Cell Competition and the regulative nature of early mammalian development

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Abstract

The mammalian embryo exhibits a remarkable plasticity that allows it to correct for the presence of aberrant cells, adjust its growth so that its size is in accordance with its developmental stage, or integrate cells of another species to form fully functional organs. Here, we will discuss the contribution that cell competition, a quality control that eliminates viable cells that are less fit than their neighbours, makes to this plasticity. We will do this by reviewing the roles that cell competition plays in the early mammalian embryo and how they contribute to ensure normal development of the embryo.

1. Cell competition, a conserved fitness quality control mechanism.

One of the most remarkable features of the early mammalian embryo is its plasticity. This plasticity manifests itself from the cellular to the organism level. Cellular plasticity is most clearly illustrated by the length of time that cells of the early embryo retain a very broad developmental potential, first when totipotent and then as they transit to pluripotency. In the case of the mouse, pluripotent features are present in at least some cells of the embryo until 8 days of gestation (Osorno et al., 2012) and in the human, pluripotent cells can still be found in 16-19 day old embryos (Tyser et al., 2021). Importantly, even when these cells initiate differentiation, they retain for some time the ability to change fates, for example by having the potential to reverse their lineage trajectory if they find themselves in the wrong environment (Plusa et al., 2008) or after ablation of cells from another lineage (Guo et al., 2021; Hogan and Tilly, 1978). But despite this potential, some cells still end up making errors during the differentiation process. In these cases, a second form of plasticity helps the embryo adjust to these errors, revealing itself at the tissue level by the elimination of aberrant cells and the activation of mechanisms of compensatory growth (Diaz-Diaz et al., 2017; Hashimoto and Sasaki, 2019; Lima et al., 2021). Similarly, when cells during early development make mitotic errors that result in karyotypic abnormalities, the embryo has the potential to self-correct these errors by eliminating the aberrant cells and adjusting growth to compensate for their loss (Bolton et al., 2016). The extreme nature of this plasticity can be revealed when the size of the pre-implantation embryo is halved or doubled through experimental manipulation. In both these cases the embryo adjusts its growth, so that by the early post-implantation stage, embryo size is similar to that of unmanipulated embryos (Lewis and Rossant, 1982). What the elimination of aberrant cells and the embryo size experiments tell us is that there must be one or multiple mechanisms by which cells can sense their proliferation rates and fitness status relative to those of neighbouring cells, and then act on this information to regulate growth and tissue fitness. Over the last few years, cell competition has emerged as one such mechanism. Here we will review its roles and discuss possible mechanisms by which cell competition acts.

Probably the first implication that cells may be subjected to selective pressures was proposed by the philosopher Wilhelm Roux, who argued that similar evolutionary pressures acted on

cells as they do on wild animals and that these pressures augment organismal fitness (Roux, 1881). It took another century, however, for the concept of cell competition to emerge as such. This was through the seminal work by Morata and Ripoll in *Drosophila* analysing the behaviour of clones of cells that were heterozygous for mutations in ribosomal genes (M*inute* mutants)(Morata and Ripoll, 1975). They observed that whilst M*inute*$^{+/-}$ animals were viable, fertile, and showed only mild phenotypic abnormalities, when M*inute*$^{+/-}$ clones were induced in the imaginal wing disc, surrounded by wild-type cells, they were eliminated by apoptosis. This context-dependant elimination led to the identification of cell competition as a fitness quality control that eliminates cells, that although are potentially viable, are less fit than their neighbours. Subsequently, a wide range of different types of viable defective cells were found to be eliminated by cell competition, including cells with signalling, polarity or metabolic defects –reviewed for example in (Bowling et al., 2019). An essential element of the nature of cell competition is that cell fitness is relative to the fitness of neighbouring cells; it took another three decades for this to be irrefutably demonstrated, again in *Drosophila*, showing that cells over-expressing the proto-oncogene *c-Myc* eliminate neighbouring wild-type cells (de la Cova et al., 2004; Moreno and Basler, 2004). This highlighted the concept that in competition, context is crucial: a wild-type cell can eliminate a defective one, but in the wrong environment selective pressures can lead to its own out-competition.

The first suggestion that cell competition is conserved in mammals came around the same time, also through the analysis of ribosomal mutations. It was found that animals carrying heterozygous mutations in the ribosomal gene *Rpl24* were viable, with pigmentation defects and a kinked tail, amongst other patterning defects. In contrast to this, when heterozygous cells were in chimeras with wild-type cells, the mutant cells were lost primarily sometime between late gestation and weaning (Oliver et al., 2004). However, in these chimeras it was not possible to establish if the fate of the lost cells was due to their slower proliferation or if they were actively eliminated. Eight years later conclusive evidence for the conservation of cell competition emerged, first by the apoptotic apical extrusion of polarity deficient Madin-Darby canine kidney (MDCK) cells when mixed with wild-type cells (Norman et al., 2012), and then by the elimination during early mouse development of wild-type cells driven by *c-Myc* over-expressing cells (Claveria et al., 2013) and of BMP signalling defective, tetraploid or autophagy-deficient cells by wild-type cells (Sancho et al., 2013). The evolutionary conservation not only of the principles of cell competition, but also of key players, placed it as a mechanism that could be regulating cell fitness in a broad range of contexts. Here we will focus on those that relate to the early mammalian embryo; its importance in other contexts is excellently covered elsewhere, for example in metabolism (Esteban-Martinez and Torres, 2021), neurodegeneration (Costa-Rodrigues et al., 2021) or cancer (Morata and Calleja, 2020; Tanimura and Fujita, 2020; Vishwakarma and Piddini, 2020).

2. Roles of cell competition in the elimination of defective cells.

Most of the original studies of cell competition involved the analysis of how defective cells are eliminated by wild-type ones, see for example the out-competition of *Minute* cells (Morata and Ripoll, 1975) or of polarity deficient cells (Agrawal et al., 1995; Brumby and Richardson, 2003). This led to the concept that cell competition was acting to optimise tissue fitness through the elimination of unfit cells. This idea fits well with what we are learning about cell competition through study of the early mammalian embryo, where those cells that are eliminated can be broadly categorised into three groups: (i) those showing a disparity in their differentiation status, (ii) karyotypically abnormal cells, and (iii) cells with mitochondrial dysfunction. Here we review what is known about the fate of the cells that fall into these three groups.

(i) Elimination of cells showing a disparity in differentiation status

Development of the pre-implantation mammalian embryo results in the establishment of three lineages, the epiblast, that will give rise to all embryonic cell types, the trophoblast that will form the placenta, and hypoblast or primitive endoderm, that will form the yolk sac (Figure 1). The specification of the first of these lineages, the trophoblast, is intimately dependent on the position of the cells in the embryo. The outer cells of the morula become specified as trophectoderm by a combination of positional, transcriptional and mechanical cues, and the remaining cells form what is known as the inner cell mass (ICM) - for review see (Chan and Hiiragi, 2020; Chazaud and Yamanaka, 2016). Regulation of the trophectoderm population is influenced by FGF signalling emanating from the enclosed ICM acting on the polar trophectoderm (Nichols et al., 2009), but little cell death has been observed in this tissue (Copp, 1978). This prompts the hypothesis that cells not fated to contribute to the foetus need not be scrutinised for their fitness for prolonged viability.

