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厚生労働科学研究費補助金

再生医療実用化研究事業

ヒト胚性幹細胞を用いた臨床利用の安全性検証のための試料保存と分析システムの構築

平成２６年度　総括研究報告書

研究代表者　　末盛　博文

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研究代表者　末盛　博文　　京都大学　再生医科学研究所　准教授

研究要旨：本研究はヒトES細胞の臨床利用において、安全性を担保する上で特に必要である、研究の各段階での試料の保存と分析を行うシステムを確立し、将来的により安全な再生医療を実現する研究基盤を構築することを目的とする。その実現のため、試料保存システムの運用の規則、SOPなどの整備を行うとともに、品質管理の手法、規格の検証が必要である。また、試料保存システムの適切な稼働を担保するため、機能の確認を順次実施し最終的に保存及び品質管理システムを確立する。本年度はヒトES細胞樹立研究指針の改定や再生医療法に関連した省令通知などに対応した、新規細胞株樹立の研究申請の準備に着手するとともに、実際の樹立やバンク化に伴い必要となる品質管理や試料等の保存システムの検証を行った。品質管理に関しては細胞の品質管理で重要な項目である、未分化状態の評価を未分化特異的遺伝子の定量PCR法により実施し、暫定的な指標値を定めた。保存システムについて継続的な動作確認を行い、正常な稼働を確認した。

A．研究目的

本研究はES細胞を用いた臨床研究において特に安全性を担保する上で必要となる研究の各段階での試料の保存と分析を行うシステムを確立し、将来的により安全な再生医療を実現する研究基盤を構築することを目的とする。

ヒトES細胞を用いた再生医療はすでに欧米では臨床試験が開始されており、今後より広範な医療領域での利用が期待されている。しかしながら、従来の細胞移植医療と異なり、ES細胞を用いた移植医療では、１ドナーに由来する細胞を様々な疾患を対象として多数の患者に適用することになる。そのため移植組織

細胞の腫瘍化や、これに由来する感染性因子の伝搬は、被験者/患者のみならず公衆衛生上の問題を生じうるのではないかとの懸念がもたれており、これらの課題への対応は、ES細胞を含めて幹細胞の医療利用の普及を促進する上で、重要性・必要性が非常に高いものである。

従来のヒト幹細胞の臨床利用に関わる指針に代わり、再生医療安全確保法に基づく省令/通知等により、ヒトES細胞の臨床利用にかかわる各種の規定が定められ、併せてヒトES細胞の樹立に関わる指針も改訂された。これらに適合したES細胞作成研究の申請にむけての作業が平成27年には開始される。本研究では提供医療機関と連携し、ドナー情報等の取扱/保存について検討、また、実施が想定される細胞の検査法の感度や精度について検証を行うことにより、将来のヒトES細胞を用いた再生医療の安全性の担保を推進する他の事業に資することができると考えられる。

B．研究方法

現在ヒトES/iPS細胞の臨床利用を目指し多くの機関が基礎/前臨床研究を進めており、我々は早期の再生医療の実現を目指して臨床用ES細胞作製の基盤研究を他の研究経費により進めている。本研究計画と別途進めている臨床用ES細胞株の樹立と分配では、臨床研究機関に細胞を分配するためのセルバンクが構築される。本事業ではこの時、臨床研究機関へ供給されるものと同一ロットの複数サンプルを将来の検証が必要になった場合に備え保存する。また、保存が適切に行われるように、その検証システムを整備する。

臨床研究機関での未分化細胞の増幅/凍結保存が行われることも想定されるが、その場合は培養履歴とともに一部試料の寄託を求めるよう調整に努める。分化誘導後移植に用いる機能細胞が作出されるが、この途中段階で組織幹細胞や前駆細胞が作出される場合は必要に応じてその一部の寄託を求める。

臨床試験において有害事象が発生した場合に、品質管理上の問題の有無について、必要に応じて寄託された試料のウイルス等の検査や遺伝子解析を行えるよう解析技術の開発検証を行う。

（倫理面への配慮）

ヒトES細胞を用いる研究に関しては、文部科学省「ヒトES細胞の樹立および分配に関する指針」「ヒトES細胞の使用に関する指針」に従い実施された。なお両指針は平成26年11月24日にそれぞれ「ヒトES細胞の樹立に関する指針」および「ヒトES細胞の分配と使用に関する指針」に改訂された。

C．研究結果

本研究を実施する京都大学再生医科学研究所では、基礎研究用のヒトES細胞株の樹立と特性解析を行ってきた。このような研究の過程で蓄積した培養/解析技術をもとに、臨床用ヒトES細胞株を作成・利用する上で必要となる細胞バンク構築を別途実施

している。本事業はこの臨床用ヒトES細胞樹立研究及び医療機関等での臨床研究/治験と連動して行う。

新規細胞株の樹立計画については、指針の整備等が想定よりも大幅に遅れたため、26年度中の研究計画の申請は行われなかった。そのため、樹立関連の試料/細胞の保存は実施しないこととし、品質基準の検証に注力することとした。

再生医療法の実施に関して規制の詳細を定めた省令や通知は平成26年 11 月に施行されている。これは当初の想定よりも大幅に時間を要することとなった。またこれにあわせてヒト ES を臨床利用に対応させるための ES 指針の改定も行われた。これらに基づき、樹立研究計画の作成に着手した。これまでに、機関の倫理委員会に臨床用 ES 細胞の作製に着手を計画していることや今後の手続きについて概要説明を行い、樹立の方針に関しては理解が得られている。

バンキングにおける安全性試験にかかわる基準の検証については、基本的に別途進められている臨床用ヒト ES 細胞のバンキングシステムの実証試験のなかで実施している。本事業では、安全性にかかわる 12 の大項目のうち、異種動物由来成分残存試験、マーカー遺伝子発現解析についてプロトコールの検討および基準値の妥当性を検証した。細胞株の樹立過程で持いられた異種由来成分はバンク化の初期段階で検出限界以下になり、その後の同様の工程により理論的にはほぼ存在を否定できるレベルに達すると推定された。マーカー発現による細胞の特性解析は、無菌試験と並びバンクの品質管理として重要である。遺伝子発現に関しては、未分化マーカーとして NANOG など4つ、分化マーカー6つを選択しqPCR による定量を行った。細胞バンク作製を複数回実施しロット間でのデータの変動を検証した。

