation and apoptosis, and performed immunofluorescence staining for cell type-specific markers on in vitro cultivated blastocysts from Arp3$^{WT/GT}$ intercrosses (Fig. 6). We observed that the majority of blastocysts expanded normally during the 24-h culture period, but that approximately 25% of the blastocysts collapsed and showed aberrant morphology. Expression of the pluripotency markers Oct4 and Nanog was spatially restricted to the inner cell mass (ICM) of normal looking blastocysts, while both markers appeared in virtually all cells of the collapsed blastocysts (Fig. 6c,c′,f,f′). In contrast, expression of the trophectoderm (TE) marker Cdx2 was markedly reduced in collapsed blastocysts as compared to those of normal appearance (Fig. 6i,i′).

Significant reduction in E-cadherin expression was also observed in blastocysts of the mutant phenotype, suggesting that the integrity of the trophoblast was impaired (Fig. 6l,l′). Taken together, these observations imply that loss of Arp3 is likely to interfere with normal development of the trophoblast, while the inner cell mass appears expanded rather than reduced. Comparable incorporation of BrdU in blastocysts of wild-type and mutant phenotype indicated that cell proliferation was probably not dramatically altered (Fig. 6o,o′). Significantly, however, blastocysts of the mutant phenotype were subject to widespread apoptosis based on the presence of active Caspase3, whereas normal embryos showed essentially no programmed cell death at this stage (Fig. 6r,r′). At least some of the Caspase3-positive cells also expressed Cdx2 (Fig. 6s′).

4. Discussion

The vital role of Arp3 and its functional requirement for lamellipodia, translational locomotion, cell spreading, and actin assembly has been challenged recently in cultured mouse fibroblasts that were treated with RNAi to silence 90% of Arp3 expression [19]. This study indeed suggests that even minor expression of Arp3 may suffice to rescue the knockdown cells. However, the gene trap mutation of the mouse Arp3 gene described here reveals the essential developmental role of Arp3 whose function is not compensated by another gene. The null mutation leads to arrest of early embryonic development and death of preimplantation embryos. Heterozygous animals with only 50% of normal Arp3 protein levels are viable and exhibit no apparent phenotype. The recessive lethal phenotype argues that the gene trap integration constitutes a genuine loss of function mutation. Based on our marker analysis and the arrested development of mutant blastocysts in vitro, we assume that Arp3-deficient embryos may suffer from abnormalities in the trophectoderm that appear to be unable to support normal blastocyst morphology.

It is, however, difficult to ascertain whether Arp3 function is essential in early embryos or only after the formation of blastocysts, because Arp3$^{GT/GT}$ morulae and E3.5 blastocysts retain maternal Arp3 protein that may rescue an even earlier mutant phenotype. In keeping with the hypothesis that Arp3 serves a later-stage functional role, we observe impaired development of Arp3-deficient blastocysts in vitro, particularly lack of migration of trophoblasts away from the inner cell mass to form the typical outgrowth. Interestingly, affected embryos tend to show stronger expression of the ICM markers Oct4 and Nanog, while Cdx2 expression is much weaker. These data together with reduced expression of E-cadherin are consistent with the notion that loss of Arp3 causes trophoblast cells to deteriorate. It has been shown that Cdx2 is required to prevent expression of Oct4 and Nanog in the outer cells of the blastocyst and these cells undergo apoptosis in the absence of Cdx2 [25].

Trophoblasts in vivo mediate hatching and the implantation of the embryo. In agreement with the observed caspase3 activity, we believe that Arp3-deficient blastocysts may lose trophoblasts by apoptosis and therefore fail to form or maintain proper trophectoderm. Consequently, these embryos are unable to implant. Alternatively, Arp3$^{GT/GT}$mutant embryos may die either due to impaired endocytosis and uptake of nutrients, or defects in intracellular transport, since the Arp2/3 complex has also been implicated recently in endocytosis of clathrin-coated vesicles [13,26].

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