Embryonic stem cells (ESC) are a set of pluripotent cells unique in character which are obtained from the 'inner cell mass of the preimplantation embryo'. They can then undergo asymmetric divisions whereby they either duplicate themselves or differentiate themselves into another cell type. It is important to identify genes involved in the regulation of stem cell function to examine the effects of altered gene expression in ES and other stem cells e.g. core networks of transcription factors (TF’s) such as Oct4, Nanog, and Sox2, govern key gene regulatory pathways/networks for the maintenance of self-renewal and pluripotency of mouse and human cells. These TF’s are modulated by specific external factors through signal transduction pathways e.g. leukemia inhibitory factor (LIF/Stat3, mitogen activated protein kinase1/3 (Mapk1/3)/TGFB superfamily and Wnt/glycogen synthase kinase 3β (Gsk3b) [1]. Although human ESC’s got isolated in 1998 from the in vitro fertilized blastocysts [2], their clinical use was limited as they were known to induce an immune response upon transplantation of these hESC’s into a patient [3].

Gene Transfer in Genetic Diseases

Majority of imprinted genes associated human disorders are genetic diseases [4]. Examples of these are Prader Willi syndrome, Angelman Syndrome, Silver Russel Syndrome, Beckwith Wiedemann Syndrome [5]. It is crucial to investigate whether there is genetic mutant within the imprinting control centre of imprinted gene especially in the embryos that are derived from assisted fertilization. In addition since imprinted genes are expressed in a monoallelic Pattern and DNA methylation plays a key role in the maintenance of genome imprinting [6], it is also important to investigate the ectopic expression of DNA methyl transferases (DNMT’s) and histone methyl transferases (HMT’s) levels in the prenatal genetic diagnosis processes, as both the DNMT’s and HMT’s are essential for the maintenance of DNA methylation at imprinted loci [7].

Gene Transfer is an area in therapeutics in which the active agent is a nucleic acid, in contrast to a protein or small molecules. Delivery of a DNA or RNA to a cell is an inefficient process; most gene transfers are carried out using a vector or gene delivery vehicle. These vehicles have been engineered from viruses by deleting some or all of the viral genome and replacing it with the therapeutic gene of interest under the control of a suitable promoter. There are three essential elements in gene transfer strategies i) a vector, ii) a gene to be delivered known as the transgene, iii) a relevant target cell to which DNA or RNA is delivered. Transduction is the series of steps in which the donated DNA enters the target cell and expresses the transgene. Gene delivery can be done in vivo, where the vector is directly injected into the patient [8] or, in case of hematopoietic and some other target cell, where removal of target cells from the patient is followed by return of modified autologous cells after gene transfer in the laboratory back to the patient [9]. This approach gives opportunities to integrate gene transfer techniques with cellular therapies.

Therapeutic cloning was introduced by Dr. Hwang in 2004 and to use it medically, Rideout used homologous recombination to replace defective gene in a Rag2-/- mice and replaced the defective Rag2 allele in nuclear transfer embryonic stem cell (nESC) with a functional allele. Genetically corrected nESCs were then differentiated into hematopoietic cells and transplanted into Rag2-/- mice [10]. Therapeutic cloning and gene therapy have also been used to cure murine equivalent of Parkinson’s disease as shown by Barberi et al. [11].

Subsequently in an attempt to develop spindle transfer (ST) oocytes for mitochondrial DNA replacement and develop ESC’s, Tachibana et al. [12,13] found that unfertilized metaphase II oocytes from mammals are sensitive to mechanical/physical stimuli where pressure, osmolarity, flux or temperature change can cause spontaneous activation in meiotic resumption and parthenogenetic development. Besides spindle transfer, pronuclear transfer between embryos has been proposed to prevent serious mitochondrial disease getting transmitted maternally which result from mitochondrial DNA’s in patients oocytes [14]. For mitochondrial replacement/ooplasmic transfer oocyte donation is required and hence their risks associated with it like ovarian hyperstimulation in young oocyte donors. US FDA considers the advantages of mitochondrial replacement [15].

