Metabolic heterogeneity during preimplantation development: the missing link?

Daniel R. Brison¹, Roger G. Sturmey², and Henry J. Leese²*  

¹Department of Reproductive Medicine, Old St Mary’s Hospital, Central Manchester University Hospitals NHS Foundation Trust, Manchester Academic Health Sciences Centre, University of Manchester, Oxford Road, Manchester M13 9WL, UK ²Centre for Cardiovascular and Metabolic Research, Hull York Medical School, University of Hull, Hull HU6 7RX, UK  

*Correspondence address. E-mail: henry.leese@hyms.ac.uk  

Submitted on May 30, 2013; resubmitted on February 21, 2014; accepted on March 4, 2014

TABLE OF CONTENTS

- Introduction
- Methods
- Cellular heterogeneity
- Metabolic heterogeneity in the early embryo
  - Are there ‘functional’ differences between early blastomeres?
  - When during preimplantation development is the embryo most sensitive to environmental perturbation?
  - Studies on ablation of gap junction formation and function
- Consequences for human-assisted conception
- Conclusions

BACKGROUND: Most tissues in the body rely on the presence of gap junctions in order to couple their component cells electrically and metabolically via intercellular transport of ions, metabolites and signalling agents. As a result, cells within tissues achieve a high degree of, ‘metabolic homogeneity’ which enables them to develop in an integrated way and co-ordinate their response to physiological signals and environmental cues. Unusually, the developing mammalian preimplantation embryo does not form functional gap junctions until it has divided into 8 or more cells. We discuss the implications of this ‘missing link’ during the first few days of development for the maintenance of homogeneity between embryonic cells and for the co-ordination of the embryonic response to intrinsic genetic damage and external environmental signals.

METHODS: No systematic review has been carried out. The physiology of preimplantation development and the general nature of gap junctions have been reviewed briefly before examining experimental evidence which addresses the following points: (i) whether there are functional differences between early blastomeres; (ii) when during preimplantation development the embryo is most sensitive to environmental perturbation and (iii) the consequences for early embryos of ablating gap junction formation and function.

RESULTS AND CONCLUSIONS: General conclusions are confounded by species differences, especially in the timing of embryonic genome activation (EGA) and the extent of intrinsic genotypic and phenotypic variation (low in embryos from inbred mice; high in human embryos). Nevertheless, we propose that the absence of gap junctions requires cleavage stage mammalian embryos to behave cell autonomously in a metabolic sense, contributes to their heightened sensitivity to environmental perturbation compared with the later stages of preimplantation development and poses more problems in the early human embryo, where there is a high degree of heterogeneity between the blastomeres. We argue that the legacy of metabolic heterogeneity, in part generated by the absence of gap junctions, is ‘rescued’ by the onset of apoptosis following EGA. In the context of human-assisted conception, since early embryos lacking gap junctions are more sensitive to environmental stress during cleavage, this would support transfer to the natural environment as early as possible after fertilization.

Key words: metabolism / preimplantation embryo / gap junctions
**Introduction**

During the early stages of mammalian preimplantation development, the fertilized egg (zygote) undergoes three rounds of cell division to form the 8-cell embryo. Up to this stage, the cell–cell contact between the individual blastomeres is minimal. The situation then changes with the onset of compaction, when the membranes of the blastomeres flatten on one another and establish adherens junctions and gap junctions (Hardy and Handyside, 1996; Fleming et al., 2001; Bloor et al., 2002). At the conclusion of this stage of development, the embryo is termed a morula and intercellular communication is well established (Fig. 1). This is then further facilitated by the appearance of desmosomes, and of tight junctions, associated with the formation of the first epithelium during blastocyst formation.

