The New Biomedicine: A Critical Appraisal

Barbara B. Knowles, Ph.D.*, Davor Solter, M.D., Ph.D., Surapol Issaragrissil, M.D., FRCP, FACP, FRCPA, FRCPath*****

Professor Emerita, The Jackson Laboratory, Bar Harbor, ME, USA,Director Emeritus, Max- Planck Institute of Immunbiology and Epigenetics, Germany, ***Director Siriraj Center for Excellence in Stem Cell Research, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand.*

ABSTRACT

Mahidol University's namesake, H.R.H. Prince Mahidol, stated the University's universal view of higher education as follows: 'True success is not in the learning, but in its application to the benefit of mankind'.[1] It is thus fitting that the following commentary speaks to the University's goal. The acquisition of basic scientific knowledge by scientists working in genetics, embryology, developmental biology, immunology and virology throughout the twentieth century led to its application to clinical problems in the twenty-first. The technology to repair genetically-deficient pluripotent stem cells, treating diseased adults or ensuring the birth of healthy babies now is almost within our power. However, the application of this knowledge to living organisms, using cells that can mutate and selectively evolve, makes clinical application tricky while social and ethical issues arising from the eventual use of these technologies requires thought. Future attention to trends in basic science research should make stem cell therapy applicable to all.

Keywords: Biomedicine; critical appraisal (Siriraj Med J 2018;70: 529-539)

PRELUDE

Arguably, the most important basic science development of relevance to the pluripotent stem cell field in the twentieth century was Leroy Stevens' description of mouse teratocarcinoma[2], tumors composed of an infinite number of differentiated cell types and tissues but also containing nests of undifferentiated, "embryonal" carcinoma cells. His sequentially, animal-passaged teratocarcinomas gave access to an experimental test of the cancer stem cell hypothesis. Indeed, injection of single embryonal carcinoma cells (ECC) into mice give rise to tumors containing the spectrum of cell types found in the original teratocarcinoma.[3] Cell lines derived from both mouse[4] and human teratocarcinomas [5] were derived, providing an even more accessible platform to dissect the characteristics of pluripotency.[6] In addition, the quest to determine the normal pluripotent stem cell (PSC)-equivalent to the ECC, was accomplished by transplanting day 3 to 6 day-old mouse embryos into ectopic sites in syngeneic mice, analyzing the tumors arising for the pluripotent phenotype. Such transplants gave rise to teratomas containing the characteristic complexity of differentiated cell types.[7,8] This finding encouraged. the derivation and characterization of mouse embryonic stem cell lines (mESCs) in cell culture[9,10] Some years later human embryonic stem cells (hESCs)[11] were isolated and the ability of multiple hESCs cell lines to differentiate into the multiple human differentiated cell types was characterized.[11]

In parallel with these findings the search for replacement tissue and cell therapies in medicine passed from organ, tissue and cell transplantation in animal

Correspondence to: Surapol Issaragrissil
E-mail: surapol.iss@mahidol.ac.th
Received 11 April 2018 Revised 17 April 2018 Accepted 2 May 2018
doi:10.14456/smj.2018.85

models to clinical trials. As the principal immunologic requirements for successful transplantation and their control evolved, the first human corneal, kidney, heart and liver transplants, as well as replacement of the whole or parts of the hematopoietic system took place. That epithelial cells cultured *in vitro*, could be grown and expanded *in vivo* led to the idea that specific cell types could be isolated and quickly expanded in quantities useful for transplantation. Pioneering work to this effect was first performed by isolating and growing patient's epidermal keratinocytes and then using them to provide autologous transplants to provide coverage for burn victims.[12] The availability of PSCs further expanded the possibility to provide, through their differentiation other progenitor, precursor or differentiated cell type for clinical use.

Concomitantly, the emergence of DNA sequencing and its application to gene editing by homologous recombination[13,14] changed genetics from observational to experimental branch of science. This rapid evolution led to an understanding of the phenotypic consequences of dysfunctional genes presaging the first efforts towards gene replacement therapy in humans to effect cures. Indeed, the latter years of the 20th century became a hot bed for correcting genes first *in vitro*, then in animal models *in vivo*, and then translating these advances for clinical use.

The first reported gene therapy clinical trials, reported before the turn of the century, involved transgenesis of two adenosine deaminase (ADA)-deficient severe combined immune-deficient (SCID) patients. In the first study they were transfused with their own peripheral blood T-lymphocytes (PBL) which had been transfected *in vitro* to Moloney murine leukemia virus (MoMLV) driving a recombinant ADA cDNA.[15] Four years after the transfusions took place the patient's ADA levels were reported to be normal, however these patients were also being treated with pegylated ADA. Others, also using *in vitro* transfection of an MoMLV-ADAcDNA vector into the patient's PBLs and bone marrow-derived cells and subsequent transfusion, reported short term reconstitution after transfusion of either cell type but only bone marrow progenitor cells provided long term reconstitution.[16] Again these patients were also continuously treated with pegylated human ADA, which muddied the results. Another very early clinical trial in a completely different organ system, reported a very small, persistent dimunition of lipoprotein levels in a hypercholesterolaemic patient. The patient was partially hepatectomized, his dissociated liver cells, exposed to a recombinant MoMLV-chicken actin-promoted human LDL receptor-cDNA vector *in vitro* and his cells were then transfused back to autologous, partially hepatectomized, enzyme-deficient host.[17] On the face of it these trials established the relative safety of vector-mediated gene therapy and hinted at the efficacy of transducing long term progenitor cells.

Regenerative Medicine in the Twenty First Century Genetic Engineering

Follow-ups to these Phase 1/2 clincal trials produced initially promising results. Two studies were initiated. $CD4^+$ bone marrow-derived cells, transduced with a replication-defective recombinant MoMLV retrovirus containing the relevant human transgene, were transplanted into X-linked SCID and ADA-SCID patients. Long-term and sufficient levels of transgene expression were reported.[18,19] However, after some time patients in these and other similar trials presented with leukemias, a predictable consequence of viral vector integration into active host genes. Retroviral integration into oncogenes leads to their overexpression, resulting in uncontrolled growth of these cells and subsequent progession to leukemia.[20,21] Around the same time a pilot study to study the effect of an intravascular dose of a liver-directed adenovirus-5 vector containing ornithine transcarbamylase (*OTC*) to a partially OTC-deficient patient also produced disturbing results. The enzyme-deficient patient had an acute, uncontrollable inflammatory response to the adeno-5 viral vector and died.[22] Accordingly, a recess on viral-directed gene therapy was declared to allow time for vector biology/pharmacology to be addressed and to explore other methods to supply enzymes and other gene products to ailing human hosts.

