DNA barcode to trace the development and differentiation of cord blood stem cells (Review)

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Abstract. Umbilical cord blood transplantation was first reported in 1980. Since then, additional research has indicated that umbilical cord blood stem cells (UCBSCs) have various advantages, such as multi-lineage differentiation potential and potent renewal activity, which may be induced to promote their differentiation into a variety of seed cells for tissue engineering and the treatment of clinical and metabolic diseases. Recent studies suggested that UCBSCs are able to differentiate into nerve cells, chondrocytes, hepatocyte-like cells, fat cells and osteoblasts. The culture of UCBSCs has developed from feeder-layer to feeder-free culture systems. The classical techniques of cell labeling and tracing by gene transfection and fluorescent dye and nucleic acid analogs have evolved to DNA barcode technology mediated by transposon/retrovirus, cyclization recombination-recombinase and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 strategies. DNA barcoding for cell development tracing has advanced to include single cells and single nucleic acid mutations. In the present study, the latest research findings on the development and differentiation, culture techniques and labeling and tracing of UCBSCs are reviewed. The present study may increase the current understanding of UCBSC biology and its clinical applications.

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Abbreviations: UCBSC, umbilical cord blood stem cells; HSCs, hematopoietic stem cells; MSCs, mesenchymal stem cells; GFP, green fluorescent protein; Cre, cyclization recombination; GESTALT, genome editing of synthetic target arrays for lineage tracing; CARLIN, CRISPR array repair lineage tracing; mSCRIBe, mammalian synthetic cell recorder integrating biological events; MEMOIR, memory by engineered mutagenesis with optical *in situ* readout

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Key words: umbilical cord blood stem cells, development and differentiation, cell Labeling and lineage tracing, DNA barcode

Contents

1. Introduction

Cord blood, also known as placental blood or umbilical cord blood (UCB), is the blood in the umbilical cord and blood vessels near the fetal side of the placenta, which may be collected when the fetus is born. In 1989, experiments by Broxmeyer *et al* (1) indicated that UCB is a potential source of hematopoietic stem/progenitor cells. UCB stem cells (UCBSCs) are a type of primitive undifferentiated cells that have the same multidirectional differentiation potential as bone marrow stem cells. UCBSCs are able to self-renew and proliferate. They may differentiate into various cell or tissue

types under the influence or induction of specific factors (2-5). UCBSCs have a wide range of sources and their application is not limited by ethics concerns and/or guidelines (6). Therefore, they are considered an important source of stem cells for transplantation and have huge potential to be widely used in clinical tissue engineering and other stem cell therapies (7,8).

The detailed investigation and understanding of the functions of UCBSCs have laid a foundation for their successful clinical application. However, the establishment of UCB cells and their differentiation *in vivo* remain incomplete (9,10). Although tracing technology is usually used to understand cell function, it exhibits several limitations. The traditional tracer technique cannot differentiate the following generations of cells from the primary cells at a large scale and the lineage relationship between the cells is not clear (11,12). Recently, DNA barcode technology used for cell development tracing has achieved lineage tracing at single-cell and single-nucleic acid mutation resolution (13). DNA barcoding combined with sequencing technology clearly demonstrated the relationship among splinter cells by labeling cells with DNA barcodes, tracing their developmental history and stacking them to form a lineage development tree in order to identify their origin, development and differentiation. This is an effective strategy for tracking large numbers of cells both spatially and temporally (14). This may aid the understanding of the self-renewal mechanism of UCBSCs and lays a foundation for their clinical application.

In the present study, the differentiation characteristics of UCBSCs were reviewed, including the research progress of the latest methods of DNA barcode technology. This information aims to increase the current understanding of the biological roles and clinical applications of these cells.

