CELL & GENE THERAPY INSIGHTS

TRANSLATION & APPLICATION OF GENOME EDITING

SPOTLIGHT

EXPERT INSIGHT

Stem cell-derived organoid cultures and genome editing tools

Amanda Andersson-Rolf & Bon-Kyoung Koo

The term stem cell was first used in the late 19th century to describe 'the ancestor' unicellular organism, the origin from which all multicellular organisms evolved. Our definition today is that of a cell characterized by two properties: self-renewal (the capacity to generate new stem cells) and multipotency (the ability to differentiate into different cell lineages) [1–4]. Due to these characteristics, stem cells hold great potential for clinical use by providing an unlimited source of cells for cell therapy in regenerative and/or personalized medicine. To realize this potential, the development of stem cell culture systems, as well as stem cell genome editing tools, has been of paramount importance. Here, recent advances in culture systems and genome editing tools will be discussed. This Expert Insight will provide an overview of current, state-of-the-art stem cell culture systems, with a focus on the recent progress in 3D tissue culture of both embryonic and adult stem cells, as well as the genome editing tools present today. Finally, we will discuss how stem cell and genome editing technologies can be combined to gain insights into human development and disease, and to fulfil the promises of stem cell research in the clinic.

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PLURIPOTENT STEM CELLS & ORGANOID CULTURE

Since the existence of pluripotent stem cells (PSCs) was definitively proven, there have been many groundbreaking achievements. Principally, three main steps have advanced the field. First is the establishment of mouse and human embryonic stem cell (ESC) cultures. In 1981, Evans and Martin derived mouse ESCs (mESCs), which retained pluripotency through culture on feeder cells, from the inner cell mass of the mouse blastocyst [5,6]. Leukemia inhibitory factor (LIF) was then identified as a key factor for maintaining pluripotency during long-term, feeder-free culture of mESCs [7,8]. Human ESCs (hESCs) were derived a decade

later [9], and in 2014, two groups reported conditions allowing the culture of naïve hESCs closely resembling mouse naïve cells [10,11]. The second achievement addressed most ethical concerns surrounding the use of fertilized human embryos to generate pluripotent human cells. In 2006, Professor Shinya Yamanka generated induced pluripotent stem cells (iPSCs) – an equivalent of ESCs – from mouse somatic cells by inducing the expression of four transcription factors: Oct4, Sox2, Klf4 and c-Myc [12]. A year later, the successful generation of human iPSCs was reported [13]. Today, iPSCs can be generated from many different species, and a series of protocols for the differentiation of ESCs and iPSCs into a diverse range of specific cell types has been established [14].

The third major advance was the establishment of stem cell-derived 3D organ cultures – often called organoid cultures. Organoids can be generated either from PSCs or adult stem cells (AdSCs), and are generally defined as 3D, self-organizing cellular structures fully or partially resembling their *in vivo* counterpart in function as well as in cell type composition [15,16]. Pioneered by the groups of Professors Yoshiki Sasai and Hans Clevers, the first organoid cultures were created almost 7 years ago by generating self-organizing cortical tissues from ESC-derived 3D aggregates [17,18]. Shortly thereafter, the adult intestinal stem cell was also utilized to generate a self-organizing epithelial structure [19]. A defined combination of extracellular matrices, chemicals and growth factors mimicking the *in vivo* niche has enabled a growing list of 3D organ cultures to be established from both PSCs and AdSCs. From PSCs, the list includes organ buds/organoids of retina [20], pituitary [21], cerebrum [22], ureteric bud [23], small intestine [24], thyroid [25], stomach [26] and liver [27]; whilst from AdSCs organoids have been formed from small intestine [19,28], colon [29], liver [30], prostate [31], pancreas [32,33], stomach [34] and lung [35]. The establishment of these 3D cultures has provided a novel platform for studying stem cell behavior, tissue patterning and organ formation in a petri dish. Moreover, these organoids open new avenues for regenerative and personalized medicine. Patient-derived or human iPSC-derived organoids may act as a transplantable cell source and/or as a system in which rapid drug screening can be performed [36,37].

GENOME EDITING TOOLS

In 1987 Professor Mario Capecchi published a method for homologous recombination (HR) in mESCs. HR is dependent on homologous template DNA. Normally this is the sister chromatid; however the introduction of an exogenous DNA template (a targeting vector) has allowed researchers to introduce specific, site-directed mutations or insertions [38]. In these targeting vectors the DNA to be inserted/edited e.g., a tag or loxP site(s), is flanked by DNA sequences homologous to the genomic insertion site. HR whilst very efficient in mESCs, was found to occur with an extremely low frequency in other mammalian systems. The solution, based on the pioneering work of Dr Maria Jasin, was to introduce double strand breaks (DSBs) into a desired site of the genomic DNA

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to facilitate HR-mediated repair of the DNA [39,40]. This strategy is applied in the three genome engineering tools described below.