平成 24 年度補助事業において整備された保存・培養設備の運転状況のモニタリングシステムの動作の検証を昨年度に引き続き継続し、基本的動作に問題がないことを確認した。

臨床用細胞バンクの構築を目指す他機関の担当者との連携を含め、バンク構築の標準化について欧米を中心に国際的な状況に関する調査を進めている。その一環として 2014 年 6 月に開催された International Stem Cell Banking Initiative 会議に参加し、情報交換を行った。この会議の成果は過去数年間にわたる活動のとりまとめをふくめて、臨床用ヒト多能性幹細胞のバンキングにおける品質管理に関する国際的なガイダンスとして 2015 年 2 月に論文発表された。

D．考察

異種動物由来成分については、きわめて短期間に検出限界以下まで除去されることが明らかにされた。バンク化の時点においてはその存在は無視しうるものと考えられる。よって、異種動物由来のウイルスについて試験を行うことが重要となる。

未分化マーカーについては比較的変動がすくなく規格値を決定で

きるものと考えられた。分化マーカーの発現解析については、発現量が小さいこともあり他の遺伝子を用いることも含めた検討が必要である。

バンクにおける品質管理基準に関してはISCBIより公開されたガイダンスに基づきつつ日本国内の各種規制に適合させる様に定めていく必要がある。

平成26年11月施行の再生医療等の安全性確保に関する法律に関連した省令等によりES細胞の樹立に使用する施設に関しても「細胞加工施設」としての許可を要求されることになった。このような要求は全く想定されておらず、従来無かった制度であり、要求水準や手続きに要する時間の予測が困難である。現に保有する施設はすでに要求を満たしているものと考えられるが、追加的な施設整備が必要となれば、新たな予算措置が必要になることも考えられる。27年度中に樹立計画の申請を行い、できるだけ早く樹立を開始したい。

E. 結論

ヒトES細胞の臨床利用までには長期間にわたる基盤的研究が必要である。細胞製品の品質保証においては、製造工程を含めてその管理のために、様々な検査法について、一定の水準で適切に実施されることが、長期的な評価に必要である。そのため今後は臨床研究機関との連携をどのように進めていくかが重要である。

F. 健康危険情報

該当なし。

G. 研究発表

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A new method for the detection of residual mouse feeder cells contaminated in the products of human plulipotent stem cells.
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H.　知的財産権の出願・登録状況（予定を含む。）

該当なし

Ⅱ．研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
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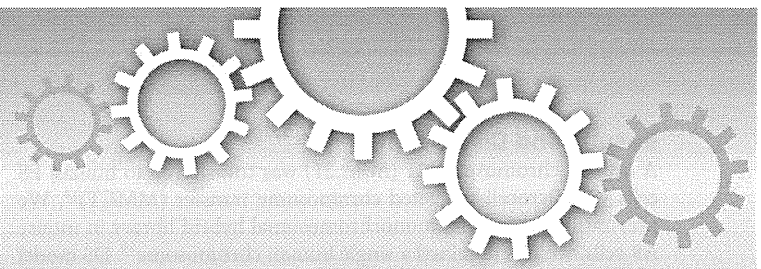
Ⅲ．研究成果の刊行物・別刷り

SCIENTIFIC REPORTS



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Down syndrome-associated haematopoiesis abnormalities created by chromosome transfer and genome editing technologies

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Infants with Down syndrome (DS) are at a high risk of developing transient abnormal myelopoiesis (TAM). A *GATA1* mutation leading to the production of N-terminally truncated GATA1 (GATA1s) in early megakaryocyte/erythroid progenitors is linked to the onset of TAM and cooperated with the effect of trisomy 21 (Ts21). To gain insights into the underlying mechanisms of the progression to TAM in DS patients, we generated human pluripotent stem cells harbouring Ts21 and/or GATA1s by combining microcell-mediated chromosome transfer and genome editing technologies. In vitro haematopoietic differentiation assays showed that the GATA1s mutation blocked erythropoiesis irrespective of an extra chromosome 21, while Ts21 and the GATA1s mutation independently perturbed megakaryopoiesis and the combination of Ts21 and the GATA1s mutation synergistically contributed to an aberrant accumulation of skewed megakaryocytes. Thus, the DS model cells generated by these two technologies are useful in assessing how GATA1s mutation is involved in the onset of TAM in patients with DS.

Down syndrome (DS), or trisomy 21 (Ts21), is the most frequent live-born aneuploidy syndrome in humans[1], and new-born infants with DS are at a high risk of developing transient abnormal myelopoiesis (TAM)[2]. In most cases, TAM resolves spontaneously within 3 months. However, DS-related acute megakaryoblastic leukaemia (DS-AMKL) subsequently develops within 4 years in approximately 20–30% of cases with a history of TAM[3–5]. Therefore, TAM has been considered as a preleukaemic stage. Acquired mutations in the N-terminal activation domain of the megakaryocyte transcription factor GATA1, leading to the expression of a GATA1 isoform (GATA1s), have been reported in DS-TAM and DS-AMKL[6–8]. Furthermore, it has been reported that DS-TAM is most likely caused by a combination of the single GATA1 mutation and constitutive Ts21, and DS-AMKL evolved from a TAM clone that acquired additional mutation(s)[9]. However, the precise mechanisms in the progression process have not been clarified yet.

Patient-derived pluripotent stem cells, including embryonic stem (ES) and induced pluripotent stem (iPS) cells, are important tools to model pathology[10–14]. Although in vitro studies using DS-ES and DS-iPS cells reproduced the haematopoietic abnormalities in DS[15–17], DS-derived pluripotent stem cells with an acquired *GATA1* mutation have not been generated. In this study, we generated novel Ts21, GATA1s, and GATA1s/Ts21 human ES cells by combining chromosome transfer and genome editing technologies.