Segregation of the epiblast and primitive endoderm is a fascinating event in the mouse embryo. When the early embryo consists of around 32 cells, individual internal cells begin to assume the transcriptional identity of either epiblast or primitive endoderm, whilst existing in an apparently random 'salt and pepper' arrangement within the ICM (Chazaud et al., 2006). FGF signalling again plays a key role in this specification event, with those cells seeing the highest level of FGF4 signalling becoming primitive endoderm (Saiz et al., 2016; Yamanaka et al., 2010). The prospective primitive endoderm is then sorted from the epiblast and these cells come to line the cavity that lies between ICM and the mural trophectoderm (Figure 1). This sorting is caused by higher cell surface fluctuations of the primitive endoderm cells, that together with differences in cellular fluidity, leads to these cells becoming localised to the surface lining the blastocoel (Yanagida et al., 2022). At least some ICM cells retain the ability to switch fate even after these different cell populations have sorted (Grabarek et al., 2012), but despite this potential, there is increasing evidence that those cells that do not sort properly are eliminated through cell competition.

Cell death is evident in the ICM during blastocyst expansion (Copp, 1978) which was attributed to misplacement of cells as they acquire either an epiblast or a primitive endoderm identity (Plusa *et al.*, 2008)(Figure 1). Importantly, these misplaced cells display cytoplasmic YAP expression and low TEAD activity (Hashimoto and Sasaki, 2019), hallmarks of Hippo pathway activation. The Hippo pathway is a known regulator of cell competition in *Drosophila* (Neto-Silva et al., 2010; Tyler et al., 2007; Ziosi et al., 2010), and Hashimoto and Sasaki observed that *Tead1* or *Yap1* mutant embryonic stem cells (ESCS) show low pluripotency factor expression, low MYC expression and are eliminated in chimeras by wild-type cells, indicating that low TEAD activity is sufficient to induce cell competition. Therefore, in the unperturbed embryo, during the time-window when the epiblast and primitive endoderm are being sorted, those cells that remain unspecified will activate the Hippo pathway, leading to their elimination through cell competition. However, how unspecified cells are identified as potential losers and what leads to cytoplasmic YAP sequestration in these cells is unknown.

The advent of ESCs and their application to protocols for introducing specific genetic alterations into mice provided a system with which to manipulate artificially the number of cells within an ICM. A pioneering study uncovered a further role for cell elimination during blastocyst development (Wang and Jaenisch, 2004). By injecting mixtures of individually identifiable clonal ESC lines and analysing the resulting chimeras postnatally, the authors established that of the 5-15 donor ESCs, progeny of only 1-3 were represented, suggesting a strong cell selection process. This result inspired an additional study, which took advantage of a refinement to ESC culture media, known as '2i' for inhibition of MEK and GSK3 signalling. In 2i plus LIF, ESCs are more homogeneously maintained in the naïve state of pluripotency and are more akin to the pluripotency status of the host pre-implantation embryo cells (Ying et al., 2008). In contrast to this, cells maintained in the traditional serum-containing medium plus LIF show heterogeneous expression of pluripotency genes, and a proportion of cells are closer to differentiation, with their transcriptional profile aligning with early post-implantation epiblast cells (Boroviak et al., 2014; Kolodziejczyk et al., 2015). When live cell imaging was used to compare the behaviour of injected ESCs maintained in both these conditions, significantly more death was observed for those cells cultured in serum plus LIF (Alexandrova et al., 2016). These results are in agreement with previous studies suggesting that naïve pluripotent cells are better at generating chimeras (Toyooka et al., 2008), and suggest that cell elimination occurs when cells are in different developmental states. MYC is a conserved read-out of competition with low MYC cells being the losers of competition (Claveria *et al.*, 2013; de la Cova *et al.*, 2004; Moreno and Basler, 2004). Interestingly, cells cultured in 2i plus LIF have lower MYC expression than cells cultured in serum plus LIF, and in the latter condition differential MYC expression was not observed between more differentiated and less differentiated cells. This suggests that in this context, competition may not be triggered by differential MYC expression.

Further evidence that a disparity in cell state induces cell elimination comes from the analysis of chimeras generated with epiblast stem cells. These cells are considered to be in a primed state of pluripotency, resembling the post-implantation embryo (Nichols and Smith, 2009), and are unable to generate chimeras when injected into pre-implantation embryos (Brons et al., 2007; Tesar et al., 2007). This was initially postulated to be because epiblast stem cells were unable to integrate into the host embryo due to a developmental stage inconsistency. However, if these cells are induced to constitutively express the anti-apoptotic factor BCL2, they now can readily generate chimeras (Masaki et al., 2016), indicating that they have no trouble integrating into the host embryo, but instead are eliminated when surrounded by cells of a different developmental stage. These results are suggestive of a role for cell competition, and conclusive evidence was provided by analysing the behaviour of cells with different levels of MYC expression. Live cell imaging of GFP-MYC ESCs revealed that MYC-high ESCs eliminate ESCs with low MYC expression and this elimination was due to these low-MYC cells being closer to the differentiated state (Diaz-Diaz *et al.*, 2017).

Together, the above results suggest that an important role for cell competition in the early mammalian embryo is to ensure that the development of cells within a tissue occurs in an orchestrated manner, ensuring in this way that differentiation is relatively homogeneous.

(ii) Cell competition and the fate of cells with karyotypic abnormalities

Karyotypic abnormalities can be lethal during late human development, however, despite this fact, aneuploidy is surprisingly common in the early human embryo with 50-80% of embryos showing mosaic aneuploidy at pre-implantation stages of development (van Echten-Arends et al., 2011). In contrast to this, later in gestation the levels of aneuploidy are drastically reduced (Kalousek et al., 1991), indicating that aneuploid cells are removed as development progresses.

Data from mouse embryos and human pluripotent stem cells points to this selection occurring early in the process of differentiation (Bolton *et al.*, 2016; Sancho *et al.*, 2013; Singla et al., 2020)(Figure 1). For example, when chimeras are generated between tetraploid and normal cells, the tetraploid cells are eliminated by apoptosis during the early post-implantation period (Sancho *et al.*, 2013), and this elimination requires p53 and repression of the mTOR pathway, as mutation of *p53* or of the mTOR repressor *Tsc2*, rescues this elimination (Bowling et al., 2018; Horii et al., 2015). Importantly, by analysing the behaviour of tetraploid ESCs, it was shown that the elimination of these cells was through cell competition, as their apoptosis was specifically induced by the presence of wild-type cells (Bowling *et al.*, 2018; Sancho *et al.*, 2013).

The above observations parallel what is found when mouse embryo chimeras are generated with a mixture of euploid cells and cells that have been treated with reversine, that induces aneuploidy by inhibiting the spindle assembly checkpoint. In these embryos it was found that the aneuploid cells are eliminated by apoptosis in the embryonic lineage (Bolton *et al.*, 2016).

