Figure 3. Generation and applications of induced pluripotent stem cells (iPSCs). A somatic cell source is isolated from the patient. Pluripotency-associated genes are delivered to reprogramme the cell. Applications of iPSCs include cell therapy and studies of pathogenesis.
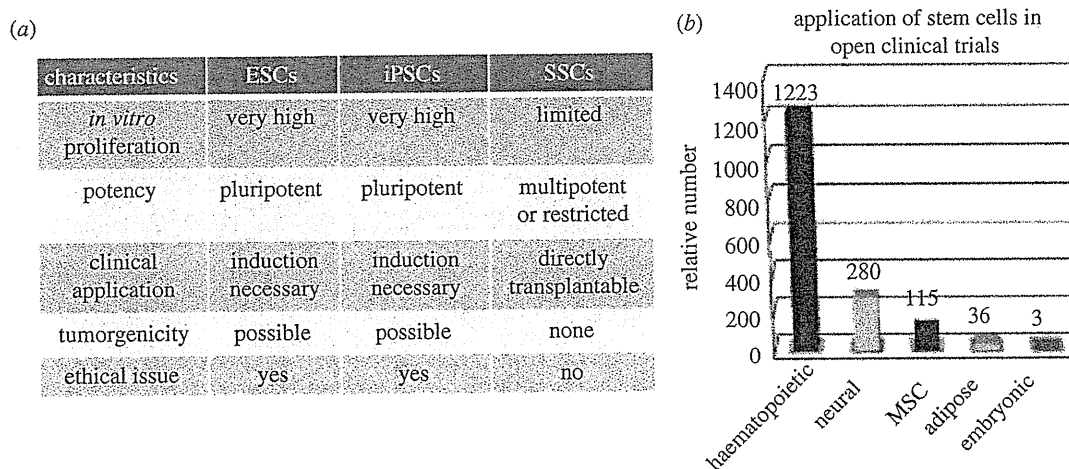

Figure 4. A comparison of stem cell characteristics and major categories of interventional trials. (a) Table comparing stem cell characteristics between embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs) and somatic stem cells (SSCs). (b) Bar graph comparing the relative numbers of open trials of the major stem cell categories (www.clinicaltrials.gov and modified from [43]). MSC, mesenchymal stem cell.

date, there are no ongoing clinical trials employing the use of iPSCs, and similar to ESCs, unless the safety profile of these cells can be firmly established, for the immediate future at least, it may be challenging to bring iPSCs into clinical trial (see figure 4 for a comparison between ESCs, iPSCs and SSCs and the categories of interventional open trials that they have been employed in).

Of all the open FDA-approved clinical trials, some 75 per cent are for HSCs but only 3 per cent involving ESCs and none involving iPSCs [43]. Those that make use of SSCs, despite the exclusion of MSCs, account for nearly 90 per cent. This pattern is informative in a number of ways and one could speculate upon the meaning of it. The tiny proportion of trials employing pluripotent stem cells may be a reflection of the difficulty in convincing the relevant regulatory

body that the risks of tumorigenicity following transplantation can be thoroughly contained. In this regard, it may be unreasonable to question the lack of iPSC-based therapies in such a manner because they are still a relatively new discovery. Bear in mind it took an entire decade to pass between the first *in vitro* derivations of hESCs to the first clinical trial in 2011, which proved to be a monumental exercise in overcoming regulatory hurdles. Should iPSCs-based therapies eventually reach clinical trial, it is likely that these cells will be subjected to the same set of tight regulations as ESCs. As mentioned previously, there are numerous different ways in which induced pluripotency could be accomplished, but there are also discrepancies between institutions in how iPSCs are maintained [44]. Combined with existing technical limitations, it has not yet been possible to have a clear epigenetic profile or even a

molecular definition of these cells. These are questions that must be addressed in order to be able to firmly establish their safety and to promote their use clinically. In the following sections, specific features of each category of stem cells shall be discussed in more detail alongside examples of potential applications and ongoing trials.

3. Somatic stem cells

Compared with the few ESC-related clinical trials that have taken place, SSC-based therapies very much dominate the current landscape. They may be considered as being more advantageous in that they are lineage-restricted and can theoretically be transplanted into patients with less prior *ex vivo* manipulation. However, in most cases, the identity of SSCs is not yet clearly defined whether in terms of phenotypic appearance or immunophenotype. Therefore, the isolation of a purified population of these cells can be technically challenging. Another issue lies in having sufficiently large enough numbers of cells to treat the disease in question. SSCs generally have more limited self-renewal capacity, whose precise mechanism remains to be clearly identified. This means that these cells undergo only a limited number of self-renewing divisions before differentiating into more committed cell progenitors [45]. In the case of HSCs, it has not been possible to expand them *in vitro* without a loss in potency. This represents a limitation of their regenerative potentials. Another limiting factor is that not all tissues possess an endogenous pool of stem cells. A notable example is the endocrine pancreas in diabetes [46]. For other categories such as NSCs, the endogenous stem cell may not spontaneously regenerate injured areas in response to damage. With EpSCs, there has been some clinical success in transplanting 'sheets' of epithelia following *in vitro* cultivation [47,48]. These tend to act as a temporary scaffold because epithelial tissue is subject to high rates of cell turnover and the multi-layered 'sheets' are eventually replaced by epithelia derived from the resident tissue EpSCs of the recipient. On the other hand, MSCs are adherent and expandable cells in culture. They show promise in that cells can be harvested in large numbers in the case of ASCs. Indeed, they are multipotent, but differentiation occurs only following considerable manipulation by cytokines *in vitro*, the extent of which may extend beyond what is clinically acceptable for therapeutic purposes. Here we discuss the more important clinical aspects of notable examples of SSCs.

(a) Haematopoietic stem cells

The field began with an investigation into the haematopoietic system following the seminal work of Till & McCulloch. Some may even argue that more so than the much-publicized ESCs and iPSCs, HSCs have defined the stem cell therapy field like no other. These cells exemplify all the properties characteristic of a so-called stem cell. Part of the simplistic elegance of the haematopoietic system is that cells can exist and function at a singular level unlike other systems where complex cell-to-cell interactions may be required. For these reasons, it has been possible to isolate highly purified populations of HSCs [49] and to use them to demonstrate at least in part, the hierarchy of mammalian development in relation to stem cells, which may also be applicable to other cellular systems. The regenerative powers of HSCs were epitomized in a very elegant and understated fashion by demonstrating that the

transplantation and successful engraftment of only one single HSC is capable of reconstituting haematopoiesis in a recipient [50,51]. At the top of the haematopoietic hierarchy are the multipotent long-term HSCs (LT-HSCs) so called because they can reconstitute haematopoiesis upon transplantation into a primary recipient and subsequently into a secondary recipient [52]. These can either self-renew to produce another LT-HSC or become a short-term HSCs (ST-HSCs), which retains the multilineage potential but is no longer able to reconstitute long-term haematopoiesis [49]. Descending down the hierarchical system, HSCs eventually become committed progenitors of the major haematopoietic lineages lymphoid, myeloid and erythroid. Finally, committed progenitors become terminally differentiated cells [53].

The normal environment for HSCs is the BM, but it can also be derived from peripheral blood or cord blood. Bone marrow transplants (BMTs) have been successful for over 30 years. It has become by far the most successful and widely performed stem cell therapy, becoming standardized treatment for hematological diseases such as leukaemia, aplastic anaemia and immunodeficient disorders [54]. Despite having such a wide range of indications, its prognostic outcome is yet very poor with nearly 50 per cent 5-year overall mortality. BMT can either be autologous (self) or allogeneic (from donor). Allogeneic transplantation can be problematic because similar to organ transplantation, both the donor and the recipient have to be human leucocyte antigen (HLA) matched [55]. So for all its elegance and simplicity, the issue of donor availability can also be a limiting factor to the therapeutic potential of HSCs. Furthermore, a vigorous myeloablative pretreatment also know as 'conditioning' precludes the use of HSC transplantation for autoimmune or other non-fatal diseases that can be treated otherwise [56]. There are various theories on the matter but in general it is believed that the conditioning procedure can lead to overt, or excess damage to the immediate environment and the cells that constitute the BM 'niche'. Consequently, this may compromise HSCs functions such as engraftment within the niche or the ability of cells to maintain its 'stemness', in other words, the typical characteristics of a stem cell. Efforts are ongoing into optimizing the conditions to facilitate the outcomes of BMT.

Putting these challenges aside, as mentioned previously, there are numerous ongoing examples of clinical trials involving HSCs. Standard BMT does not involve modification of the HSCs found within the BM before transplantation. In other cases, HSCs are taken from the peripheral blood and isolated by the identification of the surface CD34$^+$ antigen. Strictly speaking, this antigen alone does not confer the identity of true LT-HSCs, but rather a heterogeneous population of LT-HSCs, ST-HSCs and committed progenitors are isolated. A viral vector can then used to correct or compensate for a defective gene before transplanting into patients of the genetically modified cells. Collectively, the transplantation of gene-modified HSCs is called gene therapy (GT) [57]. Some of the most notable examples involve the treatment of PIDs. These include severe combined immunodeficiency (SCID) [58], chronic granulomatous disease (CGD) [59] and Wiskott–Aldrich syndrome [60]. There has been some notable successes in the GT field but unfortunately, a number of clinical trials have been marred by incidences of insertional mutagenesis owing to activation of an oncogene leading to clonal expansion or frank leukaemia [61]. Additionally, harvesting HSCs from the BM is an invasive

procedure that carries with it a certain degree of morbidity and mortality. The other alternative is to stimulate cell release from the BM by granulocyte colony stimulating factor, but some believe that this procedure can compromise the quality and function of the cells after it enters the peripheral blood. Currently, it is not possible to expand HSCs while retaining a steady population of LT-HSCs that are capable of reconstituting haematopoiesis upon serial transplantation. Efforts are still ongoing to understand the milieu of factors and conditions within the BM that allows HSCs to self-renew.

(b) Neural stem cells

Neurodegenerative disorders or injury involving the central and peripheral nervous systems have also raised considerable interest owing to the devastation they wreak on the quality of life that patients experience. Well-published examples include Parkinsons' disease (PD), amyotrophic lateral sclerosis (ALS), Alzheimer's disease (AD) and stroke. NSCs are the second most common stem cells that are currently used in clinical trials. However, where HSCs may be considered as a symbol of elegance and simplicity, the organization of neuronal tissue architecture is perhaps the most complex in the body. More so than other organs, the brain and other neuronal components are composed of a wide range of highly specified sub cell types (neuronal and non-neuronal) with a degree of interconnectedness that requires computational power to model. This level of complexity of structural organization makes it even more challenging to understand the precise disease pathophysiology or to formulate effective treatments. In examples such as PD and ALS, broadly speaking, the disease phenotype can be traced to the defective function of a single type of neuron (dopaminergic and motor). On the other hand, AD is characterized by neuronal loss in the cerebral cortex and other subcortical areas. In a stroke, or cerebrovascular accident, there is cell death (neuronal and non-neuronal) of entire areas [62]. Therefore, the challenges involved in treatments include not only generating enough numbers of cells, but also regeneration of the correct cell type. NSCs have been obtained from foetal and adult brain, and can be expanded in culture. In the adult brain, they are located in the dentate gyrus of the hippocampus and in the lateral ventricle wall of the olfactory bulb [19]. Despite the existence of NSCs in the adult brain, unlike haematopoiesis, neurogenesis may not be a lifelong process, as it appears to occur primarily during development until adulthood, when the roles of NSCs become much less clear [63]. NSCs show developmental-stage-dependent differentiation potentials. Early NSCs can make more neurons than glial cells, but late NSCs tend to make glial cells but not neurons. To date, neural differentiation from NSCs has been demonstrated for both neurons and glial cells albeit to a fairly limited repertoire. Unlike HSCs however, injections would have to be given invasively to specific areas of the brain. Combined together, these factors severely restrict the clinical potential of NSCs.

