Aneuploidy during the onset of mouse embryo development

Tereza Pauerova^{1,2}, Lenka Radonova^{1,2}, Kristina Kovacovicova¹, Lucia Novakova^{1,2}, Michal Skultety^{1,2} and Martin Anger^{1,2}

¹Department of Genetics and Reproduction, Central European Institute of Technology, Veterinary Research Institute, Brno, Czech Republic and ²Institute of Animal Physiology and Genetics, Czech Academy of Sciences, Libeňov, Czech Republic

Correspondence should be addressed to M Anger; Email: anger@vri.cz

Abstract

Aneuploidy is the most frequent single cause leading into the termination of early development in human and animal reproduction. Although the mouse is frequently used as a model organism for studying the aneuploidy, we have only incomplete information about the frequency of numerical chromosomal aberrations throughout development, usually limited to a particular stage or assumed from the occurrence of micronuclei. In our study, we systematically scored aneuploidy in *in vivo* mouse embryos, from zygotes up to 16-cell stage, using kinetochore counting assay. We show here that the frequency of aneuploidy per blastomere remains relatively similar from zygotes until 8-cell embryos and then increases in 16-cell embryos. Due to the accumulation of blastomeres, aneuploidy per embryo increases gradually during this developmental period. Our data also revealed that the aneuploidy from zygotes and 2-cell embryos does not propagate further into later developmental stages, suggesting that embryos suffering from aneuploidy are eliminated at this stage. Experiments with reconstituted live embryos revealed, that hyperploid blastomeres survive early development, although they exhibit slower cell cycle progression and suffer frequently from DNA fragmentation and cell cycle arrest.

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Introduction

Chromosome segregation during early development is in mammals highly error prone and frequently results in embryos carrying aneuploid blastomeres (Hassold & Hunt 2001, Vázquez-Diez & FitzHarris 2018, Radonova *et al.* 2019). Numerous reports from IVF clinics showed that the aneuploidy affects more than half of human embryos developing *in vitro* (Carbone & Chavez 2015), although the reported data are sometimes highly variable due to differences in sample preparation and scoring methods between clinical centers (Treff & Zimmerman 2017, Swain 2019). On the other hand, comprehensive studies concerning the aneuploidy in early embryos of non-human mammalian species are scarce (Mantikou *et al.* 2012, Carbone & Chavez 2015). Nevertheless from the available reports we know, for example, that the frequency of aneuploidy in bovine cleavage embryos varies from 18.8% in *in vivo* embryos up to 84.6% in embryos after *in vitro* oocyte maturation and fertilization (Destouni *et al.* 2016, Hornak *et al.* 2016, Tšuiiko *et al.* 2017). This is similar to the porcine *in vivo* embryos, of which 46.8% carry aneuploid blastomeres (Hornak *et al.* 2012). Since the reported aneuploidy in eggs from young animals is 12.1%, the pig embryos show an increased frequency of aneuploidy in comparison to the oocytes (Hornak *et al.* 2011). And

the recent report revealed quite high aneuploidy in embryos of rhesus monkey (Daughtry *et al.* 2019).

The initial studies indicated a surprisingly low level of aneuploidy in mouse oocytes (Lightfoot *et al.* 2006). However, the results obtained later showed 3–8% aneuploidy in *in vitro* matured eggs in this species, which is similar to the frequency of aneuploidy in human oocytes (Duncan *et al.* 2009, Danylevska *et al.* 2014). Surprisingly little is known about the aneuploidy in mouse embryos, which at first led into conclusion, that this species has a low level of aneuploidy during early development (Tšuiiko *et al.* 2019). In contrast to this, sky karyotyping revealed that 25% of mouse embryos at day E3.5 carry aneuploid blastomeres (Lightfoot *et al.* 2006) and FISH yielded 17% aneuploidy concerning three chromosomes in blastomeres at a similar stage (Bolton *et al.* 2016). Another argument for a higher aneuploidy rate in mouse embryos came from a recent publication showing that mouse blastomeres frequently carry a micronuclei, signifying a loss of genetic material (Vázquez-Diez *et al.* 2016).