This apoptosis was shown to occur during the peri-implantation/early post-implantation period and to be dependent on p53 and on autophagy, a pathway that acts downstream of mTOR (Singla *et al.*, 2020). In contrast to this, the aneuploidy in the future placental lineage led to slower proliferation, rather than cell death. Importantly, once the aneuploid cells were eliminated from the embryonic lineage, these mosaic aneuploid chimeras developed normally, a situation that contrasts with fully aneuploid embryos (Bolton *et al.*, 2016; Singla *et al.*, 2020). This suggests that the embryo can self-correct by eliminating aneuploid cells.

Similar findings have been made by inducing aneuploidies in human embryonic stem cells (hESCs) with reversine treatment. When these cells are used to generate micropatterns, allowing them to differentiate in an organised fashion, the aneuploid cells showed increased apoptosis and abnormal patterning of embryonic lineages, but survived normally in the pluripotent state and in the extra-embryonic lineages (Yang et al., 2021). This finding suggests that the apoptosis of aneuploid cells is cell intrinsic, therefore not induced by wild-type cells. However, reversine treatment does not generate a homogeneous aneuploid cell population, and it is possible that either some cells are not affected or only minimally affected by the reversine treatment and that these are outcompeting the eliminated cells. When these micropatterned assays were performed in the presence of 25% euploid cells, they generated structures that were properly patterned, with the aneuploid cells contributing preferentially to extra-embryonic rather than embryonic lineages. The authors also observed a drop in aneuploid rate from 82% in E3 human pre-implantation embryos to 5% by E7 (Yang *et al.*, 2021), strengthening the case for the elimination of aneuploid cells during development. Interestingly, analysis of single cell transcriptomic data of aneuploid cells found in human embryos at these stages of development revealed that aneuploid cells show a signature of MYC targets and mTOR signalling (Starostik et al., 2020), two hallmarks of cell competition in the mouse embryo (Bowling *et al.*, 2018; Claveria *et al.*, 2013; Sancho *et al.*, 2013). This dramatic reduction in the prevalence of aneuploidy as human development progresses together with the cell competition signature, suggests that through the elimination of karyotypically abnormal cells, cell competition could be contributing to the ability of the embryo to self-correct errors that arise early in development. Supporting this possibility is the finding that transfers of human embryos with mosaic aneuploidy led to babies that were healthy (van Echten-Arends *et al.*, 2011; Vanneste et al., 2009).

Despite these negative effects of aneuploidy, between 12.5% and 34% of human pluripotent stem lines have acquired chromosome abnormalities over time - reviewed in (Na et al., 2014). This suggests that some types of aneuploidies provide pluripotent cells with a selective advantage rather than a disadvantage. Indeed, it was shown that hESCs with these culture-acquired aneuploidies induce mechanical compression in wild-type cells and that this compression activates the HIPPO pathway, leading to cytoplasmic YAP localization and the elimination of the wild-type cells through cell competition (Price et al., 2021). Therefore, it is likely that the type of aneuploidy determines the outcome of cell competition, with some

aneuploidies turning cells into losers, whilst others can make cells into winners. Future work is required to untangle how aneuploidies cause this change in competitive behaviour and if the same pathways operate in both contexts.

(iii) Elimination of cells with mitochondrial dysfunction.

Upon implantation, the cells of the mouse embryo initiate a phase of rapid proliferation, with the average cell cycle time reducing from about 11.5 hours for epiblast cells at E5.5, to only 4.4 hours for some cells of the E7 gastrulating embryo (Snow, 1977). Concomitant with the acceleration of proliferation, epiblast cells start to exit the pluripotent state and acquire mesoderm, endoderm, or ectoderm identity, a process that involves a rewiring of the cells' epigenetic, metabolic, and signalling landscape. The dimension of these changes creates a large potential for the emergence of abnormal cells that need to be removed to prevent them from contributing to the embryo, or to the germline, that is specified around this time. In agreement with the need for a cell-clean-up around the time of gastrulation, a wave of cell death has been shown to take place at around E6.5 in the mouse embryo (Manova et al., 1998; Spruce et al., 2010), with about 35% of epiblast cells being eliminated within a 18 hour time-window (Bowling *et al.*, 2018)(Figure 1). The first indication that this elimination is likely to occur through competition came from the observation that those cells fated to be eliminated showed one of the hallmarks of cell competition: c-MYC levels that were low relative to neighbouring cells (Claveria *et al.*, 2013; Sancho *et al.*, 2013). Importantly, if MYC was over-expressed at E6.5 in a proportion of epiblast cells, these MYC-overexpressing cells induced the apoptotic elimination of low-MYC expressing cells through cell competition (Claveria *et al.*, 2013). Similarly, when viable but defective ESCs are induced to differentiate in a co-culture with wild-type cells, they are also eliminated through cell competition (Sancho *et al.*, 2013). These results suggested that in the early post-implantation embryo cell competition may be acting as a quality control to eliminate less-fit/low MYC expressing cells.

The first insight into the pathways that govern these competitive interactions came from analysis of the signalling events occurring in loser cells when they are surrounded by winner cells. It was found that both *in vitro* and *in vivo* defective cells show elevated p53 expression and when they are surrounded by wild-type cells, p53 changes its activity to repress the mTOR pathway, that induces their elimination. Importantly mutation of either *p53* or of the mTOR repressor *Tsc2*, is not only sufficient to rescue defective cell elimination, but also makes cells into super-competitors, that induce the elimination of neighbouring wild-type cells (Bowling *et al.*, 2018; Montero et al., 2022).

The above results indicate that MYC, P53 and mTOR signalling are all key for determining the competitive ability of post-implantation epiblast cells. But what is regulating these pathways during normal development to induce cell competition? An avenue into this question came from single cell RNA sequencing of cells from E6.5 stage embryos in which apoptosis had been blocked for 18 hours, to allow to capture those cells normally eliminated. When the

transcriptional profile of cells fated for elimination was compared to normal cells, they were found to be depleted for MYC and mTOR targets and enriched in P53 targets, indicating that they would likely be eliminated by cell competition if cell death had not been suppressed. Further analysis revealed that these cells displayed patterning defects, showed mitochondrial dysfunction, and accumulated mutations in their mitochondrial DNA (mtDNA)(Lima *et al.*, 2021). Importantly, disruption of mitochondrial dynamics or even introducing a small number of non-pathological mtDNA changes was sufficient to convert cells into losers, indicating that even subtle differences in mitochondrial activity can induce cell competition. These results allow to hypothesize that in the early post-implantation embryo, cell competition is promoting epiblast fitness through the elimination of cells that have lower mitochondrial activity than their neighbours. The fact that mitochondrial dysfunction can be found in a wide range of different defective cell types, including those with metabolic, signalling or karyotypic abnormalities (Lima *et al.*, 2021), suggests that this dysfunction could be a common feature of loser cells that is recognised during cell competition (Figure 2).

3. Size regulation in the mammalian embryo: an unlikely role for cell competition?

As reviewed above, many of the models used to study cell competition have involved the elimination of different types of defective cells by wild-type cells. This has led to cell competition primarily being thought of as a quality control that maintains tissue fitness by eliminating unfit cells. However, the emergence of the concept of super-competition, first demonstrated in *Drosophila* by showing that cells over-expressing *Myc* out-compete wild-type cells in a phenotypically silent manner (de la Cova *et al.*, 2004; Moreno and Basler, 2004), suggested that cell competition may also have other roles in addition to the elimination of damaged cells, such as the regulation of tissue size. When considering the early mammalian embryo, this potential role becomes a very attractive possibility that could contribute to its plasticity and regulative nature.