For more complex CNS disorders, to achieve optimum recovery, direct replacement of dead cells or tissue reconstruction may be required. However, given the complexity of CNS disorders in mind, this may be a difficult strategy to attempt. With current strategies, scientists are leaning towards neuroprotection, rather than towards neuroregeneration, based on the idea of *tissue plasticity*. This can be defined

as the innate response which SSCs have at being able to adapt and reprogramme their functionality in response to pathophysiological changes to the environment in which they are subjected to [64]. Another aspect is that certain categories of NSCs may display latent activity, where, for example, dormant cells become physiologically active upon injury. When applied to NSCs, there is evidence to suggest that following the injection of NSCs into a site of damage, there is a concerted release of neuroprotective molecules that includes immunomodulatory substances, neutrotrophic growth factors and stem cell regulators, which combine to promote repair of the damaged region. Similar behaviour has also been demonstrated for HSCs and MSCs. Until such times that the anatomical arrangement of neural tissue is better understood as well as the function and derivation of these cells, for the moment at least NSC treatment will be aimed at neural protection. The transplantation of cultured human NSCs have been attempted for Batten's disease, Pelizaeus–Merzbacher disease, chronic spinal cord injuries, strokes, amyotropic lateral sclerosis and Parkinson's disease. Current clinical trials involving NSCs concern largely that of a phase-1 safety issue.

(c) Epithelial stem cells

Epithelial stem cells refer broadly to the resident stem cell population residing within epithelial tissue. In brief, epithelia are densely packed cells connected by tight junctions and desmosomes that form continuous sheets over tissue surfaces. Their functions include physical protection against external environments, selective absorption of water and nutrients, as well as producing and secreting glandular secretions. The functions of epithelia are dependent on the tissue that it overlies [13]. Notable examples include the skin epidermis, the gut epithelium and the corneal epithelium. Unlike other somatic tissues, epithelia are usually subject to relatively high rates of cell turnover, and hence the role of EpSCs is crucial for maintaining tissue homeostasis in replacing damaged or dead cells.

Among the most well-studied EpSCs are the epidermal stem cells, which include the basal, bulge and sebaceous gland stem cells. The epidermis is made up of numerous pilo-sebaceous units with each unit consisting of a hair follicle, a sebaceous gland and the more superficial interfollicular epidermis (IFE). Under normal physiological conditions, collectively these epidermal stem cells are responsible for the homeostatic regulation of maintaining the cellular layers of the IFE and for overseeing rounds of hair growth [65]. There is evidence to suggest the existence of some form of functional overlap between the different stem cell populations. For example, sustained activation of the WNT pathway can convert basal stem cells into hair follicle stem cells [66]. Additionally, there also appears to be some plasticity in their functions in response to changes in the host environment. For example, IFE and sebaceous gland stem cells from donor mice became hair follicle cells following transplantation [67]. Bulge cells, normally responsible for the maintenance of the hair follicle, can also contribute to IFE repair upon injury. In any case, these innate mechanisms of repair are limited to incidences where the cut or injury is relatively small such that the wound site can be sufficiently covered by granulation tissue on top of which new epithelium is built.

For more severe cases of skin trauma, epidermal stem cells are particularly important because these formed the

basis for the first demonstration *in vitro* of highly expandable stem cells that could subsequently be applied to regenerate tissue. The first demonstration occurred in 1984 by Howard Green and colleagues in treating two patients with extensive skin damage caused by third-degree burns [68]. Keratinocytes, a generic name for epithelial cells in a multi-layered epithelium, were derived from patients without sorting, subsequently expanded *in vitro* and grown to a sheet, which was then transplanted over the sites of skin damage. As a continuation of this, the concept of cultivating keratinocytes *in vitro* was extended for the treatment of the genetic disease called junctional epidermolysis bullosa caused by a defect with the Laminin 5-β3 gene. It affects the ability of the basal layer of keratinocytes to adhere to the basal membrane causing blistering of the skin. The strategy involved using a retroviral vector to introduce Laminin 5-β3 cDNA into cultivated epidermal cells. This rescued the adherent properties of the cells and following subsequent transplantation successful engraftment was observed and the patient was cured [69].

Another tissue to have attracted a lot of attention in stem cell biology and regenerative medicine is the intestinal epithelium. This is a lining covering the luminal surface of the gut extending through the small to large intestines. It is composed of numerous functional units of crypts and villi. Crypts are invaginations that appear to 'burrow' into the lamina propria layer of the gut, which runs in continuation with the 'finger-like' projections of the villi [15]. The crypt-villus unit has been described as one of the simplest representations of a self-renewing entity. It is much simpler in structural organization in comparison with epidermal epithelia. Gut stem cells are found at the base of each crypt flanked by paneth cells and undergo self-renewal to produce more vigorously proliferating transient activating (TA) cells. These cells divide further and extend upwards along the walls of the crypt. At regular intervals, paneth cells insert into this vertically rising columnar procession. Upon reaching the villus junction, differentiation is initiated whereby TA cells become enterocytes or absorptive/secretive cells. Differentiated cells continue to be pushed upwards along the walls of the villus, where upon reaching the tip, cells undergo apoptotic death and shedding [70]. This regenerative process is very similar to what occurs in the IFE of the epidermis. However, as the intestinal epithelium is only one single-cell-thick, it is possible to observe the processes in a precise sequential order. This makes it much easier to identify and observe the basic cellular events encompassing tissue renewal.

Most recently, there has been exciting development where, using the Lgr5 protein as a marker to identify gut stem cells under defined culture conditions, single sorted cells became so-called gut organoids [71]. These are three-dimensional structures that are composed of gut stem cells as well as other cell types that can be found along the crypt–villus axis. In a group of recipient mice, injury was artificially induced to the colon. Following the delivery of donor-derived organoids through an enema, damaged areas were regenerated. Under histological examination, the regenerated areas displayed the appearance of normal gut epithelia. Most of all, it appeared that the transplanted cells of the organoids supported long-term epithelial regeneration by remaining engrafted after more than six months [72]. It was also demonstrated during the same study that these 'gut organoids' could be produced in large numbers from relatively few gut stem cells. These results hold promise for the treatment of diseases such as Crohn's disease, inflammatory bowel syndrome or other disorders of the gastrointestinal tract where there can be chronic damage to the gut epithelium [73]. Although it remains to be demonstrated whether these organoid-regenerated portions are fully functional, it remains an elegant demonstration of the clinical potential of EpSCs without the inevitable complications of using pluripotent cells.

Regeneration of the cornea is another example where there has been experience of clinical success. The cornea is a multi-layered avascular tissue with optical functions such as the maintenance of visual acuity and acting as a physical barrier to protect the inner eye. On its outer surface is a layer of corneal epithelium, which extends in continuation into an area of the adult cornea, called the limbus [74]. This is a transitional area between the corneal and conjunctival epithelium, where so-called limbal stem cells are found. These are believed to be the primary stem cell population involved in corneal regeneration. Allogeneic corneal transplantation, also known as keratoplasty, is already an established therapy to treat damage to the cornea [75]. The success of such a therapy appears to depend on whether the limbus remains intact following injury. If damage to the cornea is extensive and does indeed extend to the limbus, then there may be a need, for example, to supplement it with a transplantation of limbal stem cells that are firstly derived from the opposite uninjured eye and then expanded by *in vitro* culture [76]. This clinical strategy has generated some interest, as the use of cultured limbal epithelial cells is already an established part of the treatment of ocular surface diseases.

(d) Mesenchymal stem/stromal cells

The study of MSCs began with a misunderstanding over their nomenclature and functions. They were initially named mesenchymal stem cells, as they were derived from BM stroma and that the expanded progeny cells possessed osteo- and chondrogenic potential [77]. It was inaccurate to call these 'mesenchymal', which by definition refers to cells of the haematopoietic lineage that MSCs are not capable of differentiating into. In addition, the earliest studies of MSCs were not clonal in experimental design and hence without demonstrating that the cells could self-renew, it was perhaps inappropriate to call them stem cells. Nowadays they are more correctly defined as stromal cells as some, but not all, MSCs can be defined as stem cells [78]. They have been characterized as fibroblastic, adherent cells that are expandable in culture and are positive for markers including CD90 and CD105 [79]. Worth noting is that there are no single characteristic marker that will allow for the prospective isolation of a highly purified population of these cells. For this reason, the precise identity of MSCs continues to remain under debate as do comparisons between different studies. Nonetheless MSCs can be derived from BM aspirates or in larger quantities from adipose tissue. They are also found in amniotic fluid, Wharton's jelly of the umbilical cord, umbilical cord blood, dental pulp of deciduous teeth and skeletal muscle [80].

Compared with pluripotent ESCs or iPSCs, MSCs have a greater biosafety profile and lower risk of tumorigenicity [81]. MSCs are believed to be multipotent for chondrogenic, osteogenic and adipogenic differentiation [82]. However, it is

important to emphasize that demonstration of this multipotent differentiation potential occurred following extreme manipulation of *in vitro* culturing environments [83]. *In vivo*, there has been no firm suggestion to indicate that MSCs partake in the repair of these tissues; hence, their normal physiological roles remain unclear. Unfortunately, the 'stem cell' part of the name has continued to stick, leading to the often incorrect and erroneous assumption that whatever beneficial effects MSCs may have are provided through cell replacement. Gradually, there is increasing evidence to suggest that MSCs can secrete paracrine signals or act as immune suppressors to stimulate tissue recovery. Yet, at the same time, there is also contradicting evidence to suggest that this is not the case [84]. Despite this ongoing uncertainty, numerous MSC-based therapies have made it to clinical trial, perhaps in part due to the fact that they are much less immunogenic compared with other types of stem cell. The range of clinical trials that are ongoing include those attempting the treatment of bone and cartilage repair, myocardial infarct occlusive vascular disease, liver cirrhosis and strokes. Whether it is actually able to exert such effects, attempts have also been made to harness the suspected immunomodulatry effects of MSCs in treating graft versus host disease and autoimmune diseases such as multiple sclerosis, systemic lupus erythematosis and Crohn's disease. Again, the outcome has been mixed, and therapeutic benefits difficult to determine. The fact of the matter is probably that a number of the clinical trials that have taken place should not have been allowed to do so in the first place. For future MSC-therapies, the precise characterization of MSCs, better understanding of therapeutic mechanism and careful assessment of the efficacy of clinical trials are necessary.

4. Embryonic stem cells

Two clinical trials involving ESCs have so far taken place. In 2011, California-based venture company Geron Corporation started a first-in-man phase 1 clinical study, using ES cell-derived oligodendrocytes to treat patients with spinal cord injury. Unfortunately, the trial was discontinued for unknown business decisions. There has been no formal publication on the findings and no insight on either the efficacy of the proposed treatment or its safety. What was apparent from the trial was the stringent implementation of regulatory protocols, which some considered as 'oversight' before the trial was eventually approved. The other clinical trial involved the transplantation of ESC-derived retinal epthelium in patients with Stargardt's macular dystrophy and dry age-related macular degeneration. The company Advance Cell Technologies sponsored the trial, but clinical benefits were difficult to determine owing to the short four-month duration and that each trial contained only one patient. These are two of only a very limited number of examples of ESC-based therapies.

