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Regulation of the cell cycle in early mammalian embryos and its clinical implications

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ABSTRACT Early embryonic development is characterized by a plethora of very complex and simultaneously operating processes, which are constantly changing cellular morphology and behaviour. After fertilization, blastomeres of the newly created embryo undergo global epigenetic changes and simultaneously initiate transcription from the zygotic genome and differentiation forming separate cell lineages. Some of these mechanisms were extensively studied during the last several decades and valuable insight was gained into how these processes are regulated at the molecular level. We have, however, a still very limited understanding of how multiple events are coordinated during rapid development of an early mammalian embryo. In this review, we discuss some aspects of early embryonic development in mammals, namely the fidelity of chromosome segregation and occurrence of aneuploidy, as well as the clinical applications of cell cycle monitoring in human embryos.

KEY WORDS: *embryo, development, cell cycle, aneuploidy*

Aneuploidy and the chromosome segregation errors in developing embryos

Aneuploidy is the leading cause of developmental and mental disorders in human. Incidence of aneuploidy in newborns is about 0.3% (Hassold *et al.*, 1996), in stillbirths and spontaneous abortions the frequency of aneuploidy is even higher ~ 4% and ~ 35% respectively (Hassold and Hunt, 2001). Most of the aneuploidy originates from the oocyte, in sperm the incidence is much lower, about 2% (Hassold and Hunt, 2001). In preimplantation embryos, the frequency of aneuploidy is also extremely high. Data combined from 36 studies analysing 815 human embryos altogether showed that only 22% of the embryos were diploid, 73% were mosaic and 5% harboured other abnormalities (van Echten-Arends *et al.*, 2011).

During early embryonic development, chromosomal abnormalities seem to be generally tolerated, until the onset of transcription from the zygotic genome, which in human takes place at 4-8 cell stage (Fragouli *et al.*, 2013). In general, the aneuploidy in embryonic blastomeres might result from mitotic errors in primordial germ cells, meiotic errors in oocytes or sperm or mitotic errors after fertilization. Whereas the aneuploidy originating in germ cells will affect all blastomeres in the newly forming embryo, the aneuploidy resulting from divisions of blastomeres after fertilization causes mosaic aneuploidy, unless the segregation defect occurred during the first mitosis (Jones and Lane, 2013; Lee and Kiessling, 2017). In case of whole embryo aneuploidy, or aneuploidy carried from the germ cells, only three trisomies of autosomal chromosomes 13 (Patau syndrome), 18 (Edwards syndrome), 21 (Down syndrome) and aneuploidy of sex chromosomes are partially compatible with the further foetal development (Jones and Lane, 2013; Lee and Kiessling, 2017). Embryos with aneuploidy occurring later and resulting in mosaicism might still have full developmental potential, if the number of euploid cells in the embryo is sufficient (Taylor *et al.*, 2014; Bolton *et al.*, 2016).

The reported frequency of aneuploidy in human embryos varies

Abbreviations used in this paper: APC/C, anaphase promoting complex/cyclosome; ATM, ataxia telangiectasia mutated; ATR, ataxia telangiectasia mutated and Rad3-related; BUB, budding uninhibited by benzimidazole; CDK, (cyclin dependent kinase; CGH, comparative genome hybridisation; CHK, checkpoint kinase; FISH, fluorescence *in situ* hybridisation; GV, germinal vesicle; ICM, inner cell mass; MAD, mitotic arrest deficient; MBT, midblastula transition; MCC, mitotic checkpoint complex; MPF, maturation promoting factor; MTOCs, microtubule organizing centres; RAN, RAs-related nuclear protein; SAC, spindle assembly checkpoint; SCMC, subcortical maternal complex; PGS, preimplantation genetic screening; TL, time-lapse; ZGA, zygotic genome activation.

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significantly. For example, scoring aneuploidy by fluorescence *in situ* hybridization (FISH) in 216 human embryos showed that 48.1% of embryos were mosaic and the frequency of aneuploidy was 15.2% in 2-4 cell, 49.4% in 5-8 cell and 58.1% in morula stage (Bielanska *et al.*, 2002). Comparative genomic hybridisation (CGH) analysis and FISH analysis of 30 human frozen-thawed embryos at day 3 and day 5 showed an increase of chromosomal abnormalities between these two developmental stages (Daphnis *et al.*, 2008). CGH and whole genome amplification analysis of 158 blastocysts showed that the frequency of aneuploidy in blastocysts stage is significantly lower – 38.8% in comparison to the early stages, where the aneuploidy affected 51% of embryos (Fragouli *et al.*, 2008). Although the lower frequency of aneuploidy in blastocysts could be caused by a relatively lower number of analysed cells, decline of aneuploidy at the blastocyst stage or later was reported in human embryos (Evsikov and Verlinsky, 1998) and recently also in mouse embryos (Bolton *et al.*, 2016). The variance among published data is largely caused by differences between techniques used for scoring, and it is further emphasized by the variability of procedures in each laboratory, quality of analysed embryos, freezing-thawing protocols and other factors (van Echten-Arends *et al.*, 2011). Therefore, it is always important to compare results obtained by the same method.