Results and Discussion

A human chromosome 21 (hChr.21) was transferred to human ES cells via microcell-mediated chromosome transfer (MMCT)[18]. We previously generated a monochromosomal hybrid library in mouse A9 cells, which contained a single human chromosome[19]. DS model mice were generated by transferring an extra hChr.21 into mouse ES cells using the A9 library via MMCT[20,21]. Similarly, we generated human ES cells containing an extra hChr.21, creating Ts21. A pSTneo-tagged hChr.21 was transferred to human ES (KhES-1)-derived subclones (designated as WT-ES) via MMCT (Fig. 1a). Twelve G418-resistant clones from 3 independent experiments were obtained. Six clones contained an additional hChr.21 (Ts21), and 6 clones contained 2 additional copies of hChr.21 (tetrasomy 21) (Supplementary Fig. 1). Multicolour fluorescence in situ hybridisation (mFISH) analysis indicated that the hChr.21 was successfully transferred into wild-type (WT)-ES cells and that the karyotype was 47,XX,+21 (Fig. 1b, c). FISH analysis of the exogenous hChr.21 showed that the pSTneo-derived signal was in a single hChr.21 (Supplementary Fig. 2). To determine whether Ts21-ES cells could differentiate into all 3 embryonic germ layers, Ts21-ES lines were injected into testes of severe combined immunodeficiency (SCID) mice. Histological analyses revealed all 3 embryonic germ layers in all teratomas (Fig. 1d). Microarray analyses revealed that genes on hChr.21 in Ts21-ES cells were globally overexpressed, but gene expression from hChr.18 was comparable with that in WT-ES cells (Fig. 1e). These data suggest that the exogenous hChr.21 was successfully transferred to WT-ES cells and that the Ts21-ES cells have differentiation potential.

The *GATA1* mutation was generated via one of the genome editing technologies, zinc-finger nucleases (ZFNs), which were used previously to modify the endogenous genome of several species[22]. mRNAs or plasmids encoding a ZFN targeting exon 2 of *GATA1* DNA were transfected into WT-ES cells. A mutation detection assay (Cel1 assay) showed that 5 of 384 clones and 2 of 96 clones using the mRNAs and plasmids, respectively, were positive for the mutation (Supplementary Fig. 3). The mutation-positive mRNA-transfected clones were subcloned to reduce the possibility of heterogeneous populations. A restriction fragment length polymorphism (RFLP) assay using BsiHKAI enzyme showed that 1 (pZ7) of 19 clones (17 mRNA-transfected subclones and 2 plasmid-transfected clones) contained the different deletions in both alleles of exon 2 of *GATA1* (Supplementary Fig. 4); sequence analyses revealed that 2 clones (pZ19-2 and pZ28-5) contained heterozygous insertion/deletion (or deletion) in the *GATA1* gene and 1 clone (pZ7) contained different deletions (8 bp and 17bp) in both alleles, resulting in a premature TGA stop codon in exon 2 (Supplementary Figs. 5 and 6 and Fig. 2a). The clones with the premature stop codon in exon 2 had normal karyotypes (46,XX) and differentiation potential to 3

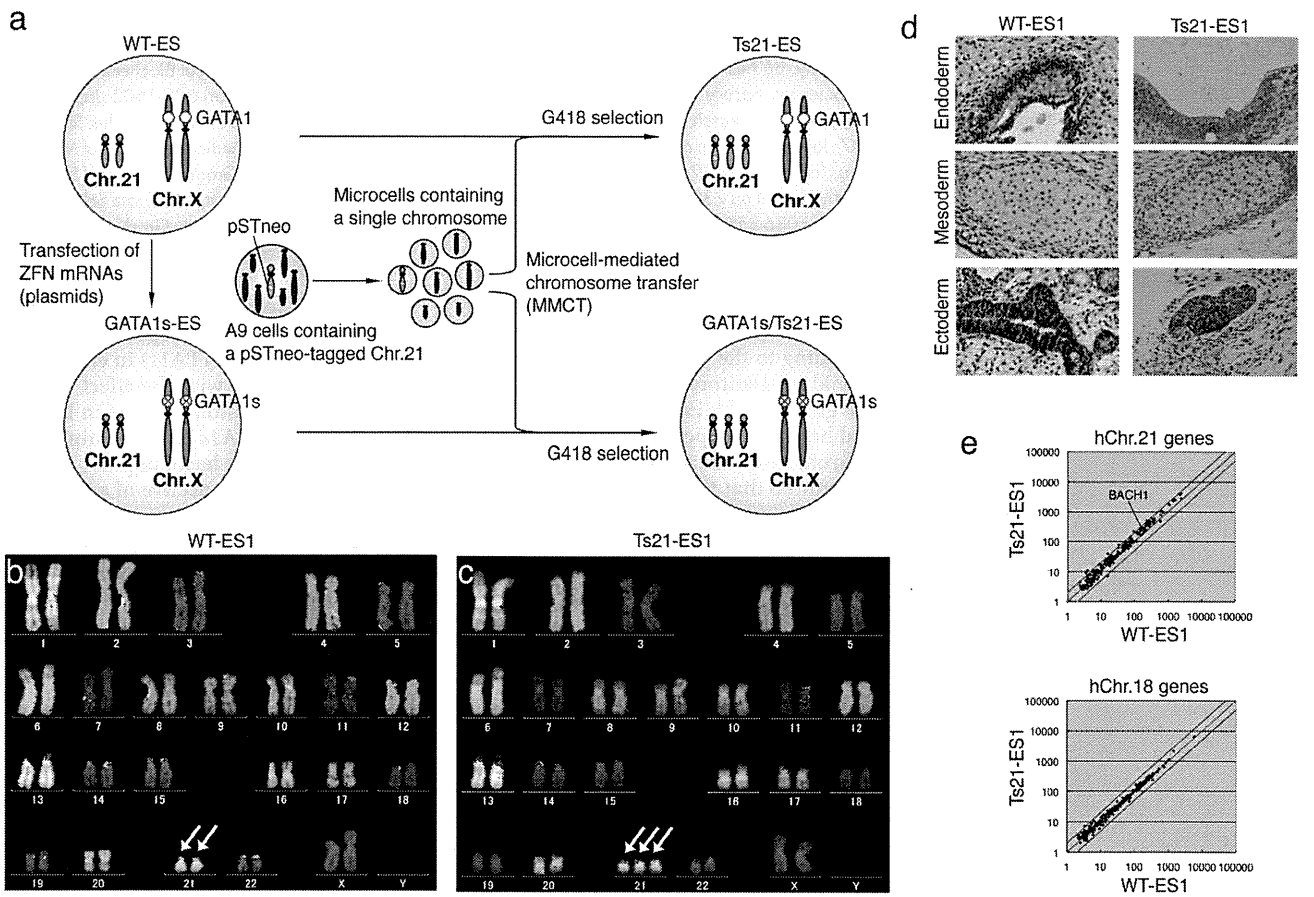

Figure 1 | MMCT of hChr.21 into human ES cells. (a) Diagram of the generation of Ts21, GATA1s, and GATA1s/Ts21 in human ES cells. (b, c) mFISH analyses in WT-ES and Ts21-ES cells. Arrows indicate hChr.21. (d) Teratomas derived from WT-ES and Ts21-ES cells. Eight weeks after cell transplantation, structures originating from all 3 germ layers were found in the teratoma. (e) Microarray analyses of WT-ES and Ts21-ES cells. Representative comparison data of genes on hChr.21 (upper panel) and hChr.18 (lower panel) between WT-ES and Ts21-ES cells are shown. The dots between blue and red lines, and the dots on red line show genes with 2-fold differences in expression and equal expression, respectively. The red dot indicates *BACH1*, a representative gene from hChr.21 (upper panel).