Much of the focus on ESCs has been largely on that of its potential clinical applications. However, as seen in the examples mentioned earlier, there remain considerable difficulties in assessing the efficacy of ESC-based therapies along with overcoming regulatory hurdles. Perhaps what has been overlooked slightly is the crucial role that ESCs have played as a comparative control alongside iPSCs in studies of the regulatory mechanisms that underlie pluripotency and differentiation. ESCs are considered to be derivatives of naturally occurring ICM cells and can pass stringent assays for assessing contribution to development. Therefore, ESCs are considered as reliable *in vitro* representations of mammalian development suitable for the aforementioned purposes. For example, techniques such as global gene-expression analysis and bisulphite genomic sequencing have been carried out to identify differences between ESCs and iPSCs during differentiation in terms of gene-expression profiles and epigenetic marks [40]. These types of comparative experiment were to prove very informative. The first of these insightful pieces of information concerns the concept of 'somatic' or 'epigenetic memory' [85]. These are residual configurations of the epigenome that were retained following the reprogramming process. By broad definition, 'epigenome' refers to the precise types of DNA methylation and histone modification, the combinatory effect of which can influence gene expression. Reprogramming usually results in the demethylation of most previously methylated regions. Methylation can have a repressive effect on gene expression, depending on its loci. This is not a permanent modification but as long as the epigenetic features that make up the 'somatic memory' remain, there are implications upon the propensity of somatic cells to be reprogrammed or that of iPSCs to undergo differentiation. For example, a failure to demethylate pluripotency-associated genes has been proposed as a mechanism that explains the partial reprogramming in iPSCs or cases where reprogramming efficiency is low. It is also possible that retention of this epigenetic memory may give preferential bias in differentiation into a certain lineage. This was observed in cases where haematopoietic differentiation was deemed to be less efficient from iPSCs that were derived from fibroblasts and neural progenitors [85]. These preferential biases may be avoided through either a second round of reprogramming (secondary reprogramming) [85] or through long-term culture *in vitro* that can remove the remaining traces of residual methylation. The repression may also be lifted by the treatment of cells with demethylating agents. On a functional level, these repressive effects of the epigenome on either reprogramming or cellular differentiation will need to be carefully considered when attempting to use iPSCs for clinical purposes.

Another important observation arising from similar studies is that during the reprogramming process, there is potential for the inadvertent capturing or retention of genomic regions from the somatic state that are prone to repression by methylation. These captured regions continue to be transferred to differentiated progenies; hence, pluripotency reprogramming can lead to some kind of a secondary accumulating effect. Studies that compared the DNA methylomes of human ESCs and iPSCs initially identified these regions as areas of aberrant iPSC-specific differential methylation [40]. Eventually, these were termed differentially methylated regions (DMRs). It was also found that this occurred at a higher frequency in iPSCs, and it appears to be a permanent defect that affects the actual genomic structure and not just the epigenome; hence it could not be erased through passaging [86]. Current evidence seems to identify the accumulation of DMRs as a stochastic process that is not caused by aberrant or random mutation. What is known is that the frequency of DMRs in ESCs and progenitor somatic cells are both lower compared with the frequency in iPSCs. It suggests that accumulation does not begin until after the cell moves away from the somatic state and yet it does not seem to be caused by intrinsic or extrinsic factors arising

from being in a pluripotent state either as frequency in ESCs is also low. Therefore, the reprogramming process could be the cause of this DMR phenomenon. The functional consequences are unclear, but one could hypothesis that there may be a profound effect on the expression of genes in the proximity of the DMR region.

In general, ESCs have a relatively established role as a comparative control in relation to iPSCs. This role is supported by the assertion that ESCs are derivatives of the naturally occurring ICM as opposed to being a product of artificial reprogramming. However, it may be questionable to consider ESCs as 'direct derivatives' of the ICM, considering the methodology of ESC derivation that involves removal of cells from the natural *in vivo* environment without clear knowledge of the effect that it may have. Additionally, there is also the discrepancy in derivation between ESCs and iPSCs, which could have implications in accounting for the inherent heterogeneity within each population. Although both are characterized as pluripotent cells, only iPSC derivation methods account for sub-clonal distinction from an original donor source. Therefore, questions may be targeted at addressing the degree of 'equivalence' not only between cells of the ICM and ESCs, but also that between ESCs and iPSCs. Firstly, let us consider the methods of ESC derivation. The natural procession of embryonic development *in vivo* is likely to be in part, a product of dynamic cellular interactions including that between the ICM and the trophectoderm (TE). This is the extra-embryonic layer that surrounds the ICM but for the *in vitro* derivation of ESCs, TE removal can enhance the efficiency of ESC establishment and facilitate its maintenance [87]. It remains unclear what the exact consequences might be of disrupting this dynamism. There are, however, several lines of evidence; some more speculative than others at suggesting differences between ICM cells and ESCS. For example, critical genes involved in developmental signalling pathways, including transforming growth factor beta (TGFβ), insulin growth factor and the MAP kinase pathways were identified to be uniquely expressed in ICM cells but not in ESCs [88]. Other circumspective indications include findings to suggest that ESCs are thought to have a higher degree of global DNA methylation [89,90] and have longer telomere lengths [89]. Additionally, ERas, a transforming oncogene, is highly expressed in mouse ESCs but not in embryos [91]. Taken together, these findings highlight the need for closer scrutiny of the resemblance between ICM cells and ESCs.

Another important point to consider is that there is a fundamental difference between how ESCs and iPSCs are derived. In brief, ESCs are usually cells of the ICM plated onto mitotically inactivated mouse embryonic fibroblasts (MEFs). Under the appropriate culture conditions, cells expand and aggregate to form colonies, which are subsequently expanded further. With the derivation of iPSCs, somatic cells are reprogrammed and similarly plated onto MEFs. The crucial difference is that there is usually a subcloning step involved where once iPSC colonies appear, they are individually transferred onto fresh MEFs and expanded separately. Therefore, iPSCs are often referred to as 'clones' of a given cell line, whereas ESCs are purely referred to in relation to the original embryo from which it is derived without further clonal distinction. It is therefore reasonable to suspect that there could be heterogeneity within each ESC line that may become apparent for example in the efficacy and efficiency of differentiation into a specified lineage [92]. There is also the

possibility that certain clones within a population of colonies may be afforded some kind of growth advantage, which means that after a certain number of passages, only a few dominant ones will remain. This is speculative, however, and can only be determined if there is also a subcloning step involved sufficiently early during ESC derivation. Therefore, returning to the question of equivalence between ESCs and iPSCs, the simple answer would be 'neither'. Current opinions seem to confer on the notion that these are two distinctly different classes of cells each with considerable amounts of inherent heterogeneity but at the same time, there is also significant overlapping similarities between the two [40]. While the degree of resemblance between ICM cells and ESCs has yet to be clearly defined, for the moment at least, ESCs remain the closest *in vitro* recapitulation of human embryonic development. Nonetheless, a more cautious approach is warranted when interpreting results of epigenetic studies involving ESCs, where it must be considered in the appropriate context particularly in comparison with iPSCs.

5. Induced pluripotent stem cells

With the challenges faced in the application of ESCs, the stem cell therapy field appeared to be stuck at a bottleneck as the search began for an alternative pluripotent cell source. In humans, this was achieved in 2007 with the derivation of hiPSCs [35]. Since then, much research has been undertaken using these cells but thus far, there are no ongoing iPSC-based clinical trials. Already in the previous section, there has been a discussion on some of the technical limitations relating to reprogramming by defined factors and differentiation from a pluripotent state that had been discovered in comparative studies alongside ESCs. In addition, one can imagine that iPSC-based therapy would encounter similar regulatory obstacles to those that were encountered in bringing forward their ESC counterpart. It is therefore worth foreseeing what these might be and how clinical translation may be accelerated. The first thing to consider is that as arrogant as it may sound, dare one say it, perhaps induced pluripotency came about too easily. Earlier attempts at cellular reprogramming to pluripotency were achieved either through SCNT or cellular fusion with ECCs and ESCs. Both of these techniques were difficult to perform and are limited by even lower levels of efficiency than reprogramming by defined factors [93,94]. SCNT has always been associated with some negative social and ethical issues. With cellular fusion, the resulting hybrid cell is tetraploid, thus precluding its application for clinical purposes. The ectopic co-expression of defined transcription factors to achieve pluripotency is considerably simpler on many levels and much more reproducible. Numerous other viral and non-viral methods of co-expressing sets of defined factors in different somatic cells have also been attempted and were successful. It led some researchers to think that perhaps the difficult part in achieving cellular therapy for patients is over. The truth is there are other similarly difficult and equally as important challenges to overcome in addition to pluripotency reprogramming.

One of the main difficulties in iPSC-based therapies may well be in the directed differentiation from pluripotent to functional somatic cells. This is possible *in vitro* by manipulating the culture conditions using cytokines critical to the development of that specific tissue. However, the *in vivo* development of cells and tissue systems is a product of dynamic interactions between cells and its environment.

The supplementation of cytokines represents only part of this dynamism and it remains a tremendous technical challenge to fully replicate this *in vitro*. Many differentiated cells have been tested for characteristic functions but very rarely in a physiologically relevant context to comprehensively prove that they are functionally comparable to their *in vivo* counterparts. Therefore, casting aside all other considerations for the moment, a reasonable question to ask might therefore be whether transplanted cells are able to partake in the homeostatic regulation of tissue functions *in vivo*? There are numerous such examples, one of them being the differentiation from pluripotent cells into transplantable β-pancreatic cells for the treatment of diabetes [95]. Although there are now established protocols for producing these cells, none has yet been able to demonstrate the same regulated secretion of insulin in response to glucose stimulation as observed *in vivo* [96]. What this example highlights is that basic functional tests are sufficient for characterization purposes, but more comprehensive assessments will be necessary, perhaps involving transplantation into an animal host, to predict *in vivo* functioning [97]. Also applicable to both ESCs and iPS cells, there is inherent instability of the genome over long-term culture [98]. Whether it is reprogramming or differentiation into functional somatic tissue, both processes takes place in relatively harsh culture environments that subject cells to stressful conditions on its genome, which could lead to aberrant gene mutations. Therefore, to make sure that cells are clinically safe for transplantation, it may be necessary to screen each and every clone for signs of epigenetic aberrations or gene mutations [99]. These are all important considerations to keep in mind during the ongoing efforts of generating not only transplantable, but also physiologically functional cells.

One of the much-trumpeted features of iPSCs is that they can give rise to more personalized therapy to patients. This can take the form of disease modelling, where autologous iPSCs are generated for applications such as the study of disease pathophysiology, drug screening and toxicology [100]. For example, patient autologous iPSCs can be generated from those with an inherited disease. The genetic defect would be transferred to differentiated cells, which may be reflected as an observable disease phenotype or be detectable at a molecular level. These concepts may be applicable to monogenic diseases caused by a single mutation to a given gene but perhaps not in more complicated circumstances where iPSCs may not be able to faithfully recapitulate the disease phenotype. Fragile X syndrome, the most common form of inherited mental retardation, is an epigenetic disorder occurring as a result of silencing of the FMR1 gene. The disease phenotype is the result of an accumulation in aberrant DNA methylation and repressive histone marks [101]. It was found that reprogramming to pluripotency could not restore the epigenetic aberrations, but in ESCs derived from embryos during preimplantation diagnosis, the FMR1 gene was expressed in its unrepressed form and that transcriptional repression occurred only after differentiation [102]. Another challenge relating to disease modelling is the issue of clonal variation. To simplify, assuming that reprogramming to pluripotency and subsequent differentiation has been achieved, clonal variation may exist in the propensity of cell to display the disease phenotype. There are a number of possible explanations, one of them being heterogeneity in the starting somatic cells. Again with reference to

Fragile X syndrome, in one study, the fibroblasts obtained from a patient contained a mixture of cells displaying the functional FMR1 gene but also those where the gene was repressed to different extents [103]. This heterogeneity in the starting somatic cell population became apparent during the characterization of differentiated neurons and glial cells from patient autologous iPSCs. In this particular example, it demonstrates the 'infidelity' of the reprogramming process by demonstrating that induced pluripotency can be the result non-discriminately targeted reprogramming [104]. Together with the relative inefficiency of reprogramming and possible remnants of somatic memory, there are numerous factors, the combinative effect of which may lead to significant variation in the presentation of the disease phenotype. Therefore in attempting to model human diseases using iPSCs, it is important to consider the molecular basis of the disease. For more complicated examples such as Fragile X syndrome, it may be necessary to start reprogramming from an isogenic starting population of somatic clones.

6. Novel developments and future prospects of stem cell therapy

It is clear that there is much about the regenerative powers of stem cells that science may attempt to harness for clinical purposes. Of course, there are obstacles that must be overcome before this goal is realized with some challenges being more difficult than others. However, it must not dampen the infectious enthusiasm that is behind all the ongoing efforts in driving the field forward. Other than the examples of clinical trials that have been given, there are a number of ongoing developments that are worth noting, some of which will be highlighted here.