Gap junctions are likely to be the most critical of the four types of junction in terms of metabolic homeostasis and the response to changes in the external environment since they permit the intercellular passage of ions (‘electrical coupling’) and small non-electrolytes (‘metabolic coupling’) up to \( \sim 1.5 \text{kDa} \). Compounds that can pass through gap junctions include nutrients, such as glucose and signalling agents: cyclic AMP, ATP, inositol triphosphate, glutamate and Ca\(^{2+}\). Gap junctions are made up of connexin proteins, of which there are 21 families in humans, classified according to their molecular weights (thus, connxin C43 has a molecular mass of \( \sim 43 \text{kDa} \)). Six connexins combine to form two ‘connexons’ or ‘hemichannels’ on adjacent cells, which come together to form gap junctions. The expression and role of gap junctions during preimplantation development has been well summarized by Houghton (2005), and more recently in a review on tight junction biogenesis by Eckert and Fleming (2008). Preimplantation embryos express multiple connexins, the pattern of which differs between species and with the origin of the embryos. For example, Bloor et al. (2004) reported that human preimplantation embryos express transcripts and/or protein for several connexin isoforms throughout development (Cx26, Cx31, Cx43 and Cx45) of which Cx43 is the predominant form and that there is heterogeneity in connexin expression between individual embryos (Fig. 1). Expression of Cx43 in the bovine differs between embryos produced in vivo and in vitro (Houghton, 2005), while expression in adult systems may be under neuroendocrine as well as growth factor and cytokine control. Major findings on gap junction physiology and function in somatic cells in recent years may help clarify the formation and role of gap junctions in the early embryo. Such discoveries include (i) the presence of a further family of gap junction proteins; the pannexins, in addition to the connexin (Fig. 2) (ii) the realization that gap junctions are not mere ‘passive channels’ but demonstrate considerable selectivity to ions and non-electrolytes which may be modified by a variety of signalling agents, notably, by phosphorylation/dephosphorylation mechanisms and (iii) the scope for epigenetic modification of gap junction expression during development. Herve and Derangeon (2013) have provided a succinct account of these new developments and Herve (2012) has edited a comprehensive series of reviews of the role of gap junctions in a variety of somatic cells. Thus far, such wider functions have not been explored in early embryos.

The extent to which ‘metabolic homogeneity’ is maintained between individual blastomeres, in the absence of gap junction intercellular communication (GJIC) has not previously been considered. In the context of this review, metabolic homogeneity is defined as similarity of metabolic function between distinct blastomeres. The converse is metabolic heterogeneity; the degree to which metabolic function varies between blastomeres within an individual embryo. After the first cell division, the cytoplasmic contents of the zygote are partitioned, and there are no connections between individual cells, a consequence of which is limited exchange of molecules between the blastomeres up to the 4–8-cell stage. Interestingly, despite the absence of GJIC at this stage, mammalian embryos tend to divide synchronously during the first one or two cleavage divisions, only losing this synchronicity after the 8-cell stage when GJIC becomes established. This emphasizes the fact that embryos are likely to have alternative systems for co-ordinating cell division. Any breakdown in this co-ordination has negative consequences for embryo viability (see Conclusion), and it is plausible to suppose that

---

**Figure 1** Confocal images of fixed human blastocysts showing protein localization of the connexons Cx26, Cx31 and Cx45 (shown in red) and colocalization (shown in yellow) with Cx43 (shown in green) (Cx26/43, Cx31/43, Cx45/43). Nuclei are shown in blue. Negative control images of blastocysts incubated with mouse immunoglobulin (IgG) (mIgG) and rabbit pre-immune serum (RPI). Scale bars = 25 \( \mu \text{m} \). From Bloor et al. (2004).
embryonic sensitivity to this breakdown is exacerbated by the absence of GJIC. Overall homogeneity between the blastomeres prior to formation of the GJIC is presumably driven by the extent to which the partition of zygotic products (including transcripts, proteins and organelles such as mitochondria) occurs equally between the daughter blastomeres. Moreover, in some species the embryonic genome activates [embryonic genome activation (EGA)] prior to the 8-cell stage and generates a new wave of transcripts and proteins. In humans EGA occurs largely at the 4–8-cell stage, with a minor activation at the 2-cell stage (Braude et al., 1988; Vassena et al., 2011) and so heterogeneity is also driven by the timing and extent of genome activation in each blastomere. This has functional consequences for cell fate. Thus, while there is compelling evidence that 4-cell and 8-cell stage blastomeres of mouse and humans remain pluripotent, commitment to future cell lineages starts to occur around this stage; for example, the polarization of blastomeres and their position in the embryo as either inner cells (which predisposes towards the embryonic [inner cell mass (ICM) lineages] or outer cells (which predisposes towards placental [trophectoderm (TE) lineages] (Johnson, 2009). It has also been suggested that cell fate decisions may have their origins much earlier in development, from pre-patterning information in the zygote which is transmitted through the first two cleavage divisions (Zernicka-Goetz, 2004). Either way, the extent of homogeneity between blastomeres, including metabolic homogeneity, has profound functional consequences for cell fate and embryonic development (see below). A caveat to this assumption is the potential for autocrine and juxtacrine interactions including the possibility of communication via microvesicles; a phenomenon well documented for somatic cells (Dragovic et al., 2011) including reproductive tissues such as the ovarian follicle (da Silveira et al., 2012) and endometrium (Ng et al., 2013), although not yet for the preimplantation embryo.