Genome Editing Technologies

Clustered regulatory interspersed short palindromic repeats (CRISPR/Cas) system is one of the genome editing technologies besides zinc finger nuclease (ZFN) and Transcription activator like effector nucleases (TALEN). ZFN has gone up to clinical research stage in AIDS therapy based on administration of human chemokine like receptor 5 (CCR5) modified T cells [16]. Gene correction by ZFN’s has also been reported in iPSC derived somatic cell biopsies in patients with sickle cell disease, α1antitrypsin deficiency and Parkinson’s disease [17-20]. CRISPR/Cas9 has been used to correct mutation in intestinal stem cells derived from patients with cystic fibrosis [21]. Two reports further cited that microinjection of Cas9 and TALEN into 1 cell stage embryos lead to efficient generation of targeted gene modified from nonhuman primates (NHP) [22,23]. In genome editing of mammals targeted gene modification is frequently carried out by microinjecting gene editing system which consists of the nuclease mRNA, single guide RNAs (sgRNA’s for Cas 9, and a homology containing donor DNA template (if necessary) into animal embryos made by IVF or ICSI.

*Corresponding author: Kulvinder Kochar Kaur, Dr. Kulvinder Kaur Centre for Human Reproduction, 721, G.T.B. Nagar, Jalandhar-144001, Punjab, India, Tel: 91-181-9501358180; 91-181-4613422; Fax: 91-181-4613422; E-mail: kulvinder.dr@gmail.com

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Mammalian ESC’s, including human ESC’ have been more effectively modified by genome editing [28,29,35-38]. Genome editing technology is more likely to come into use in medicine to prevent a genetic disease if corrective genome editing is integrated into artificial reproductive technology (ART) including in vitro fertilization (IVF) and intracytoplasmic transfer (ICSI). One important difference is that genome editing does not require oocyte donation as in ooplasmic transfer or as is required for mitochondrial replacement.

Mechanism

Genome editing technologies are more effective genetic engineering technologies that can directly modify a gene within a genome in various organisms. This is obtained by a microorganism in which a nuclease is engineered and meant to cause double stranded breaks (DSB’s) at a targeted sequence and induce DNA repair through non-homologous end joining (NHEJ) or homology directed repair (HDR). The NHEJ is a DSB repair pathway which ligates or joins 2 broken ends together without a homologous template for repair and thus leading to the introduction of small insertions or deletions also known as indels, at the site of the DSB. The HDR is a DNA template dependent pathway for DSB repair, using a technology containing donor template along with a site specific genome editing nuclease, enabling the insertion of single or multiple transgenes (gene addition) in addition to single nucleotide substitutions in which an amino acid substitution of a protein occur, i.e., gene modification, or complete repair of a mutation in the resultant organism genome also known as gene corrections. Indications of integrating corrective genome editing into ART includes those with congenital anomalies which are caused by chromosomal, multifactorial, monogenic, environmental or teratogenic factors [39]. Hence genome editing maybe beneficial for monogenic disease since a genome editing can efficiently repair such a small mutation in the human germline. One should be selective in using genome editing in cases of monogenic disease and use it only where it appears that the benefit of using will be greater than that involved in using the appropriate genetic interventions or its side effects. For e.g., if there is evidence of inheritance very likely, because there is an autonomous recessive disease with both parents being homozygous for e.g., cystic fibrosis [40], or phenylketonuria [41], or if there is an autosomal dominant disease with one parent atleast being homozygous and affected, i.e., Huntington’s disease, familial adenomatous polyposis [42], one needs to consider using it.

If one attempts to repair a mutation directly in oocytes or embryos by means of an older homologous recombination technique, this attempt is likely to fail because of its low efficiency. Hence, genome editing mediated gene corrections in ESC's which are derived from a parents embryo made by IVF/ICSI could represent an alternative approach. Advantages of self-renewal of ESC’s, in vitro expansion and cryopreservation of ESC’s helps to repeatedly correct a mutation in a specific gene by genome editing. The efficiency of indel and gene addition is 14-91% by Cas9 and 0-83.49% by ZFN or TALEN’s in a specific gene by genome editing. The efficiency of indel and cryopreservation of ESC’s helps to repeatedly correct a mutation in a specific gene by genome editing. The efficiency of indel and gene addition is 14-91% by Cas9 and 0-83.49% by ZFN or TALEN’s respectively.