In this study, we present, for the first time, the frequency of aneuploidy in each stage from zygotes up to 16-cell mouse embryos. The aneuploidy was directly measured by kinetochore counting technique inside the intact embryonic blastomeres. Since up to the 8-cell stage this technique allowed to score aneuploidy in all

blastomeres of each individual embryo, our experiments also revealed the frequency of embryos carrying aneuploid blastomeres during early development. The results also revealed that embryos with aneuploidy are arrested by the 2-cell stage. Using micromanipulation and live-cell imaging, we studied the fate of hyperploid blastomeres in live embryos during early development. Our results showed that the majority of hyperploid blastomeres survive early development, albeit their cell cycle length and survival rates are affected.

Materials and methods

Animals

Mice were purchased from commercial breeders (Anlab and Masaryk University). Embryos were obtained from mating of CD-1/BDF1 females and BDF1 males or CD-1 females and BDF1 males. The females of age between two to five months were stimulated with pregnant mare serum gonadotropin (PMSG, 5 IU, Merck) followed 44–48 h later by stimulation with human chorionic gonadotropin (hCG, 5 IU, Merck) prior to mating. The work with animals was conducted according to Act No 246/1992 Coll. on the protection of animals against cruelty and was approved by the Central Commission for Animal Welfare, approval ID 51/2015.

Embryo isolation, microinjection, blastomere fusion and transfer

Zygotes, 2-cell, 4-cell, 8-cell and 16-cell embryos were isolated at 18–21, 44–47, 58–60, 65–68 and 74–77 h after hCG stimulation into M2 media (Merck) and subsequently cultured in KSOM+AA (Caisson Laboratories) under mineral oil (Merck) at 37°C, 5% CO₂. The cumulus cells from zygotes were removed by mechanical pipetting in M2 media (Merck), supplemented with 0.05% hyaluronidase (Merck). Complementary RNAs for microinjection were prepared by *in vitro* transcription (mMESSAGE mMACHIGENE and Poly(A) Tailing kit, Life Technologies) of ORFs of desired proteins. Embryos were microinjected in M2 media (Merck) using I-10 microinjector (Narishige) and Leica DMIL inverted microscope. For induction of hyperploidy zygotes microinjected with H2B-EGFP were transferred into M2 media (Merck) containing 0.3 µg/ml phytohemagglutinin (Merck) for 30 min and fused with their polar body. The fusion of agglutinated cells was performed in 1 mm fusion chambers with 2 pulses of 90 V/75 µs using Multiporator (Eppendorf). Fused cells were then cultured in KSOM+AA (Caisson Laboratories) until the 4-cell stage (37°C, 5% CO₂). In control experiments, zygotes were microinjected and cultured in KSOM+AA (Caisson Laboratories) without fusion. Blastomere transfer was performed on Leica DMI3000 B inverted microscope equipped with Eppendorf InjectMan® NI 2 Micromanipulator (Eppendorf). 4-cell embryos were transferred into M2 media (Merck) supplemented with 10 µg/mL cytochalasin B (Merck) for 10 min prior to micromanipulation. From control 4-cell embryos one blastomere was removed using a piezo drill-assisted micromanipulation. Blastomeres in hyperploid or euploid four-cell embryos were separated and

then transferred into euploid embryos. Reconstituted 4-cell embryos were then transferred into KSOM+AA (Caisson labs) supplemented with 0.3 µg/mL phytohemagglutinin (Merck) for about 1.5 h. In case of control experiments, blastomeres were transferred without fusing with their polar body.