Methods

No systematic review has been carried out. We have examined experimental evidence that considers the general question of cellular heterogeneity: metabolic heterogeneity in the early embryo in terms of functional differences between early blastomeres; when during preimplantation development the embryo is most sensitive to environmental perturbation and the consequences for early embryos of ablating gap junction formation and function. Finally, we consider the consequences for human-assisted conception.

Cellular heterogeneity

The question of whether ‘all blastomeres are equal’ is an example of the wider consideration of how far cells of the same genotype differ from one another phenotypically; a question that can be asked at all levels of organization in living things. The theoretical physicist, Walter Elsasser, addressed the issue in a seminal article entitled ‘Outline of a theory of cellular heterogeneity’ (Elsasser, 1984). The premise was that cells of a given genotype, whether they are free living or grouped together in a tissue, are never truly identical in a phenotypic sense. Thus, Elsasser pointed to the enormity of the number of atoms in a cell; of the order of several millions, and that the chance of them all being combined into exactly the same chemical bonding possibilities is inconceivably small. While the amount of ‘order’ increases, as one moves up from atoms and molecules to cells, no two cells in a tissue are absolutely identical phenotypically though any differences may well be below the limits of detection. In a recent attempt to develop a molecular model of bacterial cell cytoplasm, McGuffee and Elcock (2010) noted that within any cytoplasm, all molecules are highly crowded, and by virtue of this crowding will experience unintended random interactions with other molecules. The impact of such interactions may be negligible, such as the temporary unfolding of a protein, leading to a short-term effect on a given reaction. Within the cytoplasm, however, containing many millions of molecules, such individual interactions may be amplified to contribute to heterogeneity within a cell population, arising from stochastic molecular interactions. The implications of this phenomenon may be demonstrated experimentally by taking ‘normal’ cells which appear identical and explanting them in culture as individual clones, whereupon they rapidly express a range of heterogeneity (Rubin, 1984).
As summarized by Rubin:

> it seems apparent – that cells organized into a tissue are under the hierarchical control of the tissue and the organism that regulates their function and composition. When they are removed from their organismal context, they exhibit their intrinsic capacity for variation

Since these reports in 1984 by Elsasser and Rubin, the notion that individual cells with the same genotype can express a degree of heterogeneity has hardened; indeed, Altschuler and Wu (2010) were able to state that

After decades of probing, measuring and analyzing the behaviours of single cells, it has become clear that the challenge is no longer to demonstrate that populations of ‘seemingly identical’ cells are heterogeneous – rather it is to determine which – if any – components of observed cellular heterogeneity serve a biological function or contain meaningful information.

As pointed out in an excellent review by Raj and van Oudenaarden (2008), many earlier studies on this topic were carried out on bacteria. Thus, Elowitz et al. (2002) quantified the variability in gene expression between individual Escherichia coli following the introduction of specific promoters linked to reporter genes. Work on eukaryotes began with yeast before moving to higher organisms once methodological difficulties had been overcome. For example, Raj et al. (2006) reported large cell-to-cell variation in RNA content measured in individual mammalian cells by in situ hybridization and Sigal et al. (2007) examined variability in protein levels in human cells using fluorescent tagging. These studies have led to a large body of research on differences between cancer cells within the same tumour (Marusyk and Polyak, 2013).

