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Human germline genome editing

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With the advent of efficient, easy-to-use genome editing by CRISPR–Cas9, editing human embryos is now possible, providing tremendous opportunities to study gene function and cell fate in early human development. The technique can also be used to modify the human germline. Unresolved questions about pre-implantation human development could be addressed by basic research using CRISPR–Cas9. In this Perspective, we discuss advances in human genome editing and consider ethical questions and potential clinical implications of this technology.

The ability to simply and efficiently target any region of the human genome using the clustered regularly interspaced short palindromic repeats (CRISPR)–CRISPR-associated (Cas) gene editing technology[1–3] has transformed biology. Targeted gene disruption or modification has been achieved in a variety of cells, and diverse uses of catalytically inactive, 'dead' Cas9 have been developed, including localising fluorescent tags and epigenetic regulation of target genes[4]. The ease of generating target-specific guide RNAs (gRNA) compared to engineering other programmed nucleases (zinc finger nucleases[5], transcription activator-like effector nucleases[6] and meganucleases[7]) made the notion of modifying the human germline genome more practicable. Thus, only two years after CRISPR–Cas9 was described as a molecular biological tool[8,9], it was applied to human embryos with the intent of assessing the clinical feasibility of gene correction[10–12].

These initial studies focused on understanding how CRISPR–Cas9 performs in human embryos, assessing mutation efficiency, off-target editing, rates of mosaicism and compatibility with continued preimplantation development[10–12] (Table 1). Many studies used non-viable tripronuclear embryos to abate ethical objections. However, the use of such material, which would be clinically discarded and wherein DNA repair mechanisms may be dysfunctional, makes it impossible to interpret experimental outcomes, as such embryos develop abnormally[13–15]. Most groups focused on achieving homology-directed DNA repair (HDR) to introduce designed edits into the human germline genome, as proof of principle for clinical application[10–12,16]. However, as we will discuss, many technical limitations exist that make HDR-based editing of human embryos difficult, as evidenced by the generally low efficiency of mutation 'repair' to date[10–12].

The specificity and efficiency of CRISPR–Cas9-based genome editing is ever-improving[17]. However, several fundamental aspects of human development, including timing of early cell cycles and mechanisms of DNA damage repair, have yet to be elucidated and will determine how the human embryo responds to CRISPR–Cas9-based genome editing. Basic research into these mechanisms will be fundamental to improving our proficiency in human-embryo genome editing, which will lead to a better understanding of our own early biology and inform the debate about potential safe and effective clinical uses of this technology.

Below, we summarise the progress already made in applying CRISPR–Cas9 to human embryos and consider the current limitations to more ambitious applications of this technology, as well as ethical implications. We discuss potential clinical applications of human germline genome editing, proposing a workflow for safe and efficacious medical research. Further, we look to the promising future of this technology in elucidating fundamental aspects of early human biology.

Utility of genome editing to understand early human development

Although the above work aimed to assess the preclinical capability of CRISPR–Cas9 to correct pathological sequences in human embryos[11,12,16], gene editing has also been applied to investigate the basic regulation of early human embryogenesis[18]. Our lab recently used CRISPR–Cas9-induced insertion or deletion (indel) mutations to disrupt the pluripotency factor OCT4 (gene name *POU5F1*) in human zygotes donated as surplus to infertility treatment[18]. The function of OCT4 has been thoroughly investigated through traditional genetic approaches in mice[19,20] and proven essential for maintenance of the pluripotent inner-cell mass by inhibiting acquisition of trophectoderm fate. With CRISPR–Cas9 technology, we showed that the function of OCT4 in early embryogenesis is not conserved among mice and humans[18]. Murine *Pou5f1*$^{-/-}$ embryos develop to the blastocyst stage but consist of only extraembryonic CDX2-expressing trophectoderm cells. However, the *POU5F1*-targeted human embryos showed a substantial defect in blastocyst formation, related at the single-cell level to a defect in the formation of all three cell lineages[18]. Intriguingly, a recent paper applying CRISPR–Cas9 to ablate *POU5F1* in bovine embryos[21] revealed a strikingly similar phenotype to that reported in human. These findings highlight that although rodent studies will continue to be transformative to our understanding of mammalian development, certain species-specific aspects can only be ascertained by performing functional studies directly in human embryos and non-rodent species. These results emphasise the power of genome editing to enable the study of gene function in previously inaccessible developmental contexts.

Alternative methods to understand gene function in human development

Though alternative techniques such as RNA or protein knockdown, pharmacological inhibition or provision of exogenous dominant-negative or overexpression constructs can allow modulation of gene expression without modifying the germline genome, there are several factors that make CRISPR–Cas9 revolutionary. Foremost is the ease with which genes of interest can be targeted for highly efficient and specific disruption, as the reliability of gene knockout reduces the number of human embryos required to come to meaningful conclusions about gene function. Furthermore, small-molecule inhibitors often have low specificity, perhaps affecting a whole family of signalling receptors[22]. The branched nature of signalling pathways complicates analysis, as downstream readouts may reflect effects on parallel pathways. Additionally, RNA knockdown studies often do not recapitulate the phenotype of that seen with a full genetic knockout[23–25], owing to a combination of effects including off-target gene modulation[26,27], activation of a p53 response[28,29], incomplete inhibition of gene expression and compensation by redundant genes[30].