The first evidence for conservation of super-competition in the mouse embryo came from some very elegant experiments by the Torres lab, that over-expressed *c-Myc* in a mosaic manner during mouse development. They found that when this was done in the early post-implantation embryo (Claveria *et al.*, 2013) or later in the heart (Villa Del Campo et al., 2014), the *c-Myc* over-expressing cells not only eliminated the wild-type cells, but did so without leading to a smaller sized embryo. This replacement of wild-type cells without disrupting embryo size, indicated that the compensatory proliferation of *c-Myc* over-expressing cells that takes place in response to the elimination of wild-type cells must be regulated within some defined parameters that allow to adjust the growth of embryo to its stage of development.

The concept of the regulative nature of the mammalian embryo is not new and has been around since a series of landmark experiments generating over- and undersized embryos starting in the 60's and then continued in the 70's and 80's. For example, when two pre-

implantation embryos are aggregated together at the egg or morula stage, the embryos merge to form a single embryo that has roughly double the number of cells than control embryos (Figure 3)(Buehr and McLaren, 1974; Lewis and Rossant, 1982; Mintz, 1965; Tarkowski, 1961). In these double sized embryos, the embryonic lineages segregate in a proportional manner, such that the ratio of primitive endoderm to epiblast is maintained (Saiz et al., 2020; Saiz *et al.*, 2016), highlighting the robustness of these first lineage choices in the pre-implantation embryo. However, when these double sized embryos are analysed at post-implantation stages, they have scaled and have a similar size and cell number to non-aggregated littermates (Buehr et al., 1993; Lewis and Rossant, 1982). Similar is observed when 4 morulas are aggregated to form quadruple embryos (Figure 3)(Rands, 1986a). Size regulation can also be seen in chimeras generated by injecting ESCs into morulas or blastocysts (Beddington and Robertson, 1989). For example, when up to double the number of ESCs than epiblast cells making up the host epiblast are injected, the ESCs colonise the epiblast and induce a proportional primitive endoderm expansion at the late blastocyst stage (Saiz *et al.*, 2020), but by mid-gestation the embryos are of an equivalent size to un-injected controls (Figure 3) (Beddington and Robertson, 1989).

Evidence for size regulation can also be found when half-sized embryos are generated by removing one blastomere at the two-cell stage. These half-sized embryos progress through pre-implantation development with half the number of cells than unmanipulated embryos, but by the time they reach mid-gestation they are a similar size to controls (Figure 3)(Rands, 1986b). Similarly, if embryo size is reduced by 85% by teratogen treatment at E5.0, treated embryos have accelerated their growth and are indistinguishable from controls at mid-gestation (Figure 3)(Snow and Tam, 1979). Together with the analysis of over-sized embryos, these findings indicate that there is a cell non-autonomous mechanism whereby individual cells sense if the embryo is of the appropriate size for its developmental stage and in response to these signals these cells adjust their proliferation/apoptosis levels accordingly.

The potential of cells to adjust their proliferation and apoptosis rates in response to the nature or number of their neighbours raises the question if cell competition, or a similar mechanism to it, could be contributing to this early embryo scaling, specifically to the adjustment of size that occurs in over-sized embryos. Much of the available evidence points against such a role. For example, the original analysis of double sized embryos found no evidence for increased apoptosis at the time-points analysed, and instead a lengthening of the cell cycle was observed (Buehr and McLaren, 1974; Lewis and Rossant, 1982). Similarly, if apoptosis is blocked in some of the cells of over-sized chimeras, size regulation still occurs (Orietti et al., 2021). However, the finding that blocking apoptosis between E5.5 and E6.5 leads to an oversized epiblast that is a third bigger than controls and the extra cells display the typical hallmarks of cell competition (Bowling *et al.*, 2018; Lima *et al.*, 2021), together with the phenotypically silent replacement of wild-type cells seen when MYC, known for promoting growth, is over-expressed in a mosaic fashion (Claveria *et al.*, 2013; Villa Del

Campo *et al.*, 2014), suggests that cell competition could be contributing to the elimination of supernumerary cells. This elimination could just reflect the removal of defective cells, but an alternative possibility is that during the early post-implantation period, the embryo can only support a certain number of epiblast cells, and the weakest third are pruned to fine-tune embryo size prior to gastrulation. This cell number pruning may be the most evolutionarily efficient process for regulating embryo size, because initially forming the exact number of cells would require dramatically increased genetic complexity (Penaloza et al., 2006). A careful analysis of the dynamics of apoptosis, proliferation, the activity of the pathways that regulate cell competition and the timing of onset of gastrulation in over-sized and undersized embryos, as well as testing the effects of inhibiting cell death throughout these embryos will be required to establish the precise importance of cell competition as size regulation mechanism.

4. Interspecies chimeras and the mechanisms of interspecies barriers.

As discussed above, some of the roles of cell competition can be inferred from studies using chimeras made up of cells from different embryos of the same species. But, particularly fascinating have been the analyses of interspecies chimeras generated with ESCs derived from one animal species and injected into the embryo of another. Here we do not aim to provide review of interspecies chimeras, as there are excellent recent reviews of this area already available, for example (Suchy and Nakauchi, 2018; Wu and Barbaric, 2021; Zheng et al., 2021a). Instead, we attempt to provide an overview and discuss the implications of these chimeras for our understanding of the roles of cell competition.

Early embryo chimeras have been primarily used to study the developmental potential of ESCs. By injecting these cells and following their ability to contribute to the different embryonic lineages, including the germline, it has been possible to infer the potency of these cells (Beddington and Robertson, 1989). The observation that pluripotent cells can contribute to all organs raised the possibility of utilising these ESCs to complement the host embryo and help overcome any potential deficit it may accrue in organogenesis. This was first demonstrated by rescuing the hematopoietic defects of immunodeficient *Rag2* deficient mice. Here the injection of embryonic stem cells into mutant embryos gave rise to functional lymphocytes (Chen et al., 1993). A similar blastocyst complementation approach has subsequently been applied to a range of non-hematopoietic organs, including the lens, liver, pancreas, lungs, or kidneys –reviewed in (Zheng *et al.*, 2021a).

The findings that functional organs primarily derived from ESCs from one species can be generated in animals of another, highlights the potential importance of this technique for regenerative medicine, as reviewed (Suchy and Nakauchi, 2018; Zheng *et al.*, 2021a). Detailed examples demonstrating this potential have nicely been discussed elsewhere (Zheng *et al.*, 2021a) and here we provide only an overview. Pioneering work by the Nakauchi lab provided a proof of concept that blastocyst complementation could be used to generate an organ of

one species in another. They exploited the fact that *Pdx1* mutant mice are apancreatic, by injecting rat ESCs into *Pdx1* mutant blastocysts and allowing these to be born. The resulting mice had a mouse sized pancreas, but this organ was rat derived, and normally regulated glucose levels (Kobayashi et al., 2010). Furthermore, they also showed that the reverse experiment could be performed, whereby mouse ESCs were injected into *Pdx1* mutant rats. In this case the pancreas was rat sized, but primarily derived from mouse cells that secreted insulin and regulated glucose levels appropriately. Importantly, they also demonstrated that islets could be taken from these chimeric animals and when transplanted back into mouse models of diabetes, this rescued their diabetes (Yamaguchi et al., 2017).