Mechanistically, aneuploidy is caused by chromosome segregation errors during cell division and it was shown that certain situations during this process might lead into aneuploidy more frequently (Taylor *et al.*, 2014). For example, the lagging chromatids during anaphase, caused in somatic cells by erroneous microtubule to kinetochore attachment, frequently lead into their missegregation. Lagging chromatids are sometimes detected in dividing blastomeres of the early embryo. However, it is not clear whether the mechanism of their origin is similar to the somatic cells. From somatic cells it is known that the connection between kinetochores and the spindle apparatus is critical for the accurate chromosome segregation into daughter cells. Amphitelic attachment, when sister kinetochores are connected to the opposite spindle poles, is a prerequisite for faithful chromosome segregation. Kinetochores attached by syntelic (both sister kinetochores attached to the same spindle pole) or merotelic (one kinetochore is attached to both spindle poles) attachments increase chance of chromosome segregation defects, such as the lagging chromatids during anaphase (Khodjakov and Pines, 2010). Particularly the merotelic attachment, which escapes detection by the spindle assembly checkpoint (SAC), may last until anaphase and result in lagging chromatids (Gregan *et al.*, 2011).

Aneuploidy might also arise from an unscheduled separation of sister chromatids. In physiological conditions, sister chromatids separate at anaphase, after separase-dependent removal of cohesion between their centromeres. Results from somatic cells showed that a precocious separation of sister chromatids before anaphase, caused by cohesion defects, resulted in aneuploidy (Mirkovic *et al.*, 2015). In oocytes, a failure to protect cohesion between the sister chromatids during the first meiotic anaphase has similar consequences (Yun *et al.*, 2014). Aneuploidy also arises, when both homologous chromosomes during meiosis I in oocytes, or sister chromatids during mitosis, co-segregate into one cell (Kuliev and Verlinsky, 2004). A less frequent causes of aneuploidy, sometimes detected in embryos, include premature division of unreplicated chromosomes, cytokinesis defects leading to tetraploidization, cell fusion, endoreduplication and chromosome breakage (Mantikou *et al.*, 2012; Taylor *et al.*, 2014). A phenomenon called chromothripsis, which involves lagging chromatids encapsulated by micronuclei, might lead to severe damage of the genetic material in somatic cells. Although it is possible that chromothripsis could also compromise the development of early embryos (Pellestor *et al.*, 2014), recently it has been shown that the sequestration of micronuclei from the remaining genetic material probably prevents initiation of chromothripsis in mouse embryos (Vázquez-Diez *et al.*, 2016).

Cleavage cycles of developing embryos – the first mitoses are unique

After fertilization, zygote engages into series of cleavage cycles, which are in various aspects significantly different from somatic mitoses. For example, the first cell cycle in *Xenopus* is three times longer than the following twelve 30-minute synchronous cycles, after which the cycles are slower again (Newport and Kirschner, 1982; O'Farrell *et al.*, 2004). In mouse embryos, the first two cell cycles are both approximately 18-20 hours long. However, the duration of individual phases of the cell cycle is significantly different (Fig. 1). The most dramatic difference between these two cycles is in the length of the G2-phase. While in the first mitosis G2 lasts about 4 hours, which in comparison to the somatic cell cycle is still quite long, during the second division blastomeres spend on average 15 hours in G2 (Sawicki *et al.*, 1978; Howlett and Bolton, 1985). Such prolonged G2 might be required for an activation of the zygotic genome (ZGA), which begins in G2-phase of the first cell cycle, but the main activation occurs in the longer G2-phase of the second mitotic division (Flach *et al.*, 1982; Bouniol *et al.*, 1995). It would be interesting to know, whether species with later ZGA, such as human or cattle, exhibit comparably prolonged G2-phase during their ZGA.

Another remarkable difference between the first and the second cell cycle, which is conserved among several species, including