(a) *In vitro* differentiation of pluripotent cells into haematopoietic stem cells and blood products

The holy grail of the haematopoietic field continues to remain that of obtaining a continuous supply of cells that may be used in transplantation therapies or be manipulated *ex vivo* for gene therapy. Efforts of generating HSCs capable of long-term engraftment *in vivo* began soon after the first establishment of hESCs. Previously in a mouse model of sickle cell anaemia, it was found that haematopoietic progenitors generated from gene corrected iPSCs were capable of long-term reconstitution of the recipient haematopoietic system several weeks following transplantation, which rescued the disease phenotype [105]. It is important, however, to consider that this was in part achieved through ectopic HoxB4 expression, which enhances the engraftment potential of haematopoietic progenitors derived from pluripotent cells. Therefore, a considerable amount of genetic manipulation was required to achieve the results noted during the study. Unfortunately, the same level of success has not been matched in humans, where it has only been possible to generate haematopoietic progenitors from ESCs that produced transient benefits in a mouse model but were not transplantable in the long term [106]. This could be due to shortcomings in replicating the *in vivo* environment to support haematopoietic differentiation. For the most part, stem cell differentiation is a stochastic process. Relatively speaking, much is known about haematopoetic differentiation from HSCs to mature

blood cells but less so before that. Perhaps revisiting the mechanisms at an epigenetic level [107] may help to identify the epigenome of LT-HSCs and to develop a strategy to arrest differentiation at that specific point during haematopoiesis.

Given the simplistic nature in the functioning of haematopoietic cells, there is hope in the development of 'off-the-shelf' products that are 'ready to use'. Candidate products include those consisting of platelets (PLTs) and red blood cells (RBCs). Normally donated blood products have a limited shelf life, with packaged platelets lasting only around 5 days and RBCs approximately 42 days. Not only that, in all countries, donor availability continues to remain a problem. Pluripotent stem cell-derived PLTs or RBCs may potentially represent a safe and stable supply of these cells. Although the technology is not yet ready for clinical application, there have already been reports of erythrocytes [108] and platelet [109,110] derivation from both ESCs and iPS cells. It will, however, have to be achievable on a large enough scale to meet the numbers that are required clinically per transfusion. Nonetheless, success in generating these products would greatly benefit the treatment of patients with hematological disorders or to supply those requiring a blood transfusion.

(b) Direct lineage conversion

Direct lineage conversion or forward reprogramming is a technique that converts one cell type directly to another through the ectopic expression of defined factors without an intermediate pluripotency step. In other words, it is a one-step as opposed to a two-step process. This technique is not limited only to conversion between somatic cells, but it can also occur between different germ cells. There are notable advantages over iPS reprogramming for example where the dynamics of reprogramming are faster and the efficiency of reprogramming is much higher [111]. However, there may be difficulties with applications in cell transplantation owing to issues with scalability. More recently, it has been suggested that direct reprogramming could be applied *in situ* for the treatment of heart disease at areas of myocardial infarcted damage [112]. Theoretically at least, this technique seems promising, but there are some important questions that need to be addressed. First of all, there is the possibility that similar to differentiation from an induced pluripotent state, epigenetic memory can impact upon the efficiency of conversion between two cell types. Perhaps even residual amount of epigenetic memory retained from the previous state could affect the functioning of the newly converted cell [112]. Another critical but more clinically relevant question is why conversion between human cells is more difficult than in mice? To date, there have been direct reports of conversion into neurons [113] and multilineage blood progenitors [114] from fibroblasts. However, demonstrating the effective functionality of these converted cells would be quite a different matter. As mentioned earlier, clinical benefits of therapies treating neurodegenerative disorders can be difficult to assess and in the latter report, the converted blood progenitors are not LT-HSCs; hence they would not be able to reconstitute the haematopoietic system in a recipient. Nonetheless, direct conversion is a technique that seems promising not only in transplanting therapy, but also as a more simplified and direct method of studying the mechanisms of transcription factor action.

(c) Embryonic stem cell- or induced pluripotent stem cell-derived organs in xenogenic animals via blastocyst complementation

As mentioned previously, most current therapeutic strategies in stem cell therapy target diseases that are amenable to cell transplantation only. Certain chronic diseases will ultimately lead to organ failure. In the current climate, there is an absolute shortage of donor organs. The differentiation potential of pluripotent cells (ESCs or iPSCs) have been well documented at the cellular level. However, the generation of a whole organ is considerably more difficult. This is because organogenesis is a complex process that involves the establishment of complex cell–cell interactions and recapturing of the conditions that naturally occur *in vivo* during embryonic development. A solution may be to use a xenogenic host. The Nakauchi laboratory was the first to demonstrate the generation of functionally normal rat pancreas into a $Pdx1^{-/-}$ (pancreatogenesis-disabled) mouse [115]. Wild-type rat iPSCs were injected into the blastocysts of $Pdx1^{-/-}$ mice resulting in a chimaeric animal with a pancreas entirely derived from the rat iPSCs. Transplantation of mature β cells from the chimaeric mice into a rat with streptozocin-induced diabetes was able to reinstate glucose regulatory capacity. Attempts are currently ongoing at replicating this strategy in larger mammals. There may be further technical and ethical challenges in the future before this technology is ready for clinical application, but it is certainly one of the most enterprising strategies at addressing donor organ shortages.

7. Premature clinical translation and risks of progress retardation

For purposes that are pure and others that are morally questionable, there is great eagerness in translating stem cell therapies into clinical practice. In some regards, the initial public furore stirred up by the media regarding the therapeutic potentials of ESCs and iPSCs may have been somewhat dampened a little. The mammoth regulatory hurdles faced by Geron [116] and Advanced Cell Technologies [117] were well documented. Clear evidence of clinical benefits from these trials remains elusive, certainly not enough to satisfy the ever-hungry public and media. Unfortunately, this meant that there has been an alarming rise in the appearance of the so-called cell banks all around the world. These are generally private institutions advertising the therapeutic benefits of MSCs, harvesting them from cord blood or adipose tissue and falsely promising numerous cures ranging from neurodegenerative diseases to diabetes [118]. As mentioned in an earlier section, MSCs are ill-defined not only in the identity of these cells, but also in their therapeutic benefits. However, stem cell therapy in general has been so overly romanticized by the media that naive members of the public have been made vulnerable to dishonest businesses.

Again, these are issues that may not appear to be scientifically relevant, but there are real concerns that because of them, further progress in the field may become compromised. Currently, there is neither a concrete set of guidelines on how to classify stem cell-based products nor a consensus on how they should be classified. Unfortunately, this lack of agreement may have led to the appearance of fraudulent clinics and for

every bit of negative publicity that they attract, it becomes more and more damaging to the scientific community in multiple folds. One might speculate that such cases contributed significantly to the 'regulatory oversight' encountered with the ESC trials [43]. Tough regulations are positive within reasonable proportions but not at the expense of stifling scientific progress. Already in a rather regrettable example, the European Court of Justice has formally banned the patenting of technologies derived from hESCs [119]. Although the decision was made on ethical grounds, it is quite reasonable to assume that such negative examples raised here would have influenced that decision. Therefore, unless the issue is urgently addressed, not only would further progress stall, there is a real risk of regression. It is clear that there needs to be tighter regulations by internal regulatory boards (IRBs) in all countries participating in stem cell research.

8. Conclusion

So where are we now? The field of stem cell therapy is currently riding on a wave of rapid growth and excitement. However, perhaps one should be inclined to call for some restraint while stressing the need to proceed with prudence.

Many conceptual therapeutic ideas have been demonstrated only by 'proof of principal'. Patience is required to allow for the completion of a comprehensive pre-clinical assessment into the safety and efficacy of these treatments before their eventual translation into the clinic. Even with HSC-based therapies, the area that has shown the most advancement, it took half a century from the first attempts at BMT before the field reached its current level of progress. The modern perception of stem cells is that of 'the solution'. It appears to many that this is some kind of transplantable differentiated cell. However, until key questions have been satisfactorily addressed, for the present moment at least, the main application especially that of pluripotent cells will likely be as a 'tool' to understand with precision, *in vivo* developmental mechanisms or disease pathophysiological development. With a view to stimulate further advancements, the field now hopes to revisit the fundamentals of stem cell biology at an epigenetic level. Epigenetic studies into pluripotency, cellular reprogramming, lineage fate decisions and other such related areas have already provided the knowledge to define the field as it is known today. Ultimately, the dream is to achieve the ambitious goal of establishing safe and effective stem cell therapy.

References

1. Briggs R, King TJ. 1952 Transplantation of living nuclei from blastula cells into enucleated frogs' eggs. *Proc. Natl Acad. Sci. USA* 38, 455–463. (doi:10.1073/pnas.38.5.455)

2. Miller RA, Ruddle FH. 1976 Pluripotent teratocarcinoma-thymus somatic cell hybrids. *Cell* 9, 45–55. (doi:10.1016/0092-8674(76)90051-9)

3. Foshay KM et al. 2012 Embryonic stem cells induce pluripotency in somatic cell fusion through biphasic reprogramming. *Mol. Cell* 46, 159–170. (doi:10.1016/j.molcel.2012.02.013)

4. Till JE, McCulloch CE. 1961 A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. *Radiat. Res.* 14, 213–222. (doi:10.2307/3570892)

5. Metcalf D. 2007 Concise review: hematopoietic stem cells and tissue stem cells: current concepts and unanswered questions. *Stem Cells* 25, 2390–2395. (doi:10.1634/stemcells.2007–0544)

6. Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM 1998 Embryonic stem cell lines derived from human blastocysts. *Science* 282, 1145–1147. (doi:10.1126/science.282.5391.1145)

7. Evans M. 2011 Discovering pluripotency: 30 years of mouse embryonic stem cells. *Nat. Rev. Mol. Cell Biol.* 12, 680–686. (doi:10.1038/nrm3190)

8. Takahashi K, Yamanaka S. 2006 Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 126, 663–676. (doi:10.1016/j.cell.2006.07.024)

9. Weissman IL. 2000 Stem cells: units of development, units of regeneration, and units in evolution. *Cell* 100, 157–168. (doi:10.1016/S0092-8674(00)81692-X)

10. Seita J, Rossi DJ, Weissman IL. 2010 Differential DNA damage response in stem and progenitor cells. *Cell Stem Cell* 7, 145–147. (doi:10.1016/j.stem.2010.07.006)

11. Martino G, Pluchino S. 2006 The therapeutic potential of neural stem cells. *Nat. Rev. Neurosci.* 7, 395–406. (doi:10.1038/nrn1908)

12. Van Keymeulen A, Blanpain C. 2012 Tracing epithelial stem cells during development, homeostasis, and repair. *J. Cell Biol.* 197, 575–584. (doi:10.1083/jcb.201201041)

13. Blanpain C, Horsley V, Fuchs E. 2007 Epithelial stem cells: turning over new leaves. *Cell* 128, 445–458. (doi:10.1016/j.cell.2007.01.014)

14. Pinnamaneni N, Funderburgh JL. 2012 Concise review: stem cells in the corneal stroma. *Stem Cells* 30, 1059–1063. (doi:10.1002/stem.1100)

15. Barker N, van de Wetering M, Clevers H. 2008 The intestinal stem cell. *Genes Dev.* 22, 1856–1864. (doi:10.1101/gad.1674008)

16. Nakada D, Levi BP, Morrison SJ. 2011 Integrating physiological regulation with stem cell and tissue homeostasis. *Neuron* 70, 703–718. (doi:10.1016/j.neuron.2011.05.011)

17. Jones DL, Wagers AJ. 2008 No place like home: anatomy and function of the stem cell niche. *Nat. Rev. Mol. Cell Biol.* 9, 11–21. (doi:10.1038/nrm2319)

18. Wagers AJ. 2012 The stem cell niche in regenerative medicine. *Cell Stem Cell* 10, 362–369. (doi:10.1016/j.stem.2012.02.018)

19. Goritz C, Frisen J. 2012 Neural stem cells and neurogenesis in the adult. *Cell Stem Cell* 10, 657–659. (doi:10.1016/j.stem.2012.04.005)

20. Keating A. 2012 Mesenchymal stromal cells: new directions. *Cell Stem Cell* 10, 709–716. (doi:10.1016/j.stem.2012.05.015)