The efficiency of genome editing improved steadily in the early part of this century. First, zinc finger proteins (ZFNs) were used to successfully target the specific gene recognition of DNA nucleases. These targeted nucleases could induce double strand breaks at specific sites in the genome and proper gene repair by homologous recombination was ensured by introducing a vector-born gene template for successful repair.[23,24] The transcription activator-like effector nucleases (TALENs) provided a more cost-effect way to mediate gene recognition by the DNA nucleases[25,26] and were also applied to human gene correction. However, the discovery of the bacterial defense system against bacteriophages, i.e. clustered regularly interspaced short palindromic repeats (CRISPR) , which uses the CRISPR-associated nuclease (Cas9) targeted to the genome by short RNA guides[27], opened mammalian genome repair to a wide range of scientists because of the simplicity of targeting and ease of operartion.[28,29] Corrective repair of the nick in the genome is repaired by

host-induced non-homologous end joining or, if a DNA template is furnished, by homologous recombination. The basic science discoveries underlying this fast moving field have been recently reviewed.[30]

The clinical use of viral vectors for gene therapy has also evolved. Due to their ease of delivery and their ability to infect a broad range of dividing and nondividing cells the adenoviral and recombinant adeno-associated viral vectors (rAAV) have been favored. However, because of the possibility of a strong immune response to the adenoviral vectors their use is currently somewhat limited mainly to oncolytic tumor therapies or to therapies in immunologically privileged sites. rAAV vectors, which can integrate into the host genome but are primarily episomal vectors, are now being actively used to transduce single human genes therapeutically.[31] Indeed, intravenous delivery of a chicken b-actin promoted-survival motor neuron1 (SMN1) transgene, delivered in an rAAV vector, resulted in improvement in the survival and motor function of fifteen young children diagnosed with spinal muscular atrophy, who were screened at the outset of treatment and found free of antibodies to AAV serotype 9. However, most of these patients eventually did develop elevated liver enzymes, perhaps as a result of the immune response to AAV, which returned to normal levels after prednisolone treatment.[32] A subsequent report of elevated liver enzymes and also neuronal toxicity following high level of AAV/SMN1 administration to nonhuman primates and piglets[33] predicts this viral vector will also prove unsafe for future therapeutic purposes. When long term expression via integration into a largely non-dividing cell population is required the g-retroviral vectors (largely MoMULV-based) cannot be used, however, modified lentiviral vectors can transduce genes into both dividing and nondividing cells. Current lentiviral vectors take advantage of the broad tropism of the pseudotyped VSV glycoprotein so they are versatile, albeit difficult to manufacture under the GMP-conditions necessary for the high titers needed for gene therapy.[34] The reader is directed to a comprehensive review on emerging themes in gene therapy, targeting various organ systems, by the currently used viral vectors[22]

Correction of host genes has now been directly effected *in vivo*. A phase 3 clinical trial demonstrated the clinical efficacy of the FDA-approved drug of Nursinerin, a mixture of 18-mer oligonucleotides, designed to activate the paralogous SMN2 gene, by rearranged splicing, to produce SMN1 protein. Infants who had been previously diagnosed with SMA were treated by multiple Nursinerin injections, directly into their cerebral spinal fluids. Forty-one percent of the treatment group and none of the control group reached their motor milestone responses a year after the initiation of the trial.[35] The next quest will be to identify SMN1 mutations at birth, start Nursinerin treatment immediately and determine whether the entire dSMA disease entity can be prevented.

Successful therapy by direct gene editing has recently been accomplished in Beethoven mice, a mouse model of human deafness resulting from mutation of a gene component of the mechano-transduction channels in mammalian hair cells. A mixture of Lipofectamine 2000 and a Cas9 nuclease-guideRNA ribonucleprotein complex were delivered by direct injection into the cochlea. A therapeutic effect was noted, measured by acoustic startle responses, in the treated, versus the untreated, ear of the same animals.[36] The potential of off-target effects, found to be negligible in this example, is always an area of concern for *in vivo* gene repair therapies. In addition, the possibility of antibodies to the bacterial CAS9 nuclease in human subjects has been raised and is now under investigation.[37] The efficacy of gene therapy, be it by supply or correction, has survived the proof of principle in animal models and clinical medicine but a safe, reliable delivery system requires further investigation. Perhaps nanoparticle or lipid-based delivery systems currently in development will prove effective. Nonetheless, deafness has been a long-term social problem in our ever enlarging aging populations, comprised of individuals often willing to enter into clinical trials, so this may well be the next frontier for gene correction therapy.

Stem Cell Therapy

In 2006, Shinya Yamanaka and his colleagues accomplished an experimental breakthrough, making use of a technique that targets transcription factor expression within a given cell type to induce its transdifferentiation into a different one.[38] They showed that forced expression of a combination of relevant transcription factors (OCT4, SOX2, KLF4 and MYC) in differentiated mouse[39] or human[40] cells could induce them to pluripotency. These induced-pluripotent stem cells (iPSC), like embryo-derived mouse and human ESCs, were capable of differentiating into essentially all cell types of the adult organism when differentiated via an *in vitro*, embryoid body assay or via an *in vivo*, teratoma assay in immundeficient mice. iPSCs, reprogrammed *in vitro*, may differ epigenetically from ESCs, derived from embryos that have undergone natural reprogramming *in vivo*. Most genomic methylation marks are flexibly reconstituted however, the methylation of imprinted genes may be at issue, setting up potential differences between ESCs and iPSCs. Whether any epigenetic differences remain between these two types of PSC and

whether more genetic perturbations occur because of this additional manipulation is under investigation but no global differences specific to one or the other cell type have been found.[41]

As soon as PSCs were isolated *in vitro* they were regarded as integral components of regenerative medicine. The esistence of iPSCs brought the concept of individualized regenerative medicine. One's own cells could be isolated, reprogrammed to pluripotency, then differentiated into suitable precursor, progenitor, or differentiated cells for autologous transplantation to treat diseases or traumatic injuries, without fear of immune rejection. Unfortunately, the possibility of cure by stem cells, be they pluri-, multi- or uni-potent, was seized upon by unscrupulous practitioners who marketed their use through clinics fraudulently claiming cures of various diseases. Because of this malpractice, individuals and the International Stem Society for Stem Cell Research called for governmental regulation of the field of regenerative medicine. Meanwhile, genuine clinical trials had been initiated with PSCs and the results are now being published in the scientific literature. As of November, 2017 the US Food and Drug Administration has the oversight of the use of human stem cells as drugs. The World Health Organization has been asked to collaborate with international groups active in cell therapy with the goal of providing guidelines to promote the development of safe and effective gene therapies within an international regulatory framework; a role that has yet to be effected.[42]

In 2003, the International Stem Cell Forum funded the International Stem Cell Initiative (ISCI) to define the human PSC by investigating their basic properties[6] and performing collaborative experiments related to their use in human medicine. PSCs proliferate (self-renew), they can differentiate, or they can die. Mutations occur in the genome of any living cell. If this mutation affects its robustness or proliferation it might grow selectively after any potentially disruptive event, e.g. ESC-isolation, iPSC-reprogramming, or even some random cell culture event, potentially conferring tumorigenic potential on its progeny. Unintentional malignancy is the worry of cell therapeutics.