2. Differentiation of UCBSCs

Stem cells possess the potential for self-renewal and multi-differentiation, which may be used to replace damaged cells and exert significant therapeutic potential in regenerative medicine. Several types of stem cells have been detected in UCB, including the following: Umbilical cord hematopoietic stem cells (HSCs), endothelial progenitor cells, mesenchymal stem cells (MSCs), unrestricted somatic stem cells and multipotent progenitor cells. HSCs, which have a relatively high content in UCB, may be divided into two cell types, namely $CD34^+$ and $CD34^-$, among which $CD34^+$ cells account for >95% of the population. MSCs are mainly derived from UCB and the bone marrow and their cell phenotypes include CD133, CD34 and CD45. Although MSCs are rarely found in cord blood, their differentiation ability is potent. Studies have indicated that UCBSCs may be induced to differentiate into nerve cells, chondrocytes, hepatocyte-like cells, fat cells, osteoblasts and islet-like cells under appropriate microenvironmental conditions. In 2003, Mitchell *et al* (15) induced the differentiation of UCBSCs using β-mercaptoethanol, antioxidants and dimethylsulfoxide. It was indicated that 80% of the cells exhibited a neuron-like appearance. Furthermore, a unique Nissl body structure of neuron cells was noted following 12 h of incubation. Fu *et al* (16) cultured UCBSCs together with the primary cortex of mice for 4 days. In total,~50% of the cells developed into neural cells, ~33% of the cells differentiated into astrocytes and ~10% into oligodendrocytes. This finding indicated the presence of neural stem cells in UCBSCs, which were able to differentiate into neural cells. A similar study demonstrated that UCBSCs that were injected into a rat model at the site of spinal cord injury caused significant functional improvement following six weeks. These stem cells were able to differentiate into nerve cells following transplantation (17). Wang *et al* (18) studied the possibility of the differentiation of UCBSCs into cardiomyocytes following their treatment with 5-azacytidine or their culture in cardiomyocyte-conditioned medium. The data indicated that both conditions resulted in the induction of the expression of the cardiomyocyte markers N-cadherin and cardiac troponin I. Furthermore, the multi-lineage potential of UCBSCs was also validated, indicating that they may be induced into the chondrogenic, osteogenic and adipogenic lineages *in vitro*. To date, multiple studies suggested that UCBSCs differentiate into hepatocyte-like cells following their transplantation into liver-damaged mice (19,20). Growth factors are usually added during these experiments. Kakinuma *et al* (19) indicated that mice with partial hepatectomy that received UCBSC transplantation exhibited transplanted cells in the liver; these cells were differentiated into hepatocytes and secreted albumin for a year. Similar results were obtained in animal models of chemically-induced liver disease, in which infused UCBSCs differentiated into mature hepatocytes (20). The results of this study indicated that UCBSCs had the tendency to differentiate into hepatocyte-like cells under certain conditions.

In addition to the aforementioned findings, Mayani *et al* (21) demonstrated that UCBSCs were able to differentiate into nerve cells, chondrocytes and liver-like cells. To date, the environmental conditions that are required for the process of differentiation, the accuracy of induced differentiation, the assessment of the relationship between differentiated and normal cells and the mechanism of differentiation have remained to be determined. In addition, the majority of experiments that assessed the induction of differentiation were performed *in vitro*, which cannot completely simulate the *in vivo* conditions. The differentiation of UCBSCs is still unknown and uncontrollable. The potential to track the differentiation of stem cells in space and time by using a more powerful tracer tool will enhance the current understanding of the biological characteristics of UCBSCs.

In order to investigate the differentiation of UCBSCs, their *in vitro* culture is required. Studies have indicated that UCB contains HSCs and several types of MSC. At present, the isolation and culture of UCBSCs are mainly focused on HSCs. The culture methods of UCBSCs mainly include the feeder layer and the feeder-free culture systems. Shetty *et al* (22) suggested that the feeder layer maintains the undifferentiated state of the cells and promotes self-renewal of UCBSCs. Mouse embryonic fibroblasts inactivated by γ-rays or mitomycin C are commonly used as feeder layers for UCBSC culture. Han *et al* (23) used DMEM with other substances added to the FBS supplement. In a subsequent study, Hutton *et al* (24) replaced the FBS with a serum substitute and added basic fibroblast growth factor. Demerdash *et al* (25) indicated that the number of expansion times of the feeder culture system were limited and that the

Table I. Labeling and tracing technology of umbilical cord blood stem cells.