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Table 1 | A summary of human embryo CRISPR–Cas9-mediated genome editing experiments

	Basic biology	Preclinical												
Technique	NHEJ	NHEJ with two guides	HDR							Base editing				
Mode of editing	Indels	Large deletions	Precise genetic changes							Single base-pair changes				
Starting material	2PN zygotes		3PN zygotes			2PN zygotes (from ICSI with het sperm donor)[b]			MII-phase oocytes during ICSI	3PN zygotes			Nuclear transfer embryos	2PN zygotes (from ICSI with het sperm)[b]
Gene target	*POU5F1*	*CCR5*	*HBB*	*HBB*	*G6PD*	*HBB*	*G6PD*	*MYBPC3*	*MYBPC3*	*RNF2*	*HBB*	*FANCF*	*HBB*	*FBN1*
On-target efficiency (≥1 allele)[a]	45% of cleavage-stage embryos with complete editing[c]	15.4% with Δ32 mutation of total analysed	6.7% with Δ32 mutation of total analysed	9.7% HDR with ssDNA of total amplified	10% HDR	20% HDR	25% HDR	100% HDR	44.4% proposed HDR with maternal allele[d]	5.9% HDR with ssODN	87.5% C-T conversion around target site	36.8% with desired nonsense mutation	100% with targeted mutation (58.8% include additional mutations)	40.9% G-A conversion at correct position	100% G-A conversion
% non-mosaicism	0%	50%	0%	0%	0%	100%	0%	50%	0%	0%	Not reported	0%	17.6%	0%	90.9%
Off-target effects	None	None	None	100% analysed by whole exome-seq	Not reported	Not reported	Not reported	None in one embryo analysed	None in three embryos analysed	None in two embryos analysed	Not reported	Not reported	One off-target in 33.3% analysed by WGS	Not reported	None
Reference	Fogarty et al., 2017	Kang et al., 2016		Liang et al., 2015	Tang et al., 2017				Ma et al., 2017		Li et al., 2017	Zhou et al., 2017		Liang et al., 2017	Zeng et al., 2018

[a]If multiple experiments were performed, the maximum efficiency is reported. Efficiency is calculated as the number of embryos containing at least one allele with the desired edit out of the total number of embryos used in that experiment. [b]Where a heterozygous sperm donor is used to create embryos carrying a mutation to be 'repaired', efficiency is calculated as the number of embryos containing at least one allele with the desired edit out of the total number of confirmed mutant embryos. [c]Of 11 embryos arrested or collected during the cleavage-stage of development (pre-compaction), 5 had no detectable wild-type *POU5F1* alleles across all cells. [d]In this experiment, it has not been proven unequivocally that HDR with the maternal allele as a repair template has occurred (see main text). NHEJ, non-homologous end joining; HDR, homology-directed repair; 2PN, two pronuclear; 3PN, tripronuclear; ICSI, intracytoplasmic sperm injection.

An alternative is to use the Trim-Away technique to target proteins for ubiquitin-mediated degradation[31]. Although innovative, it is unclear whether this method could lead to more than transient knockdown of a protein of interest, and nuclear-localised factors may be difficult to target efficiently because of their compartmentalisation. In general, any exogenously introduced construct is limited by perdurance. For these reasons, genetic knockouts are the gold-standard for understanding gene function. Such experiments are now possible in the context of human embryogenesis owing to the simplicity and efficiency of CRISPR–Cas9 technology.

Challenges of genome editing in human embryos

Rates of development of human embryos following assisted reproductive technologies, such as in vitro fertilisation (IVF), are poor. Approximately 42% of fertilised zygotes reach the blastocyst stage[32], and only 51.4% of those lead to successful implantation when transferred to a patient[33]. The initial wave of developmental arrest occurs during the period of cleavage division at the 4-to-8-cell stage, coinciding with the timing of embryonic genome activation (EGA)[34,35]. Therefore, problems with the initiation of EGA, or within the gene complement of the embryo due to aneuploidy, are most likely to manifest in arrest at this stage. Moreover, as the optimal in vitro culture requirements for human embryos are still unknown, this can further exacerbate issues of development, and promote embryo arrest, despite normal EGA[36,37].

The second point of developmental drop-off can be attributed to problems around the blastocyst stage, as a properly formed blastocyst must expand and 'hatch' to interact with the maternal endometrium and implant for further development[38]. Natural pregnancies are also thought to often end at this stage[39], though the reasons why are unclear and likely diverse. Of those embryos that do implant successfully, only roughly 47.7% make it past the third month of gestation[33].

Aneuploidy

Aneuploidy, wherein a cell contains an incorrect chromosomal complement, is quite prevalent in human embryos[40], initiating either during meiosis or during cleavage in the embryo itself[41,42]. It is estimated that 5–20% of human oocytes have undergone incorrect meiosis[43], but mitosis during embryogenesis may be even more error-prone[44,45]. The high rates of aneuploidy, persisting throughout preimplantation development and even beyond[46], suggest a decreased checkpoint activity during embryonic cleavages compared to somatic cell divisions. This point, discussed further below, is important to consider in the context of CRISPR–Cas9-mediated mutagenesis, as human embryos may not respond to DNA damage in a similar way to well-studied cellular models. On the other hand, it seems possible that some level of karyotypic abnormality may be compatible with successful development or that there are corrective mechanisms in place.

Expression of cell cycle and DNA repair factors

Indeed, DNA repair may be active in early human embryos[47], but control of cell cycle progression appears to differ greatly from that in somatic cells[48,49]. Comparative gene expression analysis between high- and poor-quality embryos revealed substantial overexpression of genes involved in DNA repair and cell cycle control in embryos carrying complex aneuploidies, including *RAD50*, a component specific to DNA double-strand-break (DSB) repair[47]. Conversely, no enrichment of genes involved in cell cycle checkpoints or apoptosis was observed in these inferior embryos, implying that DNA repair is attempted without cell cycle arrest[47]. Consistent with this finding, canonical checkpoint genes *RB1* and *WEE1* are expressed at low levels in human 8-cell embryos, whereas genes involved in promoting cell cycle progression (e.g., *CCNE1* (encoding cyclin E), *CCNB1* (encoding cyclin B), *CDC25B*, and *MYC*) are comparatively overrepresented[48,49]. Intriguingly, elements of the circadian clock are also upregulated in 8-cell embryos, suggesting that cleavage divisions may proceed periodically, without accounting for the DNA status of cells and without requirement for external growth factors[48]. Lack of checkpoint activity is also suggested by the observation that even amongst embryos with multinucleated blastomeres, caspase activity is rarely detected[50]. The absence of cell cycle checkpoints is reminiscent of early pre-EGA embryos of organisms such as the frog and fly[51], and may be advantageous if lack of checkpoint activity avoids p53-dependent arrest and cell death seen in somatic cells. Conversely, it is easy to imagine how such a situation would lead to increased levels of DNA damage and aneuploidy as development progresses, perhaps leading to eventual arrest around the blastocyst stage, when apoptosis reportedly becomes more active[52].