Cell cycle synchronization and immunofluorescence

For determination of aneuploidy *in vivo*, isolated embryos were synchronized in metaphase by 0.025 µg/mL colcemid (Merck) diluted in KSOM+AA (Caisson labs). Subsequently the embryos were treated by 10 µM Mg132 (Merck) for 1 h followed by treatment with 5 µM dimethylenastron (Merck) together with 10 µM Mg132 (Merck) also for 1 h in KSOM+AA (Caisson labs). The 1- to 8-cell embryos were then fixed in 2% paraformaldehyde (Merck) for 20 min, permeabilized with 0.1% Triton X-100 (Merck) for 15 min and blocked (0.1% BSA) overnight at 4°C. The 16-cell embryos were fixed in 1.5% paraformaldehyde (Merck) for 20 min, permeabilized with 0.5% Triton X-100 (Merck) for 1 h and blocked (0.5% BSA) overnight at 4°C. Kinetochores were visualized by human antiserum against kinetochores – CREST (1:500 dilution, Immunovision) and Alexa Fluor 488 goat anti-human (1:500 dilution, ThermoFisher Scientific) antibody. For mounting, Vectashield with DAPI (Vector Laboratories) was used.

Microscopy and live-cell imaging

Fixed embryos were scanned using Leica SP5 and Olympus FluoView 3000 confocal microscope equipped with HCX PL APO 63x/1.4 oil and UPLSAPO 100XS/1.35 silicone oil objectives. 405 and 488 nm excitation wavelengths were used for detection of DAPI and Alexa Fluor 488. Time-lapse experiments with reconstituted embryos were performed on Leica SP5 confocal microscope, equipped with an EMBL incubator (37°C, 5% CO₂). Excitation wavelengths 488 and 561 nm, HCX PL APO 40x/1.1 water objective and HyD detectors were used for detection of fluorescent signal.

Image analysis

Image analysis, kinetochore and micronuclei counting and blastomere tracking was performed using the following software: ImageJ 1.52m (NIH), LAS AF 2.7.3 (<http://www.leica-microsystems.com>) and Imaris software 9.2.1 (www.bitplane.com).

Statistical analysis

Graphpad Prism 5 for Mac OS X (<https://www.graphpad.com/scientific-software/prism/>) was used for statistical analysis, in particular, D'Agostino & Pearson omnibus normality test, one-tailed Student's *t*-test, Mann–Whitney *U* test and Chi-square test were used.

Results

Aneuploidy of mouse early embryos increases from 8- to 16-cell stage

The systematic study focused on the aneuploidy in various stages of mouse embryo development is not

available. In order to assess aneuploidy in zygotes, 2-cell, 4-cell, 8-cell and 16-cell embryos mice were stimulated by PMSG followed by hCG and mating and embryos at 18–21, 44–47, 58–60, 65–68 and 74–77 h post-hCG were isolated. To process the embryos for scoring chromosomes, we used protocol modified from Duncan *et al.* (2009), and from our previous assessment of aneuploidy in oocytes (Danylevska *et al.* 2014). The procedure additionally involved synchronization of embryos in metaphase by colcemid, followed by brief treatment with Mg132 allowing to restore the spindle and to prevent anaphase, and final synchronization by dimethylenastron simultaneously with Mg132, which led into metaphase cells with monopolar spindles with scattered chromosomes. Embryos were then processed for kinetochore staining and scanning as previously (Duncan *et al.* 2009, Danylevska *et al.* 2014) (Fig. 1A). This procedure was used to analyze 125, 128, 208, 296 and 181 individual blastomeres from zygotes, 2-cell, 4-cell, 8-cell and 16-cell embryos, respectively (Fig. 1B). Our data showed that the frequency of aneuploidy

per blastomere from zygotes until 8-cell embryos was relatively similar, ranging from 4.00 to 6.76% (Fig. 1B). In 16-cell stage embryos, the aneuploidy per blastomere increased to 11.05%. With the exception of 16-cell embryos, the data shown in Fig. 1B are only from embryos, in which all blastomeres within individual embryos were scored. With this method, we were unable to resolve all blastomeres at 16-cell stage, and therefore in our report, we show only blastomeres from embryos, in which we were able to score 8 or more blastomeres per embryo. Since the whole embryos were processed for scoring, the experiments also directly revealed the frequency of aneuploidy per embryo (with the exception of the 16-cell embryos) (Fig. 1C). Our data clearly showed that the elevating level of aneuploidy per embryo, reaching in 8-cell embryos 18.92%, is mostly caused by increasing number of cells within the embryos. In 16-cell embryos, the aneuploidy per embryo increased to 68.42% (Fig. 1C). This frequency is significantly higher than in the previous stages, but at this stage we simultaneously detected an increase