The earliest technology involved zinc finger (ZF) nucleases (ZFNs), enzymes which consist of a DNA-binding ZF domain, which binds to DNA with a specificity of 9–18 base pairs (bp), and the DNA-cleaving domain of the FokI restriction endonuclease [41]. The first use of the ZF–FokI fusion protein came in 1996, and 7 years later it was used for HR-mediated gene targeting in human cells [42]. Transcription activator-like effector nucleases (TALENs) are an alternative technology that enables genome editing [43,44]. Both ZFNs and TALENs are designed in pairs, since the FokI domain needs to dimerize in order to cleave the DNA. Whereas the ZFN DNA-binding domain consists of 3–6 ZFs each recognizing a 3 bp sequence, the TALEN DNA-binding domain is composed of several modules of tandem repeats consisting of 33–35 amino acids, each recognizing 1 bp of genomic DNA. To generate new ZFNs/TALENs, modules of known specificity are combined. However, the specificity of the individual ZF modules are affected by interactions between different modules, a phenomenon called context dependency. As a result the TALEN DNA-binding domain is both more modular and easier to design compared to the ZFN [45–48].

The most recently introduced technology is the clustered interspersed palindromic repeat (CRISPR)/CRISPR-associated (Cas) system. Unlike the two previous technologies, the endonuclease is not fused to a DNA-binding domain, and is instead guided to the genomic sequence of interest by a guide RNA sequence (gRNA) to generate a DSB. Designing a gRNA for CRISPR genome editing is both faster and easier than the generation of a functional pair of ZFNs or TALENs. Designing either of the latter requires extensive knowledge of molecular cloning and protein engineering, while the gRNAs can be ordered as oligonucleotides. This has allowed genome-wide loss-of-function screens using a CRISPR/Cas library to be performed in mouse diploid and haploid ESCs, as well as hESCs [49–51]. Since a gRNA library can be constructed directly with synthesized oligonucleotides (target sequence), the candidate genes can be identified by next-generation sequencing of the oligonucleotide part of gRNAs that serve as barcodes. This technique creates new possibilities for identifying novel gene functions in an unprecedentedly rapid manner (reviewed in [52,53]). The three technologies all have their advantages and disadvantages (reviewed in [54,55] and by Andrew Bassett within this Spotlight issue). Considering the rapid increase in publications using genome editing in recent years, CRISPR/Cas is currently the most popular option, though off-target effects will still need to be minimized if this technique is to be used in the clinic [56–58].

COUPLING STEM CELL & ORGANOID CULTURE WITH GENOME EDITING

Genome editing tools have been widely utilized in stem cell and organoid cultures, and this article will focus on some specific examples. Precise genome editing through DSBs makes it possible to generate a disease-related mutation, thus generating isogenic stem cell

lines where ideally the parental cell line has the same genotype, with the exception of the modified site; however, clonal heterogeneity acquired in culture still makes this challenging. Using ZFNs, Soldner *et al* introduced a dominant mutation strongly linked to familial Parkinson's disease (A53T mutation) into the α-*synuclein* gene in healthy donor-derived hESCs, elegantly demonstrating the use of genetic modifications in isogenic cell lines for disease modelling [59]. CRISPR/Cas tools and organoid culture have also been used for *in vitro* tumor modelling. Intestinal organoids derived from human donors underwent genome editing using the CRISPR/Cas system to introduce known cancer-causing mutations into the four genes (*KRAS*, *APC*, *TP53* and *SMAD4*) that are most frequently mutated in colorectal cancer. The resultant mutant organoids could grow independently of all stem cell niche factors, and when xenotransplanted into mice the quadruple mutant organoids were tumorigenic and displayed features of invasive adenocarcinoma [28,60].