DNA repair following CRISPR–Cas9

The above considerations with regard to DNA repair raise the question of how and when Cas9-induced DSBs are repaired in the embryo. Although the method of DSB repair depends on cell cycle stage[53], attempts have been made to bypass cell cycle dependency by introducing exogenous repair factors or inhibitors to favour HDR. This has been a promising approach in many cell-types, through inhibition of 53BP1 (ref. [54]) or overexpression of RAD51 in human pluripotent stem cells[55] and mouse zygotes[56], and may also benefit human embryo genome editing.

To date, the efficacy of introducing designed mutations into human embryos by HDR has been very low, and the formation of indel mutations predominates despite provision of a repair template[10–12,16]. Although evidence points towards the expression of crucial HDR components such as RAD50 in human embryos[47], whether expression of such components is sufficient for functional repair is unknown. Additionally, genome editing of human embryos is typically performed by microinjection of CRISPR–Cas9 components into fertilised zygotes, suggesting that DSBs may occur (and presumably be repaired) long before EGA and thus under the control of maternally deposited factors. There is simply not enough data at present to understand the capability of early blastomeres to repair DNA.

An alternative explanation for poor HDR success is lack of control over when DSBs occur. Microinjection can be performed into the cytoplasm or into the two visible pronuclei (Fig. 1a). Pronuclear appearance and fading typically herald the beginning and end of the zygotic S phase, respectively, with subsequent progression to G2 and mitosis[57,58]. However, depending on the approach (i.e., Cas9 mRNA or protein), there can be a variable delay between injection, transcription and translation (approximately a 6–12-h delay for Cas9 mRNA versus protein), DSB formation and Cas9 protein degradation (approximately>72 h post-Cas9 mRNA injection compared to 24 h for protein)[59]. This process may be further hindered by condensation of sister chromatids in preparation for mitosis, potentially making the Cas9 target sequence inaccessible, as chromatin state has been demonstrated to impact Cas9 activity[60,61]. Thus, it is difficult to determine when in the cell cycle the DSBs will be formed, and therefore whether the HDR machinery would be available for repair. One could envision using the geminin-fused Cas9 construct[62] in human embryos to promote HDR over non-homologous end joining by limiting DSB formation to the S, G2 and M phases.

The timing of microinjection has proven useful for favouring HDR in mouse embryos[63]. Coordinating the injection of CRISPR–Cas9 components with EGA, which seems to promote a long HDR-permissive G2 phase, vastly improved the success of HDR-based gene editing in mouse embryos[63]. However, it is yet to be determined whether attempting CRISPR–Cas9 microinjection around the time of human EGA will have the same dramatic effect on the cell's choice of repair mechanism, nor is it known when there is a prolonged G2 phase in human embryos (Fig. 1a).