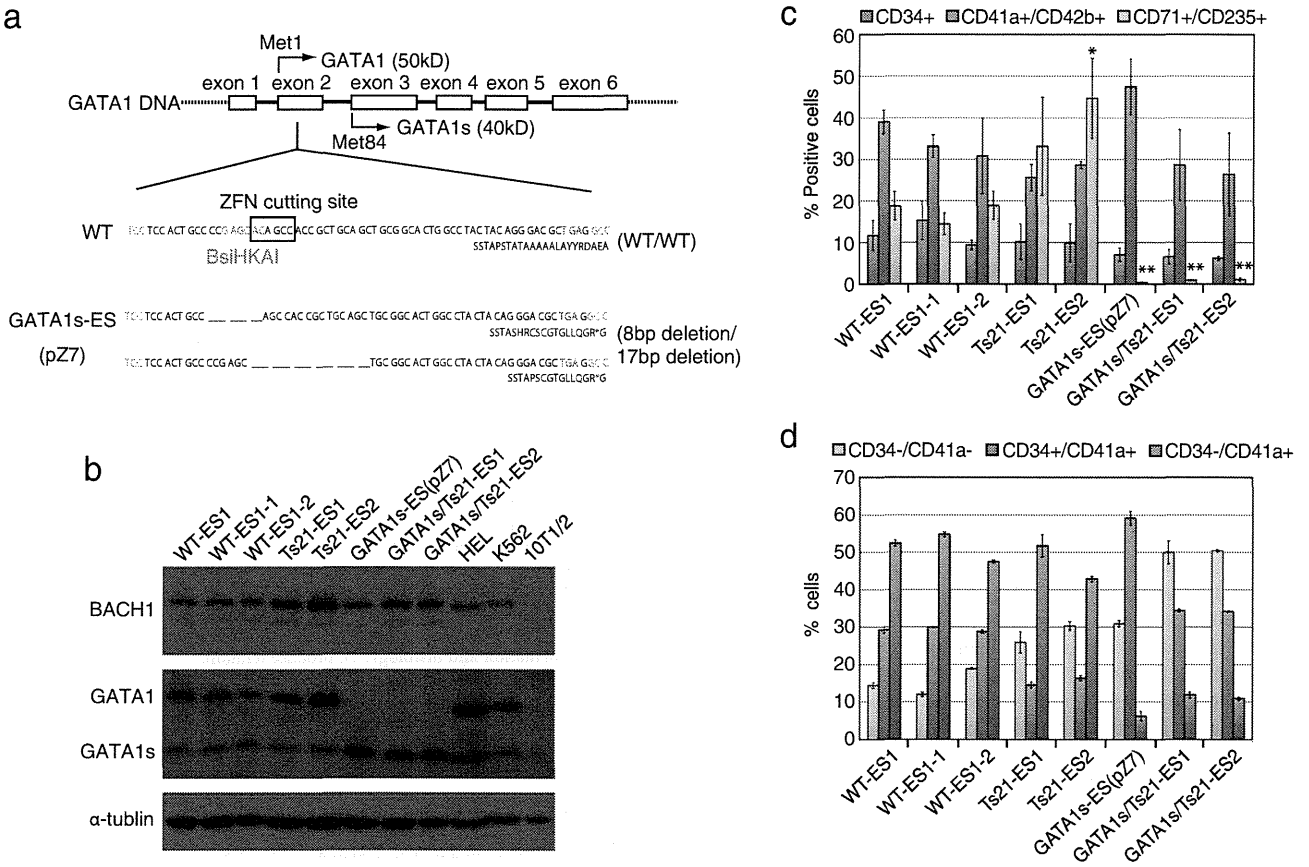

Figure 2 | Characterisation of GATA1s-ES and GATA1s/Ts21-ES cells. (a) Sequence analyses of GATA1s-ES cells. One allele had an 8-bp deletion and the other had a 17-bp deletion; both resulted in a TGA stop codon in exon 2 of *GATA1*. The *GATA1* nucleotide (upper line) and amino acid (lower line) sequences are shown for WT-ES and GATA1s-ES cells. An asterisk shows the stop codon. (b) Western blot analyses of erythroid lineage-differentiated cells derived from WT-ES, WT-ES sublines, Ts21-ES lines, GATA1s-ES, and GATA1s/Ts21-ES lines. Anti-BACH1 was used to detect the gene-dosage effect on hChr.21. Anti-GATA1 recognised the C-terminus of both GATA1 and GATA1s protein. Anti-α-tubulin was used as an internal control. HEL cell lysate and K562 nuclear extract were used as positive controls. 10T1/2 whole cell lysate was used as a negative control. Cropped blots were used in this figure. Original full-length blots are shown in supplementary figure 12. (c, d) Haematopoietic differentiation analyses using WT-ES, WT-ES sublines, Ts21-ES lines, GATA1s-ES, and GATA1s/Ts21-ES lines. Data are the means of 3 independent experiments (±S.D.). The percentage of CD34+, CD41a+/CD42b+, and CD71+/CD235+ cells are shown in each differentiation stage (ES-sac (day 14), megakaryocyte (day 20), and erythroid (day 20)) (c). Statistical analyses were performed by comparison with WT-ES cells (WT-ES1, WT-ES1-1 and WT-ES1-2). *$p < 0.05$, **$p < 0.01$ by two-tailed Student's t test. The percentage of CD34-/CD41a-, CD34+/CD41a+ and CD34-/CD41a+ cells are shown in the megakaryocyte stage (d).

embryonic germ layers (Supplementary Figs. 7 and 8). The pZ7 clone (designated GATA1s-ES) with the deletions in both alleles of exon 2 of *GATA1* was used for further analyses.