Labeling method	Labeling agent	Labeling content	Advantage	Disadvantages	Readout	(Refs.)
Gene transfection labeling	Protein	DNA	Stable	The half-life is unclear	Microscopy	(11)
Fluorescent dye labeling	CM-Dil	Cell membrane lipids	Accurate	Easily degradable	Microscopy	(12)
Nucleic acid labeling	Pyrimidine analog	DNA	High sensitivity	Signal easily lost	IHC	(40)
DNA barcode	DNA, Cre/Cas9 cell lineage	DNA/RNA	Accurate tracking of optimization	Sequencing technology requires	scRNA-seq; Illumina	(42-45)

CM-Dil, chloromethyl-benzamidodialkylcarbocyanine; IHC, immunohistochemistry; Cre, cyclization recombination; scRNA-seq, single-cell RNA sequencing; Cas9, Cre-recombinase-based and clustered regularly interspaced short palindromic repeats-associated protein 9.

growth ability of UCBSCs was reduced following differentiation for a certain number of generations. Furthermore, the authors of that study demonstrated that feeder layer cells weakened the effect of exogenous factors on UCBSCs and the animal/human-derived feeder layer was unable to achieve its clinical effect due to contamination with heterogenic/allogeneic antigens (23,26-29).

The development of optimized feeder-free culture systems has been the focus of UCBSC culture. The regeneration of UCBSCs requires the activation of various signaling pathways, such as fibroblast growth factor 2, ERK and PI3K/AKT, insulin growth factor/insulin, 1-phosphate-sphingosine/platelet-derived growth factor and TGF-β/activin/nodulation protein A/SMAD2/3, by receptor tyrosine kinases. Zhang *et al* (30) indicated that the Wnt/β-catenin and TNF receptor superfamily of proteins were associated with the survival rate of UCBSCs, while inhibition of bone morphogenetic protein markers may prevent their independent differentiation. Several studies have produced feeder-free culture media, such as TeSR1 that contains certain recombinant growth factors, which aim to enhance the ability of UCBSCs to regenerate (31,32). Zhou *et al* (33) demonstrated that the combination of activin inhibitors and MEK/ERK inhibitors increased the induction rate of UCBSCs. The current focus is to identify a feeder-free culture system that is able to maintain the normal karyotype of the cells (34). Naka *et al* (35) demonstrated that retaining the culture system in a state of low oxygen pressure reduces the differentiation of UCBSCs and the development of chromosomal abnormalities (36). Lee (37) highlighted that certain small molecular weight compounds are able to regulate the biological function of stem cells by maintaining stem cell self-renewal, inhibiting differentiation and the activation of the withering pathway. For instance, stem regenin 1, an antagonist of the aryl hydrocarbon receptor (38) and the pyrimidodiole derivative UM1717 (39), may be used to safely and effectively expand UCBSCs *in vitro*. Although these results are preliminary, it is suggested that similar strategies may be employed in UCBSC culture to reduce the production and proliferation of abnormal cells.

3. DNA barcoding

The current understanding of the biological function of UCBSCs has significantly improved. However, the mechanism of self-renewal of UCB-derived HSCs remains to be fully elucidated. Basic and clinical research has focused on investigating the trajectory inference of UCBSCs, which usually requires labeling technology. Gene transfection and fluorescent dyes are classical cell tracing techniques (11,12), which may be easily detected in viable cells using microscopy imaging technology. The double-labeled experiments may be performed using a variety of fluorescent reagents. However, at present, the half-life of the green fluorescent protein (GFP) is not sufficiently clear to accurately assess the time period required in the experiments; furthermore, physical factors, such as high temperature, and chemical factors, such as strong acid and strong alkali conditions, may cause structural damage and decomposition. The mechanism of action of fluorescent dyes is focused on achieving labeling through the cell membrane. The majority of the fluorescent agents are degraded following cell proliferation and division, which may lead to the inability of fluorescent dye labeling to maintain a high labeling rate for a long time. Seghatoleslam *et al* (40) used bromodeoxyuracil nucleoside (BrdU) for UCBSC labeling (Table I). BrdU is a pyrimidine analog, which competes with endogenous thymidine during DNA replication. Prior to or during UCBSC transplantation, the cells proliferate in the presence of BrdU. Subsequently, labeled UCBSCs continue to proliferate without BrdU and are finally stained by immunohistochemical methods. However, certain defects are present in the nucleic acid labeling method, such as the instability of BrdU during labeling. When the labeling time increases, the unlabeled surrounding cells are also labeled due to apoptosis and phagocytosis, which affects the signal strength of the transplanted UCBSCs.

Although gene transfection labeling and fluorescent dye labeling are able to mark primary cells during the process of cell division or differentiation, the offspring cells cannot be distinguished from the primary cells and the lineage relationship between cells is not clear. DNA barcoding is an emerging

Table II. Overview of barcoding techniques.