The origin of heterogeneity in the early embryo is unclear, and much evidence pointing to variations in the phenotype of individual blastomeres in the cleavage stage embryo has been arrived at in studies that have sought to understand embryonic pre-patterning. During attempts to uncover the mechanism of TE derivation, Niwa et al. (2005) reported that in the mouse the gene products of transcription factors Cdx2 and Oct 4 are present in all of the blastomeres of the 8-cell embryo, although there appear to be ‘expression domains’ in which Cdx2 expression is reduced in the inner cells while levels of Oct 4 remain relatively high. In contrast, the outer cells express higher levels of Cdx2 and reduced expression of Oct 4. On the basis of the roles of these gene products (Cdx2 associated with TE differentiation and Oct 4 important for maintenance of pluripotency of ICM cells), these differences in expression domains, and the apparent reciprocal regulation between these genes, have been proposed to play an important role in cellular differentiation. It is now well established that outer cells show increased expression of Eomes as well as Cdx2, whereas inner cells show increased expression of the pluripotency factors Nanog and Sox2 in addition to Oct4 (reviewed recently in Skamagki et al., 2013). The data therefore offer a neat demonstration of cellular heterogeneity in the early developing embryo and highlight the potential functional significance.

The cause of heterogeneity of expression of gene products may originate in earlier development. Torres-Padilla et al. (2007) reported differences in histone methylation patterns apparent as early as the 2-cell stage in mouse development, related to the plane of first blastomere cleavage. Thus, the driver of this heterogeneity may be traced back to the egg. Asymmetrical partitioning of gene products is established in cleavage. Thus, the driver of this heterogeneity may be traced back to stage in mouse development, related to the plane of first blastomere

Metabolic heterogeneity in the early embryo

In light of the idea that blastomeres within an embryo are autonomous and heterogeneous, the extent to which individual blastomeres express different metabolic phenotypes has not been considered. This is a particularly interesting concept in the cleavage embryo devoid of GJIC prior to compaction, which has an interesting parallel to aneuploidy. Thus, it is reported that during preimplantation development in the human — about three-quarters of human preimplantation embryos show aneuploidy and are chromosomally mosaic at day three of development (Mantikou et al., 2012 citing van Echten-Arends et al., 2011). These and other authors (Chavez et al., 2012; Nagaoka et al., 2012) have provided excellent accounts of the causes and extent of the various forms of aneuploidy in early human embryos. It is impossible to quantify the contribution which aneuploidy makes to the intrinsic intra-embryo variation directly; however, we can only surmise that there is even more metabolic heterogeneity than in euploid cells and that the absence of GJIC makes it more difficult for the blastomeres to operate in any co-ordinated
manner. While direct evidence for this is lacking, there are three types of indirect evidence that may help clarify the issue.

**Are there ‘functional’ differences between early blastomeres?**

This question has not been addressed, as far as we are aware, at the metabolic or cellular level; rather, almost all research is at the level of gene expression, well illustrated by the work of Roberts et al. (2011) who compared the gene transcript content of individual twin blastomeres obtained by splitting 2-cell mouse embryos. When this was done, sister blastomeres showed relatively modest differences in mRNA composition with only 178 genes showing a greater than 1.4-fold difference; most genes were distributed randomly across blastomere pairs, with little evidence of pre-patterning. Also supportive of this is the work of VerMye et al. (2011), who concluded that despite evidence of transcript pre-patterning in the oocyte, there was no transcriptome asymmetry between the sister blastomeres of early embryos in the mouse (see above). One possibility is that data such as these arise as a consequence of working on genetically inbred strains of mice; however, Held et al. (2012) have shown 2-cell stage bovine blastomeres exhibit relatively little heterogeneity in transcriptome; similar to that observed in inbred mice. Via an elegant experimental design, these authors were also able to show that the transcriptome even at this early stage of development has functional significance, since the transcriptome of one blastomere was strongly predictive of the developmental competence of the remaining twin blastomere (Held et al., 2012).

Consistent with this conclusion is the work of Guo et al. (2010) who measured the expression of 48 genes for transcription factors in individual cells during mouse preimplantation development and found—little consistent difference in the expression of the 48 genes—among individual blastomeres up to and including the uncompacted 8-cell stage. Lorthongpanich et al. (2012) have more recently shown that when mouse blastomeres are separated mechanically, the gene expression profiles tended to be unique and random, albeit with a tendency towards a TE gene profile, with a default cell fate tending towards TE. This suggests that positional cues in the embryo are likely required for the determination of blastomere heterogeneity in terms of transcript profiles and ultimately cell fate. In experiments on later stage human embryos, Galan et al. (2010) used single-cell cDNA analysis to examine the expression of genes with ‘signatures’ characteristic of ‘Inner Cell Mass’, ‘sternness’ and ‘trophoderm’ in blastomeres from 5- to 8-cell human embryos and also found that all blastomeres displayed a ‘common’ signature, with no evidence of pre-patterning at this stage. Wong et al. (2010) examined individual blastomeres from 8-cell human embryos and found significant differences in the expression of a limited number of maternal versus embryonic (zygotic) genes, concluding that ‘each blastomere may develop in a cell-autonomous manner’. This finding in individual blastomeres is corroborated by whole transcriptome studies showing extensive heterogeneity between human oocytes and embryos at the same developmental stage (Shaw et al., 2013).