An additional hChr.21 was transferred to GATA1s-ES cells via MMCT. Cytogenetic and histological analyses showed that the clones in the GATA1s genetic background contained Ts21, and 2 clones with Ts21 and GATA1s with differentiation potential to 3 embryonic germ layers in the teratoma were randomly selected for further analyses (designated GATA1s/Ts21-ES) (Supplementary Fig. 9). Microarray analyses revealed that genes on hChr.21 in GATA1s/Ts21-ES cells were globally overexpressed, but gene expression from hChr.18 was comparable with that in GATA1s-ES cells (Supplementary Fig. 10).

ES-sac–mediated in vitro haematopoietic differentiation analyses were performed (Supplementary Fig. 11). Western blot analyses of the erythroid lineage showed that a representative protein from hChr.21, BACH1[23], was overexpressed in the Ts21-ES and GATA1s/Ts21-ES lines compared with the WT-ES and GATA1s-ES cells (Supplementary Fig. 12 and Fig. 2b). Western blot analyses using a GATA1 antibody to recognise the C-terminus of GATA1/GATA1s protein showed that full-length GATA1 protein was expressed in WT-ES and Ts21-ES lines, but not in GATA1s-ES and GATA1s/Ts21-ES lines (Supplementary Fig. 12 and Fig. 2b). Additionally, GATA1s protein was remarkably increased in the GATA1s-ES and GATA1s/Ts21-ES lines. These results were also confirmed in the megakaryocytic differentiation from the ES cell lines (data not shown). These data suggest that the protein expression pattern in the genetically engineered ES-derived haematopoietic cells was comparable to that of DS and DS-TAM patients[6].

Flow cytometry analyses showed that the frequency of ES-sac–mediated erythroid (CD71+/CD235+) cells was higher in Ts21-ES cells than WT-ES cells. This is comparable to the results of a previous report by Chou et al., which demonstrated the enhanced erythropoiesis in iPS cells derived from Ts21 subjects[16]. In contrast, the frequency of erythroid cells in GATA1s-ES and GATA1s/Ts21-ES cells was significantly lower (Supplementary Fig. 13 and Fig. 2c). This is comparable to the results of previous reports by Hallanda et al. and Sankaran et al., which demonstrated the impaired erythropoiesis in the subjects with inherited *GATA1* mutations in the absence of Ts21[24,25]. Thus, the eyrhroid differentiation seems to be reciprocally affected by GATA1s or Ts21, and the effect of the GATA1s mutation overcomes the enhancing activity of erythropoiesis in Ts21-ES cells.

Curiously, we found that the population of ES-sac–mediated megakaryocytic (CD41a+/CD42b+) cells from GATA1s-ES cells was higher than that from WT-ES cells, although the further introduction of an additional hChr.21 into each cell line (WT-ES and GATA1s-ES cells) resulted in a slightly reducted frequency. (Supplementary Fig. 14 and Fig. 2c). Further analysis of the megakaryocytic differentiation showed that the ratio of immature (CD34+/CD41a+) to mature (CD34-/CD41a+) megakaryocytic cells derived from GATA1s-ES and GATA1s/Ts21-ES cells was significantly higher than that from WT-ES and Ts21-ES cells, respectively, suggesting that GATA1s disturbs the maturation of megakaryocytes and/or enhances the proliferation of immature megakaryocytes (Supplementary Fig. 15 and Fig. 2d). Furthermore, CD34-/CD41a− cells accumulated in Ts21-ES cell cultures with a reduced frequency of CD34+/CD41a+ cells, and the additional GATA1s mutation worsened the phenotype of Ts21 (Supplementary Fig. 15 and Fig. 2d). Consequently, the efficiency of CD41a+/CD42b+ cells in GATA1s/Ts21-ES cells was seemingly comparable to that in WT-ES cells. We concluded that Ts21 and GATA1s mutation differentially affect the megakaryocyte differentiation, and the combination of Ts21 and GATA1s mutations synergistically influences the process of megakaryocyte differentiation. Our in vitro differentiation system revealed for the first time that Ts21 disturbs the differentiation of megakaryocytes and further GATA1s mutation intricately perturbs the process of megakaryopoiesis in combination with increasing the dosage of genes located on hChr.21.

Taken together, our novel system combined MMCT and ZFN technologies to generate DS model cells. The combination of chromosome transfer and genome editing technologies could therefore enable the generation of in vitro chromosome abnormality syndrome models with multiple genetic alterations. Progression from TAM to DS-AMKL requires additional mutations in genes including cohesin/*CTCF*, *EZH2*, other epigenetic regulators, and *RAS*/signal transducing molecules[9]. However, the function or mechanism of each class of mutation on the leukaemogenesis remains uncertain. Our methods can aid in resolving these questions, because the desired mutations in addition to the GATA1s can be inserted to the GATA1s/Ts21-ES cells using genome editing technologies. Importantly, all of the developed ES cell lines were isogenic and genetically defined. MMCT using other chromosome donor A9 cells will enable the generation of pluripotent stem cell-derived models for different trisomy syndromes including Ts18 and Ts13 in the same genetic background. Human chromosomes can be efficiently modified in the homologous recombination-proficient chicken DT40 cells, which can be used as a shuttle system to transfer the modified chromosome to other cells[26]. Defined genomic regions can be also cloned into human artificial chromosomes[27,28]. Thus, the genes responsible for phenotypes may be identified by transferring the modified chromosome via MMCT.