21. Grompe M. 2012 Tissue stem cells: new tools and functional diversity. *Cell Stem Cell* 10, 685–689. (doi:10.1016/j.stem.2012.04.006)

22. Martin GR. 1981 Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc. Natl Acad. Sci. USA* 78, 7634–7638. (doi:10.1073/pnas.78.12.7634)

23. Evans MJ, Kaufman MH. 1981 Establishment in culture of pluripotential cells from mouse embryos. *Nature* 292, 154–156. (doi:10.1038/292154a0)

24. Stadtfeld M, Hochedlinger K. 2010 Induced pluripotency: history, mechanisms, and applications. *Genes Dev.* 24, 2239–2263. (doi:10.1101/gad.1963910)

25. Zhang SC, Wernig M, Duncan ID, Brustle O, Thomson JA. 2001 In vitro differentiation of transplantable neural precursors from human embryonic stem cells. *Nat. Biotechnol.* 19, 1129–1133. (doi:10.1038/nbt1201–1129)

26. Takayama K et al.. 2012 Efficient generation of functional hepatocytes from human embryonic stem cells and induced pluripotent stem cells by HNF4alpha transduction. *Mol. Ther.* 20, 127–137. (doi:10.1038/mt.2011.234)

27. Chadwick K, Wang L, Li L, Menendez P, Murdoch B, Rouleau A et al. 2003 Cytokines and BMP-4 promote hematopoietic differentiation of human embryonic stem cells. *Blood* 102, 906–915. (doi:10.1182/blood-2003–03–0832)

28. Ben-David U, Benvenisty N. 2011 The tumorigenicity of human embryonic and induced pluripotent stem

cells. *Nat. Rev. Cancer* **11**, 268–277. (doi:10.1038/nrc3034)

29. Swijnenburg RJ et al. 2008 Immunosuppressive therapy mitigates immunological rejection of human embryonic stem cell xenografts. *Proc. Natl Acad. Sci. USA* **105**, 12 991–12 996. (doi:10.1073/pnas.0805802105)

30. Rosenthal E. 2006 Excommunication is sought for stem cell researchers. *NY Times* (Print), A3. 1st July, 2006.

31. Tanne JH. 2006 Bush vetoes bill to expand stem cell research. *Br. Med. J.* **333**, 216. (doi:10.1136/bmj.333.7561.216-b)

32. Gurdon JB, Wilmut I. 2011 Nuclear transfer to eggs and oocytes. *Cold Spring Harb. Perspect. Biol.* **3**, pii: a002659. (doi:10.1101/cshperspect.a002659)

33. Tada M, Takahama Y, Abe K, Nakatsuji N, Tada T. 2001 Nuclear reprogramming of somatic cells by *in vitro* hybridization with ES cells. *Curr. Biol.* **11**, 1553–1558. (doi:10.1016/S0960-9822(01)00459-6)

34. Masui S et al. 2007 Pluripotency governed by Sox2 via regulation of Oct3/4 expression in mouse embryonic stem cells. *Nat. Cell Biol.* **9**, 625–635. (doi:10.1038/ncb1589)

35. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S. 2007 Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* **131**, 861–872. (doi:10.1016/j.cell.2007.11.019)

36. Yu J et al. 2007 Induced pluripotent stem cell lines derived from human somatic cells. *Science* **318**, 1917–1920. (doi:10.1126/science.1151526)

37. Stadtfeld M, Nagaya M, Utikal J, Weir G, Hochedlinger K. 2008 Induced pluripotent stem cells generated without viral integration. *Science* **322**, 945–949. (doi:10.1126/science.1162494)

38. Fusaki N, Ban H, Nishiyama A, Saeki K, Hasegawa M. 2009 Efficient induction of transgene-free human pluripotent stem cells using a vector based on Sendai virus, an RNA virus that does not integrate into the host genome. *Proc. Jpn. Acad. Ser. B. Phys. Biol. Sci.* **85**, 348–362. (doi:10.2183/pjab.85.348)

39. Liu H, Ye Z, Kim Y, Sharkis S, Jang YY. 2010 Generation of endoderm-derived human induced pluripotent stem cells from primary hepatocytes. *Hepatology* **51**, 1810–1819. (doi:10.1002/hep.23626)

40. Robinton DA, Daley GQ. 2012 The promise of induced pluripotent stem cells in research and therapy. *Nature* **481**, 295–305. (doi:10.1038/nature10761)

41. Booth C, Gaspar HB, Thrasher AJ. 2011 Gene therapy for primary immunodeficiency. *Curr. Opin. Pediatr.* **23**, 659–666. (doi:10.1097/MOP.0b013e32834cd67a)

42. Colman A, Dreesen O. 2009 Pluripotent stem cells and disease modeling. *Cell Stem Cell* **5**, 244–247. (doi:10.1016/j.stem.2009.08.010)

43. Daley GQ. 2012 The promise and perils of stem cell therapeutics. *Cell Stem Cell* **10**, 740–749. (doi:10.1016/j.stem.2012.05.010)

44. Newman AM, Cooper JB. 2010 Lab-specific gene expression signatures in pluripotent stem cells. *Cell Stem Cell* **7**, 258–262. (doi:10.1016/j.stem.2010.06.016)

45. He S, Nakada D, Morrison SJ. 2009 Mechanisms of stem cell self-renewal. *Annu. Rev. Cell Dev. Biol.* **25**, 377–406. (doi:10.1146/annurev.cellbio.042308.113248)

46. Lechner A, Habener JF. 2003 Stem/progenitor cells derived from adult tissues: potential for the treatment of diabetes mellitus. *Am. J. Physiol. Endocrinol. Metab.* **284**, E259–E266. (doi:10.1152/ajpendo.00393.2002)

47. Shortt AJ, Secker GA, Notara MD, Limb GA, Khaw PT, Tuft SJ, Daniels JT. 2007 Transplantation of ex vivo cultured limbal epithelial stem cells: a review of techniques and clinical results. *Surv. Ophthalmol.* **52**, 483–502. (doi:10.1016/j.survophthal.2007.06.013)

48. Fuchs E. 2008 Skin stem cells: rising to the surface. *J. Cell Biol.* **180**, 273–284. (doi:10.1083/jcb.200708185)

49. Weissman IL, Shizuru JA. 2008 The origins of the identification and isolation of hematopoietic stem cells, and their capability to induce donor-specific transplantation tolerance and treat autoimmune diseases. *Blood* **112**, 3543–3553. (doi:10.1182/blood-2008-08-078220)

50. Okabe M et al. 2009 Definitive proof for direct reprogramming of hematopoietic cells to pluripotency. *Blood* **114**, 1764–1767. (doi:10.1182/blood-2009-02-203695)

51. Osawa M, Hanada K, Hamada H, Nakauchi H. 1996 Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. *Science* **273**, 242–245. (doi:10.1126/science.273.5272.242)

52. Orkin SH, Zon LI. 2008 Hematopoiesis: an evolving paradigm for stem cell biology. *Cell* **132**, 631–644. (doi:10.1016/j.cell.2008.01.025)

53. Seita J, Weissman IL. 2010 Hematopoietic stem cell: self-renewal versus differentiation. *Wiley Interdiscip. Rev. Syst. Biol. Med.* **2**, 640–653. (doi:10.1002/wsbm.86)

54. Copelan EA. 2006 Hematopoietic stem-cell transplantation. *N. Engl. J. Med.* **354**, 1813–1826. (doi:10.1056/NEJMra052638)

55. Little MT, Storb R. 2002 History of haematopoietic stem-cell transplantation. *Nat. Rev. Cancer* **2**, 231–238. (doi:10.1038/nrc748)

56. Doulatov S, Notta F, Laurenti E, Dick JE. 2012 Hematopoiesis: a human perspective. *Cell Stem Cell* **10**, 120–136. (doi:10.1016/j.stem.2012.01.006)

57. Greenberger JS. 2008 Gene therapy approaches for stem cell protection. *Gene Ther.* **15**, 100–108. (doi:10.1038/sj.gt.3303004)

58. Gaspar HB et al. 2011 Hematopoietic stem cell gene therapy for adenosine deaminase-deficient severe combined immunodeficiency leads to long-term immunological recovery and metabolic correction. *Sci. Transl. Med.* **3**, 97ra80. (doi:10.1126/scitranslmed.3002716)

59. Kang HJ et al. 2011 Retroviral gene therapy for X-linked chronic granulomatous disease: results from phase I/II trial. *Mol. Ther.* **19**, 2092–2101. (doi:10.1038/mt.2011.166)

60. Galy A, Thrasher AJ. 2011 Gene therapy for the Wiskott–Aldrich syndrome. *Curr. Opin. Allergy Clin. Immunol.* **11**, 545–550. (doi:10.1097/ACI.0b013e32834c230c)

61. Hacein-Bey-Abina S et al. 2003 LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. *Science* **302**, 415–419. (doi:10.1126/science.1088547)

62. Lindvall O. 2012 Why is it taking so long to develop clinically competitive stem cell therapies for CNS disorders? *Cell Stem Cell* **10**, 660–662. (doi:10.1016/j.stem.2012.04.004)

63. Fuentealba LC, Obernier K, Alvarez-Buylla A. 2012 Adult neural stem cells bridge their niche. *Cell Stem Cell* **10**, 698–708. (doi:10.1016/j.stem.2012.05.012)

64. Martino G, Bacigaluppi M, Peruzzotti-Jametti L. 2011 Therapeutic stem cell plasticity orchestrates tissue plasticity. *Brain* **134**, 1585–1587. (doi:10.1093/brain/awr115)

65. Blanpain C, Fuchs E. 2006 Epidermal stem cells of the skin. *Annu. Rev. Cell Dev. Biol.* **22**, 339–373. (doi:10.1146/annurev.cellbio.22.010305.104357)

66. Nguyen H, Merrill BJ, Polak L, Nikolova M, Rendl M, Shaver TM, Pasolli HA, Fuchs E. 2009 Tcf3 and Tcf4 are essential for long-term homeostasis of skin epithelia. *Nat. Genet.* **41**, 1068–1075. (doi:10.1038/ng.431)

67. Arwert EN, Hoste E, Watt FM. 2012 Epithelial stem cells, wound healing and cancer. *Nat. Rev. Cancer* **12**, 170–180. (doi:10.1038/nrc3217)

68. Green H. 2008 The birth of therapy with cultured cells. *Bioessays* **30**, 897–903. (doi:10.1002/bies.20797)

69. Mavilio F et al. 2006 Correction of junctional epidermolysis bullosa by transplantation of genetically modified epidermal stem cells. *Nat. Med.* **12**, 1397–1402. (doi:10.1038/nm1504)

70. Radtke F, Clevers H. 2005 Self-renewal and cancer of the gut: two sides of a coin. *Science* **307**, 1904–1909. (doi:10.1126/science.1104815)

71. Sato T et al. 2009 Single Lgr5 stem cells build crypt-villus structures *in vitro* without a mesenchymal niche. *Nature* **459**, 262–265. (doi:10.1038/nature07935)

72. Yui S et al. 2012 Functional engraftment of colon epithelium expanded *in vitro* from a single adult Lgr5(+) stem cell. *Nat. Med.* **18**, 618–623. (doi:10.1038/nm.2695)

73. Shaker A, Rubin DC. 2012 Stem cells: one step closer to gut repair. *Nature* **485**, 181–182. (doi:10.1038/485181a)

74. Takacs L, Toth E, Berta A, Vereb G. 2009 Stem cells of the adult cornea: from cytometric markers to therapeutic applications. *Cytometry A* **75**, 54–66. (doi:10.1002/cyto.a.20671)

75. Daniels JT, Dart JK, Tuft SJ, Khaw PT. 2001 Corneal stem cells in review. *Wound Repair Regen.* **9**, 483–494. (doi:10.1046/j.1524-475x.2001.00483.x)

76. Pellegrini G, Rama P, Mavilio F, De Luca M. 2009 Epithelial stem cells in corneal regeneration and epidermal gene therapy. *J. Pathol.* **217**, 217–228. (doi:10.1002/path.2441)