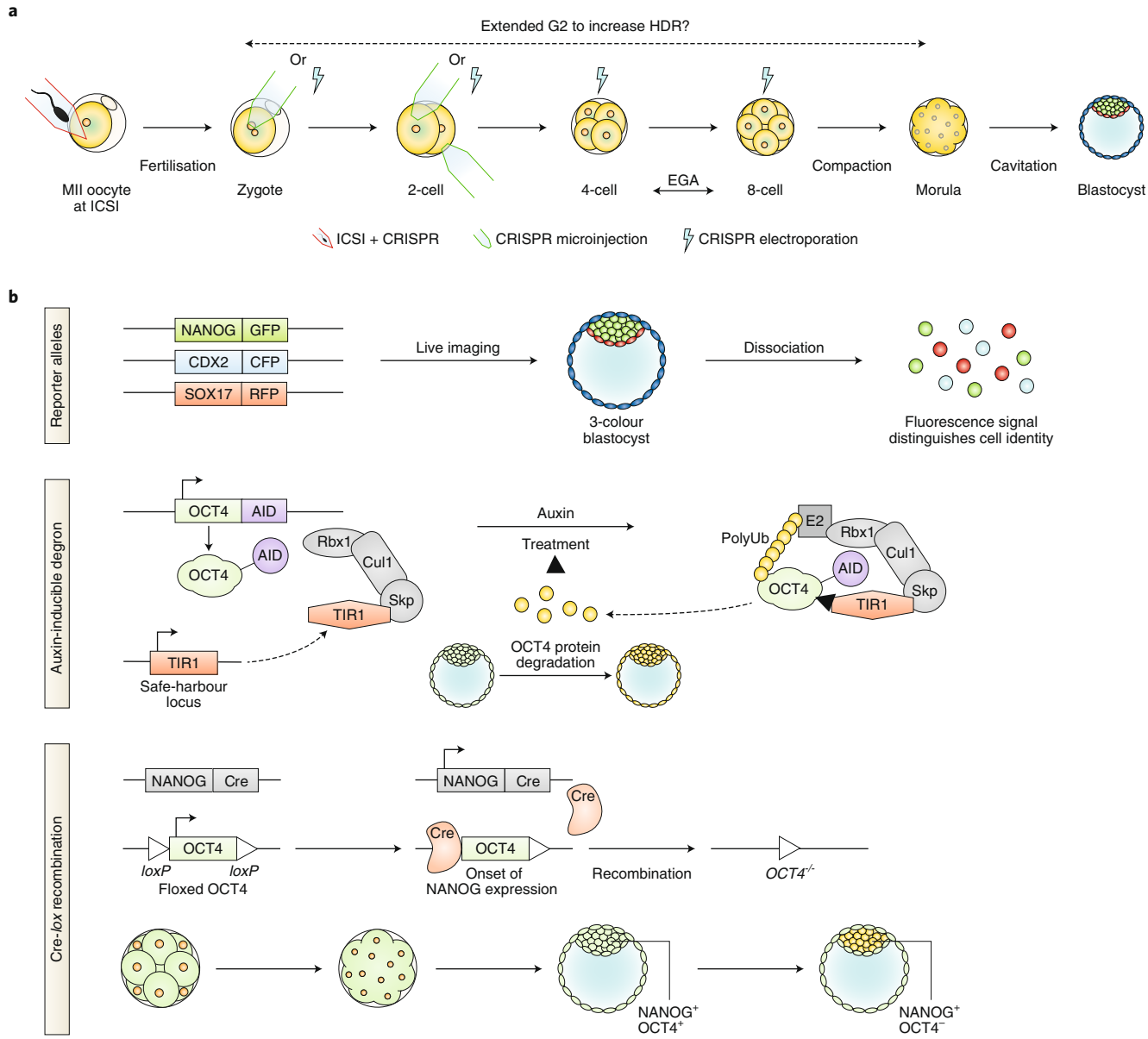

Fig. 1 | Techniques for introducing and utilising genome editing of human embryos. a, CRISPR–Cas9 components, as either a ribonucleoprotein complex or DNA or RNA templates, can be introduced into human embryos at various stages using differing methods: injection coincident with fertilisation by intracytoplasmic sperm injection (ICSI) in MII (metaphase II stage) oocytes; microinjection at the zygote stage, into the cytoplasm or the two pronuclei; or electroporation, which may open the door to genome editing at later stages. Whereas introducing CRISPR–Cas9 during a long G2 phase in mouse embryogenesis apparently vastly improves rates of homology-directed DNA repair (HDR)[63], it is unclear whether and when there is a corresponding event in human preimplantation development. EGA, embryonic genome activation. **b**, Examples of how HDR-based genome editing could be applied in future basic research to generate insight into human preimplantation embryogenesis. Top, targeting of fluorescent reporters to lineage-specific genes (e.g., *NANOG* for the epiblast, *SOX17* for the primitive endoderm and *CDX2* for the trophectoderm) to investigate cell fate in human blastocysts. This could be useful for maintaining location and cell identity information following single-cell dissociation. Middle, applying the auxin-inducible degron (AID) system to genetically tag a gene of interest with an AID sequence and introduce a constitutively expressed TIR1 construct into a safe-harbour site for auxin-inducible degradation of a target protein[114]. TIR1 will form a complex with endogenous ubiquitin ligase components (E2, Rbx1, Cul1, Skp) and facilitate specific ubiquitin-mediated degradation of a protein of interest tagged with AID. Shown is an example of AID tagging of the locus encoding OCT4. OCT4 is normally expressed in all cells of the blastocyst. Upon auxin treatment of the embryo, OCT4 protein would be reversibly destroyed, allowing for assessment of its function with temporal specificity. PolyUb, polyubiquitylation. Bottom, leveraging the Cre–*lox* recombination system to study gene function in a temporally controlled manner by inducibly deleting a gene of interest flanked by *loxP* sites (a 'floxed' gene)[115,116]. In this example, the OCT4 locus is 'floxed'. Introduction of a Cre recombinase transgene under the control of the *NANOG* promoter leads to specific deletion of OCT4 within the pluripotent NANOG-positive epiblast cells. OCT4 expression is therefore lost only from the epiblast, allowing assessment of its pluripotency-specific role.

Detailed information about the timing of early cell cycle progression in human embryos is not available. To understand whether a similar prolonged G2 phase exists in human preimplantation development, live embryo imaging and cell cycle reporters such as the FUCCI system[64,65] or chromosome painting (achieved recently using dCas9 (ref. [66])) would be required. Further consideration is

needed about the current limitations of such experiments including both the difficulty of imaging fairly large human preimplantation embryos (the human blastocyst is approximately 0.1–0.2 mm in diameter, ~4× larger than the mouse blastocyst) and maintaining their normal in vitro development in a microscope chamber, as well as the potential toxicity of introducing cell cycle reporters. Such experiments have, however, been achieved in the mouse by tracking fluorescently labelled chromosomes (H2B-mCherry) and kinetochores (EGFP-Cenpc)[67] or by genetically introducing the FUCCI system[68].

An alternative approach for introducing Cas9-gRNA ribonucleoprotein complexes and a repair template into oocytes at the second meiotic metaphase (MII) coincident with fertilisation by intracytoplasmic sperm injection (ICSI) has recently been suggested to increase HDR in human embryos[16]. The rationale for this experiment was that introducing CRISPR–Cas9 at this early stage would lead to DSB induction and repair prior to the first cell division, and indeed, the investigators report a dramatically reduced incidence of mosaicism, though this differs from a previous report in mice[69]. However, the authors also report a considerable increase in HDR, with the proportion of homozygous wild-type embryos increased from 66.7% by zygote microinjection to 72.4% by MII injection. Still, 20–30% of targeted embryos carried indels at the on-target site, regardless of whether a repair template was provided[16]. This finding may severely limit applicability in a future clinical context, because mosaic embryos may harbour undesired indel mutations.