What can one conclude from these data, most of which are on mouse, and on gene expression in individual blastomeres from the same embryo? One could argue that in the absence of GJIC, it is better to have blastomeres as homogeneous as possible—i.e. with near identical gene expression patterns. In this ‘metabolic homogeneity’ scenario the downstream consequence of any differential expression in a given cell, which will comprise the generation of low-molecular-weight products of transduction, is confined to that cell only; a picture that seems to reflect the data on mouse blastomeres. The alternative possibility—which seems especially to apply to human blastomeres, with their high rates of aneuploidy and differential expression of maternal and zygotic genes—is that there is heterogeneity in blastomere gene expression. This ‘metabolic heterogeneity’ scenario in the absence of GJIC will limit the capacity of the embryo to correct for the consequences of differential gene expression, especially the low molecular weight products of translation.

It is however possible that even if differences in gene expression between individual blastomeres are generated randomly, what variation there is could set in train functional differences which appeared downstream especially in response to some external stimulus. One example of such a stimulus might be positional cues in the embryo arising from cell-to-cell contact or position within the embryo, which determine cell fate. Alternatively, if blastomeres are heterogeneous at this stage, at least with respect to some of the key determinants of cell fate as a result of pre-patterning, then the absence of GJIC will act to maintain and protect that heterogeneity and fate determination. In this context, Houghton (2005) made the important point with regard to embryos in their natural environment that ‘maternally derived molecules will need to be distributed rapidly and evenly between the cells of the early embryo to ensure a consistent response’. Such distribution would obviously be limited prior to GJIC, therefore the embryonic response to external stimuli can only be consistent if blastomeres are homogeneous.

The absence of GJIC prior to the 8-cell stage therefore both requires blastomere homogeneity in order to ensure an appropriate response to external stimuli, whilst also protecting heterogeneity which might be important in driving cell fate.

**When during preimplantation development is the embryo most sensitive to environmental perturbation?**

A number of studies have addressed this issue. Zander et al. (2006) sought to discover when early mouse embryos were most sensitive to the detrimental effects of ammonium ions. Embryos were exposed to 300-μM ammonium between the zygote and 2-cell stage, 2–8-cell stage or post-compaction. Blastocyst formation was monitored and the embryos transferred to recipients. Blastocyst total cell and ICM number were reduced in embryos exposed between the zygote and 2-cell and 2–8-cell stages and apoptosis was increased in the zygote-2-cell exposure group. Implantation rates were not affected by ammonium exposure at any stage, but there were negative effects on fetal development following zygote-2-cell and 2–8-cell exposure. The authors concluded that their results—clearly indicate that the precompaction stage, in particular between the zygote and 2-cell, is extremely sensitive to ammonium, which implies that this is a crucial stage of development for environmental stress’.

Data consistent with this conclusion had earlier been reported by Lonergan et al. (2003) who examined the temporal sensitivity to different culture environments in domestic animal embryos and concluded that ‘—modification of the culture environment or inadequate culture conditions during the period of culture immediately after fertilization can result
in severe consequences in terms of the normality of the blastocysts’. In particular, the period around the time of EGA was shown to be the most sensitive to the impact of in vitro culture (Lonergan et al., 2003). In bovine, Gad et al. (2012) found that embryonic transcript profiles were strongly influenced by the in vitro environment especially around the time of EGA and were predictive of developmental competence (Gad et al., 2012 and references therein). Rooke et al. (2010) showed that serum added to sheep zygotes during the first 2 days of culture retarded development to blastocysts but when added post-compaction gave rise to a greater number of blastocysts. Lane and Gardner (2005) documented further such examples: mouse and hamster zygotes have limited capacity to regulate intracellular pH and calcium concentration and are more sensitive to reactive oxygen species (Nasr-Esfahani and Johnson, 1992).

In summary, there is general agreement that the early cleavage stages of preimplantation development are especially vulnerable to environmental stress but less discussion of why this is the case.