Technology	DNA editing system	Barcode length (bp)	Diversity	Species	*In vivo*	Readout	(Refs.)
TracerSeq	Tol2	20	NR	Zebrafish	Yes	Illumina	(52)
Embedded viral	Retrovirus	33	NR	Mouse	Yes	Illumina	(54)
CellTag	Retrovirus	8	NR	Human	No	scRNA-seq; Illumina	(53)
Polylox	Cre-loxP	1,942	849	Mouse	Yes	Pacbio	(55,56)
GESTALT	Cas9	266	4195	Zebrafish	Yes	Illumina	(58)
mSCRIBe	Cas9	70	1890	Zebrafish	Yes	scRNA-seq; Illumina	(60)
Homing barcodes	Cas9	240	NR	Mouse	Yes	Illumina	(61,62)
MEMOIR	Cas9	256	-256	Mouse	Yes	FISH	(59)
CARLIN	Cas9	276	4,400	Mouse	Yes	scRNA-seq; Illumina	(64)

NR, not reported; GESTALT, genome editing of synthetic target arrays for lineage tracing; CARLIN, CRISPR array repair lineage tracing; mSCRIBe, mammalian synthetic cell recorder integrating biological events; CRISPR, Cre-recombinase-based and clustered regularly interspaced short palindromic repeats; Cas9, CRISPR-associated protein 9; MEMOIR, memory by engineered mutagenesis with optical *in situ* readout.

technology that uses unique nucleic acid sequences to label individual cells. The genetic barcodes are inserted into the genomic DNA of the cells at a specific time-point and the cells divide to produce progeny cells, forming cell clones whose progeny inherit the barcodes. The progeny of the labeled cells is identified by reading the tags (41). This process is termed genetic barcoding. This provides insight into cell behavior regarding space and time. Barcodes are created by using a special nucleic acid sequence as a permanent or dynamic marker for a single cell. The number of barcode sequences that may be used is theoretically infinite and a large population of cells may be efficiently labeled and tracked at the single-cell level (Table I) (42-48).

Development of the DNA barcode. Initially, the DNA barcode technology was based on the identification of unique retroviral integration sites and barcode identities, such as those identified using southern blot or PCR assays (49,50). As the development of sequencing technology has promoted the progress of barcode technology, numerous approaches have created and deployed more complex DNA barcodes for lineage tracing (Table II). The basic concept of these methods is lineage tracing by DNA barcodes and the assessment of the changes induced in their targets, including whole-genome or mitochondrial-genome sequencing data (51). The methods based on targeted barcode approaches are generally divided into the three following categories (Fig. 1): Transposon/retrovirus, Cre-recombinase-based and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9)-methods, which mediate *in vivo* integration of transgenic DNA targets, *in vivo* recombination of transgenic DNA cassettes and *in vivo* editing of transgenic DNA targets. In all these methods, the DNA barcode alters the genome of a single cell, whose progeny inherits the barcode and may be considered a cloning unit. DNA barcodes may subsequently be recorded and measured in high throughput so that thousands of different cloning units may be tracked in parallel.

Transposon/retrovirus-based DNA barcode. Certain studies have used a predefined library of barcodes that are easy to interpret and track by sequencing. For instance, TracerSeq is a clonal barcode method demonstrated in zebrafish (52). TracerSeq utilizes ongoing transposase activity to continuously integrate a predefined library of barcodes into embryos as an injection plasmid library. Progressive integration of plasmids provides clonal and subclone heritable tags for the genome. CellTag is an integration method of 8-nt barcodes based on lentiviral delivery (53). In this design, the location of the cell tag is the 3'untranslated region of the GFP gene, followed by the simian virus 40 polyadenylation signaling sequence. By allowing >1 barcode to mark each cell, the diversity of the combination is expanded. Bramlett *et al* (54) developed a detailed protocol for a viral barcoding program (Fig. 1A). The barcode was 33-bp in length and included a 6-bp library ID and a random 27-bp barcode. In theory, 427-bp barcodes may be generated. New users may use this protocol to create custom barcode libraries in their laboratories and easily set barcodes at low cost (54). However, these methods limit the possibility of using additional experimental methods and reduce the diversity of the barcodes. These strategies are also limited to short-term culture and tolerant cell separation systems.