Methods

Cell culture. Mouse A9 cells containing hChr.21 (A9(21-16)) that were used as fusion donors for chromosome transfer were established as described previously[19]. The A9(21-16) cells were maintained in Dulbecco's modified Eagle's medium plus 10% foetal bovine serum (FBS) with 800 μg/mL G418 (Promega, Tokyo, Japan). A human ES line, a KhES-1-derived subline[29], was used following the human ES cell research guidelines of the Japanese government. Because the subline contained chromosomal abnormalities in chromosome 1q, the subline was further subcloned (designated as WT-ES1). WT-ES1 was used for MMCT and ZFN transfection experiments. WT-ES1 was further subcloned for the control cell lines (designated as WT-ES1-1 and WT-ES1-2). The parental human ES cell line and microcell hybrid clones were maintained on mitomycin C (Sigma-Aldrich, Tokyo, Japan)-treated Jcl:ICR (CLEA Japan, Tokyo, Japan) mouse embryonic fibroblasts as feeder layers in primate ES medium (ReproCell, Tokyo, Japan) supplemented with 4 ng/mL recombinant basic fibroblast growth factor (WAKO, Osaka, Japan). The mouse C3H10T1/2 cell line that was purchased from RIKEN BioResource Center (Tsukuba, Japan) was cultured in Eagle basal medium (Life Technologies, Carlsbad, CA, USA) containing inactivated 10% FBS and 2 mM L-glutamine (Life Technologies). The human ES cell differentiation medium was Iscove's modified Dulbecco's medium (Sigma-Aldrich) supplemented with an insulin-transferrin-sodium selenite cocktail (Life Technologies), 2 mM L-glutamine, 0.45 mM α-monothioglycerol (Sigma-Aldrich), 50 mg/mL ascorbic acid (Sigma-Aldrich), and 15% FBS.

MMCT. MMCT was performed as described previously[20]. A9 cells containing hChr.21 were used as donor microcell hybrids. Briefly, WT-ES1 and GATA1s-ES (pZ7) cells were fused with microcells prepared from donor hybrid A9 (21-16) cells and selected with G418 (50 μg/mL). The transferred hChr.21 in each line was characterised by cytogenetic analyses.

Microarray analyses. Total RNA from WT-ES, Ts21-ES, GATA1s-ES and GATA1s/Ts21-ES cells was prepared using RNeasy (Qiagen, Hilden, Germany) according to the manufacturer instructions. Microarray analyses were performed using a 3D-Gene Human Oligo chip 25k (Toray Industries Inc., Tokyo, Japan). Microarray slides were scanned using a 3D-Gene Scanner (Toray Industries) and processed by 3D-Gene Extraction software (Toray Industries).

Cytogenetic analyses. Slides of microcell hybrids and ZFN-transfected clones were stained with quinacrine mustard and Hoechst 33258 to enumerate chromosomes. Images were captured using an AxioImagerZ2 fluorescence microscope (Carl Zeiss GmbH, Jena, Germany). FISH analyses were performed using fixed metaphases of microcell hybrids using digoxigenin-labelled (Roche, Basel, Switzerland) human Cot-1 DNA (Life Technologies) and biotin-labelled (Roche) pSTneo plasmid DNA essentially as described previously[20]. Chromosomal DNA was counterstained with DAPI (Sigma-Aldrich). Images were captured using the NIS-Elements system (Nikon, Tokyo, Japan). mFISH analyses were performed in accordance with the manufacturer instructions (MetaSystems, Altlussheim, Germany). Human mFISH probes were purchased from MetaSystems GmbH. Metaphase images were captured digitally with a CoolCubeI CCD camera and the ISIS mFISH software program (MetaSystems).

Teratoma formation and histology. To produce teratomas, 1×10^6 WT-ES, Ts21-ES, GATA1s-ES, and GATA1s/Ts21-ES cells were subcutaneously injected into testes of SCID mice (Charles River, Yokohama, Japan). After 8 weeks, resected teratomas were fixed in 20% formalin, processed for paraffin sectioning, and then stained with haematoxylin and eosin. All animal experiments were approved by the Institutional Animal Care and Use Committee of Tottori University.

Transfection. Custom-designed ZFN plasmids and ZFN-encoding mRNA for targeted mutation of the human *GATA1* gene were generously provided by Sigma-Aldrich. The design, cloning, and validation of the ZFNs were performed by Sigma-Aldrich. Human ES cells (2×10^6) were collected in 100 μL Nucleofector solution (Lonza, Tokyo, Japan) with 2.5 μg of each ZFN plasmid and 2.5 μg of pCX-EGFP (gift from Dr. Okabe, Osaka University) or with 2 μg of each ZFN-encoding mRNA and 2.5 μg of pCX-EGFP and were transfected using Nucleofector (Lonza). Twenty-four hours before and after transfection and after sorting, a Rock inhibitor, 10 μM Y-27632, was added to the culture medium. The GFP-positive fraction of the transfected cells was sorted by fluorescence-activated cell sorting 2 days after transfection. Three hundred eighty-four clones using the mRNAs and 96 clones using the plasmids were picked and expanded for further analyses.

Genomic PCR and mutation analyses. Genomic DNA was extracted from human ES cell lines that were transfected with ZFN plasmids or ZFN-encoding mRNAs using a genomic extraction kit (Gentra System, Minneapolis, MN, USA), and PCR was performed using primers as follows. Primer pairs for the SURVEYOR mutation detection assay (Cel1 assay) and RFLP analyses using BsiHKAI restriction enzyme to detect the mutation in the *GATA1* region were GATA1-F/GATA1-R (347 bp), 5′-TTTCTGTGTCTGAGGACCCC-3′ and 5′-GACCTAGCCAAGGATCTCCA-3′. The Cel1 assay was performed using SURVEYOR Mutation Detection Kits (Transgenomic, Omaha, NE, USA) in accordance with the manufacturer instructions. PCR products were purified by QIAquick PCR Purification Kit (Qiagen), digested with the enzyme, electrophoresed on a 2% agarose gel, and stained with ethidium bromide. Furthermore, the PCR products were subcloned into the pCR4-TOPO vector (Life Technologies), and the vector DNA was sequenced by a 3130xL Genetic Analyzer (Life Technologies) sequencer.

Haematopoietic differentiation of human ES cells. The differentiation of human ES cells into haematopoietic cells was performed as described previously[30]. In brief, small clumps of human ES cells were transferred onto mitomycin C-treated C3H10T1/2 cells and co-cultured in haematopoietic cell differentiation medium supplemented with 20 ng/mL human vascular endothelial growth factor (R&D Systems, Minneapolis, MN, USA), which was replaced every 3 days. On day 14 of culture, the haematopoietic progenitor cells (HPCs) within the ES-sacs were collected and then transferred onto fresh mitomycin C-treated C3H10T1/2 cells and further cultivated in differentiation medium supplemented with human thrombopoietin (R&D systems) and combinations of other cytokines/mediators (human stem cell factor (R&D systems), heparin sodium (Ajinomoto Pharmaceuticals Co, Tokyo, Japan), and human erythropoietin (Prospec-Tany TechnoGene, East Brunswick, NJ, USA)). On day 17, an equal volume of the medium was added, and cells were further incubated for 3 days.