For this reason the ISCI initiated a large study to compare 120 cell line pairs, from both early and late *in vitro* passage. The goal was to determine whether any common genomic changes appeared during their culture. It was already known that PSCs tended to accumulate particular chromosome abnormalities *in vitro*. Indeed, ~14% of the early passage cell lines and 33% of the high passage cell lines exhibited increased ploidy of chromosomes 1q, 12p, 17 and centromeric 20.[43] Further, single nucleotide polymorphism (SNP) analysis of the paired early and late passage cells revealed common amplification of an area of chromosome 20q11.21 in ~20% of the karyotypically normal cell pairs tested.[43] Analysis of the genes in the minimal 20q amplicon revealed *BCL2XL* as one of the three genes in this region that were expressed in PSCs. Overexpression of the BCL2L1 (BCL-XL isoform), and not the other two gene products, in normal PSCs conferred the rapid growth characteristics of the copy-number-variant PSCs, while inhibition of BCL-XL suppressed the PSCs growth advantage.[44] From these data BCL-XL was demonstrated to be a selected, potential driver mutation in some PSCs *in vitro*. Furthermore, most of the human embryonal carcinoma cell lines tested, and a human teratocarcinoma biopsy exhibited 20q11.21 amplification, circumstantially linking this mutation with malignant transformation[44]. A different large scale study of PSCs looked for and identified mutations in *TP53* in 14/257 cell lines tested.[45] Others exposed PSCs with p53 mutations to culture stress and found these cells exhibited increased cell proliferation, decreased apopotosis, decreased cell differentiation and enhanced clonogenic efficiency.[46]

Combine these studies show that mutations affecting two known drivers of human cancer, BCL-XL and p53, give rise to selective PSC growth *in vitro*. Whereas no known mutation is known to cause human teratocarcinoma (a common tumor in young men) studies of 129-strain mouse teratocarcinomas suggest a missed differentiation cue in some cells of the male embryonic germ line results in their continued proliferation and increases their opportunity for acquiring transformative mutations leading to testicular cancer.[47]

The ISCI also conducted a study to determine which of the commonly used *in vitro* or *in vivo* differentiation assays best predict PSC malignant potential. Only the teratoma assay, when analyzed by histology and immunohistochemistry to reveal nests embryonal carcinoma-like cells and yolk sac elements, or when analyzed by RNA-seq analysis and Teratoscore, to reveal molecular markers of pluripotency and yolk sac, was effective.[48] These findings suggest that random mutation and selective proliferation will be an underlying threat to PSCs as they are currently maintained. Although therapeutic transplants will focus on differentiated, or adult stem, cells derived from PSCs, the absence of malignant potential in the starting cell population is a requirement for the initial PSC cell line. The International Stem Cell Initiative has discharged its experimental mandate, having set standards for both the PSC field and quality control imperatives for International Stem Cell Banking. Now, twenty years after isolation of

the first human PSC, a Japanese pharmaceutical company (Sumitomo Dainippon) has opened to produce cells for regenerative medicine clinical trials. More such companies will follow to service the evolving PSC industry.

Evidence for the first in human clinical trials of PSC-derivatives are all based on extensive preclinical trials in multiple mammalian model organisms. Two well known companies, Asterias and International Stem Cell Corporation, have ongoing Phase 1/2 clinical trials using ESC-derived oligodendritic cells for spinal cord injury and ESC-derived neural stem cells for Parkinson's disease and they have done or acquired preliminary evidence for trials in other organ systems. In this ESC-based industry both the ESC, and the ESC-derive adult stem cells to be transferred have been subjected to exhaustive, long-term tumorigenicity testing. Progress updates about these trials can be found on these company's websites.

The first clinical trials of PSC-derived cells in regenerative medicine appropriately focused on the macula, the functional central area of the retina, which is known to degenerate in the eyes of some elderly people. The macula, is composed of retinal pigmented epithelial cells (RPE), brown pigmented cells of cobblestone morphology, that are easily seen/identified upon spontaneous differentiation of PSCs *in vitro*, and in PSC-generated teratomas. There are several advantages to clinical transplantation of these terminally differentiated cells: the back of the eye is surgically approachable, it is generally regarded as an immunologically privileged site and the the transplant itself is confined to an accessible non-vital organ. Addtionally, blind elderly people are willing to participate in trials that might improve their chances of independent living. One of the first proper clinical trials, using suspensions of cells from differentiated RPEs from an allogeneic embryonic stem cell (ESC) line reported the safety of the procedure but very mixed results on efficacy.[50] These authors suggested some of the adverse results they found might have been associated with the short-term immunosuppression they used. Consequently, after the iPSC technology became routine, fibroblasts from two patients with macular degeneration were reprogrammed to iPSCs. After differentiation, the sheets of patient-derived RPEs were subjected to intensive genomic and epigenomic scrutiny and tumorigenicity testing. Afterwards, a sheet of autologous cells was transplanted under the first patient's fovea in the absence of immuno-suppressants. Despite signs of neovascularization and maintenance of the graft up to 1 year post-transplant, there was neither diminishment nor major improvement of the patient's visual acuity. The second patient in this clinical trial cohort was not transplanted with his own iPSC; three copy number variants were identified in the differentiated RPE cell sheets that differed from his own cells prior to reprogramming and the cells were thought to represent a potential safety risk.[51] He subsequently received an allogeneic transplant, which was not successful, most probably due to a surgical problem rather than immune rejection. A third clinical trial, consists of a cohort of ten patients, each of whom received a patch of retinal pigment epithelium differentiated from an allogeneic, stable, human ESC, grown on a human vitronectin-coated polyester membrane. This composite, cell and membrane, was transferred to the fovea using a specially manufactured tool to patients treated with a short-term immune suppressive regime, but the transplants persist without long-term immunosuppression. The reported results from two of these ten patients at a year of engraftment indicate both safety and efficacy.[52] The remaining eight patients will be reported on in future. A fourth phase 1/2 clinical trial used RPEs differentiated from another ESC line, grown on a different, parylene, substrate and then transplanted to the fovea. Here at about a year of transplant one of five patients in the trial displayed improved vision, three patients experienced worse vision and there was no change in the vision of one patient.[53] Taken together, it appears the efficacy of macular replacement depends on the methodology used but each trial reported the macular transplants themselves safe. No difference between the success of autologous (iPSC-derived) or allogeneic (ESC-derived) transplants at this particular host site were found, albeit this is regarded as an immunologically privileged site. Also, only one was an autologous (iPSC-derived) transplant.