Unexpected editing outcomes following CRISPR–Cas9 in human embryos

Interestingly, in the study discussed above[16], the increase in mutation-free embryos was not attributed to HDR using the exogenous repair template, but rather was suggested to be the result of interhomologue repair using the wild-type maternal allele[16]. This suggestion has been met with scepticism by some researchers, with suggestions that alternative explanations may account for the observed result, including allele dropout (ADO), parthenogenetic oocyte activation or the introduction of undetected large deletions or rearrangements[70,71]. One reason for these objections is the physical separation of male and female genetic material by nuclear membranes during the very early stages of post-fertilisation development[70], whereby the parental pronuclei remain distinct throughout the first interphase and DNA replication. Recent experiments in mouse zygotes have suggested that the separation may persist even longer, with the formation of independent mitotic spindles[72]. Ma and colleagues have provided data in support of their interpretation of interhomologue DSB repair by performing long-range PCR and SNP analysis[73], but outstanding questions still remain, and further studies will be required to determine whether the parental homologues possess the ability to interact prior to pronuclear fading.

The potential confounding factor of large CRISPR–Cas9-induced deletions[74] also calls into question the interpretation of studies using end-joining mechanisms for gene knockout in human embryos and other contexts. Accumulating evidence points towards a surprising incidence of large DNA deletions or rearrangements resulting from CRISPR–Cas9 mutagenesis[74]. By analysing the consequences of CRISPR–Cas9 targeting to various loci in mouse embryonic stem cells (ESCs) in depth, this study determined that the range of mutations is more complex than simple insertion or deletion, including compound mutations and translocations of nearby sequences[74]. Further, more than 20% of targeted alleles contained large deletions that stretched up to 6 kb away from the CRISPR cut site. The authors also corroborated these results in additional human and mouse cell lines, suggesting a universality to the occurrence of dramatic DNA lesions following genome editing[74].

The use of base editors in human embryos

In addition to conventional knockouts, CRISPR–Cas9-mediated base editing can be used to more precisely alter the genome while bypassing the need for DSBs that may allow unintended modifications to genomic DNA[75,76]. Given how little is understood about the repair of genetic lesions in human embryos, base editing is a promising alternative, and has been applied recently in this context[77–80] (Table 1). As with traditional CRISPR–Cas9, these studies had a preclinical focus on disease-associated mutations that could be repaired to wild-type sequences with base editing, providing a proof of principle of the technique's utility in the human germline. These studies detected little or no off-target activity, but variable efficiency of on-target base conversion, with some conversion of nearby nucleotides, some unexpected conversion patterns (e.g., C-to-A or C-to-G instead of C-to-T deamination) and a low proportion of indel mutations[77–80]. Moreover, recent studies have suggested unexpectedly high off-target editing in rice and in mouse embryos[81,82], necessitating further evaluation in a human context. If base editing in human embryos can be shown to be reliable, it could be an immensely powerful tool for introducing null mutations into genes to study their function during early development, as demonstrated in the mouse[83]. Alternatively, it may be possible in the future to use primed editing, which uses a primed editing guide RNA and a modified Cas9-nickase fused to an engineered reverse transcriptase[84]. Though this method has been used successfully to edit mutations in human cells, it has yet to be tested on mammalian embryos, and mouse embryo studies would be an informative next step.

Available tools for evaluating success

When genetically engineering model systems, undesirable alleles can be selected against to yield an organism that has only the desired genotype. Working with any human material, however, makes this impossible, and whereas undesired effects may be accounted for in basic research, they must be strictly identified and avoided in clinical applications. Additionally, working with the human germline presents its own unique set of obstacles. In particular, it is important to consider the small amount of DNA that can be used for 'diagnosis' of genome editing events in embryos. In the laboratory, it is possible to use single-cell analysis of whole, dissociated embryos to assess the range of genotypes introduced following CRISPR–Cas9-mediated mutagenesis[16,18]. However, if preparing genome-edited embryos for subsequent implantation and establishment of pregnancy, preimplantation genetic diagnosis (PGD) would be required, typically involving a relatively non-invasive biopsy of a small number of trophectoderm cells from blastocysts[85,86].

For more than two decades, the potential for mosaicism to impede accurate PGD has been a concern[43]. A recent study in sheep compared genotyping results from trophectoderm biopsies and bulk sequencing of the remaining cells from the same embryos following CRISPR–Cas9 targeting of the *PDX1* gene in MII oocytes and found a distinct lack of correlation[87]. Less than 50% of trophectoderm biopsies were found to be concordant with sequencing of the remaining embryo, with a trend for underestimating the amount of unedited PDX1 (ref. [87]). This could also be attributed to one of the other major issues encountered when sequencing low-input DNA, which is ADO[88,89]. It is standard practice to perform whole-genome amplification on low-input DNA samples, but such amplification often favours one allele over the other. Amplification bias can be random, or may clearly relate to allele length as for *PDX1* (ref. [87]), in which the shorter mutant allele is preferentially amplified. The apparent prevalence of large deletions[74] makes the latter especially worrying.

It is currently a concern that if mosaicism or ADO leads to misrepresentation of embryo genotypes in a clinical setting, the outcomes could be highly variable depending on the level of mosaicism and the nature of unidentified editing outcomes. It should be

considered that apparently correctly gene-edited embryos chosen for transfer could yield unhealthy pregnancies if the percentage of edited cells overall (or specifically in the disease-affected tissue) is too low to ameliorate symptoms or if there are large deletions or rearrangements that could impact on nearby genes. An additional concern is that outcomes may be gene specific, both in terms of the edited target and potential collateral damage on neighbouring regions of the genome. The question of how to determine whether a gene-edited embryo would be healthy is still an open one, and it may be that our current technologies are insufficient to answer it at present.