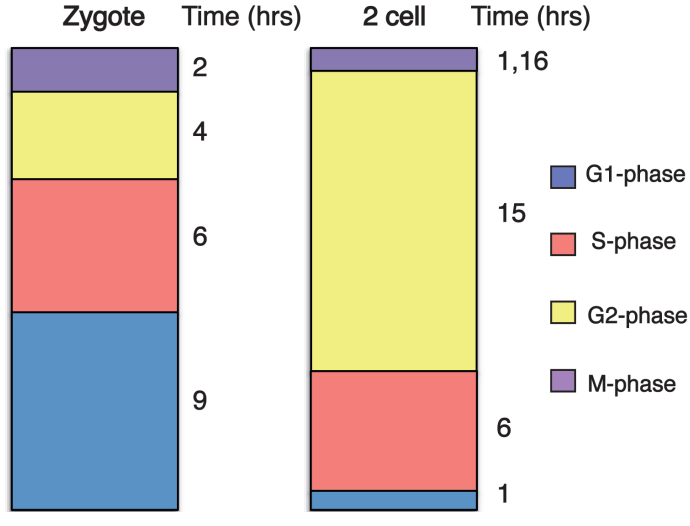

Fig. 1. The differences in duration of individual phases of the first and the second cell cycle in the mouse embryo. *Bars represent the length of the cell cycle in zygotes and 2 cell stage embryos. The duration of G1 (blue), S (red), G2 (yellow) and M phase (purple) phases are shown proportionally to the duration of each cycle. Results from Sawicki* et al.*, 1978; Howlett and Bolton, 1985 and Ciemerych* et al.*, 1999 were used for this figure.*

Caenorhabditis elegans, *Sphaerechinus granularis*, *Xenopus laevis* and *Mus musculus*, is a prolonged M-phase during the first cell cycle (Ciemerych *et al*., 1999; Chesnel *et al*., 2005; Sikora-Polaczek *et al*., 2006; Kubiak *et al*., 2008). In mouse, the first mitosis requires approximately 120 minutes, whereas the second mitosis is only about 70 minutes long (Ciemerych *et al*., 1999). This phenomenon also appears to be associated with the dynamics of the maturation promoting factor (MPF) activity, which differs significantly in both divisions. While in the first mitosis the MPF activity reaches plateau lasting approximately 40 minutes, in the second mitosis the profile of MPF activity is similar to the somatic cells, rising gradually to its maximum and then abruptly decreasing (Ciemerych *et al*., 1999). According to the recent report, the MPF activity lasts longer during the first mitosis due to the delayed activation of anaphase promoting complex/cyclosome (APC/C) (Ajduk *et al*., 2017).

The length of subsequent embryonic cell cycles in mouse is shorter, about 12 hours (Smith and Johnson, 1986) and in mammals the early cleavage cycles are not synchronized (Gamow and Prescott, 1970). It was also observed that the size differences between individual blastomeres might play role in the asynchrony of the cell divisions. In mouse, the smaller blastomeres finish their fifth cell cycle within 14 hours, whereas the bigger ones need only 12 hours to complete this division (MacQueen and Johnson, 1983). The differences in the dynamics of the cell cycle are further increased when the embryo reaches the blastocyst stage (approximately 4 days in mouse, 5 days in human). Blastocyst is composed of trophoblast cells, later forming placenta, and the inner cell mass (ICM) giving rise to the embryo body and from which embryonic stem cells can be derived. The cell cycle of ICM cells and the trophoblast differs significantly. Whereas the trophoblast giant cells after implantation undergo endoreduplication resulting in genome amplification (Varmuza *et al*., 1988), cells in ICM proliferate in rapid cell cycles with reduced G1 and G2 phases and maintain their diploid state (Savatier *et al*., 2002).

Driving forces behind cleavage cycles

Overall complexity of the cell cycle regulation in mammalian embryos is emphasized by the fact that it is initially achieved without transcription from the embryo and relies on the maternal stockpile instead. Only after the ZGA (reviewed in (Jukam *et al*., 2017)), embryos initiate a fully independent cell cycle program. In mouse, the initiation of transcription is detectable in male pronucleus in zygote and then the main ZGA follows during 2 cell stage (Aoki *et al*., 1997; Abe *et al*., 2018). In other mammalian species, the ZGA appears later, for example during 4-8 cell stage in human and cattle and during 8-16 cell stage in sheep and rabbit, and the waves of transcription are not short and focused as in mouse (Schultz and Heyner, 1992; Nothias *et al*., 1995) (Fig. 2). This means that in mammalian species with the later ZGA, regulatory molecules important for controlling the first cleavage cycles are provided from the maternal resources and therefore in some species must sustain until the 16 cell stage.

In both somatic and embryonic cells, the mechanism driving cells through the cell cycle is based on sequential activation of kinases and phosphatases (reviewed in (Hunter, 1995)). The key molecular complex, controlling events in mitosis and meiosis is called MPF, originally described by Masui and Clarke in late seventies as complex of cyclin B and CDK1 (Masui and Clarke, 1979). Throughout the years additional regulatory molecules were discovered and therefore now we recognize MPF as the activity of not only the originally described cyclin B/CDK1 complex, but also other associated kinases and phosphatases, required for controlling its activity (Hégarat *et al*., 2016). The activity of MPF rises before mitosis and it is responsible for dramatic events observed in this stage, such as dissolution of the nuclear membrane, chromosome condensation and spindle assembly. In cells approaching anaphase, MPF activity decreases, which is facilitated by targeting cyclin B for destruction by APC/C (Pines, 2011).