Figure 1 The frequency of aneuploidy during mouse early development. (A) Representative images of individual stages of early mouse embryos after immunostaining. DNA (red) is visualized by DAPI staining and kinetochores (green) by CREST antiserum. Scale bar: 10 μ m. (B) The table and chart show the frequency of aneuploidy in individual blastomeres of zygotes and 2- to 16-cell embryos. (C) The table and chart show the frequency of aneuploidy in zygotes and 2- to 16-cell embryos. Bars represent aneuploidy per embryo in each stage, line refers to aneuploidy per blastomere. Data were obtained in three to nine individual isolations in each embryo stage, the numbers of blastomeres and embryos are indicated.

of aneuploidy per blastomere and more embryos carrying aneuploid blastomere. Our results indicate that chromosome division at the 8-cell stage is more error prone than in the previous stages.

Numerical chromosomal aberrations, involving only a few chromosomes, are the prevailing type of aneuploidy

The scoring technique also revealed the number of chromosomes contributing to the aneuploidy in each individual blastomere. The polyploidy, resulting most likely from the failure of cytokinesis, was observed in one out of five aneuploid zygotes and in half of the aneuploid 2-cell embryos (Table 1A). However, beyond the 2-cell stage, we were unable to detect any polyploid blastomeres. This indicates in part that the failure of cytokinesis is not a frequent division error after the 2-cell stage. It, however, also means that the blastomeres with polyploid genome typically do not progress beyond the 2-cell stage. The aneuploidy observed in 4- to 16-cell stage embryos were exclusively numerical chromosomal aberrations, affecting up to two chromosomes. Since all embryos up to 8-cell stage were completely analyzed, our data also revealed how many cells are on average affected in each aneuploid embryo (Table 1B). As expected, aneuploidy in zygotes and 2-cell embryos affects all blastomeres. In 4-cell embryos 2 blastomeres and in 8-cell embryos 2.86 blastomeres were on average affected.

The duration of the cell cycle was longer in hyperploid blastomeres

To study the fate of blastomeres suffering from hyperploidy, zygote was fused with the polar body (PB) and then reconstituted embryos were prepared (Fig. 2). At this stage, usually, a second PB is present, whereas the first PB is degraded (Wakayama & Yanagimachi 1998). The fusion with polar body is admittedly rare during normal circumstances, but it allowed us to obtain cells, which would not suffer from a lack of gene expression. Also, since the fusion was monitored as ingested PB formed a third pronuclei inside the zygote, this method allowed to transfer only cells, which were certainly hyperploid

(Fig. 2A and B). From the time-lapse experiments, it was apparent that during the first cell division, endogenous chromosomes, as well as chromosomes from fused PB, aligned on a single spindle, similar to our previously published results (Novakova et al. 2016). The hyperploid embryos were cultured until the 4-cell stage, then the individual blastomeres were separated after zona removal and the single hyperploid blastomeres were transferred into control embryos, from which one blastomere was removed (Fig. 2C and D). Each reconstituted 4-cell embryo, therefore, contained three control blastomeres and one hyperploid blastomere. Since the zygotes before fusion and also the control zygotes were microinjected with histone tagged by different fluorescent proteins, hyperploid blastomeres in dividing embryos were distinguishable from the control blastomeres (Fig. 2D). The development of reconstituted embryos was monitored by confocal live-cell imaging. We observed that both, the hyperploid as well as control blastomeres frequently suffered from lagging chromosomes, which sometimes caused the formation of micronuclei (Vázquez-Díez et al. 2016) (Fig. 3A). Taking advantage of differently labeled control and hyperploid blastomeres, we assessed the occurrence of micronuclei in both (Fig. 3B). Although the total number of micronuclei we scored in 257 control blastomeres was lower than in 70 hyperploid blastomeres, the frequency of micronuclei per blastomere was not significantly different between both groups (3.50% vs 7.14%). We also analyzed the frequency of the ‘inner’ and ‘outer’ divisions in control and hyperploid blastomeres during the 8-cell division (Fig. 3C). Our results showed that 59.52% of control and 64.29% of hyperploid blastomeres showed ‘outer’ division, whereas the remaining cells divided as ‘inner’ division, and there was no difference between hyperploid and control blastomeres (Fig. 3D). In both results, the frequency of micronuclei and the position of the daughter cells during division in 8-cell embryos indicated that the hyperploid blastomeres behaved relatively similar in comparison to the control blastomeres. However, when we analyzed the duration of the interphase and mitosis during 8- to 16-cell, 16- to 32-cell and 32- to 64-cell divisions, hyperploid blastomeres showed slower division (Fig. 4). In all analyzed divisions, both the interphase and the mitosis