Rat ESCs have similarly been used to generate a predominantly rat thymus by injecting them into mouse *Nude* mutant embryos (Isotani et al., 2011), rat eye tissue when injected into *Pax6* mutant mouse embryos (Wu et al., 2017) or rescue the heart and growth defects of *Nkx2.5* mutant animals when injected into mutant embryos of this genotype (Wu *et al.*, 2017). Similarly, functional rat gametes have been generated when rat pluripotent stem cells are injected into mouse blastocysts (Honda et al., 2017; Isotani et al., 2016), and functional mouse gametes were generated when mouse ESCs were injected into *Prdm14* mutant embryos (Kobayashi et al., 2021). Also, mouse kidneys can be generated by injecting ESCs into *Sal1* mutant rats (Goto et al., 2019). However, in contrast to these successes, less efficient rescue of endothelial formation could be achieved when rat ESCs were injected into *Flk1* mutant mouse embryos (Hamanaka et al., 2018; Wang et al., 2020). Success in blastocyst complementation has also been reported in larger animals, with a partial rescue of endothelial formation being observed when human pluripotent stem cells over-expressing the anti-apoptotic factor BCL2 were injected into ETV2 mutant pig embryos (Das et al., 2020) and human pluripotent stem cell derived skeletal muscle being achieved when the tumour suppressor and apoptotic regulator *TP53* is mutated and the cells injected into *MYF5/MYOD/MYF6* mutant pig embryos (Maeng et al., 2021). Together these impressive series of examples of blastocyst complementation provide the exciting perspective that human organs for transplantation may, sometime in the future, be able to be generated in large animals, an achievement that would be an enormous step for regenerative medicine.

Despite the above discussed successes, one important conclusion that can be drawn from the analysis of interspecies chimeras is that there is a significant barrier preventing the co-existence of cells from different species and that this barrier increases with the evolutionary distance (Figure 4). For example, mouse and rat diverged about 20 million years ago (Kumar et al., 2017), but the generation of chimeras between these two animals is not only less efficient than chimeras made with cells from the same species, but is also associated with embryonic lethality (Kobayashi *et al.*, 2010; Wu *et al.*, 2017). If cells from animals that are more distantly related are used to generate chimeras, for example human and pig (Das *et al.*, 2020; Maeng *et al.*, 2021), human and cattle (Wu *et al.*, 2017), or human and monkey (Tan et

al., 2021), then there is a significant drop in the level of chimerism, to the extent that if rat cells are injected into pig embryos, no chimeras are recovered (Wu *et al.*, 2017).

A number of reasons have been suggested to mechanistically explain the existence of an interspecies barrier, including differences in the length of time that cells from different species require to undertake key steps of the differentiation process, cell adhesion or developmental stage differences between the donor and host cells that prevent proper donor cell integration into the host embryo, or incompatibility between the ligands and receptors of donor and host cells due to their evolutionary divergence -reviewed in (Zheng *et al.*, 2021a). One approach that has been taken to overcome this interspecies barrier was the inhibition of apoptosis. For example, enhanced chimeric contribution can be achieved when BCL2 is over-expressed or TP53 inhibited in human pluripotent cells and these used to generate chimeras with pig embryos (Das *et al.*, 2020; Maeng *et al.*, 2021).

The involvement of apoptosis in the elimination of donor cells in interspecies chimeras raises the possibility that cell competition may be mediating the barrier between species. A further suggestion of this role comes from the analysis of chimeras generated by injecting rat or mouse cells into *Igfr1* mutant mouse embryos. The authors found that the growth retardation of mutant embryos could not only be complemented by the donor cells, but that in these experiments very high contribution chimeras composed primarily of donor rat or mouse cells can be recovered at late stages of development (Nishimura et al., 2021). Given that IGF signalling is upstream of mTOR, an important nutrient sensing pathway, and that low mTOR activity determines loser fate during cell competition in the mouse embryo (Bowling *et al.*, 2018), these experiments pointed to cell competition being involved in the elimination of donor cells in interspecies chimeras.

Definitive proof for this role came with some landmark experiments by the Wu lab (Zheng et al., 2021b). They demonstrated that whilst human pluripotent stem cells grow exponentially in a homogeneous culture, they are eliminated when co-cultured with mouse or rat ESCs, but not with *Rhesus* monkey cells, and that they eliminate cow pluripotent cells in a co-culture condition. In turn, mouse cells eliminated monkey and cow stem cells. Interestingly, this elimination only occurred when cells were cultured in the primed state of pluripotency, but not in the naïve state. This context dependent elimination, that occurs when different cells are co-cultured, but not when they are cultured homogeneously, is one of the hallmarks of cell competition and therefore directly points to an involvement of this mechanism. Importantly, the authors found that mutating factors required for loser cell elimination during cell competition, such as *TP53* or components of the innate immune pathway P65 or MYD88 in the human pluripotent cells prevent their elimination by cell competition. P53 has been shown to act upstream of mTOR during cell competition (Bowling *et al.*, 2018), or to make cells sensitive to mechanical compression (Wagstaff et al., 2016), and a rewiring of the components of the innate immune pathway has been suggested to be involved in the

recognition of loser cells by winner cells during cell competition (Meyer et al., 2014). These results therefore suggest that either an ability to compete for nutrients or for space in the epithelia, or the recognition of cells of one species by another as non-self via innate immune proteins could be involved in providing a barrier that prevents cells from different species from co-existing. As discussed above, differences in mitochondrial activity trigger cell competition in mouse (Lima *et al.*, 2021). An intriguing possibility is that steep differences in this activity between cells of different species may act upstream of TP53 and P65/MYD8 in interspecies chimeras. In this way, differential mitochondrial activity could be the primary signal that is recognised when cells from different species are confronted with each other. Further work is required to test this hypothesis.

5. Mechanisms of cell competition and perspectives.

The above reviewed work suggests that cell competition plays a range of roles during early mammalian development, including the elimination of cells that are mis-patterned, karyotypically abnormal or have mitochondrial dysfunction, the regulation of embryo size or acting as an interspecies barrier. The question that then arises is if in mammals there is a common mechanism of cell competition that accounts for all these roles, or if there are multiple different types of cell competition acting within a tissue at the same time.