The core system, controlling cell cycle in somatic cells, is also preserved in mammalian embryos. However, there are important differences resulting from adaptation to sequential cell divisions. Gene knockout experiments showed that some molecules, which are required for cell cycle control in somatic cells, are also essential during the cleavage cycles of the early embryos. For example, without cyclin B1 embryos are unable to develop and die during the initial stages of development (Brandeis *et al*., 1998; Strauss *et al*., 2018). WEE1, an important regulator of MPF activity, is also essential, and its deletion is very early lethal (Tominaga *et al*., 2006). APC/C activity is required also in early cleavage cycles and deletion of *Cdc20* gene causes arrest in 2 cell stage, which eventually results in apoptosis (Li *et al*., 2007). Some cell cycle regulators are however initially dispensable, although they might be required later, during or after gastrulation. Cyclin A2 deletion showed delayed lethality after day 5 and also protein was not detectable in early embryos after 2 cell stage until blastocyst. This indicates that this cyclin is dispensable during the early embryonic development after ZGA (Murphy, 1999; Winston *et al*., 2000; Hara *et al*., 2005). Deletion of all D cyclins does not affect early development and embryos arrest only long after implantation (Kozar *et al*., 2004). All these experiments illustrate that early cleavage cycles are somewhat unique and that the cell cycle machinery seems to be modified in order to support uninterrupted divisions of the early embryo.

Monitoring of cell cycle progression by surveillance mechanisms in early embryos

Cell cycle progression is monitored by multiple pathways called checkpoints (reviewed in (Hartwell and Weinert, 1989; Harashima *et al*., 2013)). Monitoring important events during cell cycle ensures, that the genetic material transferred to a new generation is intact. The fidelity of checkpoints is also important for preventing cancer in multicellular organisms (Kastan and Bartek, 2004). The cleavage cycles of early developing embryos are however unique in many aspects. For example, DNA replication in embryonic mitoses in *Xenopus* is extremely fast and could be accomplished within 30 minutes (reviewed in (Kermi *et al*., 2017)). Therefore, it was initially not obvious, whether similar checkpoints, known from somatic cells, are operating in early embryos. And despite some progress in the recent years, our information about the function of cell cycle checkpoints in early mammalian embryos, is still very limited.

For example, somatic cells respond to DNA damage or to unreplicated chromosomes by activating pathways involving ataxia–telangiectasia-mutated (ATM) and ataxia-telangiectasia-mutated-and-Rad3-related (ATR) kinases and checkpoint kinase 1 and 2 (CHK1, CHK2) (Harrison and Haber, 2006). Their activation leads to cell cycle arrest until the problem is resolved or until

programmed cell death. In *Xenopus* embryos these pathways are not active before the mid-blastula transition (MBT) and the blastomeres in stages before MBT can enter cell cycle with unreplicated or damaged DNA (Kimelman *et al.*, 1987; Anderson *et al.*, 1997; Hensey and Gautier, 1997). Similar situation is in zebrafish, where the inhibition of DNA replication also does not prevent the cell cycle progression (Ikegami *et al.*, 1997). Mouse oocytes are able to respond to DNA damage in meiosis I, but not in meiosis II, by activation of SAC machinery and postponing activation of APC/C and anaphase (Lane *et al.*, 2017). In mouse embryos however, the response to DNA damage was not extensively studied. It was nevertheless shown that the depletion of CHK1 is lethal very early (Takai *et al.*, 2000) and the 2 cell mouse embryos exhibit sensitivity to DNA damage induced by UV light, cisplatin or laser (Mu *et al.*, 2011; Wang *et al.*, 2013). This indicates that the mouse embryo, in contrast to *Xenopus* or zebrafish, is capable of responding to DNA damage immediately after fertilization, perhaps because of the early onset of ZGA. However, we definitely need more studies to clarify this.