Evidence that addresses this issue can be drawn from cross-species comparisons of the timing of periods of sensitivity. As discussed above and widely observed previously, the time when embryos of different species experience blocks to development in culture tends to coincide with the timing of EGA (e.g. late 1-cell/2-cell stage in mouse, 4–8-cell stage in human, 8–16-cell stage in bovine). This suggests that the reorganization of the embryonic developmental programme which occurs during EGA is in itself the sensitive event. In species where GJIC has not been established by that stage, this sensitivity is likely to be enhanced by the fact that the fate of each blastomere is effectively autonomous; in other words a blastomere that has failed to undergo EGA successfully cannot be ‘rescued’ by one which has, via GJIC. If early blastomeres do fail to undergo EGA or carry other defects, such as high levels of DNA damage or aneuploidy, the incidence of which is highest during the first three mitotic divisions in humans (Mantikou et al., 2012), they may need to be eliminated from development via apoptosis. Again, this ability does not seem to be present in each species until the time of EGA or later (Boumela et al., 2011). Data from mouse suggest that apoptosis is actively suppressed in early cleavage stage embryos, such that isolated mouse blastomeres are one of the few mammalian cell types known which do not undergo apoptosis when cultured individually in the absence of cell–contact or survival signals (Raff et al., 1993 and reviewed by Brison, 2000). This is likely to have evolved as a mechanism to protect the viability of the entire embryo when it contains very few cells; however, the consequence is that prior to the start of GJIC, preimplantation embryos lack the ability to eliminate blastomeres from development in a regulated manner, such as by apoptosis. It is worth noting that the ability to execute apoptotic pathways in early embryos coincides with mitochondrial maturation such that apoptotic suppression and associated cellular survival may be a positive consequence of immature mitochondria within the cleavage stage embryo. Of course, blastomeres can be lost via fragmentation and uncontrolled cell death; perhaps this represents a fail-safe mechanism which has evolved to eliminate blastomeres with gross cellular damage and prevent ‘damaged’ cellular components being propagated to daughter cells which may otherwise form part of the intact embryo once GJIC is initiated. However, in the context of the idea being explored here, the suppression of apoptosis, whether ‘intended’ or as a by-product of mitochondrial status, ensures that any blastomere heterogeneity, metabolic or otherwise, is maximized prior to the advent of GJIC. Following EGA, GJIC and the start of apoptosis, the embryo then develops a mechanism for reducing heterogeneity by eliminating unwanted blastomeres. For further discussion of this issue see Mantikou et al. (2012).

Studies on ablation of gap junction function and formation

The issue of whether the absence of GJIC up to the 8-cell stage encourages blastomere autonomy may be approached indirectly by asking if GJIC is obligatory for preimplantation development to proceed normally. This has been addressed by ablating gap junction function using targeted gene inactivation or by applying inhibitors of GJIC. Early studies yielded conflicting results. For example, Becker et al. (1995) found that anti-peptide antibodies to Cx43 in the mouse blocked dye transfer between 8- and 16-cell blastomeres and inhibited compaction, whereas Reaume et al. (1995) reported that Cx43-null homozygous mutant embryos developed normally and gave rise to live births. The consensus view of such studies, (summarized by Houghton, 2005; Eckert and Fleming, 2008) was that GJIC was not obligatory for early embryo development. This was also the conclusion of Houghton et al. (2002a,b) who measured non-invasively the metabolism of single embryos from Cx43 null mutant mouse, and of normal mouse embryos treated with the inhibitor of GJIC, 18-glycyrrhetinic acid (AGA). In each case, impaired GJIC did not result in a change in glucose consumption, a good marker of preimplantation embryo viability, nor in the extent of apoptosis. However, Houghton et al. (2002a,b) reported the discovery of multiple connexins in mouse preimplantation embryos (as discussed above for humans and bovine) suggesting there was overlap or redundancy in function. Houghton et al. (2002a,b) also felt that experiments with inhibitors of GJIC needed to be treated with caution since specific inhibitors capable of blocking all types of channel were not available and that the effects of ablating gap junction function might not become apparent until later in the life of the embryo; specifically, that: ‘the expression of multiple connexins—allows the implanting conceptus to undergo rapid diversification of cell types required for establishment of both embryonic (i.e. fetal) and extraembryonic (i.e. yolk sac and placenta) tissues’. Moreover, research on other tissues has shown that gap junctions are often found co-localized within tight junction complexes (Kojima et al., 2007) and that some connexons influence the assembly or maintenance of tight/adherens junctions (Derangeon et al., 2009) and thus play a role in regulating cell polarity.