Each clinical trial requires extensive preclinical experience and preparation in order to obtain permission of the relevant oversight agencies. From an industrial standpoint ensuring the safety of a single PSC line for transplantation is expensive in time and money. Therefore, minimizing the number of cell lines to be permitted is particularly relevant to industrializing the processes and improving outcomes.[54,55] However, when regarding transplants to other sites in the body, especially to the hematopoietic system, immune rejection of HLA-mismatched transplants leadx to rejection. The immunologic imperative for autologous, iPSC-derived, transplants for corrective transplantation is currently neither cost nor time efficient. To minimize the potential future problem of finding a PSC with a suitable HLA match the Japanese have proposed banking PSCs with a series of population-common homozygous HLA haplotypes (typing for HLA-A,-B,-C -DRB1,-DQB1, -DPB1 and perhaps KIR), to be used to for transplantation. In such a scenario regional

cell banks would focus on collecting and characterizing iPSCs of the most common haplotypes in their region's population for future therapeutic use. The feasibility of this approach and the number of tolerable mismatches has been recently modeled by retrospective analysis of cord blood transplantation pairs in Japan.[56]

There are some accessible organ systems, such as the skin, where populations of adult stem cells were derived for autologous transplantation to correct a defect or replace a tissue lost to trauma or aging. Keratinocytes explanted from a not-yet-affected area of skin from a patient with junctional epidermolysis bullosa, a skin disease caused by *LAMB3* mutation, were isolated *in vitro*, transduced with a normal *LAMB3* cDNA under the control of the MoLV long terminal repeat. Corrected colonies were isolated, expanded and transplanted back to the patient. Because populations of gene-corrected cells in the explanted epidermis were marked by the multiple integration sites of the transgene the authors could trace the derivation of the various patches of new skin. Analysis of these mosaic skin patches revealed that "specific stems cells persist contributing to both renewal and repair by giving rise to pools of progenitors that persist for various periods of time, replenishing differentiated cells to make short term contributions to wound healing".[57] Although these epidermal stem cells were not derived from PSCs, but rather came directly from a population of adult stem cells[56], this work supports the concept that an entire organ system can be readily regenerated from autologous transplants if the proper population of gene-corrected stem cells can be identified. (As an aside transplanted skin can be readily surveyed over the life time of the individual for malignant nodules.)

Gene correction in hematopoietic cells, suitable for transplantation to patients with hematopoietic defects, has been under intense scrutiny for some time. Many consider therapy with cells corrected *ex vivo* and then transferred for permanent correction in vivo preferable to vector-driven gene replacement therapy *in vivo*, which may pose an inherent danger to the patient. In early studies, the b-globin gene, from patients with sickle cell anemia was corrected by homologous recombination in bone marrow-derived $CD34^+$ cells. Unfortunately, after transplanting the population of HSC/progenitor cells into humanized immune-compromised mice, only a limited level of corrected cells could be found, suggesting that the transplanted cells contained more mature progenitors rather than the hematopoietic stem cells (HSC) necessary for extensive repopulation *in vivo*.[59] High expression levels of the *IL2R* gene was obtained after homologous recombination in $CD34^+$ cells isolated from a SCIDX1 patient. Here, cells transplanted into humanized immunocompromised mice were functional but the authors speculate whether the limited number of HSCs and precursors obtained in vitro could ever repopulate the absent lymphoid lineages in a diseased human.[60]

If gene correction can be performed in iPSCs, which are then to be differentiated into the HSCs necessary for successful long-term engraftment, corrective transplantation becomes a simple matter of cell selection and efficacy testing. This is the current sticking point in hematopoietic stem cell therapy, regardless of the source of the gene corrected cell. CRISPR/Cas9 correction of genes in iPSCs from patients afflicted with various anemias has provided many laboratories with cells for *in vitro* disease modeling.[61,62] Unfortunately, the *in vitro* hematopoietic differentiation protocols in current usage for human iPSC preferentially induce primitive hemangiogenic differentiation rather than definitive, adult hematopoiesis. For this reason the safety and efficacy of gene-corrected cells capable of differentiating into mature elements of the hematopoietic system cannot be estimated. The transcription factors[63], the cytokines and signaling pathways[64] required for definitive human hematopoetic differentiation have now been defined, providing the basic research tools necessary to isolate the cell types capable of forming all elements of the mature definitive hematopoietic system. Experiments in mice suggest combined manipulation of transcription factors, signaling pathways and cytokines in a spatial and temporal fashion can yield the cells necessary for engraftment and lineage-specific, nonmalignant reconstitution simply using adult vascular endothelial cells as a starting point.[65] Regardless of the cell source, using patient-derived, gene-corrected hematopoietic cells for autologous transplantation eliminates the possibility of immune rejection, unless the corrected gene product is recognized as foreign, and also eliminates the possibility of graft versus host disease. Managing allotransplantation, although possible, complicates this aspect of modern biomedicine.

Defining the cell type for transplantation is of essence but the manufacturing process required for even testing the safety and cell numbers required for efficacy is also to be considered. Fine tuning the process itself, under GMP, animal product-free conditions requires: reprogramming (by chemicals, small molecules or transcription factors); editing and selecting the corrected targeted gene (by CRISPR/Cas9 technology); and differentiation, expansion and selection of the currently undefined long-term repopulating cell type for transplantation. Many aspects of process development have been addressed in the

context of therapeutic adoptive T-cell transfer. Here large numbers of patients cells are purified, modified and reinfused for cancer therapy.[66]

Effecting cures from selected, differentiated, characterized cell populations will be a giant step into the area of personalized medicine. This year the California Stem Cell Agency, which invests in stem cell-based therapies, has funded four California-based studies to target patients with severe sickle cell disease. With other funded studies in progress, this area is targeted for rapid development.

Genetic Engineering in Oocytes and Zygotes

Ever since development of homologous recombination technology[13,14] scientists have sought methods to mutate or modify genes by injecting relevant constructs directly into the oocyte or zygote. Such a direct approach would significantly shorten the time necessary to produce genetically modified animals although it is complicated by the inability to use the positive/negative selection crucial for selecting successful homologous recombinants.[67] The frequency of homologous recombination without selection, even when DNA constructs were injected directly into cells[68], is so low that such an approach in embryos was completely impractical.

All this changed with the advent of CRISPR-Cas technology which targets the relevant gene with a frequency of 10-20% or higher. In addition, it became possible to target several genes simultaneously[69] thus significantly facilitating the production of animals carrying multiple mutations and avoiding very time consuming and expensive breeding protocols. CRISPR-Cas did not conceptually change the mutagenesis approach to the study of gene function but made it much faster, cheaper and more reliable. Moreover, the high frequency of gene targeting made it possible for the first time to contemplate similar experiments using human eggs and zygotes. The first such study describing the effect of OCT4 mutation in early human development was recently published.[70] Here, loss of OCT4 affected human preimplantation development as has been previously shown in mice, though it seems that the disruption of development occurs earlier in human embryos. This conclusion is somewhat tentative since a control, using mouse zygotes injected with the similar constructs, was not included. One can envision further, similar studies using CRISPR-Cas to study the role of specific genes in human development, albeit the limitations posed by the available material and by the difficulties of performing extensive analysis will likely limit the extent of such an approach.