Cre recombinase-based DNA barcode. The Polylox system (Fig. 1B) integrated a 2.1-kb synthetic gene from Arabidopsis into the Rosa26 site of the mouse and inserted 10 loxP sites into this gene, which resulted in the induction of the transient expression of the Cre recombinase enzyme. Following exposure to Cre recombinase, these loxP sites were randomly

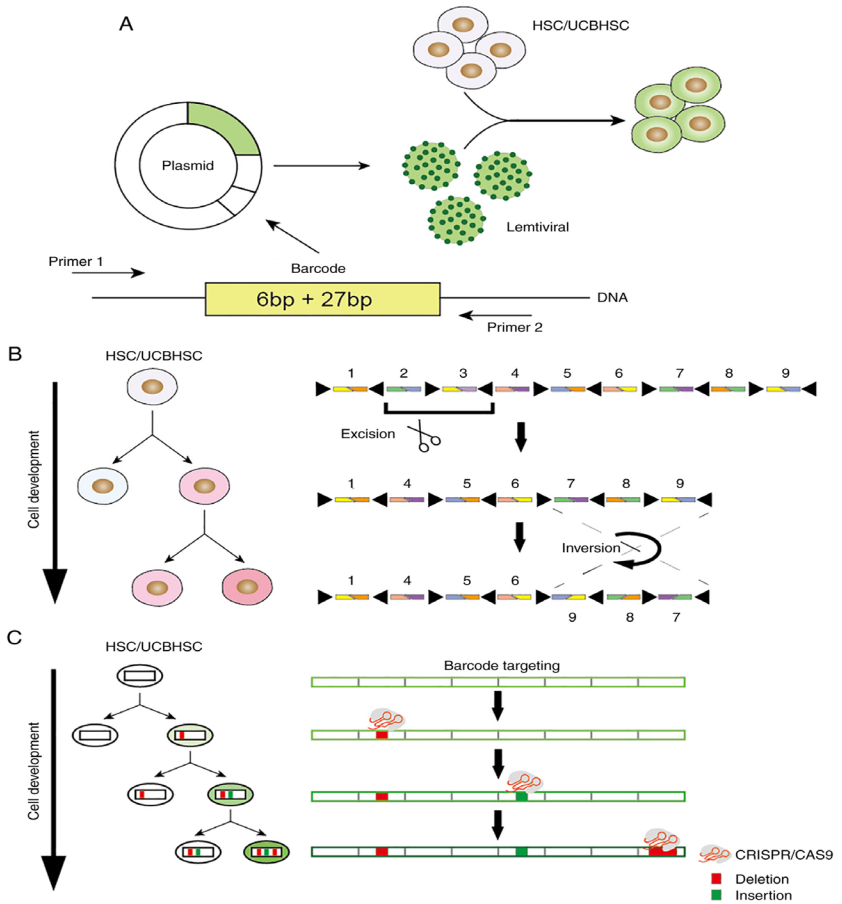

Figure 1. Strategies of barcode production *in vivo* for HSC/UCBHSC. (A) The synthesized barcode was cloned into a plasmid and then packaged into a lentiviral vector. The cells of interest are then transduced. (B) Cre recombinase recognizes the LoxP site (black triangles). If the loxP site is in the opposite direction, recombination results in inversion and the DNA region (color segments) between the loxP sites are reversed. If these sites face in the same direction, the sequence between loxP sites is deleted. (C) As cells differentiate, insertions and deletions of barcodes are produced due to incomplete NHEJ repair of Cas9-mediated double-strand breaks. UCB, umbilical cord blood; HSC, hematopoietic stem cell; CRISPR, Cre-recombinase-based and clustered regularly interspaced short palindromic repeats; Cas9, CRISPR-associated protein 9.