Predicting on-target mutations in human embryos

One possibility to simplify the analysis of on-target editing is to use predictive algorithms to aid selection of highly specific gRNA sequences and preempt the likely mutation spectrum arising from CRISPR–Cas9. Three recent papers demonstrate highly accurate predictive tools[90–92]. The success of these algorithms largely relies on training data from CRISPR–Cas9 experiments, and all report high cell-type specificity. Given that studies in human embryos are limited, it would be impossible to generate the required amount of data for accurate prediction. However, the mutation spectrum observed in human ESCs may be closely reflective of the in vivo embryo[18]. Given this, it is interesting to note that Allen et al. included pluripotent stem cell-types in their analyses and found higher rates of large modifications and a prevalence of microhomology-mediated deletions[91]. Intriguingly, microhomology-mediated small deletions are favoured at tandem repeats, leading to suggestions of possible therapeutic routes for repeat diseases like Fragile X syndrome and Huntington's disease[91,92].

Evaluating off-target effects

The issues of mosaicism and ADO also diminish our ability to evaluate off-target effects of CRISPR–Cas9 in human embryos. Although numerous techniques exist to scan for off-target editing genome-wide[93–98], they usually involve detection of Cas9 cleavage in vitro and give limited information about editing that has actually occurred in cells. One exception is Digenome-seq[93], in which in vitro cleavage of genome-edited cellular DNA pinpoints potential Cas9 off-target sites by the 'straight' alignment of high-throughput sequencing read ends flanking cut-sites, and the remaining 'staggered' reads spanning the sites can be interrogated for indel mutations. Circle-seq has the advantage of experimentally determining sites of off-target mutations in primary human cells that have been edited[95] but requires amounts of genomic DNA (~25 μg) that would preclude its use on preimplantation human embryos. Although recent methods have been developed to identify cut sites directly in vivo, BLISS[97] uses cells and tissues fixed on slides and is therefore not applicable to whole embryos, and DISCOVER-seq[98] relies on ChIP methodology, which is also greatly limited by chromatin yield from embryos.

Low-throughput methods of in silico prediction followed by targeted sequencing can be used on PCR-amplified sites of expected off-target cleavage, but it is possible that the reliability of this method may be influenced by the use of low-input DNA, particularly by mosaic editing. Additionally, off-target editing is suggested to be disproportionately hindered by chromatin compared to on-target effects, making in vitro assessment less reliable[99]. Interestingly, however, a number of studies suggest that off-target activity of CRISPR–Cas9 may be less extensive than initially feared in both cultured cells[100,101] and human embryos[16,18]. Editing at the one-cell stage may be advantageous in reducing the likelihood of off-target mutations compared to editing many more cells at later stages of development or in adults. For example, with an infrequent off-target mutation, the likelihood of incurring a mutation is higher as more cells are targeted.

Box 1 | Prerequisites for clinical human embryo genome editing

The ethics of human germline genome editing are widely discussed, both by scientists and the general public and media. In light of recent events regarding the use of CRISPR–Cas9 on human embryos bound for implantation and pregnancy, and the guidelines set out in documents published by scientific and ethical bodies, the following points present what, in our view, are the most important prerequisites for any potential future use of clinical human embryo genome editing:

1. The treatment must address an unmet medical need and be judged the most reliable method of safely sparing an individual from genetic disease[105,106];
2. The alleles introduced into patients should exist naturally in humans of a similar genetic background, given that it is very difficult to predict the potential for gene–gene interactions to impinge upon overall phenotype, and the balance of potential benefit to potential harm must be as certain as possible[105,106]. This is relevant in modifying CCR5 for HIV resistance, because inactivating mutations are sometimes present in northern European populations but very rare in others, perhaps pointing towards negative selection[131]. This argument also concerns the persistence of sickle cell anaemia in African populations, wherein heterozygotes are protected from malaria[132]. When carrying out genome editing to prevent genetic disease might increase susceptibility to other conditions, serious consideration should be given to the benefit-to-risk ratio on a case-by-case basis;
3. There must be adequate proof of safety through in-depth preclinical research, subject to peer and regulatory review, investigating the precise genome edits to be made, and any off-target effects and how they might affect overall health[105,106];
4. There should also be sufficiently robust methods in place to assess on- and off-target effects in modified embryos[105,106]. Currently available methods are severely limited by small sample size and mosacism (Table 1), therefore, it is currently not possible to unambiguously determine the genetic consequences of genome editing in human embryos;
5. The informed consent process should always be discussed with participants by an unbiased third-party agent, who is trained in the correct procedures and has no conflict of interest, rather than by members of the scientific team themselves; and
6. The work must be performed openly and conform to broad societal consensus about acceptable uses of this technology. Scientific progress in areas as controversial as human genome editing cannot proceed successfully without the trust and support of the public who it is designed to help, as proven by the general response following the surprising announcement in November 2018[133].

Possible clinical applications and alternatives to genome editing

Even before any experiments applying CRISPR–Cas9 gene editing to human embryos had been confirmed, the scientific community was well aware of the potential implications of using this technology in a clinical setting. Some suggested imposing a series of restrictions[102], whereas others called for a complete moratorium of gene editing in human embryos, especially because of fears that it may impact on the approval for somatic cell therapies[103]. Nevertheless, others quickly came out in support of such research, stressing the potential benefit that could come from it and suggesting that the scientific community would indeed be remiss in denying future generations