Genome Editing of Mammalian ESC’s

Gene correction mediated by genome editing technologies can be attained in hESCs as shown by analysis when modified ESC’s are analyzed for the occurrence of off targeted mutations. Following that a karyoplast is removed from the genetically corrected ESC and transferred into the female recipient. This can be potentially carried out in humans as similarly modified genetic cells by ZFN have been used to generate a biallelic knockdown of pigs [43]. Also, human SCNT has been used to derive blastocysts by three independent groups [44-46]. This if used only for the birth of one single child does not imply human cloning. With this there is a risk of xeno contamination in case mouse feeder cells, or fetal bovine serum, or recombinant growth factors are used from nonhuman species in human ESC medium. This can be avoided by using a xeno free culture reagents. Another thing is although electroperation or transfection agents might cause cytotoxicity [35], preliminary research could decrease such risks. Further there are problems with human ESC’s having a tendency to acquire genetic changes in the nucleus and/or in the mitochondria on prolonged culture [47-49]. Also not all colonies in a dish comprise of the same clones. Hence mutant colonies might get mixed into a colony during a subsequent NT procedure.

Zygote approach, as compared to ESC approach has fewer steps. In that genome editing system in just injected into the cytoplasm or pronucleus of zygotes to correct a mutation in a gene. Following screening of embryos, one or more embryos which have a corrected gene with no off target mutations, are then used for embryo transfer (ET). Maternal blood containing cell free DNA, used for noninvasive prenatal testing [50,51], is used to check the genetic condition of the fetus. After an invasive genetic testing like chorionic villus biopsy or amniocentesis confirms the absence of genetic mosaic mutations in the fetus, ET is done, although these carry a risk of miscarriage. Still longterm follow up is required as the effect of germine which gets modified has an effect on the entire body, even after a successful birth.

Effective gene modification results from microinjection of genome editing system into mammalian zygotes. The indel efficiency of a single gene by TALEN’s or Cas 9 ranges from 0.5-40.9% per zygote injected. The efficiency of 40.9% was attained in non-human primate embryos by the Cas 9 system composed of mRNA and sgRNA. Here a set of twin female neonates with both modified Rag and PPARγ were born. Also, Ran et al. [28] reported Cas 9 nickase treatment can induce indels in Mercep2 at 80-100% of mouse blastocysts [28]. In neonates the efficacy of indel and gene addition are 0-41.7% by TALEN’s or Cas 9 respectively. But these gene modifications results in off target modifications. Cas 9 nickase mutant resulted in less off target mutations, as compared to wild type mutations, which could not solve the target problem completely [30].

Advantages of Genome Editing Technology in Zygotes over ESC’s

Prior to undertaking an embryo transfer (ET), a prenatal genetic diagnosis (PGD) is required while doing the zygote approach whether an cleavage stage (D-3) or blastocyst stage (D-5) to make sure that no off-target mutation has developed i.e., checking the correction of the mutation has been complete. Though mutations were not detected in the defined population of target sites in nonhuman primates (NHP’s) in previous studies [22,23], hence a careful assessment is needed if PGD can definitely confirm genetic conditions in modified embryos. In PGD, zona pellucida is opened and embryonic cells removed from the embryo [52]. Thus embryo has to undergo physical manipulations twice, once during microinjection of the genome editing system and secondly at the time of biopsy required for PGD. If ICSI is done to avoid polyspermity these attempts become three. These might affect the future development of embryo both in vivo as well as in vitro.