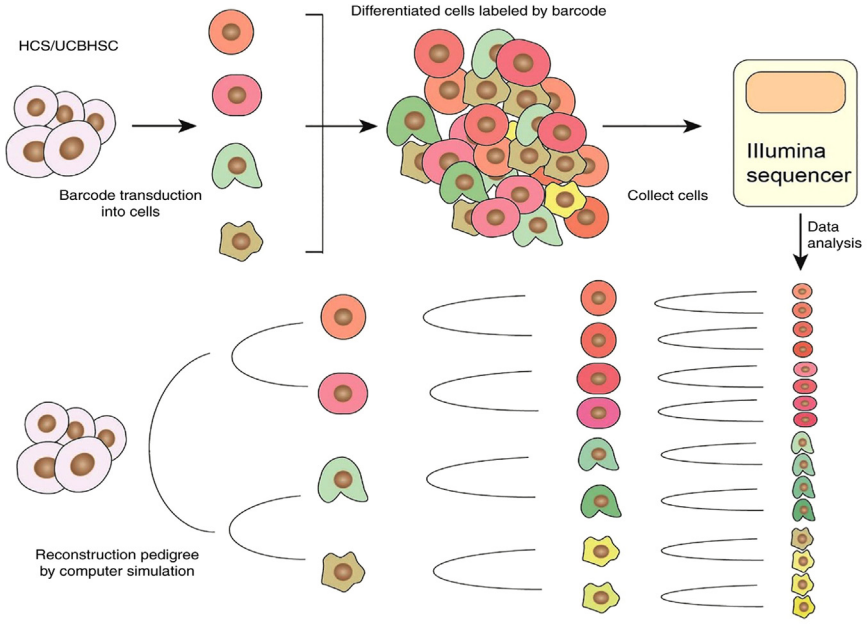

Figure 2. Schematic representation of lineage-tracing approach for HSC/UCBHSC. Different colors represent different barcodes and different shapes represent different cell types. The combination of barcode technology and single-cell sequencing technology builds a more perfect lineage tree. UCB, umbilical cord blood; HSC, hematopoietic stem cell.

excised or flipped from the DNA sequence, which subsequently generated a barcode in the HSCs, and was finally combined with sequencing technology in order to evaluate their differentiation fate *in vivo*. However, certain shortcomings may exist in the barcodes, which are based on the Cre-loxP system. Cre is inherently more prone to excision than inversion. Therefore, the size of the target array will be reduced over time. An additional disadvantage is that barcode target arrays are long and repetitive due to the low diversity of recombinase recognition sites and the nature of their minimum spacing requirements. To achieve high barcode diversity, the specific target array must contain more fragments, which requires the barcode to be read by a low-flux long read sequence. This drawback may be mitigated by future improvements in length sequencing technology (55,56).

CRISPR-based DNA barcode. The development of CRISPR/Cas9 technology has enabled the use of this novel technology instead of DNA recombinase in order to assess DNA barcoding (Table II). In the barcode scheme, which is based on CRISPR editing, CRISPR/Cas9-induced double-strand break of genomic DNA is usually repaired during cell division based on the non-homologous end joining (NHEJ) (57) mechanism common in mismatch repair. Therefore, short random insertions and deletions are gradually introduced into the barcode region of the DNA to mark cells and construct lineage relationships.

The genome editing of synthetic target arrays for lineage tracing (GESTALT) system (Fig. 1C and Table II) was the first system introduced to confirm this principle (58). The GESTALT system utilizes the CRISPR/Cas9 genome labeling technology to accumulate the combined sequence diversity and form a compact, multi-target and information-intensive barcode, which combines and accumulates the mutations generated by the dense target site array, records the cell lineage information on a large scale and is able to query the lineage information from at least hundreds of thousands of cells on a large scale. Only one barcode sequence is read in each cell and the generated barcode is used for lineage tracking of the zebrafish.

An additional similar method is memory by engineered mutagenesis with optical *in situ* readout (MEMOIR) system (Table II), a method of inducing mutation memory and optical in situ reading by designing a set of barcode recording elements, which is based on the fact that the target-induced mutation of CRISPR/Cas9 irreversibly changes the state of a given barcode recording element and subsequently reads its sequence in a single cell by multiple single molecule RNA fluorescence hybridization. This analyzes the final state of a log in a single cell (59). Therefore, lineage information may be reconstructed from the cell community. In addition, the combination of the endogenous gene expression analysis with lineage reconstruction of the same cell enables the deduction of the dynamic changing rate of embryonic stem cells between two gene expression states. Finally, computer simulation reconstruction is used to indicate the way by which a parallel MEMOIR system, which runs in the same cell, is able to record and read the history of dynamic cell events.