Table 1 Characterisation of aneuploidy during early development.

	Developmental stage				
	Zygote	2-cell	4-cell	8-cell	16-cell
A					
Number of aneuploid BLs	5	8	12	20	20
Tetraploid BLs	1	4	0	0	0
BLs with numerical chr. aberrations	4	4	12	20	20
B					
Number of embryos with aneuploidy	5	4	6	7	
Average number of aneuploid BLs per embryo	1.00	2.00	2.00	2.86	
% of aneuploid BLs per affected embryos	100.00	100.00	50.00	35.75

Figure 2 Induction of hyperploidy in zygotes and formation of reconstituted embryos. (A) Scheme of fusion between zygote and the polar body. (B) Time frames from live cell imaging experiment showing third nuclei in zygotes after fusion and division of hyperploid and control zygotes, upper panels represent control cell, lower panels represent cell after fusion with polar body. Histone H2B (red in control and green in fused zygote) is visualized by microinjected cRNA of histone H2B fused to EGFP and mCherry fluorescent proteins. 0 h = GVBD. Scale bar: 20 μ m. (C) Overview of the procedure for creation of reconstituted embryos. (D) Time frames from live cell imaging experiment showing the development of a reconstituted embryo. Histone H2B (red in control and green in hyperploid blastomeres) is visualized by microinjected cRNA of histone H2B fused to EGFP and mCherry fluorescent proteins. 0 h = first frame. Scale bar: 20 μ m.

Figure 3 Frequency of micronuclei and division type in reconstituted embryos. (A) Time frames from live cell imaging shows the formation of micronuclei from the lagging chromosomes. Upper panels represent control blastomeres, lower panels represent hyperploid blastomeres. DNA is visualized by microinjection of cRNA encoding histone H2B fused to mCherry (red, control blastomeres, upper panels) or EGFP (green, hyperploid blastomeres, lower panels) fluorescent proteins. Lagging chromosomes after mitoses (left figures) and micronuclei are indicated by frame and showed in detail. Scale bar: 20 μ m. (B) The chart represents the frequency of micronuclei in control ($n=257$) and hyperploid ($n=70$) blastomeres during the development of reconstituted embryos. 3.50% of control and 7.14% of hyperploid blastomeres carried micronuclei. Data were obtained in two independent experiments. The difference between control and hyperploid blastomeres was not statistically significant ($\alpha < 0.05$; $P=0.2006$). (C) Images illustration of the 'inner' and 'outer' divisions in control and hyperploid blastomeres. Scale bar represents 10 μ m. (D) The plot showing frequency of the 'inner' and 'outer' divisions in control ($n=42$) and hyperploid ($n=14$) blastomeres during 8-cell division. 59.52% of control and 64.29% of hyperploid blastomeres showed 'outer' division, 40.48% of control and 35.71% of hyperploid blastomeres showed 'inner' division. Data were obtained in two independent experiments. The difference between control and hyperploid blastomeres was not statistically significant ($\alpha < 0.05$; $P=0.7520$).

were longer in hyperploid cells than in the control cells. The accumulated delay in hyperploid blastomeres throughout three divisions (8–16, 16–32 and 32–64) was on average about 7 h. In order to assess whether blastomere transfer could be responsible for cell cycle delay, we performed a similar experiment but instead of transferring hyperploid blastomere, normal blastomere without fusion was transferred. Our results showed that the blastomere transfer alone is not significantly affecting the cell cycle progression (Supplementary Fig. 1, see section on [supplementary materials](#) given at the end of this article).