Broadly speaking, three distinct modes of cell competition have been suggested - reviewed for example in (Bowling *et al.*, 2019). First, competition for a limiting resource, such as nutrients or growth factors. Although this was the initially favoured model, over the years there has not been conclusive evidence supporting this type of competition. This said, the possible importance of resource competition cannot be completely discarded yet, as loss of activation of the mTOR nutrient sensing pathway mediates the elimination of cells with mitochondrial dysfunction in the post-implantation mouse embryo (Lima *et al.*, 2021) or polarity defective cells in the *Drosophila* imaginal eye epithelium (Sanaki et al., 2020). The second form of competition is mechanical competition, whereby in *Drosophila* (Levayer et al., 2016), mammalian MDCK cells (Wagstaff *et al.*, 2016) and hESCs (Price *et al.*, 2021), tissue crowding induces the elimination of loser cells. The third type of competition involves the recognition of a different fitness status by neighbouring cells, either through the differential expression of different isoform of the FLOWER protein in *Drosophila* (Merino et al., 2013; Rhiner et al., 2010) and cancer cells (Madan et al., 2019), or via activation of a re-purposed innate immune pathway in loser cells of the *Drosophila* wing imaginal disc (Meyer *et al.*, 2014).

If one takes a first look at which form of cell competition could be acting during early mammalian development, there is evidence for several having roles. As indicated, the Hippo pathway is required for the elimination of mis-patterned pre-implantation epiblast cells (Hashimoto and Sasaki, 2019) and for the elimination of wild-type hESCs by karyotypically abnormal ones through mechanical forces (Price *et al.*, 2021). In contrast to this, p53

induction and repression of the mTOR pathway leads to the elimination of cells with mitochondrial dysfunction in the early post-implantation embryo (Lima *et al.*, 2021) and elevated p53 expression has also been shown to induce cell competition later in mouse development (Bondar and Medzhitov, 2010; Zhang et al., 2017). The involvement of a p53/mTOR axis suggests that the presence of wild-type cells may enhance the stress of dysfunctional cells, and this leads to their elimination. Although, as mentioned above, nutrient or growth factor deprivation cannot be discarded as having a role in the elimination of cells with mitochondrial dysfunction. Support for the possible role of winner cells creating a toxic environment for loser cells comes from analysis of how *p53* null mutant cells eliminate wild-type cells. Here, the number of mutant neighbours that wild-type cells have correlates with their elimination, which is not dependent on space or nutrient availability (Montero *et al.*, 2022). Finally, a role for cell competition mediated by direct signalling from winner to loser cells is suggested by the analysis of competition between human and mouse pluripotent cells. Here, inhibiting components of the NFKb/innate immune pathway or p53 prevents elimination of the human cells (Zheng *et al.*, 2021b), suggesting that the mouse cells signal to the human cells via these pathways.

Together, the above results point to very different modes of loser cell elimination acting in the early mammalian embryo. But the question that remains is whether there could be a common feature that acts upstream of these pathways to initiate cell competition. If you separate competition into mechanical and biochemical (Gradeci et al., 2021), this could be true for biochemical competition. The best characterised loser cells in the mammalian embryo are those of the post-implantation embryo, as they have been transcriptionally profiled at the single cell level (Lima *et al.*, 2021). A prominent feature of these cells is mitochondrial dysfunction and the activation of the integrated stress response (ISR), a stress pathway triggered by mitochondrial dysfunction to re-establish cellular homeostasis (Pakos-Zebrucka et al., 2016). The ISR can be stimulated by a range of cues, including defects in protein homeostasis (proteostasis) and in a key study by the Piddini lab, proteotoxic stress has been demonstrated to induce cell competition in *Drosophila*, as it triggers the elimination of ribosomal mutant *Minute* or polarity defective cells in the imaginal wing disc (Baumgartner et al., 2021). Interestingly, the rate of protein turnover has been found to regulate the different speeds at which cells differentiate during mouse and human development (Rayon et al., 2020). Similarly, it has been found that the slower rate of biochemical reactions, including the rate of protein degradation, in human cells compared to mouse cells explains the longer oscillation period of the segmentation clock in humans (Matsuda et al., 2020). It is tempting therefore to speculate that differences in proteostasis may be inducing cell competition in a broad set of contexts, from the early post-implantation mouse embryo to when cells from different species are confronted with each other.

The mitochondria play a central role in orchestrating proteostatic and metabolic stress responses (Andreasson et al., 2019). We hypothesize that in biochemical cell competition, the

competitive ability of the cell is determined by its ability to respond to mitochondrial/proteotoxic stress. We postulate that two cells with steep differences in mitochondrial activity or proteostasis have very different secretomes that they are inherently adapted to. When these two different cell types are confronted, they are exposed to each other's secretome, creating a new micro-environment that poses a challenge to the proteostasis and mitochondrial homeostasis of each cell type. For example, there is evidence to suggest cell non-autonomous regulation of proteostasis and mitochondrial stress pathways (Owusu-Ansah et al., 2013; Sala et al., 2017) and this regulation is likely to be affected by changes in the micro-environment. In our model, those cells that are most able to adapt to, and survive proteotoxic or mitochondrial stress are those that would be the winners of cell competition. Interestingly, some of the key cell competition players including Myc, mTOR and p53 are known regulators of mitochondrial activity and proteostasis (Kim and Guan, 2019; Kruiswijk et al., 2015; Zhang et al., 2020), raising the possibility that they could be affecting the outcome of cell competition by regulating these processes. It is worth noting that although some studies have found cell competition in mammalian cells to be mediated by factors secreted into the media (Sancho *et al.*, 2013), others have not (Diaz-Diaz *et al.*, 2017; Montero *et al.*, 2022; Zheng *et al.*, 2021b). This would suggest that any such changes to the micro-environment would likely need to primarily affect the immediate vicinity of cell. This competition would help ensure the most efficient and robust cells contribute to the developing embryo and would allow to adjust cell numbers in the embryo if nutrients are scarce or prevent the co-existence of cells with distantly related species. This model needs to be thoroughly tested. For example, it will be important to establish precisely what effects the stronger cells have on weaker cells, how they activate/repress downstream pathways or in what contexts can this quality control become dysfunctional.

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Figure 1. Roles of cell competition in the elimination of defective cells during early mammalian embryogenesis. A - Peri-implantation developmental stages of the early mouse embryo. Cells eliminated by competition are highlighted by a different colour and marked with an "x". B - Roles (i-iii) of cell competition events that occur during the depicted stages of early mouse development. For simplicity, the competitive events observed in hESCs (ii) are shown in the equivalent timeline of the mouse embryo development.

Figure 2. Elimination of cells with mitochondrial dysfunction. Shortly after implantation, at the exit of pluripotency, epiblast cells experience dramatic changes: they show an increase in

proliferation rate, they adapt to new metabolic demands, and undergo a rewiring of their epigenetic and signalling landscape. These events occur within a narrow window of time and lead to the possibility of defective cells emerging, that need to be removed to promote healthy embryo development. A range of cellular defects emerging at the exit of pluripotency (e.g. aneuploidy, mis-patterning, or mutations in the mitochondrial DNA (mtDNA)) lead to mitochondrial defects that are sensed by the neighbouring normal cells. Once different fitness levels are established, cell competition acts as a purifying mechanism by removing these defective cells and preventing their contribution to the germ line, that is specified around E6.5 (Hancock et al., 2021).