The methods used to further increase the diversity of barcodes have been redeveloped and are included in the mammalian synthetic cell recorder integrating biological events (mSCRIBE) (60) and Homing CRISPR barcodes (Table II) (61,62). Both methods share the same principle and they are designed to target the spacer sequence of their own genome by mutated guide RNA (gRNA), which is termed self-targeting guide (stg) RNA in mSCRIBE and homing gRNA (hgRNA) in the homing barcode. Despite its different names, its working principles are interlinked. Initially, stgRNA/hgRNA cleavage is performed, while the mutated genomic DNA produces barcode diversity and subsequently, the mutated site produces new stgRNA/hgRNA, which is targeted to the mutated genomic site. New barcodes are continuously generated until the spacer is truncated to <16-18 nt or the protospacer-adjacent motif sequence is lost. The generation of new barcodes would not be terminated and may even be continuously updated by specific mouse models. Another innovation that increases the diversity of the Cas9-edited barcodes is the use of terminal deoxynucleotidyl transferase (TdT) as an additional transgenic component of the barcodes. In the presence of double-strand breaks, TdT catalyzes the random binding of nucleotides at DNA cleavage sites, which increases the insertion-based editing frequency (63).

More recently, Bowling *et al* (64) established a mouse cell line for CRISPR array repair lineage tracing (CARLIN) and its corresponding analytical tools (Table II). CARLIN is able to generate transcriptional recognition barcodes (up to 44,000 barcodes), which are compatible with sequencing barcode coding and fully genetically defined in an induced manner at any time-point in mouse development or adulthood. For the CARLIN system, 10 single guide RNAs were designed based on GESTALT tracking technology to ensure efficient cleavage of target sites in the presence of Cas9 (58). The gRNAs were designed in a quadratic iterative manner, with one expressing the gRNA driven by the U6 promoter and the second carrying the tetO operon upstream of each gRNA. Constitutive expression of molecular tracer arrays is also based on constitutive CAG promoter-driven fluorescent proteins. All these elements are integrated in mouse embryonic stem cells, which are mediated by recombinase and inserted together into the widely used Col1a1 site. Finally, doxycycline induction was used to control the expression of the Cas9 gene and promote the fragmentation of double-stranded DNA in the target array. These breaks were repaired, resulting in the expression and stable inheritance of a variety of altered DNA sequences in the CARLIN allele. This system may produce gene deletions ranging from 1 to 252 bp, as well as gene insertions up to 51 bp in length. Based on CRISPR-Cas9 gene editing technology, CARLIN may perform lineage tracing in the phylogenetic process and heterogeneity analysis on cell populations, as well as control the extent of gene editing when doxycycline is added. It is a model that allows simultaneous cell lineage tracing and single-cell level transcriptome analysis *in vivo*, providing an important tool platform for research on multiple cell lineages.

The CRISPR/Cas9 method is promising for achieving high diversity, labeling of various tissues and organs of organisms and generation of *in vivo* barcodes over time. However, the diversity generated in practice is considerably lower than that in theory due to the repair mode of the NHEJ, which selects the generation of deletions rather than insertions, leading to the gradual shortening of the CRISPR barcodes over time. However, the development of this methodology has enabled the use of multiple barcodes of a single cell compared with one single-cell barcode, which greatly increases the diversity

produced. With the rapid development of CRISPR barcoding technology, these limitations may be reduced.

Delivery and reading of DNA barcodes. The manual assignment of a single barcode (one-to-one) to the labeled cells is the most ideal delivery method, which may guarantee that they are completely and not repeatedly labeled. This delivery approach is not acceptable in terms of cost and time. The assignment of barcodes to individual cells is difficult and may only be used under limited conditions. At present, the most effective delivery methods still rely on *in vitro* production of barcode vectors, such as the use of retroviral transfection (41), as well as plasmid injection and electroporation (44). It should be noted that the nature of the cells may be altered during barcode transduction. Although several studies have indicated that lentiviral integration does not result in significant changes in transduced cells (65-69), it is still possible to randomly insert specific lentiviral vectors into certain genomic regions and alter cell behavior. Therefore, experimental replicates and controls must be used rigorously during the experiments to rule out this rare possibility.

To date, the majority of research studies that have investigated reading of the DNA barcodes depend on the extraction of nucleic acids and subsequently, the barcodes are detected or quantified *in vitro*. The method of barcode reading has also undergone tremendous changes in recent decades. It was initiated with PCR amplification and sizing, continued with Sanger sequencing (46,70) and eventually developed to high-throughput sequencing. The reduction of the sequencing cost is an important factor that promotes the development of barcode reading technology. The development of single-cell RNA sequencing provided significant progress in barcode detection. It is able to read the cell development information carried by the barcode and at the same time obtain the transcription status and cell type of the cell (Fig. 2). However, during the sequencing of a single cell, its spatial position is lost (71).