FUTURE OF MONOGENIC DISORDERS

Applying gene editing technologies to PSC cultures and derivatives allows the development of promising autologous cell sources for transplantation with unlimited expansion capacity. As discussed above, disease models can be generated by the introduction of mutations into healthy donor material. The opposite can therefore be achieved using diseased material: instead of introducing mutations, the mutation(s) causing the disease can be corrected using genome editing (Figure 1). This milestone was achieved first in PSCs and now also in AdSC-derived organoids. In PSCs the first gene to be corrected was a mutant form of *IL2GR*, which causes X-linked severe combined immunodeficiency [61,62]. Schwank *et al* repaired the disease-causing mutation in intestinal organoids derived from cystic fibrosis patients. Cystic fibrosis is an autosomal recessive disorder where both copies of the cystic fibrosis transmembrane conductance receptor (CFTR) are mutated. CFTR is an ion channel and its dysfunction results in the disturbed transport of fluid and thickening of the mucus in organs such as the lung, pancreas and small intestine [63,64]. Patient-derived organoids fail to swell in a functional assay involving application of the small molecule Forskolin. However, following HR-mediated gene correction using CRISPR/Cas, the gene-corrected patient organoids performed as well as healthy control organoids in the Forskolin swelling assay [65]. Furthermore, the *CFTR* mutation has also been corrected in human iPSCs [66]. Although the precise gene correction of *CFTR* in PSCs and AdSC-derived culture is a great achievement and provides a proof-of-concept showing that organoids and iPSCs hold great potential for cell therapy, it is important to note that in order to be 'cured' the patient would need entire tissue replacement using the gene-corrected cells in several tissues and rather large surface areas. At the moment this still represents a large technical obstacle to overcome. However, diseases where the functional complication

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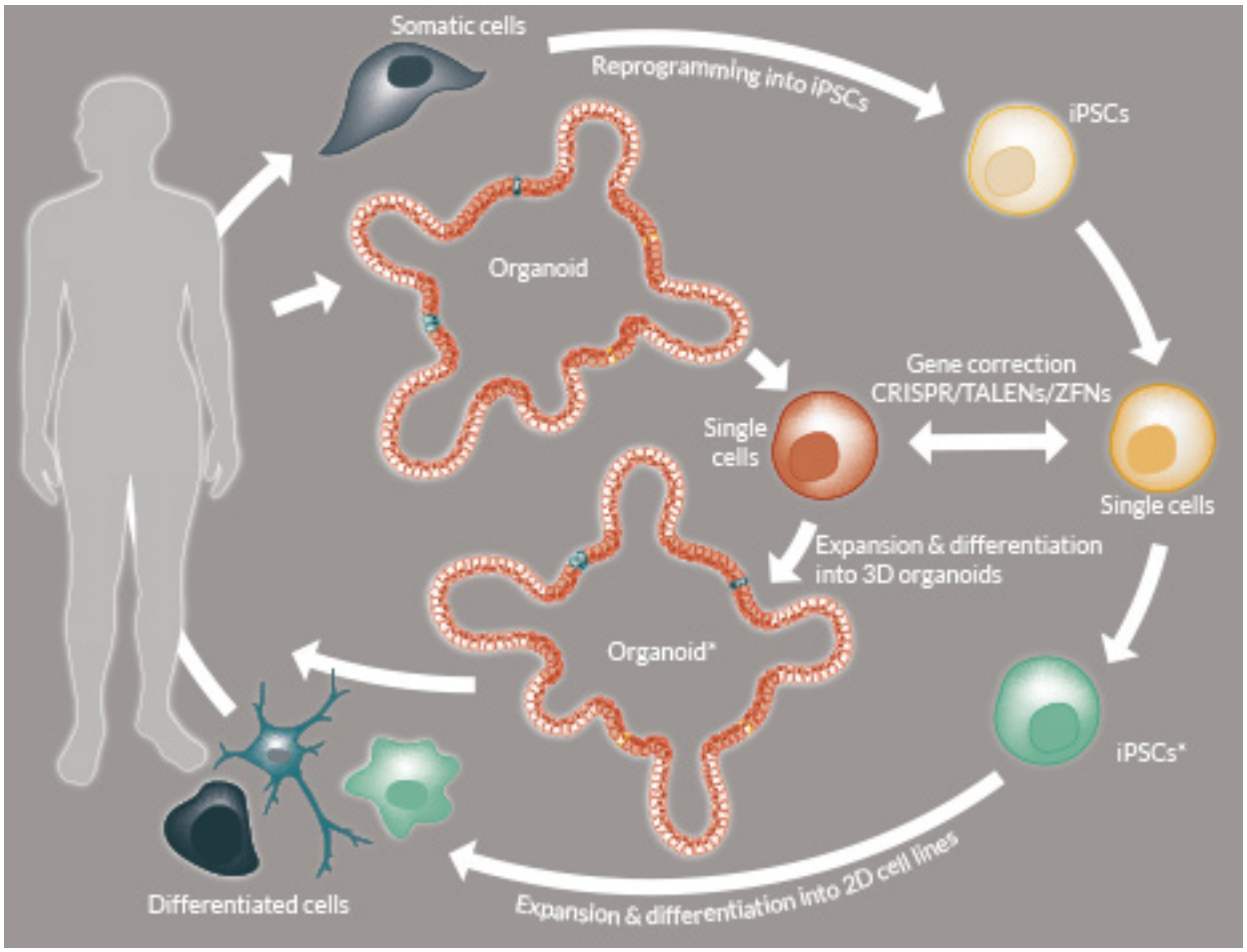
FIGURE 1

Schematic illustrating the routes of gene-edited cell therapy.