CRISPR-Cas technologies provide however another, never before available, opportunity namely targeted correction of disease-causing mutations directly in the zygote resulting in complete cure of a genetic disease. Several "proof of principle" results have been reported using mouse zygotes.[71,72] Zygotes from mice carrying a dominant mutation in the *Crygc* gene that causes cataract have been injected with Cas9 mRNA, a guide RNA and an oligonucleotide resulting in homology directed (HDR).[72] Approximately half of the newborn mice derived from injected zygotes showed successful targeting of the mutant allele while the wild type allele was not affected. Sequencing of the mutant alleles showed HDR as well as non-homologous end joining (NHEJ) repair and interestingly, in few of NHEJ cases the mutant phenotype was accidentally corrected. Similar results were obtained when attempting to correct *Dmd* gene, mutation of which results in mouse model of Duchenne muscular dystrophy (DMD).[71] Sequencing of the targeted allele showed the presence of both HDR and NHEJ and also a degree of genetic mosaicism in successfully targeted mice. The mosaicism is likely caused by the delay of CRSPR-Cas mediated targeting so that targeting occurs following one or two divisions thus affecting some but not all blastomeres of the cleaving embryo. Interestingly due to the specific mechanism of DMD even partial correction in mosaic animals resulted in significant phenotypic improvement.[71] These and other similar reports indicated that gene correction in the zygote using CRISPR-Cas is indeed possible but not without its problems. The frequency of successful targeting and correction was not 100% and mosaicism occurred relatively frequently which could have compromised the results, depending on the nature of the mutation. However, off target effects and targeting of the wild type allele were not commonm though they could not be excluded and most certainly could affect the use of this technology to correct human mutations.

The first attempt of gene targeting using human embryos was reported in 2015.[73] The authors used polyspermic, tripronuclear zygotes that are occasionally produced in fertility clinics following in vitro fertilization (IVF) and are normally discarded. They attempted targeting of the endogenous β-globin gene with mixed results. Though HDR was observed, the success rate was modest and embryos showing gene editing were mosaic. Off-target effects were also observed as well as NHEJ. Similar results were reported in an experiment attempting to introduce the naturally occurring *CCR5Δ32* allele (homozygous carriers of this allele are resistant to HIV infection). Here the authors observed a low incidence of HDR, mosaicism and possible off-target effects.[74]

Modification of the CRISPR-Cas technology, the so called "base editor" that can target and promote single base conversion, has recently been used to attempt correction of the mutation causing β-thalassemia.[75] The authors used cloned human embryos produced by enucleating human oocytesm fusing them with lymphocytes from a patient with β-thalassemia. Base editing, i.e. correction of the mutation, was observed in a relatively high number of analyzed blastomeres but mosaicism was always present. These studies though preliminary indicate that gene editing in human embryo is technically feasible though many problems remain to be solved before its use in the clinic can be contemplated.

Recently a very detailed study using both human zygotes and oocytes, attempting to correct the dominant mutation in the MYBPC3 gene that causes hypertrophic cardiomyopathy (HCM) has been published.[76] The mutation, which the authors attempted to correct, involves a four base deletion in exon 16 of the MYBPC3 gene. Sperm from a heterozygous patient with this mutation was used for IVF and either zygotes or M2 oocytes were injected with appropriate targeting constructs. In the case of M2 oocytes the targeting constructs were injected together with the sperm. Targeting constructs consisted of the Cas9 protein and guide RNA with or without single stranded donor oligonucleotide (ssODN) necessary for HDR. The authors suggested that injection of M2 oocytes eliminates mosaicism and that HDR can occur (as others have also occasionally reported) with high frequency even in embryos not injected with ssODN. This type of repair is supposedly mediated by the wild-type allele as a repair template. The report is rather optimistic indicating a high frequency (not 100%) of repair, a way of avoiding mosaicism and the absence of an off-target effect. However, there is major problem with this report and an extensive critique of it has been published.[77] Basically, the issue boils down to the lack of positive proof that any correction of the mutated allele took place. The authors of the original report[76] argued that, without any correction half of the embryos would carry a mutant allele, and that, since they observed substantial increase of embryos with the wild type allele only, they concluded that some of those embryos must represented gene correction. However, there are many possible reasons why a mutant allele was not detected in injected embryos[77] and these were not excluded in the original study. Actually, only the sequencing of the corrected allele, showing the presence of the original sequence followed by the inserted sequence (either from ssODN or from wild-type allele), followed in turn by the original sequence would represent incontrovertible evidence for gene correction. It is surprising that this evidence is lacking in the original report.[76]

Gene engineering in human embryos is obviously in its infancy and many technical problems need to be resolved if we are to see it used in clinical practice. Considering these uncertainties, we cannot but ask if this difficult and controversial technique is ever going to be necessary and justified. Novel mutations cannot be detected, thus cannot be corrected using this technique. Heterozygous parents with known recessive mutations will produce one quarter homozygous mutant embryos that can be corrected, thus avoiding the disease phenotype. Heterozygous parents with a dominant mutation will have one half of the progeny with the mutant phenotype which can also be avoided by gene correction. Nevertheless, gene correction in these situations is not essential since a much simpler, cheaper and more reliable approach, using preimplantation genetic diagnosis (PGD), can eliminate affected embryos. Some authors argued that by using gene correction there will be more embryos suitable for transfer thus increasing the chance of reproductive success.[76] This may be true, however, as we cannot distinguish which embryos need correction, we will have to target all of them with the potential for introducing off-target effects in otherwise completely normal embryos. For parents who insist on having their own genetic children there may be a situation in which gene correction in the zygote is the only possibility of having normal progeny. These are couples in which both parents are homozygous for a recessive mutation (e.g. β-thalassemia) or one or both parents are homozygous for a dominant mutation (e.g. Huntington disease). Such cases are rather rare and even for them one could envision a safer method for producing normal progeny. For example, the production of functional germ cells, derived from pluripotent stem cells, is approaching reality,[78,79] thus one could imagine deriving induced pluripotent cells from one or both parents, correcting the mutation and, following extensive study for off-target effects, deriving germ cells to be used in IVF.

In summary, genetic engineering in embryo is a fascinating possibility with many postulated uses, though it is hard to imagine its significant, massive application in human medicine.