Figure 3. Size regulation in the mammalian embryo. A - Normal stages and timings of mouse embryonic development. B – Oversizing experiments performed in the early embryo, either by aggregation of 2 or 4 embryos, or by microinjection of ESCs into morulas or blastocysts to form chimeras. For simplicity, we show only microinjection at the morula stage. Normal embryos are recovered at mid-gestation despite these procedures. C – Undersizing experiments performed in the early embryo, either by ablation of a portion of embryonic cells, or by teratogen treatment (illustrated as lightning bolts; note the smaller embryo size at E5.5). Early embryo development can be delayed but normal embryos are recovered at mid-gestation.

Figure 4. Interspecies chimeras and mechanisms of interspecies barriers- Evolutionary distance between species and the potential for the generation of interspecies chimeras through blastocyst injection with wild-type naïve donor cells. Yellow line – indicates species that can be both donor and recipient of cells in interspecies chimeras; Green line – indicates species that have been used only as donor of cells to embryos of another species; Brown line – indicates species that have only been used as recipient of cells from another species. The level of chimerism is indicated by the proportion of green stains in the embryo. Tree of life generated with iTOL (Letunic and Bork, 2021).

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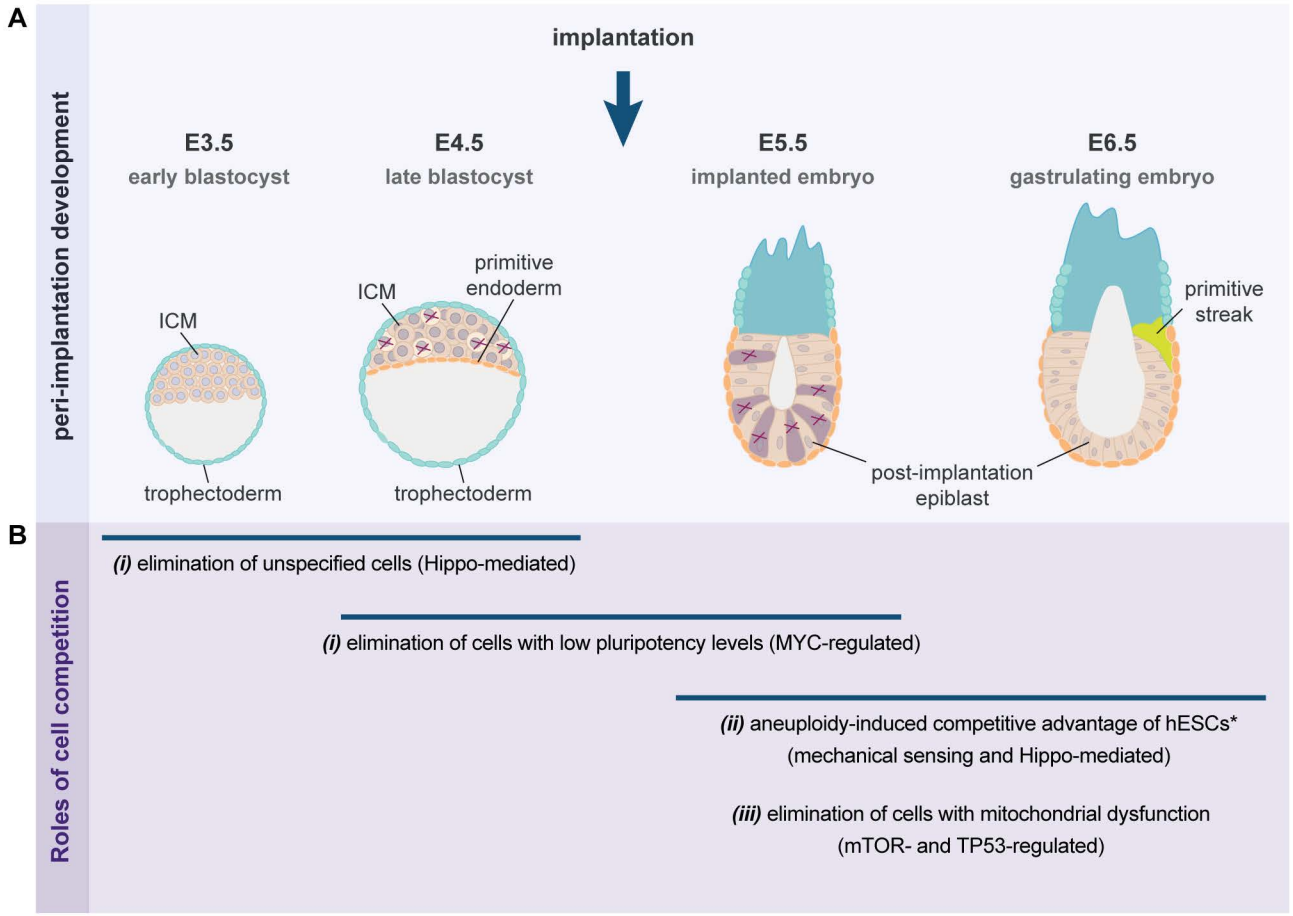
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Fig. 1