Western blot analyses. Protein extracted from the differentiated ES cells was separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis on an 8%

polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. The membrane was blocked with 5% dry milk and probed with a mouse monoclonal antibody against BACH1 (F-9 (sc-271211); Santa Cruz Biotechnology, Santa Cruz, CA, USA) or a goat polyclonal antibody against the C-terminus of GATA1 (C20 (sc-1233); Santa Cruz Biotechnology). The membrane was then incubated with a horseradish-peroxidase–conjugated secondary antibody and developed with enhanced chemiluminescence reagents (Pierce Western Blotting Substrate; Thermo, Yokohama, Japan). To confirm that the amount of protein in each lane was comparable, the membrane was stripped and probed with a monoclonal antibody against α-tubulin (DM-1A; ICN Biomedicals, Santa Ana, CA, USA). HEL 92.1.7 whole cell lysate and K562 nuclear extract were used as positive controls (sc-2130 and sc-2277, respectively; Santa Cruz Biotechnology). C3H10T1/2 whole cell lysate was used as a negative control.

Flow cytometry analyses. The expression of cell surface molecules was analysed by flow cytometry (FACSAria; Becton Dickinson, Franklin Lakes, NJ, USA). On day 14, a fraction of the HPCs within the ES-sacs were stained with CD34 antibody for 30 minutes on ice. Nonadherent cells on day 20 of culture were prepared in PBS containing 3% FBS (staining medium) and stained with combinations of antibodies for 30 minutes on ice. All samples were then washed with staining medium and analysed by flow cytometry. The following antibodies were used: allophycocyanin (APC)-conjugated anti-CD34 (Biolegend, San Diego, CA, USA), APC-conjugated anti-CD41a (integrin αIIb subunit, Biolegend), phycoerythrin (PE)-conjugated anti-CD41a (Biolegend), PE-conjugated anti-CD42b (Glycoprotein Ibα, Biolegend), PE-conjugated anti-CD71 (BD Pharmingen, San Diego, CA, USA), and APC-conjugated anti-CD235 (Glycophorin A, Biolegend).

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Author contributions

Y.K. participated in all aspects and prepared the manuscript; N.K., Y.Y. and S.Y. performed chromosome transfer and differentiation experiments; K.K. performed cytogenetic analyses; M.Osaki and S.T. performed teratoma formation and histological analyses; H.S. created human ES cell sublines; S.A., K.H. and T.S. performed mutation analyses; and T.T., R.S., H.N., T.Y. and M.Oshimura supervised the study.

Additional information

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Points to consider in the development of seed stocks of pluripotent stem cells for clinical applications: International Stem Cell Banking Initiative (ISCBI)

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1. Background and utility of this document

In 2009 the International Stem Cell Banking Initiative (ISCBI) contributors and the Ethics Working Party of the International Stem Cell Forum published a consensus on principles of best practice for the procurement, cell banking, testing and distribution of human embryonic stem cell (hESC) lines for research purposes [1], which was broadly also applicable to human induced pluripotent stem cell (hiPSC) lines. Here, we revisit this guidance to consider what the requirements would be for delivery of the early seed stocks of stem cell lines intended for future development in delivery of differentiated products of human pluripotent stem cell (hPSC) lines. The term 'seed stock' is used here to describe those cryopreserved stocks of cells established early in the passage history of a pluripotent stem cell line in the lab that derived the line or a stem cell bank, hereafter called the 'repository'. The seed stocks should provide cells with suitable documentation and provenance that would enable them to be taken forward for development in human therapeutic applications. WHO recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biologicals and for the characterization of cell banks were updated in 2010 and provide a number of definitions and guiding principles that may apply to stem cells. The term 'cell bank' is used to described a stock of vials or other containers of cells with consistent composition aliquoted from a single pool of cells of the same culture history (for other specific definitions see PAS 84 [2] and WHO [3]).

Three important assumptions have been made in the preparation of this document. First, that cell banks of hPSCs, or their differentiated progeny, intended for use in clinical trials or other specified human application, would need to be established from samples of these seed stocks in a Good Manufacturing Practice (GMP) facility with a relevant product manufacturing license and with additional risk assessment and risk mitigation focused on the new banking process/reagents and the specific intended clinical application. Second, it has been assumed that undifferentiated pluripotent stem cells would not be inoculated into patients. Third, where feeder cells are used to culture hPSC lines, their cellular nature and intimate contact with the therapeutic cells means that they should be subject to similar risk assessment and banking procedures as applied to the hPSC cells.

It is important to note that responsibility for establishing and updating national regulations for medicinal products relies on National Regulatory Authorities. Therefore, national requirements for cell therapy may vary considerably. Accordingly, it is not intended that this international consensus provides comprehensive guidance that will ensure compliance with requirements in any given jurisdiction. Rather, it is designed to aid the development of clinical grade materials by providing points to consider in the preparation of seed stocks of stem cell lines for use in cell therapy. It may arise that there are circumstances where it is not reasonably possible to meet specific procedures presented in this document. Where this is the case any alternative procedures should be justified and mitigate against any adverse consequences. Finally, this document could also serve as a useful reference to assist in the evaluation of potential sources of candidate cell lines for the development of cell-based medicines, and provide the links necessary to enable identification some of the key differences in regulatory requirements between countries.

2. Governance and ethics

2.1 General principles

Centers banking stem cell lines (hereafter called repositories) should adopt transparent and harmonized protocols for the collection, storage, access, and use of the cell lines that they curate. As part of a comprehensive governance structure, repositories should establish robust mechanisms for the authentication of *bone fide* users and should strive for equitable and transparent conditions of access and of material transfer (Appendix 1). Such protocols should be adopted according to internationally accepted principles for research ethics and in compliance

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with applicable legal, ethical and regulatory requirements (Appendix 2, 3 and 4). Furthermore, repositories should establish a system for documenting and monitoring performance with respect to such principles and requirements.

2.2 Key issues in determining provenance of pluripotent stem cell lines

Repositories should ascertain the provenance (source/origin) of the human biological specimens from which the pluripotent stem cell lines have been derived. International guidance exists for documenting the provenance of the cell lines [1,4–7].