Asterisks indicate gene corrected iPSCs and organoids. One route is to reprogramme somatic cells into iPSCs, perform gene correction using gene editing technology and subsequently differentiate them into 3D organoids or 2D cell lines before transferring them back to the patient. An alternative is to derive AdSC organoids, perform gene correction and then transfer them back into the patient. AdSCs: Adult stem cells; iPSCs: Induced pluripotent stem cells.

is restricted to a certain tissue or cell type and is caused by one gene could still be considered as a relatively easy target for gene-edited cell therapy. Potential candidates for current technologies are presented below.

Alpha 1 anti-trypsin deficiency

Alpha 1 anti-trypsin (A1AT) deficiency is an autosomal co-dominant hereditary disorder caused by an inactivating mutation in the *A1AT* gene. In healthy individuals, A1AT functions as a protease inhibitor and protects tissues primarily by inhibiting enzymes secreted by inflammatory cells. Reduction or lack of function of A1AT results in chronic tissue degradation, mainly of the lung. In addition, certain mutations can cause the misfolding and improper secretion of the protein, causing damage to the liver and eventually leading to liver cirrhosis [67,68]. Due to its liver-specific expression and monogenic cause, A1AT deficiency is a potential target for gene-edited cell therapy. Gene correction has already been performed in iPSCs using ZFNs [69].

Familial hypercholesterolemia (ApoB & PCSK9)

Hypercholesterolemia is defined by elevated levels of cholesterol in the blood which later lead to atherosclerosis and subsequently cardiovascular disease [70,71]. The enzyme proprotein convertase subtilisin type 9 (PCSK9) is mutated in familial hypercholesterolemia, which is an autosomal dominant disease. PCSK9 is expressed in the liver, where it induces degradation of the low-density lipoprotein receptor (LDLR), resulting in a reduction of the rate of degradation of low-density lipoprotein (LDL) cholesterol. Mutations causing PCSK9 to bind more efficiently to the LDLR receptor consequently result in a higher level of LDL in the blood. Due to its inhibitory function (on LDL degradation) the presence of mutant PCSK9 is sufficient to cause the disease. Therefore, while the disease will be partially alleviated following the introduction of gene-corrected cells, the presence of remaining mutant cells may cause some symptoms to persist. On the other hand, Apolipoprotein B (ApoB) binds lipids (e.g., LDL) as well as LDLR, leading to clearance of the lipid. Mutations (e.g., R3500Q) causing ApoB loss-of-function decrease LDL degradation and consequently lead to elevated LDL levels [72,73]. In this case, introduction of gene-corrected cells producing a functional form of the protein may be enough to fully eliminate disease symptoms. Antisense nucleotides targeting PCSK9 have been shown to lower LDL in non-human primates. In addition, *in vivo* genome editing of *PCSK9* using CRISPR/Cas resulted in reduction of cholesterol levels in mice, showing that introduced inactivating mutations can have beneficial effects [71,74–77]. Cell therapy may still be a valuable alternative in the future.

For organoids and PSCs to be used in clinical practice, safe transplantation back to the original patient is essential and this poses a challenge. Although gene-corrected iPSCs can be differentiated into the cell type of interest, contaminating pluripotent cells may cause tumors in the recipient [78–80]. Removal of the remaining PSCs following differentiation is therefore crucial. AdSC-derived organoids have been shown to be genetically stable and the tissue identity of these organoids also seems to be stable unless challenged by genetic modifications [81,82]. However, careful examination of tumorigenic or other malfunctions of AdSC or PSC-derived organoids is still required.

SUMMARY

The last two decades have brought significant improvements to both stem cell culture systems and genome editing tools. PSC- or AdSC-derived 3D culture systems have enabled new ways of modelling tissue patterning, organ formation and complex diseases such as cancer in an unprecedented manner. Meanwhile CRISPR/Cas technology has made gene editing simple and widely available for researchers working in various fields and model systems. Together, these two revolutionary technologies – stem cell-derived organoid cultures and genome editing tools – will bring us one step closer to fulfilling the dream of many stem cell biologists today: applying gene-edited cell therapy to routine medical practice.

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