Price Tag

The new clinical and therapeutic modalities described here also create new social and ethical problems. The cost of some clinical interventions like organ transplants or life-long maintenance therapy for certain genetic diseases is currently in the million dollar range as are

drug therapies for selected malignancies.[80,81] Are gene and stem cell therapies going to follow similar trends? [80,82] The first FDA-approved gene therapy for biallelic RPE65 mutation-associated retinal dystrophy, LUXTURNA, developed by Spark Therapeutic Inc. costs $ 850,000 per treatment.[83] One can easily argue that this price tag, for a quality of life improvement for a child who will now see, instead of being blind for life, is justified and even makes economic sense. However, at the present time we have only vague notions about the long-term success rate of these novel therapies, or how much will they cost, and who should finally pay for them. The cost of development and FDA-approval of treatment for very rare genetic diseases is in the billion dollar range and this cannot be recovered by charging the extremely small target population. It is very likely that stem cell therapies and gene engineering, the ultimate in personalized medicine, requiring highly skilled teams of molecular, cell and developmental biologists and clinicians will be even more expensive and will be available only at a very few specialized clinical centers. This brings us to the issue of access and justice[84] namely, will most of these therapies be available to the very rich only? How much is society willing to pay and what is the right cost-benefit ratio[85] for treatment? Is it fair to continue research funded by taxes, paid by all, if the products of this research will be available to only a few? At this moment most of these therapies are in the state of fascinating promises with many obvious biological and technical problems to be solved. One can only hope that by the time these problems are solved we will also have a clear notion of how to deal with the social and ethical conundrums arising.

REFERENCES

1. https://mahidol.ac.th/history
2. Stevens, LC, Little CC. Spontaneous testicular tumors in an inbred strain of mice. *Proc Natl Acad Sci USA*. 1950; 40: 1080-87; Stevens, LC. Studies of transplantable testicular teratomas of strain 129 mice. J Natl Canc Inst. 1958; 20: 1257-76; Stevens LC Embryology of testicular teratomas in strain 129 mice. J Natl Canc Inst. 1959; 23:1249-95.
3. Kleinschmidt LJ, Pierce GB, Jr. Multipotentiality of single embryonal carcinoma cells. Canc Res. 1964; 24: 1544-52; Pierce, GB Jr. Teratocarcinoma: model for a developmental concept of cancer. Cur Topics Develop Biol. 1967; 2: 223-46.
4. Kahan BW, Ephrussi B. Developmental Potentialities of Clonal *In Vitro* Cultures of Mouse Testicular Teratoma. J Natl Canc Inst.1970; 44:1015–36; Rosenthal MD, Wishnow R, Sato GH.The in vitro growth and differentiation of clonal populations of multipotential mouse cells derived from a transplantable testicular teratocarcinoma. J Natl Canc Inst. 1970; 44:1001-14
5. Andrews PW, Bronson DL, Benham F, Strickland S, Knowles BB. A comparative study of eight testicular teratocarcinoma cell lines. Int J Canc. 1980;26:269-80.
6. The International Stem Cell Initiative Consortium. Characteristization of human embryonic stem cell lines by the International Stem Cell Initiative. Nature Biotech. 2007;25:803-14.
7. Stevens LC. The development of transplantable teratocarcinomas from intratesticular grafts of pre- and postimplantation mouse embryos. Dev Biol. 1970; 21: 364-82.
8. Solter D, Skreb N, Damjanov I. Extrauterine growth of mouse egg-cylinders results in malignant teratoma. Nature. 1970;227: 503-504.
9. Martin GR. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc Natl Acad Sci USA. 1981;71:7634-8.
10. Evans MJ, Kaufman MH. Establishment in culture of pluripotential cells from mouse embryos. Nature. 1981; 292:154-6.
11. Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiegiel JJ, Marshall VS, et al. Embryonic stem cell lines derived from human blastocysts. Science. 1998;282:1145-7.
12. O'Connor NE, Mulligan JB, Banks-Schlegel S, Kehinde O, Green H. Grafting of burns with cultured epithelium prepared from autologous epidermal cells. Lancet. 1981;317:75-78.
13. Smithies O, Greg RG, Boggs Koralewski MA, Kucherlapati, RS. Insertion of DNA sequences into the human chromosomal β-globin gene locus by homologous recombination. Nature. 1985;317: 230-5.
14. Thomas KR, Capecchi MR. Site directed mutagenesis by gene targeting in mouse embryo-derived stem cells. Cell. 1987;51(3): 503-12.
15. Blaese RM, Culver KW, Miller AD, Carter CS, Fleisher T, Clerici M, et al. T-lymphocyte-directed gene therapy for ADA-SCID: Initial trial results after 4 years. Science. 1995;270:475-80.
16. Bordignon C, Notarangeio LD, Nobili N, Ferrari G, Casorati G, Panina P, et al. Gene Therapy in peripheral blood lymphocytes and bone marrow for ADA- immunodeficient patients. Science 1995; 270:470-5.
17. Grossman M, Raper SE, Kozarsky K, Stein EA, Engelhardt JF, Muller D, et al. Successful ex vivo therapy directed to liver in a patient with familial hyper-cholesterolaemia. Nature Gen 1994;6:335-41.
18. Hacein-Bey-Abina S, Le Deist F, Carlier, G, Bouneaud C, Hue BC, DeVillartay JP, et al. Sustained correction of X-linked severe combined immunodeficiency by ex vivo gene Therapy. N Engl J Med 2002;346:1185-93.
19. Aiuti A, Slavin S, Aker M, Ficara F, Deola S, Mortellaro A, et al. Correction of ADA-SCID by stem cell gene therapy combined with nonmyeloablative conditioning. Science. 2002;296(5577): 2410-3.
20. Hacein-Bey-Abina S, Von Kalle C, Schmidt M, McCormack MP, Wulffraat N, Leboulch P, et al. LMO2-Associated Clonal T Cell Proliferation in Two Patients after Gene Therapy for SCID-X1. Science. 2003;302:415-9.
21. Dunbar CE, High KA, Joung K, Kohn DB, Ozawa K, Sadelain M. Gene therapy comes of age. Science. 2018;359:175-85.
22. Raper SE, Chirmule N, Lee FS, Wivel NA, Bagg A, Gao GP, et al. Fatal systemic inflammatory response syndrome in a ornithine transcarbamylase deficient patient following adenoviral gene