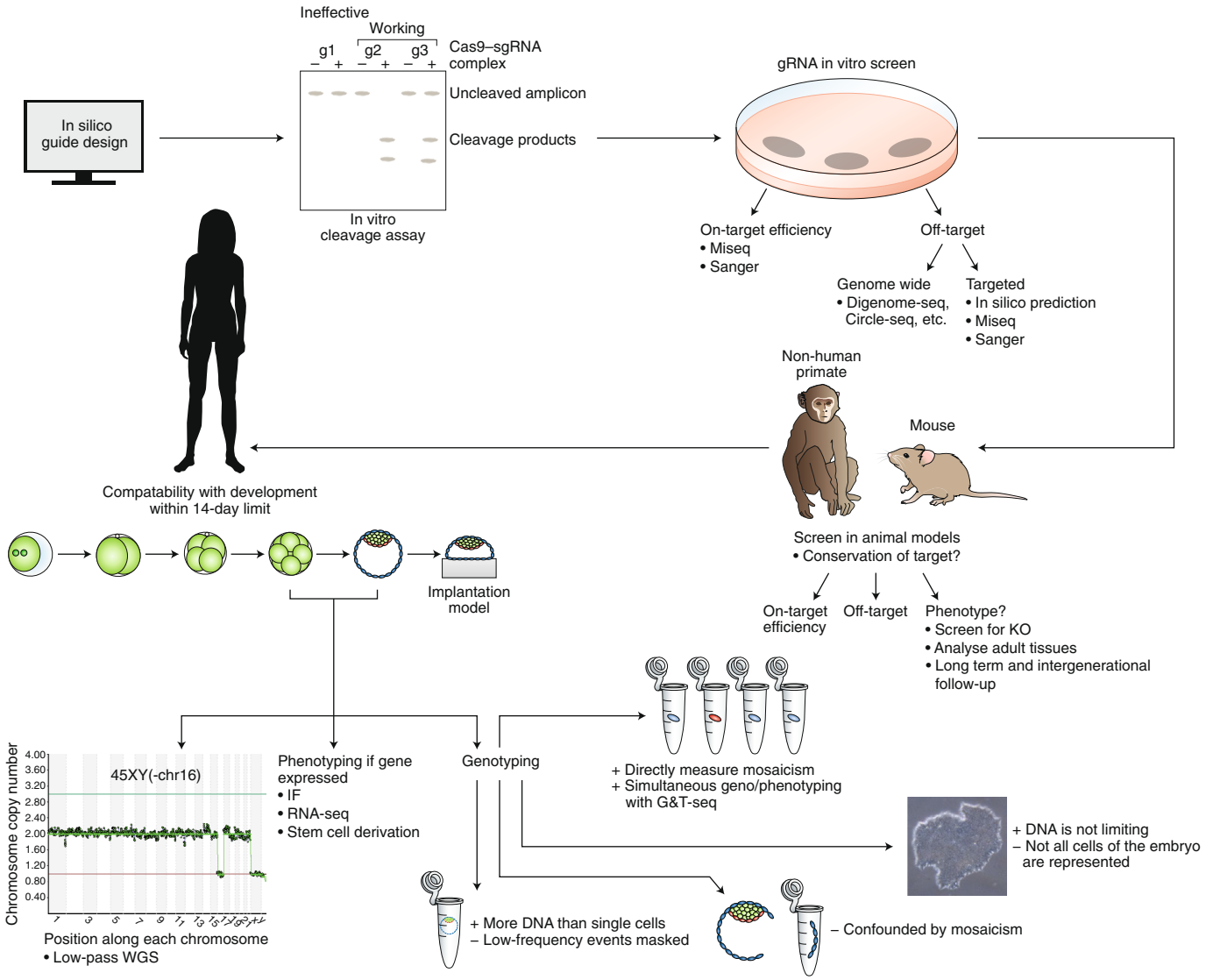

Fig. 2 | A proposed workflow for preclinical evaluation of human embryo genome-editing experiments. gRNAs (e.g., g1, g2, g3) are designed using online tools and then tested by in vitro cleavage or Sanger sequencing, revealing the (in)ability of any gRNA sequence to mediate on-target cleavage by Cas9. Guides that clearly cut the on-target site are carried forward to cellular testing (e.g., human embryonic stem cells (hESCs)). Non-transformed, karyotypically normal cells should be used to allow interpretation of editing outcomes. On-target and off-target editing can be assessed by targeted sequencing (Sanger or MiSeq), whereas genome-wide methods (Digenome-seq, Circle-seq or whole-genome sequencing (WGS)) may be useful for detecting unexpected off-target effects. gRNAs with high on-target efficiency and no evidence of off-target activity can be tested in preclinical models. Depending upon local availability and target sequence conservation, mouse or non-human primate models may be useful. On- and off-target editing must be screened by DNA sequencing. Although these events may differ between species, it is essential to evaluate the consequence of sequence changes, especially those that may not have been previously detectable in the human population, in as many cellular contexts as possible. The phenotype of introduced edits must be assessed in animal and human cell line models when possible. Alternatively, early expression would allow phenotyping in the embryo model, but adult and intergenerational follow-up are also required to ensure no long-term adverse effects. If any guides pass these screening stages, preclinical studies would be performed in human embryos. Compatibility of the desired edit with continued development should be assessed, perhaps up to the 14-d limit, using recently described methods[128,129]. On-target efficiency and off-target editing is commonly assessed at the 8-cell or blastocyst stage. Genotyping can be performed at the whole-embryo or single-cell level, or using a biopsy of a few cells. Pros and cons of these approaches are listed. Special consideration must be taken to account for potential large deletions. Samples should be assessed for karyotypic abnormalities that may potentially be induced following CRISPR–Cas9-mediated DSBs. If targeting a gene that is expressed early, preliminary phenotyping in the human embryo (e.g., by immunofluorescence (IF), RNA-seq or G&T-seq[130]) should be performed. Alternatively, hESCs could be derived from edited embryos and differentiated to a relevant cell type to investigate phenotype.

the chance to drastically reduce the burden of genetic disease[104]. Regardless of the stance taken, there is almost universal agreement that any potential application of clinical germline genome editing must come only after in-depth public and policy discussions, and should fit a number of strict ethical and safety criteria, as laid out in documents published by the US National Academies of Sciences, Engineering and Medicine[105] and the UK Nuffield Council on Bioethics[106] (Box 1).