Consequences for human-assisted conception

There is an increasing awareness of the potential impact of assisted reproduction technologies (ARTs) on early human embryo development in vitro and the long-term health of ART children (see Brison et al., 2013 for a recent review). Since culture conditions are not optimized for human embryos, this may raise considerably the risk of stress induced by in vitro environmental factors (Leese, 2012). The consideration of culture stress includes the ongoing debate as to whether IVF embryos should be transferred back to the uterus during early cleavage (Day 2 or 3) or at the blastocyst stage (Days 5–6). According to the proposition advanced here, embryos are more sensitive to environmental stress during the early stages, in part because they lack GJIC to keep them in good metabolic order, and should therefore be transferred to their natural environment as early as possible, preferably on
Day 2. We acknowledge that, ideally, this should be to the Fallopian tube but in practice, and since the inception of IVF, it has been the uterus, which the embryo would not normally enter until Day 3 or 4. This tolerance of asynchrony was demonstrated by Marston et al. (1977) who showed that pronucleate rhesus monkey embryos could be transferred to the uterus and give rise to a pregnancy. In relation to this, Hunter (2002) has long argued that the early cleaving human embryo can survive in the uterus (such asynchrony leads to embryo rejection in rodents and domestic animals) because ‘there is considerable overlap in the composition of luminal fluid in primates between the Fallopian tubes and uterus’, the explanation for which mainly lies in the highly tortuous nature of the uterotubal junction in rodents and domestic animals) because ‘there is considerable overlap in the composition of luminal fluid in primates between the Fallopian tubes and uterus’, the explanation for which mainly lies in the highly tortuous nature of the uterotubal junction in rodents and domestic animals, which limits the exchange of molecules between the two environments compared with the comparatively unrestricted conduit in the human.

The absence of GJIC has further possible implications for ART since early human embryos may be particularly sensitive to disruptions in the synchrony of early cleavage (see above). Time-lapse studies of human embryo development have established the timing and synchrony of early cell divisions, and shown that deviations from the normal pattern are negative predictors of viability (see Herrero and Meseguer, 2013 for a recent review of this subject). The absence of GJIC may mean that any disruption to the synchrony of cleavage is more difficult to rescue since the blastomeres are unable to communicate with each other. This notion is summarized in Fig. 3, which represents diagrammatically the scenario pre- and post-GJIC formation. In the embryo pre-GJIC, blastomeres act autonomously, whereas post-GJIC formation, coordinated responses are possible. In this regard, human embryos may be particularly sensitive to the loss of blastomeres by (i) apoptosis (see above), (ii) the process of cellular fragmentation in culture (common in human embryos), (iii) following freeze–thaw damage and (iv) from cell removal at the 8-cell stage by embryo biopsy for PGD or preimplantation genetic screening; all of which precede the establishment of mature gap junctions at the morula stage.

**Conclusions**

We propose that the absence of GJIC requires cleavage stage mammalian embryos to behave cell autonomously in a metabolic sense, contributes to their heightened sensitivity to environmental perturbation compared with the later stages of preimplantation development (Fig. 3), and most likely, poses more problems in the early human embryo, where there is a high degree of heterogeneity between the blastomeres. The absence of GJIC may play a role in cell fate, by modulating the response of blastomeres to external cues, whilst also protecting any pre-patterned heterogeneity. During ART procedures human embryos commonly experience loss of blastomeres, which may increase their sensitivity to external stimuli. In all species, the legacy of life without GJIC may be ‘rescuable’ with the onset of apoptosis following EGA.

**Authors’ roles**

The authors conceived and wrote the manuscript jointly.

**Funding**

R.S. acknowledges support from the Medical Research Council and the Hull York Medical School. D.B. acknowledges support from the Medical Research Council and EU FP7 Health Programme.

**Conflict of interest**

The authors have no conflict of interest to declare.

**References**


Zander DL, Thompson JG, Lane M. Perturbations in mouse embryo development and viability caused by ammonium are more severe after exposure at the cleavage stages. *Biol Reprod* 2006;74:288–294.