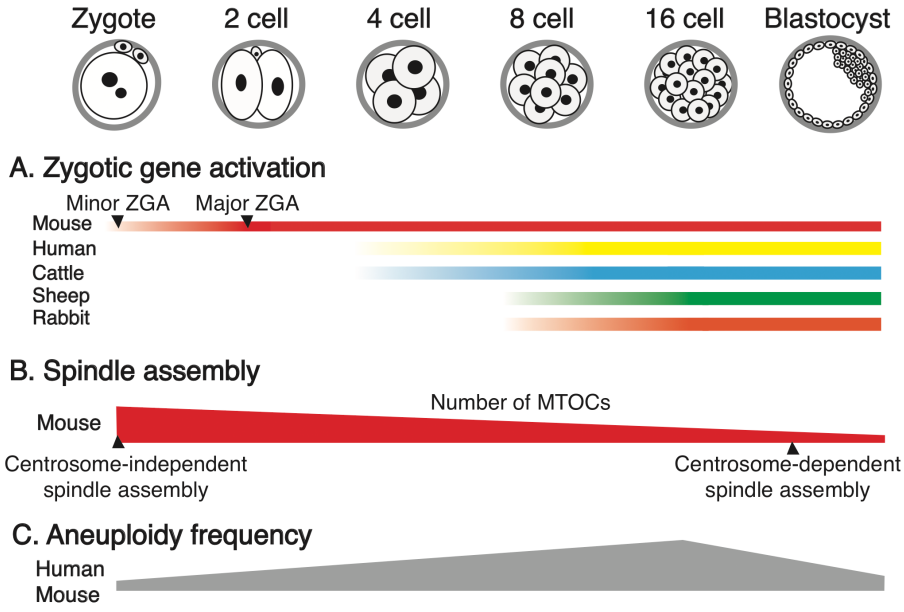

Fig. 2. The temporal relationship between zygotic gene activation, acentrosomal spindle assembly and aneuploidy frequency. *Schematic view of the timing of zygotic gene activation* **(A)***, number of MTOCs* **(B)** *and frequency of aneuploidy* **(C)** *is shown for selected species as indicated. Results from articles by Aoki* et al.*, 1997; Abe* et al.*, 2018; Schultz and Heyner, 1992; Nothias* et al.*, 1995; Gueth-Hallonet* et al.*, 1993; Courtois* et al.*, 2012; Howe and FitzHarris, 2013; Fragouli* et al.*, 2008; Evsikov and Verlinsky, 1998; Bolton* et al.*, 2016 were used for this figure.*

Another important checkpoint mechanism is SAC, which monitors the attachment of kinetochores to the spindle microtubules in mitosis, as well as in meiosis (Musacchio, 2011; Foley and Kapoor, 2013; Musacchio, 2015; Marston and Wassmann, 2017). Key molecules are proteins from the mitotic arrest deficient protein family (MAD1, MAD2, MAD3) and the budding uninhibited by benzimidazole family (BUB1, BUB3) and also other proteins, including Aurora B, PLK1, MPS1. Unattached kinetochores during prophase facilitate formation of mitotic checkpoint complex (MCC), consisting of MAD1 and BUBRI proteins, together with BUB3 and CDC20. This complex, by binding to CDC20, inhibits APC/C, which requires CDC20 for its activation. The release of CDC20, upon binding of kinetochore by spindle microtubules, leads into full APC/C activation and eventually into anaphase. Simultaneously operating pathway involving Aurora B dissolves attachments, which do not produce tension between sister kinetochores. SAC therefore ensures not only that all kinetochores are attached to the spindle microtubules, but also that kinetochores of sister chromatids are attached to the opposite poles of the spindle. Because of the high frequency of aneuploidy, this pathway was extensively studied in oocytes, unfortunately much less is known about the role of SAC in developing mammalian embryos. The deletion of *Mad2*, which in somatic cells is essential for SAC function, in mouse embryos affects mostly rapidly dividing cells in the epiblast after day 6.5 (Dobles *et al.*, 2000). Similar results were obtained with *Bub3* knockout in mouse, indicating that SAC components are required only later during embryogenesis (Kalitsis *et al.*, 2000). Human embryos treated with nocodazole at day 3 respond by apoptosis, but not before day 5. Although SAC challenged by nocodazole is functional, its activity in early human embryos, before the blastocyst stage, seems to cause a prolonged mitotic arrest instead of apoptosis (Jacobs *et al.*, 2017).

For some time, it was speculated that SAC in larger cells, such as mouse oocytes, is absent or lacks certain functions known from somatic cells. Experiments using XO females initially led to the conclusion that SAC is not operating in mouse oocytes (LeMaire-Adkins *et al.*, 1997). Experiments reported later, which involved injection of dominant-negative *Bub1* into GV oocytes (Tsurumi *et al.*, 2004), targeting of *Mad2* by morpholino (Homer *et al.*, 2005) or *Bub1* gene knockout (McGuinness *et al.*, 2009) however showed, that SAC in oocytes is functional and essential for timing of anaphase and for preventing aneuploidy. It is however still not clear, whether SAC in large mammalian gametes or embryos possesses all functions, known from somatic cells. For example, congression defects, which are able to delay anaphase in mitosis, are tolerated in meiosis I (Nagaoka *et al.*, 2011; Lane *et al.*, 2012; Sebestova *et al.*, 2012).