* shown in the equivalent mouse embryo development timeline

Fig. 2

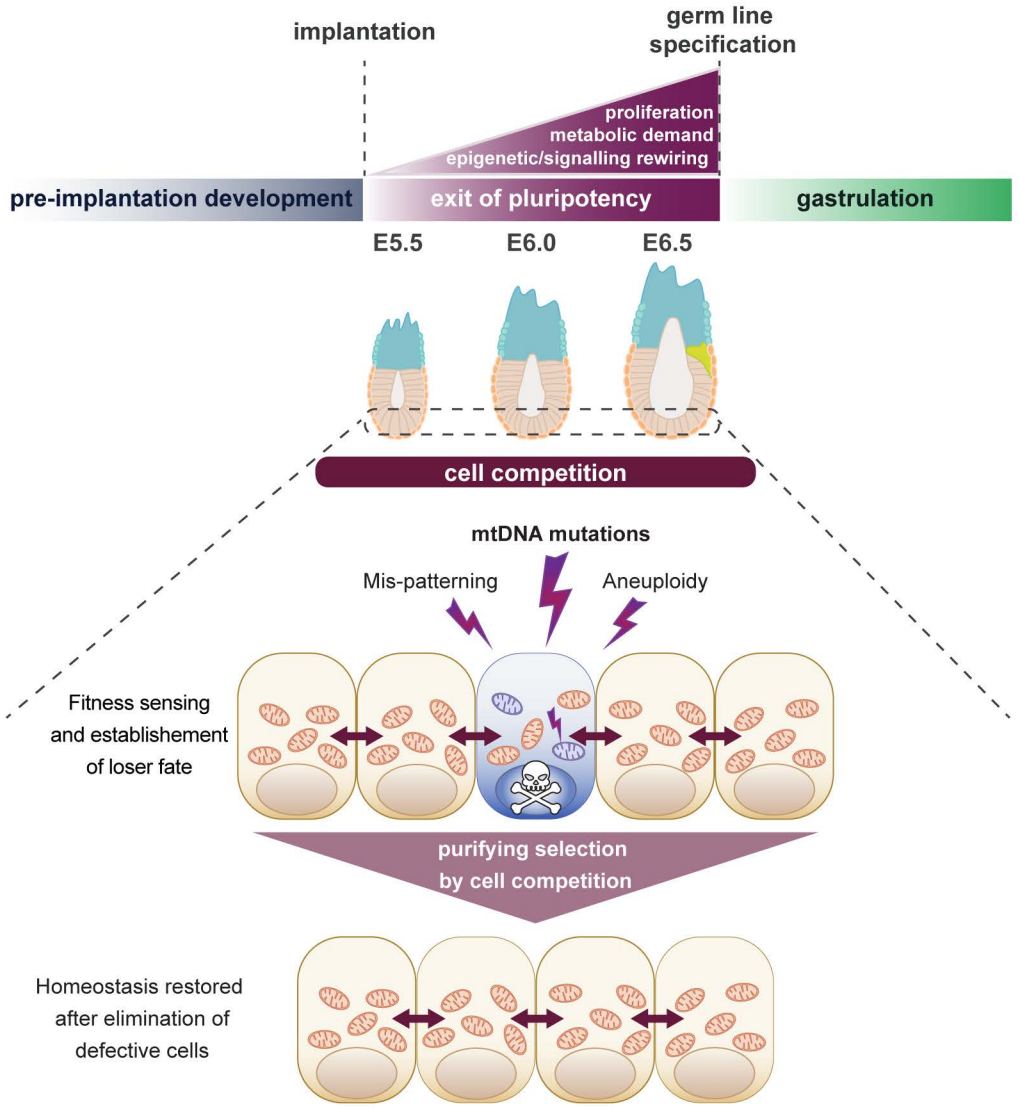

implantation
germ line specification
proliferation
metabolic demand
epigenetic/signalling rewiring
pre-implantation development
exit of pluripotency
gastrulation
E5.5
E6.0
E6.5
cell competition
mtDNA mutations
Mis-patterning
Aneuploidy
Fitness sensing and establishement of loser fate
purifying selection by cell competition
Homeostasis restored after elimination of defective cells

Fig. 3

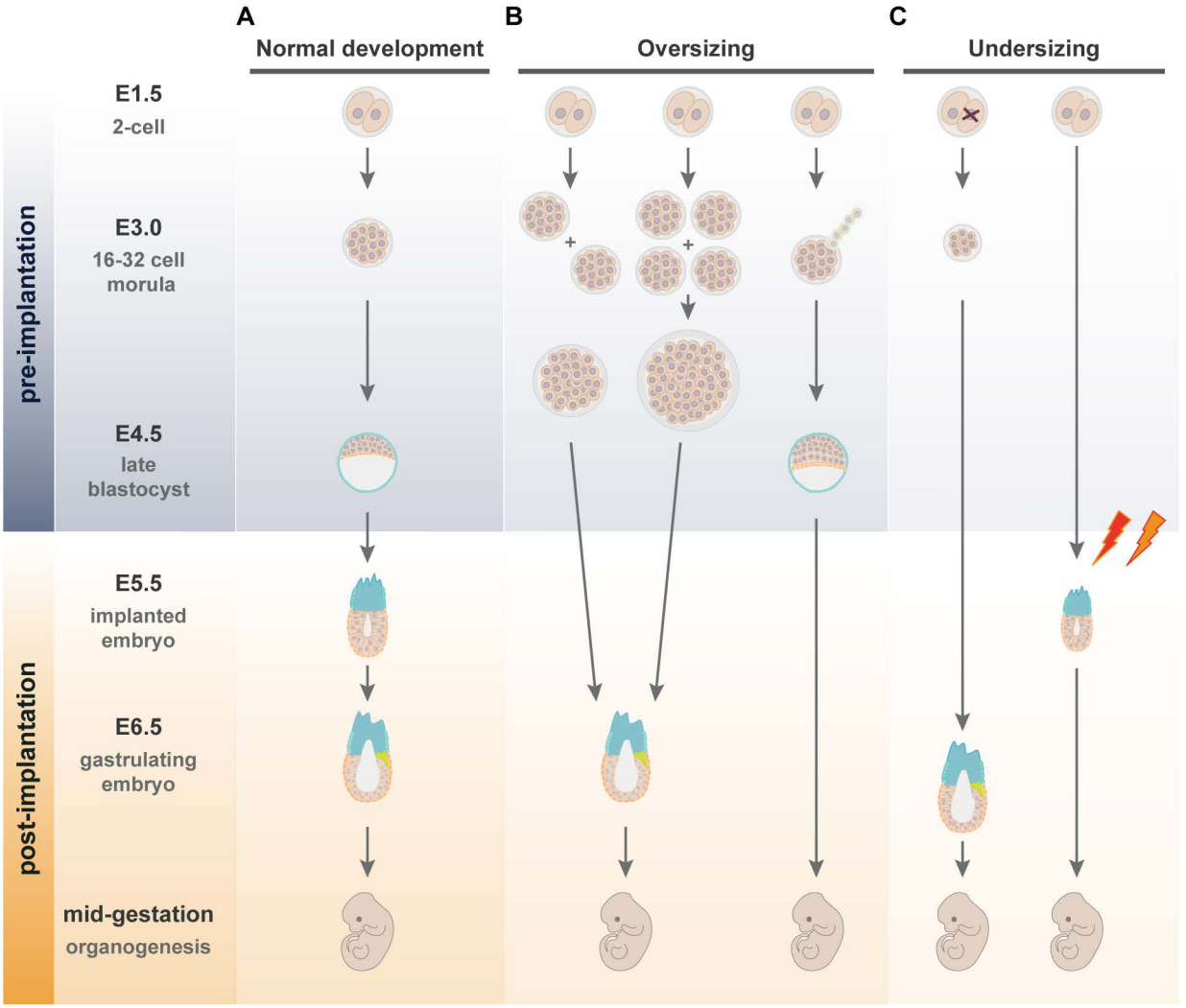
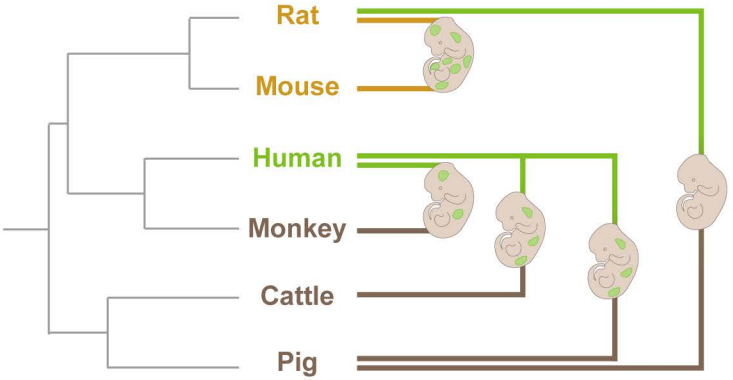

A
Normal development
B
Oversizing
C
Undersizing
pre-implantation
post-implantation
E1.5
2-cell
E3.0
16-32 cell
morula
E4.5
late
blastocyst
E5.5
implanted
embryo
E6.5
gastrulating
embryo
mid-gestation
organogenesis

Fig. 4



Barriers to interspecies chimeras (donor *vs* recipient):
Cell competition (relative fitness)
Differentiation process and timing
Cell adhesion
Developmental stage