Production of disease-free embryos

One common argument against the use of genome editing is that IVF with PGD is an already available route to ensuring that couples

carrying disease genes can bear disease-free children. However, in our view, there are notable benefits to trying to correct disease alleles, rather than selecting for disease-free embryos.

For instance, when both parents are carriers of recessive or dominant genetic disease, the number of embryos eligible for transfer to establish pregnancy is greatly reduced if avoiding affected and carrier embryos. Given the low in vitro development rates of human embryos, and the rates of successful pregnancies following IVF (~12% of fertilised eggs), it is likely that the number of cycles needed to yield a healthy, disease-free child for such parents is multiplied[107]. This can present future parents with the possibility of repeated ovarian stimulation and IVF cycles, which comes with the risk of complications[108–110].

This situation is exacerbated in the case of autosomal dominant disorders, which may not present until later in life and therefore do not always preclude an affected person from reaching reproductive age. In addition, advances in modern medicine mean that more patients of once severely life-limiting diseases are surviving to adulthood, increasing the likelihood of passing on disease-associated dominant alleles. In cases in which one parent carries two dominant disease-causing alleles, it would be impossible at present for them to produce a healthy, genetically related child. This may lead some to pursue IVF with donor gametes, an option which may not always be appealing to prospective parents.

One must also consider the potential morally objectionable aspects of current and prospective reproductive technologies. At present, selection against disease-carrying embryos involves either destruction before implantation or initiation of pregnancy followed by elective termination after in utero diagnosis. With safe and effective human germline genome editing, it should be considered that many fewer (or feasibly no) embryos would be destroyed based on their genotype. Ultimately, providing more options for patients empowers them to make the choice that is best for their family and circumstances.

Conclusions and future outlook

Future non-clinical basic biology. We envisage that future developments in gene editing technology and our understanding of its mechanism inside human embryos will open up many more avenues for understanding the biology of early human development (Fig. 1b). In particular, improving our ability to perform HDR would provide the opportunity to introduce a variety of genetic changes, such as genetic tags as reporters of gene expression[63]. In many species, reporter alleles linked to fluorescent markers have revealed the precise temporal and spatial expression profiles of genes of interest, particularly when combined with live-imaging technologies. Recently, three-colour mouse embryos were generated by HDR following CRISPR–Cas9, with genes distinguishing all three cell-types of the blastocyst[63]. In our view, having such fluorescent markers to distinguish cells of different types within the human embryo could vastly improve the efficiency of microdissection procedures needed for successful derivation of human ESCs and single-cell analyses of lineage-specific gene expression patterns. One concern is that, at present, such procedures are performed on morphology alone and cell types are assigned retrospectively and often based on transcriptional markers with *a priori* assumptions, which is an unreliable readout of cell identity.

Additionally, the auxin-inducible degron (AID) system has been employed in mammalian embryos through CRISPR–Cas9 (ref. [63]). By simultaneously introducing an AID tag to a gene-of-interest and a TIR1 construct into a "safe-harbour" locus, the degron system can be used to inducibly, yet reversibly, destroy any desired protein upon auxin treatment[111]. This method allows for temporal control of protein expression during embryogenesis, but, similar to knockdown studies, may not lead to 100% protein removal in the desired timeframe.

For inducible control at the gene level, the Cre-*lox* system[112,113] could be applied, using CRISPR–Cas9 to 'flox' genes for subsequent recombination-based deletion. However, the efficiencies required for this system to work reliably in human embryos may preclude its use.

Future potential clinical use

The announcement of the birth of 'CRISPR babies' on the eve of the second international human genome-editing summit in Hong Kong has led to renewed calls for a moratorium on human germline genome editing[114]. However, it has also apparently inspired others to follow in the same footsteps[115]. We would thus like to stress the immense importance of proper regulation and oversight of all future endeavors in this field, and provide a suggested workflow for rigorous preclinical evaluation (Fig. 2). Since November 2018, great strides have been made to ensure that any potential future use of reproductive genome editing is thoroughly vetted prior to approval and is keenly scrutinised while underway. China will soon introduce regulations to guarantee that accountability for gene editing in humans lies with those performing the technique, outlawing any experiments that undermine an individual's well-being or dignity[116]. China also recently drafted specific regulations regarding the approval process for editing human embryos[117]. Interestingly, Japan had previously released draft guidelines with a more permissive stance on human embryo genome editing, which did not outlaw germline editing for reproduction[118]. However, in the wake of the Hong Kong announcement, government officials are now discussing the introduction of strict, unambiguous legislation to prevent the implantation of genetically modified human embryos[119]. Further, the World Health Organisation has introduced an expert advisory committee to oversee global standards in human genome editing, who quickly suggested the establishment of a global registry for such research, to widespread approval[120].

Given the existing limitations in our understanding of the biology of the human embryo and how the germline genome might interact with CRISPR–Cas9-mediated DSBs, we and many others feel that the time to pursue such research clinically is not at hand[121,122]. Much more work is required to solidify our knowledge of the basic biology of human development before we consider introducing genome modification into the repertoire of treatments offered to IVF patients. One must ensure that the outcome will be the birth of healthy, disease-free children, without any potential long-term complications. The regulatory handling of mitochondrial replacement therapy, also a form of heritable genome editing that affects only the mitochondrial DNA[123,124], should be taken as an example. Many years of preclinical data went into proving the safety and efficacy of this technique prior to its consideration for use in human reproduction, and it can only be used when there is a known risk of inheriting serious mitochondrial disease[125–127]. Applying similar rigor to the handling of germline genome editing will be crucial in determining whether this is a viable clinical option in the future.

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Competing interests
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Additional information
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