Since a cleavage embryo consists of 6-8 cells, a single biopsy is used for PGD [53]. But in 15-80% of cases, mosaicism might effect and have an impact on the interpretation of PGD results [54-56]. While in the blastocyst stage, the embryo is made up of 130 cells, in the inner cell mass destined to develop the future fetus along with surrounding trophectoderm. Trophectoderm cells have been biopsied recently from...
a blastocyst for PGD which avoids the chances of damaging the embryo [53]. Though even up to blastocyst stage mosaicism remains [54-56], the results of randomized controlled trials supported that a single cell biopsy at cleavage stage causes more damage to the embryo as compared to at blastocyst stage and results in poorer clinical outcomes [57]. Further the microinjecting genome editing system into one cell stage embryo needs observation at the molecular level. During the one cell stage nuclear transitions which involve the separated oocyte and sperm pronuclei, pronuclear fusion and cleavage to the 2 cell stage. Right now pronuclear injection and cytoplasmic injection are adopted for introducing the genome editing system into mammalian zygotes. Because of this the injection method and timing of injection should be optimized as if there is incomplete gene correction this may fail to prevent a genetic disease. Also cytotoxicity caused by genome editing system which gets introduced in the case of plasmid, mRNA or protein with or without the short repair template DNA should be probed [58]. Recently it has been seen that the ZFN and TALEN proteins can cross cell membranes and induce endogenous gene disruption [23,59]. The advantage that this method offers is it can limit the time cells are exposed to nucleases, thus minimizing off target activity. The advantages which the zygote approach has over ESC approach are within terms of it being simple, which means that it is a more controllable protocol. Secondly this is not associated with the ethical issue of human cloning as is the case with ESC approach. If there are 13-15 oocytes retrieved, which is optimal for getting success with first IVF cycle, getting more effective gene correction [60,61], as compared to zygote approach is more likely to be possible in a clinical setting. Cas 9 is increasingly being used for zygote approach due to its easy preparation. Although Cas 9 is likely to cause greater number of off target mutations than ZFN and TALEN’s [28], but Cas 9 is being rapidly improved which shows that a combination of Cas 9 nuclease and paired gRNA’s, fusion of inactive Cas 9 to Fok1 nucleases improves the specificity of targeted genes modification [28,62,63]. Recently, Kang et al. [64] have used CRISPR/Cas mediated genome editing systems in human 3PN embryos. They found that although Cas 9mRNA, gRNA and donor DNA were introduced successfully with naturally occurring CCR5 delta32 allele into human embryo, in the embryos containing engineered CCR5 delta 32, the other alleles at the same locus could not be fully controlled because they either remained wild type or maintain indel mutations. Hence they concluded that although therapeutic treatments of genetic diseases get implicated by this research, there are significant technical issues which need to be addressed. Hence they advocated preventing any application regarding genome editing on the future germline till further rigorous evaluation and discussion are undertaken by the global research and ethics committees.

Legally in some countries these germline gene modifications are either banned [59], based on legislation or in 29 countries including India, China, Japan and Ireland are banned based on guidelines. Although it is not banned in USA but temporarily they are under FDA supervision, while under NIH guidelines, germline gene modifications are allowed. Countries like Brazil, Belgium, Bulgaria, Canada, Denmark, Sweden, Czech Republic ban it on the pretext that modified genes maybe inherited by the offspring.

Thus, with more experience and research and awareness gradually under strict control and supervision these genome editing technologies may be used on day successfully in parents carrying children with recurrent heritable disorders giving so much agony to them for which no answer exists currently once these technical niggles are overcome.