The number of hyperploid blastomeres declined faster than the control blastomeres

The live imaging experiments showed that the cell cycle in hyperploid blastomeres was slower than in the control blastomeres. Using the previously generated data, we

studied the proportion between hyperploid and control blastomeres during the cleavage cycles. Scoring showed that the proportion 3/1 between control and hyperploid blastomeres in newly reconstituted 4-cell embryos was maintained also in 8-cell embryos, but then it changed to 3.48/1 in 16-cell embryos and finally into 4.38/1 in 32-cell embryos (Fig. 5A). The shifted ratio between control and hyperploid blastomeres indicated that the hyperploid blastomeres more frequently arrested in development. We, therefore, analyzed the occurrence of cells with fragmented DNA or arrested in cell cycle for each line (Fig. 5B). The results showed that in 257 divisions of control blastomeres, 5% showed fragmented DNA or cell cycle arrest, whereas in 70 hyperploid blastomeres this was observed in 23%. We believe that the higher rate of cell cycle arrest and DNA fragmentation in hyperploid embryos could be responsible for shifting the proportion between control and hyperploid blastomeres observed in our experiments.

Figure 4 Length of the interphase and mitosis in hyperploid and control blastomeres. (A) Left scatterplot shows a comparison of the length of interphase in 8-cell embryonic hyperploid (12:79 h, $n = 14$) and control (09:89 h, $n = 42$) blastomeres. Right scatterplot shows a comparison of the length of mitosis in 8-cell embryonic hyperploid (00:91 h, $n = 14$) and control (00:50 h, $n = 42$) blastomeres. Red line represents mean. Data were obtained in two independent experiments. The difference between control and hyperploid blastomeres was significant in both interphase ($\alpha < 0.05$; $***P < 0.0001$) and mitosis ($\alpha < 0.05$; $***P = 0.0006$). (B) Left scatterplot shows a comparison of the length of interphase in 16-cell embryonic hyperploid (10:57 h, $n = 23$) and control (09:60 h, $n = 80$) blastomeres. Right scatterplot shows a comparison of the length of mitosis in 16-cell embryonic hyperploid (01:10 h, $n = 23$) and control (00:64 h, $n = 80$) blastomeres. Red line represents mean. Data were obtained in two independent experiments. The difference between control and hyperploid blastomeres was significant in both interphase ($\alpha < 0.05$; $*P = 0.0462$) and mitosis ($\alpha < 0.05$; $***P < 0.0001$). (C) Left scatterplot shows comparison of the length of interphase in 32-cell embryonic hyperploid (12:42 h, $n = 26$) and control (10:10 h, $n = 114$) blastomeres. Right scatterplot shows comparison of the length of mitosis in 32-cell embryonic hyperploid (00:81 h, $n = 26$) and control (00:65 h, $n = 114$) blastomeres. Red line represents mean. Data were obtained in two independent experiments. The difference between control and hyperploid blastomeres was statistically significant in both interphase ($\alpha < 0.05$; $***P < 0.0001$) and mitosis ($\alpha < 0.05$; $**P = 0.0073$).