transfer. Mol Genetics & Metab. 2003;80:148-58.
23. Urnov FD, Miller JC, Lee YL, Beausejour M, Rock JM, Augustus S, et al. Highly efficient endogenous human gene correction using designed zinc-finger nucleases. Nature. 2005; 435:646-51.
24. Carroll D. Progress and prospects: Zinc-finger nucleases as gene therapy agents. Gene Therapy. 2008;15:1463-68.
25. Christian M, Cermak T, Doyle EL, Schmidt C, Zhang F, Hummel A, et al. Targeting DNA double strand breaks with TAL effector nucleases. Genetics. 2010;186:757-61.
26. Miller JC, Tan S, Qiao G, Barlow KA, Wang J, Xia DF, et al. A TALE nuclease architecture for efficient genome editing. Nat Biotechnol. 2011;29(2):143-8.
27. Wiedenheft B, Sternberg SH, Doudna JA. RNA-guided genetic silencing systems in bacteria and archaea. Nature. 2012;482:331.
28. Jinek M. Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. A Programmable Dual-RNA–Guided DNA Endonuclease in Adaptive Bacterial Immunity. Science. 2012; 337:816-21.
29. Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, et al. Multiplex genome engineering using CRISPR/Cas systems. Science. 2013; 339:819-23.
30. Urmov FD. Genome editing B.C (before CRISPR): lasting lessons from the old testament. CRISPR J. 2018:1:32-34.
31. Keeler AM, ElMallah MK, Flotte TR. Gene Therapy 2017: Progress and future directions. Clin Transl Sci. 2017;10:242-8.
32. Mendell JR, Al-Zaidy S, Shell R, Arnold WD, Rodino-Klapac LR, Prior TW, et al. Single-dose gene-replacement therapy for spinal muscular atrophy. N Engl J Med 2017;377:1713-22.
33. Hinderer C, Katz N, Buza EL, Dyer C, Goode T, Bell P, et al. Severe toxicity in nonhuman primates and piglets following high-dose intravenous administration of an adeno-associated virus vector expressing human SMN. Hum Gene Ther. 2018;29(3):285-98.
34. Gandara C, Affleck V, Stoll EA. Manufacture of third-generation lentivirus for preclinical use, with process development considerations for translation to good manufacturing practice. A guide. Hum Gene Ther Methods. 2018;29(1):1-15.
35. Finkel RS, Mercuri E, Darras BT, Connolly AM, Kuntz NL, Kirschner J, et al. Nusinersen versus sham control in infantile-onset spinal muscular atrophy. N Engl J Med. 2017; 377:1723-32.
36. Gao X, Tao Y, Lamas V, Huang M, Yeh WH, Pan B, et al. Treatment of autosomal dominant hearing loss by in vivo delivery of genome editing agents. Nature. 2018; 553:217-221.
37. Charlesworth CT, Deshpande PS, Dever DP, Dejene B, Gomez-Ospina N, Manti S, et al. Identification of pre-existing adaptive immunity to Cas9 proteins in humans. bioRxiv preprint first posted online Jan. 5, 2018; doi: http://dx.doi.org/10.1101/243345.
38. Davis RL, Weintraub H, Lassar AB. Expression of a Single Transfected cDNA Converts Fibroblasts to Myoblasts. Cell. 1987; 51:987-1000.
39. Takahashi K, Yamanaka S. Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors. Cell 2006;126:663-76.
40. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell. 2007;131:861-72.
41. Bar S, Schachter M, Eldar-Geva T, Benvenisty N. Large-scale analysis of loss of imprinting in human plluripotent stem cells. Cell Reports. 2017;19:957-68.
42. Petricciani J, Hayakawa T, Stacey G, Trouvin JH, Knezevic I. Scientific considerations for the regulatory evaluation of cell therapy products. Biologicals. 2017; 50:20-26.
43. International Stem Cell Initiative, Amps K, Andrews PW, Anyfantis G, Armstrong L, Avery S, et al. Screening ethnically diverse human embryonic stem cells identifies a chromosome 20 minimal amplicon conferring growth advantage. Nat Biotechnol. 2011;29(12):1132-44.
44. Avery S, Hirst AJ, Baker D, Lim CY, Alagaratnam S, Skotheim RI, et al. BCL-XL mediates the strong selective advantage of a 20q11.21 amplification commonly found in human embryonic stem cell cultures. Stem Cell Reports. 2013;1(5):379-86.
45. Merkle FT, Ghosh S, Kamitaki N, Mitchell J, Avior Y, Mello C, et al. Human pluripotent stem cells recurrently acquire and expand dominant negative P53 mutations. Nature. 2017;545:229-33.
46. Amir H, Touboul T, Sabatini K, Chhabra D, Garitaonandia I, Loring JF, et al. Spontaneous single-copy loss of TP53 in human embryonic stem cells markedly increases cell proliferation and survival. Stem Cells. 2017;35:872-85.
47. Dawson EP, Lanza DG, Webster NJ, Benton SM, Suetake I, Heaney JD. Delayed male germ cell sex-specification permits transition into embryonal carcinoma cells with features of primed pluripotency. Development. 2018;145:1-12.
48. The International Stem Cell Initiative. Assessment of established techniques to determine developmental and malignant potential of human pluripotent stem cells. Nature Comms. 2018, in press.
49. Andrews PW, Ben-David U, Benvenisty N, Coffey P, Eggan K, Knowles BB, et al. Assessing the safety of human pluripotent stem cells and their derivatives for clinical applications. Stem Cell Rpts. 2017;9:1-4.
50. Schwartz SD, Regillo CD, Lam BL, Eliott D, Rosenfeld PJ, Gregor NS, et al. Human embryonic stem cell-derived retinal pigment epithelium in patients with age-related macular degeneration and Stargardt's macular dystrophy: follow up of two open-label phase 1/2 studies. Lancet. 2015; 385:509-16.
51. Mandai M, Watanabe A, Kurimoto Y, Hirami Y, Morinaga C, Daimon T, et al. Autologous induced stem-cell-derived retinal cells for macular degeneration. N Engl J Med. 2017; 376:1038-46.
52. da Cruz L, Fynes K, Georgiadis O, Kerby J, Luo YH, Ahmado A, et al. Phase 1 clinical study of an embryonic stem cell-derived retinal pigment epithelium patch in age-related macular degeneration. Nat Biotechnol. 2018;36(4):328-337.
53. Kashani AH, Lebkowski JS, Rahhal FM, Avery RL, Salehi-Had H, Dang W, et al. A bioengineered retinal pigment eptithelial monolayer for advanced, dry age-related macular degeneration. Sci Transl Med. 2018 Apr 4;10(435). pii: eaao4097.
54. Takashima K, Inoue Y, Tashiro S, Muto K. Lessons for reviewing clinical trials using induced pluripotent stem cells: examining the case of a first-in-human trial for age-related macular degeneration Regen Med. 2018;13:123-8.
55. Hassan S, Simaria AS, Varadaraju H, Gupta S, Warren K, Farid SS. Allogeneic cell therapy bioprocess economics and optimization: downstream processing decisions. Regen Med. 2015;10:591-609.
56. Morishima Y, Fumihiro A, Kashiwase K, Matsumoto K,