References
1. Nishiyama A, Xin L, Sharov AA, Thomas M, Mower G, et al. (2009) Uncovering early response of gene regulatory networks in ES cells by systematic induction of transcription factors. Cell Stem Cell 5: 420-433.
2. Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, et al. (1998) Embryonic stem cell lines derived from human blastocysts. Science 282: 1145-1147.
3. Martin MJ, Muotri A, Gage F, Varki A (2005) Human embryonic stem cells express an immunogenic nonhuman sialic acid. Nat Med 11: 229-232.
4. Butler MG (2009) Genomic imprinting disorders in humans: A mini-review. J Assist Reprod Genet 26: 477-486.
5. Walter J, Paulsen M (2003) Imprinting and disease. Semin Cell Dev Biol 14: 101-110.
6. Barlow DP, Bartolomei MS (2014) Genomic imprinting in mammals. Cold Spring Harb Perspect Biol 6.
7. Zhang T, Termanis A, Ozkan B, Bao XX, Culley J, et al. (2016) Gfla/GLP Complex maintains imprinted DNA methylation in embryonic stem cells. Cell Rep 15: 77-85.
8. Breitbach CJ, Reid T, Burke J, Bell JC, Kim DH (2010) Navigating the clinical development landscape of oncolytic viruses and other cancer therapeutics: No shortcuts on the road to approval. Cytokine Growth Factor Rev 21: 85-99.
9. Cartier N, Haoein-Bey-Abina S, Bartholomae CC, Veres G, Schmidt M, et al. (2009) Haematopoietic stem cell gene therapy with a lentiviral vector in X linked adnoleukodystrophy. Science 326: 818-823.
10. Rideout WM, Hochgedlinger K, Kyba M, Daley QG, Jaenisch R (2002) Correction of a genetic defect by nuclear transplantation and combined cell and gene therapy. Cell 109: 17-27.
11. Barberi T, Klivenyi P, Calignasaa NY, Lee H, Kawamata H, et al. (2003) Neural subtype specification of fertilization and nuclear transfer embryonic stem cells and application in parkinsonian mice. Nat Biotechnol 21: 1200-1207.
12. Tachibana M, Amato P, Sparman M, Woodward J, Sanchis DM, et al. (2013) Towards germline gene therapy of inherited mitochondrial diseases. Nature 493: 627-631.
13. Tachibana M, Sparman M, Srinanitamochahi M, Ma H, Clepper L, et al. (2009) Mitochondrial gene replacement in primate offspring and embryonic stem cell. Nature 461: 367-372.
14. Craven L, Tuppen HA, Greggain GS, Harbottle JL, Murphy JL, et al. (2010) Pronuclear transfer in human embryos to prevent transmission of mitochondrial DNA disease. Nature 465: 82-85.
15. FDA (2014) Meeting materials, cellular, tissue, and gene therapies advisory committee.
16. Tebas P, Stein D, Tang WW, Frank I, Wang SQ, et al. (2014) Gene editing of CCR5 in autologous CD4 T cells of persons infected with HIV. N Engl J Med 370: 901-910.
17. Sebastiani V, Maeder ML, Angeles JM, Haddad B, Khayr C, et al. (2011) In situ genetic correction of the sickle cell anaemia mutation in human induced pluripotent cells using engineered zinc finger nucleases. Stem Cells 29: 1717-1726.
18. Yusa K, Rashid ST, Strick-Marchand H, Varela I, Liu PQ, et al. (2011) Targeted gene correction of Fli-1 antithympy deficiency in induced pluripotent stem cells. Nature 478: 391-394.
19. Soldier F, Laganère J, Cheng AW, Hockemeyer D, Gao Q, et al. (2011) Generation of isogenic pluripotent stem cells differing exclusively at two early onset Parkinsson point mutations. Cell 146: 318-331.
20. Schwank G, Koo BK, Sasselli V, Dekkers JF, Heo I, et al. (2013) Functional repair of CFTR by CRISPR/Cas9 in intestinal stem cell organoids of cystic fibrosis patients. Cell Stem Cell 13: 653-668.
21. Bacman SR, Williams SL, Pinto M, Peralta S, Moraes CT (2013) Specific elimination of mutant mitochondrial genomes in patient-derived cells by mitoTALENs. Nat Med 19: 1111-1113.
22. Liu H, Chen Y, Niu Y, Zhang K, Kang Y, et al. (2014) TALEN-mediated gene mutagenesis in rhesus and cynomolgus monkeys. Cell Stem Cell 14: 323-328.
23. Niu Y, Shun B, Cui Y, Chen Y, Wang J, et al. (2014) Generation of gene-modified cynomolgus monkey via Cas9/RNA-mediated gene targeting in one-cell embryos. Cell 156: 836-843.
24. Wu Y, Liang D, Wang Y, Bai M, Tang W, et al. (2013) Correction of a genetic disease in mice via use of CRISPR-Cas9. Nat Stem Cell 13: 659-662.

25. Tesson L, Usal C, Ménoret S, Leung E, Niles BJ, et al. (2011) Knockout rats generated by embryo microinjection of TALENs. Nat Biotechnol 29: 695-696.

26. Yang H, Wang H, Shivilla CS, Cheng AW, Shi L, et al. (2013) One-step generation of mice carrying reporter and conditional alleles by CRISPR/Cas-mediated genome engineering. Cell 154: 1370-1379.