Applications of DNA barcoding. DNA barcode technology is a powerful pedigree tracing tool used for UCBSCs and other stem cells. Cheung *et al* (72) exposed $CD34^+$ UCBSCs to a barcode lentiviral library and subsequently inoculated two non-obese diabetic (Nod)/severe combined immunodeficient (Scid)/IL2 receptor $(IL2R)y^{-/-}$ mice with $1x10^5$ barcoded cells, respectively. This method was initially combined with sequencing analysis and it confirmed that $CD34^+$ UCBSCs were able to differentiate into human $CD3^+$ T cells. Belderbos *et al* (73) transplanted lentiviral barcode $CD34^+$ cells from 20 UCB donors into Nod/Scid/$IL2Ry^{-/-}$ mice. Following 10 weeks, human B, T and myeloid cells were detected. This indicated that UCB-derived $CD34^+$ cells were able to be traced for multiple generations by the DNA barcode in mouse xenotransplantation. This study further indicated donor-to-donor heterogeneity in the clonal dynamics of transplanted human UCBSCs in mouse xenografts. Several research groups have tracked the activity of various HSCs following single recipient transplantation, suggesting that HSCs only have a small role in daily hematopoiesis under stable conditions (42,47,74). Due to the significant reduction in the sequencing cost and the development of synthetic biology, which includes the improvement of DNA barcode technology, it is expected that additional novel barcode strategies will be widely used in research of UCBSCs. The differentiation plasticity of UCBSCs remains controversial. Research on UCB cell biology is highly significant to further the understanding of its cellular and molecular mechanisms, as well as the difficulties encountered in the clinical applications of UCBSCs. It is considered that DNA barcode tracing cannot only be applied to UCBSC research, but also extended to several fields, such as neuroanatomy, cell activity recording and cancer research (75-77). The barcode method allows the reconstruction of stem cell lineages during development based on barcode similarities and tracing of cell relationships in a single experiment (78). In fate-mapping studies, barcodes may be used to count the number of stem cell divisions in heterogeneous cell populations (79). In addition, the barcode may also aid the identification of the cellular origin of occurrence, recurrence and metastasis of cancer stem cells and the heterogeneous responses of cancer stem cells to treatment (80).

An important challenge remains to be addressed prior to reconstructing the pedigree tree with the barcodes. A sufficiently high level of barcode diversity is required so that every cell may be uniquely labeled at the end of the experiment. Insufficient diversity will either terminate pedigree tracking prior to the end-point or seriously hinder tree reconstruction, since the cells in the distant pedigrees will share the same barcode. As a result, the complexity of the DNA sequence increases exponentially with the length and diversity of the barcodes. However, the current in situ reading of the barcode or cell transcription is considerably slow, inefficient or biased (49-51,81,82). Additional technical development is required. DNA barcode technology exhibits high potential as a genealogy tracking tool and the rapid development of this field is expected to occur in the next years.

4. Summary

Currently, research on UCBSC has achieved periodical success and additional experiments are required to verify its wide clinical applications. The combination of cell barcode and single-cell sequencing technologies may continuously record the development history of cells, which is a milestone in lineage tracking technology. The mechanism of UCBSC development following transplantation may be further elucidated and the time required for these methods to be used in large-scale clinical applications is reduced. However, the current cell barcode technology is not able to generate sufficient diversity to perform specific labels on each cell. In addition, the efficiency of reading the barcode is not sufficiently high based on the current sequencing technology, the reading process exhibits certain bias and potential off-target effects are present. Additional technical development is required to develop a more simple and feasible lineage tracing technique with high sensitivity, strong specificity, low external interference, no apparent toxicity and low false-positive rate. It is expected that future tracer techniques will be able to fully describe the development of UCBSCs and their differentiation and tissue formation ability during tissue homeostasis or disease development.

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Availability of data and materials

Data sharing is not applicable to this article, as no datasets were generated or analyzed during the current study.

Authors' contributions

M-YW, X-CP and H-WX conceived and designed the present review. The manuscript was drafted by M-YW. Manuscript revisions and modifications were carried out by YZ, G-SL, QH, W-QC, Z-WH, YW, ZM, X-WW, YX, S-XF and X-CP. Final changes were made by M-YW and H-WX. All authors read and approved the final manuscript. Data authentication is not applicable.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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