Important issues to consider when evaluating provenance include:

- Evidence of free and voluntary informed consent, for the proposed research use, in conjunction with independent review and oversight, with particular attention given to disclosure of potential clinical and commercial applications.
- The extent to which reimbursement (e.g., expenses, financial incentives, monetary payments) were provided for donation of biological samples.
- The ability of the donor to withdraw original specimens, derived cell lines, data or otherwise to discontinue participation in research.
- The possibility that derived cell lines may be used for a wide range of research, possibly through a public repository.
- The establishment of robust systems for data security and traceability.
- The implementation of mechanisms for the protection of donor privacy and confidentiality. Particular attention should be given to the generation and use of genome sequence data.

Many national and more local jurisdictions have explicit policies governing the acquisition and use of human biospecimens for pluripotent stem cell derivation, particularly with regard to embryonic sources (Appendix 2 and 3). Prior to accepting a pluripotent stem cell line, a repository should determine its provenance by first documenting that the biospecimen was collected and the cell line derived in a manner broadly consistent with international standards for research ethics [4,5,6,7,201]; and second, to make a positive determination that the biospecimen was obtained in a manner consistent with applicable laws in the country of origin.

2.2.1 Provenance determination and international standards

Providers of cells should be able to demonstrate to the repository that they have met all applicable legal and ethical requirements associated with the procurement of the human biospecimen from which the pluripotent stem cell line was derived. Given the heterogeneity of national laws and regulations governing research and clinical applications, the depositor of the cell line should provide information that enables the repository governance structure to determine whether the conditions of derivation, use and distribution are broadly consistent with the repository's national regulation. Moreover, repositories should have in place a mechanism (e.g., 'horizon scanning', advisory board) to track changes in the legal and regulatory frameworks. In addition, repositories should verify and retain sufficient documentation to support a determination that each cell line has been obtained in accordance with international standards for research ethics.

Key principles include the following:

Independent review and oversight

The protocol for procurement of tissues, gametes or embryos for the purpose of generating a pluripotent stem cell line should be subject to independent scientific and ethical review. Review bodies include ethics committees, licensing bodies or committees responsible for oversight of research involving human subjects.

Voluntary informed consent

In addition to verifying appropriate informed consent, the repository should ascertain additional details regarding donor's disclosure when available (Appendix 1b). Numerous bodies and national policies recommend or require the disclosure of specific information to donors (particularly for hESC derivation). A number of jurisdictions have consent requirements that include, but are not limited to, disclosure of possible human transplantation, genetic modification, international sharing and commercial potential. Documentation of a robust informed consent process that addresses these requirements can serve to support wide distribution and utilization of the cell lines (Appendix 2 and 3). Informed consent requirements for stem cell derivation, use and banking have evolved over time and jurisdictional variations may exist for different sources of biospecimens. In addition, standards for evaluating informed consent processes may need to be flexible and allow for context-specific considerations. For example,

agreement to banking could include broad consent to future unspecified research (subject to appropriate security mechanisms and governance); whereas some protocols may be intended to develop a specific cell product. Donors should be notified of the possibility of future use in cellular therapies, commercialization of eventual products and of the international sharing of samples and of stem cell lines. Moreover, donors should also be informed of the limitations in privacy protection (see section 3.2 & 5.1) given the need to assure traceability for safety reasons (see section 6.9).

Gratuitous donation

Donors should not be paid to provide somatic cells, gametes or embryos for stem cell derivation, nor should they be reimbursed for any costs, such as tissue storage, prior to the decision to donate.

2.2.2 Compliance determination and access policies

Mechanisms should be in place to make a positive determination of compliance with both the ethical and legal requirements of the jurisdiction of biospecimen's origin, together with those of the jurisdiction where the cell line was derived, deposited, and will be used in research (Appendix 2 and 3). Furthermore, it is important to consider that there may be jurisdictional or funding agency restrictions on the types of cell lines eligible for research use as may be the case for hESC lines. To the extent feasible, the banking entity (repository) should strive to compile complete provenance information for evaluation; however, it is ultimately incumbent on the end user of the cell line to determine that its provenance meets local ethics and legal requirements.

Repositories should also adopt transparent, flexible and equitable access policies. Given the importance of international collaboration, such policies should include procedures for deposit of cell lines of foreign origin, and for the distribution of cell lines to researchers in other jurisdictions. Among the policy criteria to be considered are the following:

- Mutual recognition via 'reciprocal policy agreements' allowing for transnational sharing of cell lines provided that the cell lines were derived by, deposited in, or approved for use by a licensing entity formally recognized as having adopted consistent ethical and legal requirements.
- 'Substantial equivalency' whereby criteria for cell line derivation, use, and banking in different jurisdictions involve ethical and legal requirements that are deemed to be 'broadly' or 'substantially' equivalent, to a degree that is acceptable to the repository management and under applicable regulation.

3. Provenance and selection of donor tissue

3.1 Donor selection, screening and medical records

Eligibility criteria for embryo, cell or tissue donors intended for human transplantation are subject to national regulatory frameworks and institutional protocols in the jurisdiction of origin. As a general rule, donor eligibility determination requires screening for risk factors associated with infection and communicable disease. These are typically focused on serous human viral blood born pathogens (e.g., HIV, hepatitis B virus, hepatitis C virus) and may also include other pathogens endemic to the donor's origin (e.g., human T-cell lymphotropic virus I&II, Chaga's disease, malaria). Donor testing for these agents may be required to be carried out under national licensed facilities.

For hESCs there are a number of considerations pertaining to donor screening protocols for assisted reproduction treatment (Appendix 3). For hiPSC evaluation, inclusion and exclusion criteria represent a starting point for risk-assessment or risk mitigation. In some cases, inclusion criteria may call for the collection of cells and tissues from patient groups with specific clinical (disease) indications. Any information regarding known disease indication should be associated with specific cell and tissue samples to support risk evaluation (see section 6.3). While cell lines derived from patients with inherited disease have been recognized as having potential scientific utility, they are unlikely to be suitable for development of general clinical applications.

Finally, regulatory authorities responsible for the evaluation of biological products consistently emphasise the value of a donor medical history. It is important to note that rules adopted in some jurisdictions may require a review of donors' relevant medical records and or a medical history screening; considerations for extended medical histories have been published by [8].

3.2 Allogeneic cell transplantation

The establishment of hiPSC repositories for human leukocyte antigen (HLA) haplotype representation to facilitate immune-genetic