Discussion

Our data presented here demonstrated that the kinetochore counting assay could be used to analyze all blastomeres in individual embryos for aneuploidy up to 8-cell stage. In comparison to other methods based on microarrays, amplification of nucleic acids and sequencing, this method allows evaluation of individual blastomeres, their position within the embryo, the

Figure 5 The ratio between control and hyperploid blastomeres. (A) Individual charts show the proportion between the control and hyperploid blastomeres in 12 reconstituted embryos. In 4-cell embryos the proportion was 21:7, in 8-cell embryos 42:14, in 16-cell embryos 80:23 and in 32-cell embryos 114:26. The data are summarizing results from two independent experiments. (B) The plot represents the proportion between dividing and fragmented or arrested control ($n = 257$) and hyperploid ($n = 70$) blastomeres during the development of reconstituted embryos. The frequency of fragmented/arrested blastomeres was 5.00% in control blastomeres and 23.00% in hyperploid blastomeres. The difference between control and hyperploid blastomeres was statistically significant ($\alpha < 0.05$; $***P = 0.0002$).

integrity of the chromosomes, occurrence of precocious segregation of sister chromatids or presence of micronuclei. On the other hand, this method has also significant disadvantages. A major drawback is that it can be used only on cells synchronized in metaphase. Also the kinetochore counting requires a strong signal from kinetochores and a high-resolution confocal microscope. According to our experience, with aim to score all cells within the embryo it becomes difficult to apply this method beyond the 8-cell stage. This is mainly due to embryo compaction, increasing number of cells and their superposition. Nevertheless, we believe that this method could be improved further using microscopy techniques more suitable for thick samples, such as, for example, light-sheet microscopy.

In this study, for the first time, we present a comprehensive analysis of the aneuploidy in zygotes, 2-cell, 4-cell and 8-cell mouse embryos and also an estimation of aneuploidy in 16-cell embryos. Our data demonstrate that the frequency of aneuploidy per blastomere is similar from zygotes until the 8-cell embryos, oscillating between 4 and 7%. Such frequency

is comparable to the 3–8% aneuploidy reported previously from mouse metaphase II eggs (Duncan *et al.* 2009, Danylevska *et al.* 2014). It is also consistent with the frequency of micronuclei observed during mouse embryo development (Vázquez-Diez *et al.* 2016). The occurrence of micronuclei was shown to be proportional to chromosomal losses not only in mouse embryos but also in primate (Daughtry *et al.* 2019) and human (Kort *et al.* 2016) embryos. Our data show that the increase of aneuploidy per embryo from zygotes up to 8-cell stage is therefore mainly driven by accumulating blastomeres. This seems to change with the 16-cell embryos. In this case, the higher number of cells, is boosted by increased frequency of aneuploidy per blastomere (6.76% in 8-cell stage in comparison to 11.05% in 16-cell stage) and also by relatively more affected embryos (7 out of 37 in 8-cell stage in comparison to 13 out of 19 in 16-cell stage). The contribution of all three factors most likely contributed to the elevated frequency of aneuploidy per embryo at this stage, which reached 68.42%. Admittedly, our data from the 16-cell stage embryos are not as thorough as from the previous stages, due to the fact that we were unable to resolve and score all blastomeres within each individual embryo. The observed increase of the aneuploidy at the 16-cell stage is, however, consistent with the occurrence of micronuclei, which almost doubled between 8- and 16-cell stage (Vázquez-Diez *et al.* 2016) and also with a recent report showing aneuploidy in rhesus monkey cleavage stage embryos reaching 73.5% (Daughtry *et al.* 2019). We can only speculate what could be the underlining mechanism driving this increase of aneuploidy per blastomere and also per embryo between 8- and 16-cell embryos. However, during the 8-cell stage, substantial changes are initialized, including for example an asymmetric division of blastomeres underlying the acquisition of the cell fate. And it remains to be tested how these events are contributing to the overall aneuploidy.

Surprisingly, in our experiments, we were not able to detect embryos affected by total aneuploidy beyond the 2-cell stage. Occurrence of aneuploidy in zygotes and 2-cell embryos would inevitably lead into embryos with total aneuploidy in later stages. However, in our study, all embryos with aneuploidy after 2-cell stage were mosaic, with only several blastomeres affected. It is important to emphasize, that for analysis of aneuploidy, we used solely *in vivo* developed embryos in our study. The embryos were cultured *in vitro* only for a limited amount of time necessary for achieving synchronization in mitosis. The whole embryo aneuploidy was, however, reported in mouse blastocysts (Treff *et al.* 2016), cleavage stage embryos of rhesus monkey (Daughtry *et al.* 2019) or human cleavage stage embryos (Kort *et al.* 2016). In all these cases, however, the embryos were obtained after fertilization and development *in vitro*. Our results might indicate, that the aneuploidy affecting the whole embryo, is not compatible with *in vivo* development after