A potential explanation why large cells, such as oocytes or embryos, are unable to respond to various defects in the spindle assembly, was obtained recently. Using nocodazole to induce SAC-dependent arrest in a relatively large *C. elegans* blastomeres showed that the duration of this arrest increases with every subsequent cleavage cycle and seems to be dependent on the cell size. Or more precisely - on a ratio between the cell size, which is changing with every cycle, and the number of kinetochores, which remains constant (Galli and Morgan, 2016). Recent experiments, using mouse oocytes with cell volume altered by micromanipulation, confirmed the original results from *C. elegans* and established a link between cellular volume and SAC strength also in mammalian oocytes (Kyogoku and Kitajima, 2017). It seems that the reason why SAC is relatively weak in large cells is the disproportion between the inhibitory signal created by unattached or erroneously attached kinetochores and the large volume of cytoplasm containing excess of proteins from the APC/C pathway. In another

recently published report authors however showed that although the oocytes with reduced volume are degrading selected APC/C substrates faster, the bivalents, which failed to biorient, are still unable to induce the anaphase delay (Lane and Jones, 2017), which is not consistent with the theory that SAC should be more efficient in smaller cells. Furthermore, it was shown in *C. elegans* that the SAC strength, besides the cell volume, is also linked, by yet unknown mechanism, to the cell fate (Gerhold *et al.*, 2018). In this study authors discovered that the duration of a transient mitotic arrest induced by nocodazole was longer in blastomeres committed to the germline than those differentiating into soma. And their results could be only partially explained by the size differences between these cells. It is clear that more experimental work is needed in order to understand the functionality of SAC in oocytes and embryos.

Regulation of spindle length in mammalian oocytes and early embryos

Blastomeres of early mammalian embryos are exhibiting remarkable changes in size and morphology within a relatively short time. During the first several cleavage cycles, the size of blastomeres is halving with each division, and the size of the nucleus is reduced accordingly by a mutual, but not yet well understood, interaction (Tsichlaki and FitzHarris, 2016). In order to facilitate accurate chromosome segregation, blastomeres of early embryos also regulate the length of their spindles, although differently than in somatic cells, and the similar patterns of the regulation of spindle size can be observed in embryos across many species (Heald and Gibeaux, 2018). Unlike in somatic cells, where spindles usually adjust their size to the entire cell diameter, during the early embryonic development, the spindle size is initially significantly smaller than the cell size, which was shown in *Xenopus, C. elegans* and mouse (Wühr *et al.*, 2008; Hara and Kimura, 2009; Courtois *et al.*, 2012; Yamagata and FitzHarris, 2013). In mouse, increasing cell volume in 2 cell blastomeres by fusion of two or three cells together, increased also the spindle size, which was however still significantly smaller than the size of the cell (Novakova *et al.*, 2016). A transition to the spindles spanning throughout the entire cell size is gradual and in mouse it is completed around 8 cell stage (Courtois *et al.*, 2012) or perhaps even earlier (Yamagata and FitzHarris, 2013). However, it still remains unresolved, how the length of the spindle is regulated during initial stages of embryonic development (Mitchison *et al.*, 2015). It was shown recently that the blastomeres of *C. elegans* and *Paracentrotus lividus* adjust their spindle length to the speed of the microtubule growth, which differs between stages (Lacroix *et al.*, 2018). In parallel to other mechanisms, this might provide another means for synchronizing spindle size with cell diameter, at least in some species (reviewed in (Goshima and Scholey, 2010; Heald and Gibeaux, 2018)).

Another important change during early embryonic development is a transition from the acentrosomal to the centrosomal spindle assembly. In mouse oocytes, the centrosomes are absent and the growth of spindle microtubules is organized from microtubule organising centres (MTOCs) in the vicinity of the chromatin (Szollosi *et al.*, 1972; Calarco-Gillam *et al.*, 1983; Maro *et al.*, 1985; Dumont *et al.*, 2007; Schuh and Ellenberg, 2007). It was shown that the spindle assembly requires multiple steps of decondensation and fragmentation of MTOCs, after which they form two individual spindle poles (Clift and Schuh, 2015). In human oocytes, the spindle assembly takes significantly longer than in mouse oocytes and the process seems to be based on small GTPase RAN, rather than on MTOCs (Holubcová *et al.*, 2015). Although the RAN-mediated microtubule nucleation pathway is important for the spindle assembly also in mouse oocytes, the inhibition of this pathway does not result in a complete blockage of the spindle assembly (Dumont *et al.*, 2007; Schuh and Ellenberg, 2007; Maresca *et al.*, 2009). Knockdown of pericentrin leads to the inability to create MTOCs, disruptions of the spindle assembly and significant chromosome segregation errors (Baumann *et al.*, 2017), suggesting that in mouse oocytes the MTOCs-controlled mechanism of the spindle assembly is more important than the RAN-mediated microtubule nucleation pathway. It is conceivable that prioritizing the RAN GTPase pathway over the MTOCs during the spindle assembly renders human oocytes prone to form multipolar spindles, which are frequently observed in these cells (Holubcová *et al.*, 2015; Haverfield *et al.*, 2017). Throughout the early development the number of MTOCs in mouse embryos gradually decreases, and the centrosome-dependent spindle assembly becomes the main pathway around 64 cell stage (Fig. 2), which was shown by electron microscopy studies, as well as by a detection of centriolar components, such as gamma tubulin (Gueth-Hallonet *et al.*, 1993), pericentrin (Courtois *et al.*, 2012) and centrin-2 (Howe and FitzHarris, 2013). In mouse embryos, the crucial role in the acentrosomal MTOCs assembly is played by microtubule bridges connecting two daughter cells after cytokinesis (Zenker *et al.*, 2017). The site of the spindle assembly is spatially predetermined by microtubule bridges, which provide scaffold of the spindle assembly during the following mitosis.