77. Caplan AI. 1991 Mesenchymal stem cells. *J. Orthop. Res.* **9**, 641–650. (doi:10.1002/jor.1100090504)

78. Horwitz EM, Dominici M. 2008 How do mesenchymal stromal cells exert their therapeutic benefit? *Cytotherapy* **10**, 771–774. (doi:10.1080/14653240802618085)

79. Mizuno H, Tobita M, Uysal AC. 2012 Concise review: adipose-derived stem cells as a novel tool for future regenerative medicine. *Stem Cells* **30**, 804–810. (doi:10.1002/stem.1076)

80. da Silva Meirelles L, Chagastelles PC, Nardi NB. 2006 Mesenchymal stem cells reside in virtually all post-natal organs and tissues. *J. Cell Sci.* **119**, 2204–2213. (doi:10.1242/jcs.02932)

81. Ra JC et al. 2011 Safety of intravenous infusion of human adipose tissue-derived mesenchymal stem cells in animals and humans. *Stem Cells Dev.* **20**, 1297–1308. (doi:10.1089/scd.2010.0466)

82. Uccelli A, Moretta L, Pistoia V. 2008 Mesenchymal stem cells in health and disease. *Nat. Rev. Immunol.* **8**, 726–736. (doi:10.1038/nri2395)

83. Pittenger MF et al. 1999 Multilineage potential of adult human mesenchymal stem cells. *Science* **284**, 143–147. (doi:10.1126/science.284.5411.143)

84. Kebriaei P, Robinson S. 2011 Treatment of graft-versus-host-disease with mesenchymal stromal cells. *Cytotherapy* **13**, 262–268. (doi:10.3109/14653249.2010.549688)

85. Kim K et al. 2010 Epigenetic memory in induced pluripotent stem cells. *Nature* **467**, 285–290. (doi:10.1038/nature09342)

86. Lister R et al. 2011 Hotspots of aberrant epigenomic reprogramming in human induced pluripotent stem cells. *Nature* **471**, 68–73. (doi:10.1038/nature09798)

87. Chen AE et al. 2009 Optimal timing of inner cell mass isolation increases the efficiency of human embryonic stem cell derivation and allows generation of sibling cell lines. *Cell Stem Cell* **4**, 103–106. (doi:10.1016/j.stem.2008.12.001)

88. Niakan KK, Han J, Pedersen RA, Simon C, Pera RA. 2012 Human pre-implantation embryo development. *Development* **139**, 829–841. (doi:10.1242/dev.060426)

89. Varela E, Schneider RP, Ortega S, Blasco MA. 2011 Different telomere-length dynamics at the inner cell mass versus established embryonic stem (ES) cells. *Proc. Natl Acad. Sci. USA* **108**, 15 207–15 212. (doi:10.1073/pnas.1105414108)

90. Yamanaka S. 2012 Induced pluripotent stem cells: past, present, and future. *Cell Stem Cell* **10**, 678–684. (doi:10.1016/j.stem.2012.05.005)

91. Takahashi K, Mitsui K, Yamanaka S. 2003 Role of ERas in promoting tumour-like properties in mouse embryonic stem cells. *Nature* **423**, 541–545. (doi:10.1038/nature01646)

92. Osafune K, Caron L, Borowiak M, Martinez RJ, Fitz-Gerald CS, Sato Y, Cowan CA, Chien KR, Melton DA. 2008 Marked differences in differentiation propensity among human embryonic stem cell lines.

Nat. Biotechnol. **26**, 313–315. (doi:10.1038/nbt1383)

93. Whitworth KM, Prather RS. 2010 Somatic cell nuclear transfer efficiency: how can it be improved through nuclear remodeling and reprogramming? *Mol. Reprod. Dev.* **77**, 1001–1015. (doi:10.1002/mrd.21242)

94. Tat PA, Sumer H, Pralong D, Verma PJ. 2011 The efficiency of cell fusion-based reprogramming is affected by the somatic cell type and the in vitro age of somatic cells. *Cell Reprogram.* **13**, 331–344. (doi:10.1089/cell.2011.0002)

95. Hansson M, Madsen OD. 2010 Pluripotent stem cells, a potential source of beta-cells for diabetes therapy. *Curr. Opin. Invest. Drugs* **11**, 417–425.

96. Naujok O, Burns C, Jones PM, Lenzen S. 2011 Insulin-producing surrogate beta-cells from embryonic stem cells: are we there yet? *Mol. Ther.* **19**, 1759–1768. (doi:10.1038/mt.2011.165)

97. Kroon E et al. 2008 Pancreatic endoderm derived from human embryonic stem cells generates glucose-responsive insulin-secreting cells in vivo. *Nat. Biotechnol.* **26**, 443–452. (doi:10.1038/nbt1393)

98. Chen LW, Kuang F, Wei LC, Ding YX, Yung KK, Chan YS. 2011 Potential application of induced pluripotent stem cells in cell replacement therapy for Parkinson's disease. *CNS Neurol. Disord. Drug Targets* **10**, 449–458.

99. Baker M. 2009 Stem cells: fast and furious. *Nature* **458**, 962–965. (doi:10.1038/458962a)

100. Grskovic M, Javaherian A, Strulovici B, Daley GQ. 2011 Induced pluripotent stem cells—opportunities for disease modelling and drug discovery. *Nat. Rev. Drug Discov.* **10**, 915–929. (doi:10.1038/nrd3577)

101. Bagni C, Greenough WT. 2005 From mRNP trafficking to spine dysmorphogenesis: the roots of fragile X syndrome. *Nat. Rev. Neurosci.* **6**, 376–387. (doi:10.1038/nrn1667)

102. Urbach A, Bar-Nur O, Daley GQ, Benvenisty N. 2010 Differential modeling of fragile X syndrome by human embryonic stem cells and induced pluripotent stem cells. *Cell Stem Cell* **6**, 407–411. (doi:10.1016/j.stem.2010.04.005)

103. Sheridan SD, Theriault KM, Reis SA, Zhou F, Madison JM, Daheron L, Loring JF, Haggarty SJ, Cookson MR. 2011 Epigenetic characterization of the FMR1 gene and aberrant neurodevelopment in human induced pluripotent stem cell models of fragile X syndrome. *PLoS ONE* **6**, e26203. (doi:10.1371/journal.pone.0026203)

104. Onder TT, Daley GQ. 2012 New lessons learned from disease modeling with induced pluripotent stem cells. *Curr. Opin. Genet. Dev.* (doi:10.1016/j.gde.2012.05.005)

105. Hanna J et al. 2007 Treatment of sickle cell anemia mouse model with iPS cells generated from

autologous skin. *Science* **318**, 1920–1923. (doi:10.1126/science.1152092)

106. Wang L, Menendez P, Shojaei F, Li L, Mazurier F, Dick JE, Cerdan C, Levac K, Bhatia M. 2005 Generation of hematopoietic repopulating cells from human embryonic stem cells independent of ectopic HOXB4 expression. *J. Exp. Med.* **201**, 1603–1614. (doi:10.1084/jem.20041888)

107. Saleque S, Kim J, Rooke HM, Orkin SH. 2007 Epigenetic regulation of hematopoietic differentiation by Gfi-1 and Gfi-1b is mediated by the cofactors CoREST and LSD1. *Mol. Cell* **27**, 562–572. (doi:10.1016/j.molcel.2007.06.039)

108. Lu SJ, Feng Q, Park JS, Lanza R. 2010 Directed differentiation of red blood cells from human embryonic stem cells. *Methods Mol. Biol.* **636**, 105–121. (doi:10.1007/978-1-60761-691-7_7)

109. Takayama N, Nishikii H, Usui J, Tsukui H, Sawaguchi A, Hiroyama T et al. 2008 Generation of functional platelets from human embryonic stem cells in vitro via ES-sacs, VEGF-promoted structures that concentrate hematopoietic progenitors. *Blood* **111**, 5298–5306. (doi:10.1182/blood-2007–10-117622)

110. Takayama N et al. 2010 Transient activation of c-MYC expression is critical for efficient platelet generation from human induced pluripotent stem cells. *J. Exp. Med.* **207**, 2817–2830. (doi:10.1084/jem.20100844)

111. Vierbuchen T, Wernig M. 2011 Direct lineage conversions: unnatural but useful? *Nat. Biotechnol.* **29**, 892–907. (doi:10.1038/nbt.1946)

112. Jayawardena TM et al. 2012 MicroRNA-mediated in vitro and in vivo direct reprogramming of cardiac fibroblasts to cardiomyocytes. *Circ. Res.* **110**, 1465–1473. (doi:10.1161/CIRCRESAHA.112.269035)

113. Pang ZP et al. 2011 Induction of human neuronal cells by defined transcription factors. *Nature* **476**, 220–223. (doi:10.1038/nature10202)

114. Szabo E, Rampalli S, Risueno RM, Schnerch A, Mitchell R, Fiebig-Comyn A, Levadoux-Martin M, Bhatia M. 2010 Direct conversion of human fibroblasts to multilineage blood progenitors. *Nature* **468**, 521–526. (doi:10.1038/nature09591)

115. Kobayashi T et al. 2010 Generation of rat pancreas in mouse by interspecific blastocyst injection of pluripotent stem cells. *Cell* **142**, 787–799. (doi:10.1016/j.cell.2010.07.039)

116. Strauss S. 2010 Geron trial resumes, but standards for stem cell trials remain elusive. *Nat. Biotechnol.* **28**, 989–990. (doi:10.1038/nbt1010-989)

117. Schwartz SD et al. 2012 Embryonic stem cell trials for macular degeneration: a preliminary report. *Lancet* **379**, 713–720. (doi:10.1016/S0140-6736(12)60028-2)

118. Cyranoski D. 2012 Stem-cell therapy takes off in Texas. *Nature* **483**, 13–14. (doi:10.1038/483013a)

119. Smith A. 2011 'No' to ban on stem-cell patents. *Nature* **472**, 418. (doi:10.1038/472418a)

Rapid T-cell chimerism switch and memory T-cell expansion are associated with pre-engraftment immune reaction early after cord blood transplantation

Cord blood (CB) contains immature immune cells and is thought to be less active in inducing allogeneic immune reaction than other sources of stem cells. However, a high incidence of immune-mediated complications has been reported, such as pre-engraftment immune reaction (PIR) and haemophagocytic syndrome (HPS) early after cord blood transplantation (CBT) (Kishi et al, 2005; Narimatsu et al, 2007; Frangoul et al, 2009; Takagi et al, 2009; Patel et al, 2010). In addition, we reported that human leucocyte antigen (HLA) disparity in the graft-versus-host (GVH) direction adversely affected engraftment kinetics when single calcineurin inhibitors were used for GVH disease (GVHD) prophylaxis (Matsuno et al, 2009). These observations suggested that the GVH reaction plays a critical role in engraftment. Here, we report the engraftment kinetics of donor-derived T cells using a multicolour flow cytometry-based method (HLA-Flow method) (Watanabe et al, 2008) and also describe the results of naïve/memory T-cell phenotype analyses early after CBT.

Between November 2009 and September 2010, 73 adult patients underwent single-unit CBT at Toranomon hospital. This study reports 41 patients who were eligible for chimerism analysis using the HLA-Flow method and survived more than 14 d after CBT. Characteristics of the patients and CB are summarized in Table SI. All patients provided written informed consent, and the study was conducted in accordance with institutional review board requirements. Peripheral blood was collected at 1, 2, 3, 4, and 8 weeks after CBT. Anti-HLA monoclonal antibodies in combination with lineage-specific antibodies were used to analyse the lineage-specific chimerism as previously reported (Watanabe et al, 2008). Anti-HLA antibodies specific for donor and recipient HLA in all patients are summarized in Table SII. At 2, 4, and 8 weeks after CBT, T-cell subsets were analysed using the following monoclonal antibodies: peridinin-chlorophyll-protein − cyanin 5·5 (PerCP-Cy5·5)-CD8, phycoerythrin − cyanin 7 (PE-Cy7)-CCR7, allophycocyanin (APC)-CD4, APC-Cy7-CD3 (BD Pharmingen, San Jose, CA, USA), and Pacific Blue-CD45RA (CALTAG, Carlsbad, CA, USA). Absolute numbers of $CD4^+$ T cells ($CD3^+CD4^+$), $CD8^+$ T cells ($CD3^+CD8^+$), and naïve ($CD45RA^+CCR7^+$) and memory ($CD45RA^-CCR7^{+/-}$) T cells were calculated by multiplying the peripheral lymphocyte counts by the percentage of positive cells. PIR was characterized by non-infectious high-grade fever ($>38\cdot5°C$) coexisting with skin eruption, diarrhoea, jaundice and/or body weight gain greater than 5% of baseline, developing 6 or more days before engraftment (Kishi et al, 2005; Uchida et al, 2011). Cumulative incidence of neutrophil engraftment, PIR, and GVHD were calculated using Gray's method. Intergroup comparisons were performed using the Mann–Whitney U-test.