Orihara T, Yabe H, et al. Risk of HLA homozygous cord blood transplantation: implications for induced pluripotent stem cell banking and transplantation. Stem Cells Transl Med. 2018;7(2): 173-9.
57. Hirsch T, Rothoeft T, Teig N, Bauer JW, Pllegrini G, De Rosa L, et al. Regeneration of the entire human epidermis using transgenic stem cells. Nature. 207;551:327-32.
58. Green H, Kehinde O, Thomas J. Growth of cultured human epidermal cells into multiple epithelia suitable for grafting. Proc Natl Acad Sci USA. 1979;76:5665-68.
59. Hoban MD, Cost GJ, Mendel MC, Romero Z, Kaufman MO, Joglekar AV, et al. Correction of the sickle cell disease mutation in human hematopoietic stem/progenitor cells. Blood. 2015;125:2597-604.
60. Genovese O, Schiroli G, Escobar G, Tomaso TD, Firrito C, Calabria A, et al. Targeted genome editing in human reporulationg hematopietic stem cells. Nature. 2014;510:235-40.
61. Wattanapanitch M, Damkham N, Potirat P, Trakarnsanga K, Janan M, U-pratya Y, et al. One step genetic correction of hemoglobin E/β-thalassemia patient-derived iPSCs by the CRISPR/Cas9 system. Stem Cell Res Ther. 2018 Feb 26;9(1):46.
62. Wen Y, Jin YC, Wang Y, Pan RL, Qasbe A, Ye Z, et al. A universal approach to correct various HBB gene mutations in human stem cells for gene therapy of beta-thalassemia and sickle cell disease. Stem Cells Transl Med. 2018;7(1):87-97.
63. Sugimura R, Jha DK, Han A, Soria-Valles C, da Rocha EL, Lu YF, et al. Haematopoietic stem and progenitor cells from human pluripotent stem cells. Nature. 2017;545:432-8.
64. Leung A, Zulick E, Skvir N, Vanuystel K, Morrison TA, Naing ZH, et al. Notch and AHR signaling impact definitive hemato-poiesis from human pluripotent stem cells. Stem Cells. 2018 Mar 23. doi: 10.1002/stem.2822. [Epub ahead of print]
65. Lis R, Karrasch CC, Poulos MB, Kunar B, Redmond D, Duran JGB, et al. Conversion of adult endothelium to immunocompetent haematopoietic stem cells. Nature. 2017;545(7655):439-445.
66. June CH, O'Connor RS, Kawalekar OU, Ghassemi S, Milone MC. CAR T cell immunotherapy for human cancer. Science. 2018; 359:1361-5.
67. McCarrick III JW, Parnes JR, Seong RH, Solter D. Knowles BB. Positive-negative selection gene targeting with the diphtheria toxin A-chain gene in mouse embryonic stem cells. Transgenic Res. 1993; 2: 183-190.
68. Zimmer A, Gruss P. Production of chimaeric mice containing embryonic stem (ES) cells carrying a homeobox *Hox 1.1* allele mutated by homologous recombination. Nature. 1989:338:150-3.
69. Wang H, Yang H, Shivalila CS, Dawlaty MM, Cheng AW, Shang F, Jaenisch R. One -step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genombe engineering. Cell.2013;153:901-8.
70. Fogarty NME, McCarthy A, Snijders KE, Powell BE, Kubikova N, Blakeley P, Lea R, Elder K, Wamaitha SE, Kim D, Maciulyte V, leinjung J, Kim J-S, Wells D, Vallier L, Bertero A, Turner JMA, Niakan. Genome editing reveals a role for OCT4 in human embryogenesis. Nature. 2017;550:67-73.
71. Long, C, McAnally JR, Shelton JM, Mireault AA, Bassel-Duby R, Olson EN. Prevention of muscular dystrophy in mice by CRISPR/Cas9-mediated editing of germline DNA. Science. 2014; 345:1184-88.
72. Wu Y, Liang D, Wang Y, Bai M, Tang W, Bao S, Y:an Z, Dangsheng L Li J. Correction of a genetic deisease in mouse via use of CRISPR-Cas9. Cell Stem Cell 2013; 13:659-62.
73. Liang P, Xu Y, Zhang X, Ding C, Huang R, Zhang Z, Lv J, Xie X, Chen Y, Li Y, Sun Y, Bai Y, Songyang Z, Ma W, Zhou C, Huang J. CRISPR/Cas9-mediated gene editing in human tripronuclear zygotes. Protein Cell 2015; 6:363-72.
74. Kang X, He W, Huang Y, Yu Q, Chen Y, Gao X, Sun S, Fan Y. Introducing precise genetic modifications into human 3PN embryos by CRISPR/Cas-mediated genome editing. J Assist Reprod Genet. 2016; 33:581-88.
75. Liang P, Ding C, Sun H, Xie X, Xu Y, Zhang X, Sun Y, Xiong Y, Ma W, Liu Y, Wang Y, Fang J, Liu D, Songyang Z, Zhou C, Huang J. Correction of □-thalassemia mutant by base editor in human embryos. Protein Cell. 2017; 8:811-22.
76. Ma, H, Marti-Gutierrez N, Park S-W, Wu J, Lee Y, Suzuki K, Koski A, Ji D, Hayama T, Ahmed R, Darby H, VanDyken C, Li Ying, Kang E, Park A.-R, Kim D, Kim S-T, Gong J, Gu Y, Xu X, Battaglia D, Krieg SA, Lee DM, Wu DH, Wolf DP, Heitner SB, Belmonte JCI, Amato P, Kim J-S, Kaul S, Mitalipov S. Correction of a pathogenic gene mutation in human embryos. Nature; 548: 413-419.
77. Egli D, Zuccaro MV, Kosicki M, Church GM, Bradley A, Jasin M. Inter-homologue repair in fertilized human eggs? http://dx.doi.org/10.1101/181255.
78. Ishii T, Saitou M. Promoting In vitro gametogenesis research with a social understanding. Trends Molec. Med. 2017; 23: 985-88.
79. Saitou M, Miyauchi H. Gametogenesis from pluripotent stem cells. Cell Stem Cell. 2016; 18: 721-735.
80. Hayden EC. Gene therapies pose million dollar conundrum. Nature. 2016; 534: 305-306.
81. Dolgin E. Cancer's cost conundrum. Nature. 2018; 555: S26-29.
82. Orkin S H, Reilly P. Paying for future success in gene therapy. Science. 2016; 352: 1059-1061.
83. https://www.statnews.com/2018/03/21/gene-therapy-luxturna-launch/
84. Faden RR, Dawson L, Bateman-House AS, Mueller Agnew D, Bok H, Brock DW, Chakravarti A, Gao X-j, Greene M, Hansen JA., King PA, O'Brien SJ, Sachs DH, Schill KE, Siegel A, Solter D, Suter SM, Verfaillie CM, Walters LB, Gearhart JD. Public stem cell banks: Considerations of justice in stem c ell research and therapy. Hastings Center Report 2003; 33:13-27.
85. Hettle R, Corbett M, Hinde S, Hodgson R, Jones-Diette J, Woolacott N, et al. The assessment and appraisal of regenerative medicines and cell therapy products: an exploration of methods for review, economic evaluation and appraisal. Health Technol Assess 2017; 21:(No. 7).