The role of spindle orientation during early development

During early development, spindle positioning plays also important regulatory role. In oocytes during meiosis, the divisions are highly asymmetric, giving rise to metaphase II egg, containing most of the cytoplasm and one remarkably smaller polar body. This is facilitated by positioning of the spindle close to the cortex before division in anaphase I and also in anaphase II (reviewed in (Mogessie *et al.*, 2018)). However, during embryonic mitoses, central position of the spindle is essential for achieving equal distribution of the cytoplasm into daughter cells. In mouse zygotes, the positioning of the spindle occurs without centrosomes and astral microtubules, and depends mainly on F-actin (Chew *et al.*, 2012; Yu *et al.*, 2014; Chaigne *et al.*, 2016), regulated by subcortical maternal complex (SCMC) via cofilin protein (Yu *et al.*, 2014). F-actin-dependent mechanism is responsible mostly for a coarse positioning of the paternal and maternal pronuclei in the cell centre and the dynamics of the actin mash depends on a molecular motor Myosin Vb as in prophase I oocytes (Almonacid *et al.*, 2015; Chaigne *et al.*, 2016). After nuclear envelope break down, the actin network changes during the spindle formation into an actin cage surrounding the spindle (Chaigne *et al.*, 2016; Chen *et al.*, 2018) as in oocytes (Azoury *et al.*, 2008; Schuh and Ellenberg, 2008) and the actin is responsible for precise centring of the metaphase plate by an increasing cortical tension (Chaigne *et al.*, 2016). Surprisingly, it seems that the maintenance of the spindle in the central position is controlled mainly by passive forces created by viscosity of the cytoplasm (Chaigne *et al.*, 2016; Chaigne *et al.*, 2017). The spindle in the mouse zygote is assembled during a multistep procedure

(Reichmann *et al.*, 2018). The first step is characterized by clustering of growing microtubules near the maternal and paternal pronucleus. Second step includes assembly of two individual spindles, which then both align forming a single barrel-shaped spindle in the third step. This unique, three-step assembly of the spindle provides a mechanistic explanation for previously known spatial separation of the parental chromosomes on the metaphase plate of the zygotic spindle. It is also conceivable that such a complex procedure is more error prone. The blastomeres with two separated nuclei, sometimes observed in IVF clinics, might be a consequence of a failure during the third step of the spindle assembly. Positioning of the spindle plays an essential role also during the first cell fate decision (DeBella *et al.*, 2006). Polarity, resulting from the positioning of the cleavage furrow relative to animal–vegetal axis, starts in some species, such as *C. elegans*, already in the zygote. In mouse, the non-random distribution of cellular material appears in the second cleavage division (reviewed in (Ajduk and Zernicka-Goetz, 2015)). Recently it was shown by several laboratories that the spindle orientation plays an essential role in breaking symmetry in mouse development (Korotkevich *et al.*, 2017), reviewed in (Mihajlović and Bruce, 2017; Chen *et al.*, 2018).

The link between the cell cycle and the quality of the embryos

Preimplantation genetic screening (PGS) introduced in the late 1980s, provided the first possibility for assessing the quality of human embryos (Edwards, 1987). The techniques used for PGS are constantly developing, becoming more accurate, sensitive and also less invasive. Initially, the DNA amplification and karyotyping techniques were used for analysis of polar bodies, isolated from metaphase II eggs or blastomeres of developing embryo. Later, the DNA probes (FISH) were introduced, allowing simultaneous detection of a relatively narrow set of chromosomes (Griffin *et al.*, 1992; Delhanty *et al.*, 1993). Then the CGH and the whole genome amplification were introduced, however, both these techniques are time consuming (Nagaoka *et al.*, 2012). Currently the array-based molecular cytogenetic techniques represent a significant improvement over the previous techniques in accuracy and reliability, however they still require a biopsy of embryonic cells, which might compromise the developmental potential of embryos. Therefore, in our opinion, the future belongs to less invasive techniques, such as the time-lapse (TL) monitoring of embryonic development.