We analysed lineage-specific chimerism for 32, 40, 40, 34, and 34 patients at a median of 8 (range, 7–11; week 1), 15 (14–20; week 2), 22 (21–25; week 3), 29 (28–36; week 4), and 57 (56–62; week 8) days post-transplant, respectively. Fig 1A shows representative results for $CD4^+$ T-cell chimerism. $CD4^+$ and $CD8^+$ T-cell chimerism results in all patients are shown in Fig 1B. Of 41 enrolled patients, 37 achieved neutrophil engraftment at a median of 19 d (range, 13–38 d). Thirty-nine patients achieved donor-dominant T-cell chimerism (>90%) by 3 weeks after CBT, whereas the remaining two patients, with recipient-dominant T-cell chimerism (>90%) at every point tested, developed graft failure because of early relapse (day 14 post-transplant) and rejection, respectively. Among the 39 patients who achieved donor-dominant T-cell chimerism, two died before engraftment due to non-relapse causes on day 28 (infection) and day 25 (diffuse alveolar haemorrhage), respectively. Among those with donor-dominant chimerism, 24 (63%) of 38 evaluable patients developed PIR at a median of 8 (6–11) days after CBT. Patients who achieved donor-dominant T-cell chimerism (>90%) at 1 week had a higher incidence of PIR compared to those who did not ($P = 0\cdot017$, Fig 1C). In a representative patient at 2 weeks after CBT, rapid conversion from naïve to memory phenotype was observed in both $CD4^+$ and $CD8^+$ T cells (Fig 2A). Fig 2B shows the relative proportion of naïve $CD4^+$ and $CD8^+$ T cells at 2, 4, and 8 weeks after CBT in 37 evaluable patients who achieved donor-dominant T-cell chimerism. Patients who developed PIR had significantly more lymphocytes, $CD4^+$ T cells, $CD8^+$ T cells, $CD4^+$ memory T cells, and $CD8^+$ memory T cells at 2 weeks after CBT compared with those without PIR (Fig 2C and data not shown).

Our data confirmed that a majority of patients achieved donor-dominant T-cell chimerism around 2 weeks after CBT. We also found that early recipient-type T-cell chimerism was closely associated with graft rejection. A remarkable finding was that a rapid recipient-to donor-dominant switch
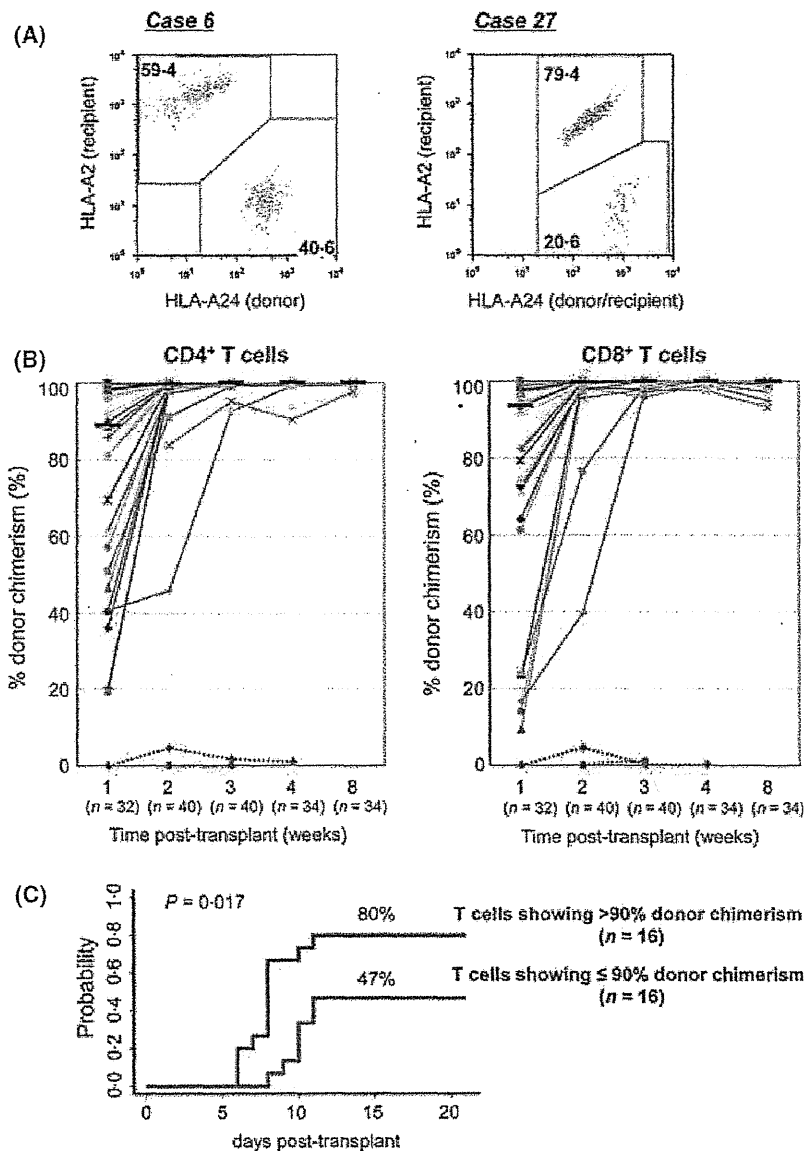
Fig 1. T-cell chimerism analysed by HLA-Flow method. (A) Chimerism analysis by the HLA-Flow method separated donor- vs. recipient-derived cells among CD4$^+$ T cells at 1 week after cord blood transplant (CBT). In Case 6, human leucocyte antigen (HLA)-A2 was recipient-specific and HLA-A24 was donor-specific. In Case 27, HLA-A2 was recipient-specific, whereas HLA-A24 was shared by both donor and recipient, indicating that HLA-A2-negative and HLA-A24-positive cells were donor-derived. (B) The median percentages of donor-derived CD4$^+$ T cells and CD8$^+$ T cells at 1 week after CBT were 88·9%, and 93·5%, respectively. Red dotted lines indicate recipient-dominant chimerism in two patients who developed graft failure. (C) Cumulative incidence of pre-engraftment immune reaction (PIR) according to chimerism status of T cells at 1 week after CBT

of T-cell chimerism at 1 week post-transplant was associated with a higher incidence of PIR, supporting a hypothesis that PIR could be an early variant form of GVH reaction caused by donor-derived T cells. CB T cells are naïve and do not include pathogen-specific effector T cells. Grindebacke et al (2009) demonstrated that about 80% of CD4$^+$ T cells kept the naïve phenotype during the first 18 months after birth. In contrast, we found a rapid conversion from naïve to memory phenotype at 2 weeks after CBT. In addition, PIR

could be associated with peripheral expansion of donor-derived memory T cells. Recently, Gutman et al (2010) reported that CD8$^+$ T cells predominately expressed effector memory or effector phenotype early after double-unit CBT, reflecting an immune response of the dominant unit against the non-engrafting unit. These findings suggest that donor-derived naïve T cells will be activated by alloantigens and differentiate into mature cells early after CBT. Most of the present patients with PIR responded promptly after a
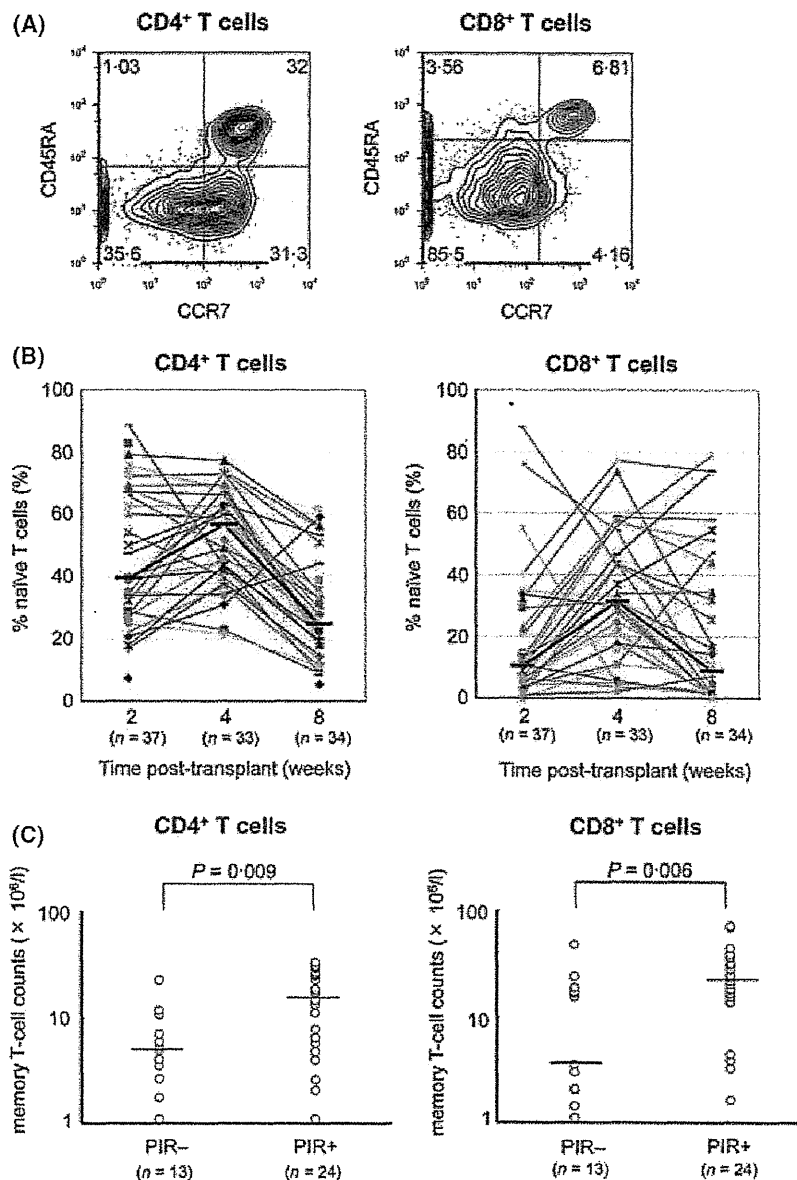
Fig 2. Conversion from naïve to memory T-cell phenotype. (A) A rapid conversion from naïve phenotype (CD45RA⁺CCR7⁺) to memory phenotype (CD45RA⁻CCR7⁺/⁻) in a representative sample at 2 weeks after cord blood transplant (CBT) (Case 5). (B) Relative proportion of naïve CD4⁺ and CD8⁺ T cells at 2, 4, and 8 weeks after CBT. Bold horizontal lines denote median values. (C) Memory T-cell counts at 2 weeks after CBT in patients with or without pre-engraftment immune reaction (PIR).

short course of steroid treatment, and none experienced graft failure due to HPS. This observation could be attributed to more intensive immunosuppression from adding mycophenolate mofetil to tacrolimus in the majority of patients (Uchida et al, 2011). Although neither the T-cell chimerism nor the memory T-cell counts affected the incidence of acute GVHD, steroid treatment for PIR could suppress the onset of acute GVHD. In conclusion, rapid T-cell chimerism switch and donor-derived memory T-cell expansion were associated with PIR, supporting a significant role of donor-derived T cells in the pathogenesis of the early immune reaction after CBT.