the 2-cell stage and it is conceivable, that such embryos might be eliminated by yet unknown mechanism. We can only speculate whether this mechanism is linked to the zygotic genome activation (ZGA), occurring simultaneously at this stage (Jukam *et al.* 2017). For example, it was hypothesized that in human embryos, in which the ZGA occurs later, this process is accompanied by increased frequency of blastomere arrest (Mantikou *et al.* 2012). It is also possible that the total aneuploidy slows down the *in vivo* developing embryos to the extent that such embryos might be absent during the collection of samples of each particular stage. This is however unlikely since the embryos with early segregation errors continue in development, at least *in vitro* (Mashiko *et al.* 2020). Similarly, we did not detect any embryos carrying polyploid blastomeres after the 2-cell stage. It indicates that *in vivo* blastomeres after the 2-cell stage are not prone to the failure of cytokinesis.

It was shown previously that the mosaic aneuploidy in the embryos is lethal if the number of normal cells is not sufficient to support embryonic development (Lightfoot *et al.* 2006, Bolton *et al.* 2016). In contrast to this, recent experiments showed that even embryos with early chromosome segregation errors are capable of completing embryonic development, although the blastocyst rate is lower (Mashiko *et al.* 2020). In our experiments, we tested whether hyperploid blastomeres, transferred under zona pellucida of control 4-cell embryos, developed similar to the control blastomeres. The extra chromosomes ensured that albeit the blastomeres had unbalanced genome, they were not limited by the absence of gene expression. Scoring of the frequency of micronuclei showed no significant difference between control and hyperploid blastomeres. We also found no difference in the distribution of hyperploid blastomeres between 'outside' and 'inside' dividing cells at the 8-cell stage. These results indicated that the hyperploid blastomeres adapted normal behavior in the microenvironments of the reconstituted embryos. Our results showed that the hyperploid blastomeres survive in mouse embryos between 8- and 64-cell stage, although their numbers are reduced in comparison to the control blastomeres. In the previously published study, it was shown that the reduction of aneuploid blastomeres started only after early blastocysts formation, and it mainly concerned the ICM lineage (Bolton *et al.* 2016). The differences between our report and the previous study might be related to the degree of the aneuploidy. In the study by Bolton *et al.* (2016), the authors used spindle assembly checkpoint (SAC) abolition to induce aneuploidy. This procedure created both hyperploid and hypoploid blastomeres, with aneuploidy affecting only a few chromosomes within the cell. In our study, we created hyperploid blastomeres, with presumably 1.5 C. Multiple extra chromosomes perhaps created a severe problem to assemble the spindle apparatus correctly, and therefore

the anaphase was delayed in comparison to the control blastomeres. This is consistent with a recent report showing that the tetraploid blastomeres suffer from congression defects and their mitosis is longer, which depends on SAC activity (Paim & FitzHarris 2019). The analysis of the cell cycle showed that the hyperploid blastomeres had an overall slower cell cycle progression. The delay in each cell cycle could also contribute to the underrepresentation of aneuploid blastomeres in the next developmental stage. Interestingly, during divisions of hyperploid blastomeres, we did not observe multipolar mitoses, which are known from human oocytes and somatic cells (Kuffer *et al.* 2013). In our live cell imaging experiments, we observed that the DNA of hyperploid blastomeres fragmented more frequently and cells remained arrested in the cell cycle. Whereas in the group of control cells we observed only arrested blastomeres and none of them showed DNA fragmentation, in hyperploid blastomeres the DNA fragmentation was seen in 50% of arrested blastomeres.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/REP-20-0086>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

T P, L R, K K and L N involved in experimental work and data acquisition. T P, L R, K K and M S involved in data analysis. M A and T P involved in writing the manuscript. M A contributed in conceptualization and supervision and funding.

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