It was observed that deviations from the average timing of cleavage cycles lead to morphological anomalies and developmental defects (Dewey *et al.*, 2015). Monitoring the length and the timing of early cleavages can therefore provide valuable information for selecting the best embryos for transfer. The TL monitoring of embryonic development was successfully used also for other species, for example for bovine embryos (Massip and Mulnard, 1980; Massip *et al.*, 1982). During TL monitoring, the morphological changes of developing embryo are recorded, during which the timing of important changes is obtained (morphokinetic parameters). It was shown that there is a correlation between developmental potential of the embryo and the observed morphokinetic parameters (Desai *et al.*, 2018). Although, not everyone is convinced that TL monitoring will replace PGS (Kaser and Racowsky, 2014; Kramer *et al.*, 2014) and there are studies indicating that euploid and aneuploid embryos do not show significant differences in their morphokinetic parameters (Rienzi *et al.*, 2015), more results show that the morphokinetic parameters can be used to convincingly distinguish euploid and aneuploid blastomeres, (Wong *et al.*, 2010; Meseguer *et al.*, 2011; Chavez *et al.*, 2012; Rubio *et al.*, 2012; Desai *et al.*, 2014; Vera-Rodriguez *et al.*, 2015). Although the automated TL monitoring is new, the morphokinetic parameters were assessed from the beginning of IVF, however only in discrete time intervals, and manually, outside of the incubator, which perhaps compromised embryo quality more than the TL monitoring. Recently, using TL monitoring, multiple cell cycle and morphological parameters, such as the morphology of the nucleus, presence of micronuclei, granularity of the cytoplasm and perhaps others, might be assessed simultaneously (Milewski and Ajduk, 2017). For assessing chromosomal abnormalities and DNA damage however TL monitoring alone is not sufficient yet. Therefore it is still advisable to combine TL monitoring with PGS to obtain the best assessment of the embryo quality (Wong *et al.*, 2010; Chavez *et al.*, 2012; Basile *et al.*, 2014).

Although the TL monitoring of human embryos is relatively recent, the data are already indicating that the good embryos are characterized by well-structured cell cycles, with none or minimal morphological defects, whereas embryos with chromosomal disorders and of lower quality, exhibit prolonged duration of cell cycles and signs of fragmentation (Chavez *et al.*, 2012; Hlinka *et al.*, 2012). Wong and co-authors (Wong *et al.*, 2010) suggested to asses namely the following parameters of human embryonic development: length of the first cytokinesis, time between the first and the second mitosis and time between the second and the third mitosis. In another study, authors analysed 75 human zygotes by TL monitoring and subsequently scored individual blastomeres for their chromosome content. They concluded that euploid embryos display tightly clustered cell cycle parameters, while embryos with chromosomal aberrations exhibit more diverse morphokinetic parameters. They also suggest that non-invasive TL monitoring of embryos could help to improve IVF outcomes (Chavez *et al.*, 2012). Retrospective analysis of TL monitoring of transferred embryos suggested that embryos, which undergo division from 2 to 3 cell within 5 hours, have significantly lower implementation rate, than embryos with normal length of the cell cycle. Short cell cycles resulted sometimes in incomplete DNA replication and higher incidence of chromosomal abnormalities (Rubio *et al.*, 2012). TL monitoring showed significant differences between good quality embryos and the embryos with limited developmental potential in parameters such as: time from insemination to syngamy, timing of the cleavage to 2 cell, 4 cell, and 8 cell stage and duration of the second cell cycle, suggesting that TL monitoring can improve selection of embryos for transfer (Desai *et al.*, 2014). Significant differences between euploid and aneuploid embryos in the timing of the nuclear envelope breakdown of pronuclei, the onset of the first cytokinesis, and the cleavage time from 2 to 4 cell stage were observed in a study in which TL monitoring, CGH and single cell assessment by quantitative reverse transcription PCR (RT-qPCR) were combined (Vera-Rodriguez *et al.*, 2015). These results confirm a hypothesis that TL monitoring can help to predict good quality embryos based on morphokinetic parameters.

Conclusion

Our understanding of the main principles of early embryonic development in mammals is still very limited and future progress

will require more studies focused on the important molecular mechanisms controlling cell cycle and differentiation, and also the mechanisms contributing to chromosome segregation errors and aneuploidy. In certain aspects, we are in a better position than several years ago, because of a recent explosion of new microscopy and molecular biology techniques, sensitive enough to study physiological processes in developing embryos, and with a resolution sufficient to study molecular interactions.

Combined effort of genetic manipulations, molecular biology approaches and imaging techniques will certainly allow to identify conditions, which play a critical role in creating chromosome segregation errors and aneuploidy in embryos. It is also clear that we need to utilize more frequently other mammalian model systems, such as porcine and bovine embryos. Mouse animal model is excellent in many aspects and we assume that it will continue to serve as a major mammalian model system. However, certain aspects of early development, such as for example timing of the onset of transcription from the embryonic genome, are simply too different in mouse, compared to human embryonic development.

The TL monitoring of morphokinetic parameters is a great non-invasive approach for assessing embryo quality. For even a broader application of this approach we definitely need standardization of this technique in terms of imaging parameters, as well as better software for fast data analysis. With faster and more sensitive instruments, together with the improved data analysis, this could be a major tool for assessing embryo quality. However, we still need to keep in mind that even when it is considered to be a very mild procedure, it might cause problems, when used inappropriately. We also need more studies focused on long-term consequences of TL monitoring, such as the effect on health parameters of adult animals.

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