27. Li W, Teng F, Li T, Zhou Q (2013) Simultaneous generation and germline transmission of multiple gene mutations in rats using CRISPR-Cas systems. Nat Biotechnol 31: 684-686.

28. Ran FA, Hsu PD, Lin CY, Gootenberg JS, Konermann S, et al. (2013) Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity. Cell 154: 1380-1389.

29. Wang H, Yang H, Shivilla CS, Dawlaty MM, Cheng AW, et al. (2013) One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. Cell 153: 910-918.

30. Li F, Cowley DO, Banner D, Holle E, Zhang L, et al. (2014) Efficient genetic manipulation of the NOD-Rag1-/- mouse by combining in vivo fertilization and CRISPR/Cas9 technology. Sci Rep 4: 5290.

31. Yasuda A, Mitsui N, Watanabe T, Sakuma T, Oyadomari S, et al. (2014) Highly efficient targeted mutagenesis in one cell mouse embryos mediated by the TALEN and CRISPR/Cas systems. Sci Rep 4: 5705.

32. Carlson DF, Tan W, Lillico SD, Viveros Martin A, Proudfoot C, et al. (2012) Efficient TALEN-mediated gene knockout in livestock. Proc Natl Acad Sci U S A 109: 17382-17387.

33. Liu Z, Zhou X, Zhu Y, Chen ZF, Yu B, et al. (2014) Generation of a monkey defective lentiviral vector delivery. Nat Biotechnol 25: 1298-1306.

34. Marmont B, Guonsev P, Beausejour CM, Colleoni S, Lee YL, et al. (2007) Gene editing in human stem cells using zinc finger nucleases and integrase-defective lentiviral vector delivery. Nat Biotechnol 25: 1298-1306.

35. Mashimo T, Kaneko T, Sakuma T, Kobayashi J, Kunihiro Y, et al. (2013) Efficient gene targeting by TALE effector nucleases co-injected with exonucleases in zygotes. Sci Rep 3: 1253.

36. Lombardo A, Genovesi P, Beausejour CM, Colleoni S, Lee YL, et al. (2007) Gene editing in human stem cells using zinc finger nucleases and integrase-defective lentiviral vector delivery. Nat Biotechnol 25: 1298-1306.

37. Hockemeyer D, Soldner F, Beard C, Gao Q, Mitajtova P, et al. (2009) Efficient targeting of expressed and silent genes in human ESCs and iPSCs using zinc-finger nucleases. Nat Biotechnol 27: 851-857.

38. Hockemeyer D, Wang H, Kiani S, Lai CS, Gao Q, et al. (2011) Genetic engineering of human pluripotent cells using TALE nucleases. Nat Biotechnol 29: 731-734.

39. Zhou J, Maeder ML, Mali P, Pruett-Miller SM, Thibodeau-Begany S, et al. (2009) Gene targeting of a disease-related gene in human induced pluripotent stem and embryonic stem cells. Cell Stem Cell 5: 97-110.

40. Li W, Li X, Li T, Jiang MG, Wan H, et al. (2014) Genetic modification and screening in rat using haploid embryonic stem cells. Cell Stem Cell 14: 404-414.

41. WHO (2014) Congenital anomalies. Fact Sheet number 370.

42. Neocleous Y, Vialleuros PK, Tanteles GA, Costi C, Moutaf M, et al. (2014) Apparent homozygosity of p.508del in CFTR due to a large gene deletion of exons 4-11. Case Rep Genet 2014: 613863.

43. Protopopov A, Tansey MZ, Kovac J, Hovnick T, Podraskaj KT, et al. (2012) Five novel mutations and two large deletions in a population analysis of the phenylalanine hydroxylation gene. Mol Genet Metab 106: 142-148.

44. Cruz-Correa M, Diaz-Algorry Y, Mendez V, Vazquez P, Lozada ME, et al. (2013) Clinical characterization and mutation spectrum in Hispanic families with adenomatous polyposis syndrome. Fam Cancer12: 555-562.

45. Hauschild J, Petersen B, Santiago Y, Queisser AL, Carnwath JW, et al. (2011) Efficient generation of a biallelic knockout in pigs using zinc-finger nucleases. Proc Natl Acad Sci U S A 108: 12013-12017.