Acknowledgements

Correspondence

Author contributions

NM, HY, NW, HN, and ST designed the study; NM, HY, and NW performed the research; NM and HY analysed data; HY, NU, HO, AN, TI, K Ishiwata, NN, MT, Y A-M, K Izutsu, KM, AW, and ST performed transplantation; AY reviewed histopathological findings; and NM, HY, NW, NU, HN, and ST contributed to writing the paper.

Competing interests

The authors have no competing interests.

Naofumi Matsuno[1,2]*
Hisashi Yamamoto[2]*
Nobukazu Watanabe[1]
Naoyuki Uchida[2]
Hikari Ota[2]
Aya Nishida[2]
Taichi Ikebe[2]
Kazuya Ishiwata[2]
Nobuaki Nakano[2]
Masanori Tsuji[2]
Yuki Asano-Mori[2]
Koji Izutsu[2]
Kazuhiro Masuoka[2]
Atsushi Wake[2]
Akiko Yoneyama[3]
Hiromitsu Nakauchi[4]
Shuichi Taniguchi[2,5]

[1]Laboratory of Diagnostic Medicine, Division of Stem Cell Therapy, Centre for Stem Cell Biology and Regenerative Medicine, the Institute of Medical Science, the University of Tokyo, Tokyo, Japan, [2]Department of Haematology, Toranomon Hospital, Tokyo, Japan, [3]Department of Infectious Diseases, Toranomon Hospital, Tokyo, Japan, [4]Division of Stem Cell Therapy, Centre for Stem Cell Biology and Regenerative Medicine, the Institute of Medical Science, the University of Tokyo, Tokyo, Japan and [5]Okinaka Memorial Institute for Medical Research, Tokyo, Japan
E-mail: nwatanab@ims.u-tokyo.ac.jp

*The first two authors contributed equally to this work.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table SI. Patient and cord blood characteristics.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

References

Frangoul, H., Wang, L., Harrell, F.E., Jr, Ho, R. & Domm, J. (2009) Preengraftment syndrome after unrelated cord blood transplant is a strong predictor of acute and chronic graft-versus-host disease. Biology of Blood and Marrow Transplantation, 15, 1485–1488.

Grindebacke, H., Stenstad, H., Quiding-Jarbrink, M., Waldenstrom, J., Adlerberth, I., Wold, A.E. & Rudin, A. (2009) Dynamic development of homing receptor expression and memory cell differentiation of infant CD4+CD25high regulatory T cells. Journal of Immunology, 183, 4360–4370.

Gutman, J.A., Turtle, C.J., Manley, T.J., Heimfeld, S., Bernstein, I.D., Riddell, S.R. & Delaney, C. (2010) Single-unit dominance after double-unit umbilical cord blood transplantation coincides with a specific CD8+ T-cell response against the nonengrafted unit. Blood, 115, 757–765.

Kishi, Y., Kami, M., Miyakoshi, S., Kanda, Y., Murashige, N., Teshima, T., Kusumi, E., Hara, S., Matsumura, T., Yuji, K., Masuoka, K., Wake, A., Morinaga, S., Kanemaru, M., Hayashi, T., Tanaka, Y. & Taniguchi, S. (2005) Early immune reaction after reduced-intensity cord-blood transplantation for adult patients. Transplantation, 80, 34–40.

Matsuno, N., Wake, A., Uchida, N., Ishiwata, K., Araoka, H., Takagi, S., Tsuji, M., Yamamoto, H., Kato, D., Matsuhashi, Y., Seo, S., Masuoka, K., Miyakoshi, S., Makino, S., Yoneyama, A., Kanda, Y. & Taniguchi, S. (2009) Impact of HLA disparity in the graft-versus-host direction on engraftment in adult patients receiving reduced-intensity cord blood transplantation. Blood, 114, 1689–1695.

Narimatsu, H., Terakura, S., Matsuo, K., Oba, T., Uchida, T., Iida, H., Hamaguchi, M., Watanabe, M., Kohno, A., Murata, M., Sawa, M., Miyamura, K. & Morishita, Y. (2007) Short-term methotrexate could reduce early immune reactions and improve outcomes in umbilical cord blood transplantation for adults. Bone Marrow Transplantation, 39, 31–39.

Patel, K.J., Rice, R.D., Hawke, R., Abboud, M., Heller, G., Scaradavou, A., Young, J.W. & Barker, J.N. (2010) Pre-engraftment syndrome after double-unit cord blood transplantation: a distinct syndrome not associated with acute graft-versus-host disease. Biology of Blood and Marrow Transplantation, 16, 435–440.

Takagi, S., Masuoka, K., Uchida, N., Ishiwata, K., Araoka, H., Tsuji, M., Yamamoto, H., Kato, D., Matsuhashi, Y., Kusumi, E., Ota, Y., Seo, S.,

Matsumura, T., Matsuno, N., Wake, A., Miyakoshi, S., Makino, S., Ohashi, K., Yoneyama, A. & Taniguchi, S. (2009) High incidence of haemophagocytic syndrome following umbilical cord blood transplantation for adults. British Journal of Haematology, 147, 543–553.

Uchida, N., Wake, A., Nakano, N., Ishiwata, K., Takagi, S., Tsuji, M., Yamamoto, H., Kato, D., Matsuno, N., Masuoka, K., Araoka, H., Asano-Mori, Y., Izutsu, K., Makino, S., Yoneyama, A. & Taniguchi, S. (2011) Mycophenolate and tacrolimus for graft-versus-host disease prophylaxis for elderly after cord blood transplantation: a matched pair comparison with tacrolimus alone. Transplantation, 92, 366–371.

Watanabe, N., Takahashi, S., Ishige, M., Ishii, Y., Ooi, J., Tomonari, A., Tsukada, N., Konuma, T., Kato, S., Sato, A., Tojo, A. & Nakauchi, H. (2008) Recipient-derived cells after cord blood transplantation: dynamics elucidated by multicolour FACS, reflecting graft failure and relapse. Biology of Blood and Marrow Transplantation, 14, 693–701.

INTERNAL🥚MEDICINE

Risk Assessment for Acute Kidney Injury after Allogeneic Hematopoietic Stem Cell Transplantation Based on Acute Kidney Injury Network Criteria

Jinichi Mori[1], Kazuteru Ohashi[1], Takuhiro Yamaguchi[2], Minoru Ando[3], Yuka Hirashima[4],
Takeshi Kobayashi[1], Kazuhiko Kakihana[1] and Hisashi Sakamaki[1]

Abstract

Objective This is a retrospective study for risk assessment of acute kidney injury after allogeneic hematopoietic stem cell transplantation (allo HSCT) based on the Acute Kidney Injury Network (AKIN) criteria.

Methods Two hundred and eighty-nine consecutive patients who received allo HSCT were studied retrospectively to identify the risk factors for AKI according to the AKIN criteria. The incidence of AKI based on AKIN staging and overall survival (OS) was evaluated using Cox proportional hazard regression models treating each AKIN stage as a time-dependent covariate.

Patients We identified a total of 180 patients who developed AKI within 100 days after allo HSCT; AKI was classified as stage 1 in 88 patients (30.5%), stage 2 in 46 patients (15.9%) and stage 3 in 46 patients (15.9%).

Results Patients who developed stage 3 AKI had a significantly worse survival compared to those who developed no AKI or lower stage AKI (HR: 7.6, 95%CI: 4.8-12.1; p<0.001). Multivariate analysis for risks for developing AKI revealed an episode of sepsis or sinusoidal obstruction syndrome (SOS) and the use of liposomal amphotericin as a major cause of the severe stage of AKI.

Conclusion On the basis of our analysis, sepsis, hemorrhagic cystitis, and acute GVHD were associated with severe AKI, and SOS was associated any stage of AKI.

Key words: hematopoietic stem cell transplantation, renal function, risk assessment, AKIN criteria

Introduction

The concept of acute renal failure (ARF) has now been changed to the new paradigm of acute kidney injury (AKI) (1). The Acute Dialysis Quality Initiative (ADQI) recently established evidence-based guidelines for the treatment and prevention of ARF (1). Recognizing the need for a uniform definition for ARF, the ADQI group also proposed new definitions, such as the Risk, Injury, Failure, Loss, and End-stage renal failure classification (RIFLE), and modification was subsequently made by the Acute Kidney Injury

Network (AKIN) (2, 3). The utility of these new definitions has been evaluated mainly in the field of emergency medicine, but few reports are available on AKI associated with allo HSCT (4). AKI is a common complication early after allo HSCT (5). Recent reports have shown that AKI results in poor long-term survival and several risk factors were identified for developing AKI following allo HSCT (6-8). However, detailed risk assessment based on each AKIN stage has not been fully investigated to date. The present study evaluated the incidence of AKI, as defined by AKIN staging (9), in patients undergoing allo HSCT. We also performed a retrospective analysis of risk assessment in trans-

Table 1. Criteria of Renal Dysfunction

AKIN classification*	sCre criteria
1	increased sCre ≥0.3mg/dL or sCre baseline ≥×1.5-2
2	increased sCre >×2-3 from baseline
3	increased sCre ≥×3 from baseline or sCre ≥4mg/dL with an acute increase of at least 0.5mg/dL

* AKI was evaluated using only GFR criteria in our study, as data for urine output could not be retrieved.

plant recipients to determine whether each stage of AKI in an allo HSCT setting can be predicted.

Materials and Methods

The subject sample of this retrospective study was comprised of 289 patients who underwent allo HSCT between August 2004 and December 2009. Preparative therapy was basically performed according to the primary disease and type of transplant. Generally, patients with lymphoid malignancy were conditioned using a total body irradiation (TBI)-containing regimen (12 Gy), including cytarabine at 8 g/m² and cyclophosphamide (CY) at 120 mg/kg. Conversely, patients with myeloid malignancy were conditioned using a non-TBI-containing regimen that included intravenous busulfan (BU) at 12.8 mg/kg and CY at 120 mg/kg. Plasma concentrations of BU were not monitored. Total lymphoid irradiation (TLI) to 7 Gy was included in BU/CY regimens in cases with mismatched or unrelated transplantation. Non-myeloablative allogeneic stem cell transplantation was performed using preparative conditioning comprising CY (120 mg/kg) and fludarabine (125 mg/m²). Cyclosporine (CyA) or tacrolimus (FK) plus short-term methotrexate were used for GVHD prophylaxis. FK was used in cases involving either unrelated or mismatched transplantation (3). Trough level of blood concentration of >400 ng/mL CyA and >20 ng/mL FK were defined as toxic levels. The CyA or FK dose was gradually reduced from day +90 until day +180. Immunosuppression was continued in patients who developed GVHD. Acute and chronic GVHD were diagnosed and graded according to previously established criteria (10). Most patients received ursodeoxycholic acid as prophylaxis for sinusoidal obstruction syndrome. Tosulfloxacin and fluconazol were orally administered 14 days before allo HSCT. trimethoprim/sulfamethoxazole was administered to prevent *Pneumocystis* pneumonia. Cytomegalo virus (CMV) infection was monitored weekly according to CMV antigenemia. Positive antigenemia, defined as >1/65,000 cells, was treated using ganciclovir twice daily until negative CMV antigenemia was obtained. Patients switched to foscarnet in case of poor response to ganciclovir or developing pancytopenia. All patients underwent a routine screening blood test including serum creatinine (Cr) at least 3 times per week. In case of any clinical event, additional blood tests were performed. Sepsis was defined as body temperature higher than 38.0℃ with positive blood culture. Sinusoidal obstruction syndrome (SOS) was considered to be possible when at least two of the following conditions occurred concomitantly after transplantation: jaundice (total bilirubin level >2.0 mg/dL), pain-

ful hepatomegaly, and fluid accumulation evidenced by ascites or unexplained body weight gain (>5% above base line weight). Hemorrhagic cystitis (HC) was defined as the presence of sustained macroscopic hematuria more than 10 days after transplantation in the absence of other conditions such as ureteral lithiasis, disseminated intravascular coagulation or multiorgan failure.

Hematopoietic cell Transplantation comorbidity index (HCT-CI) was scored (11), but the data of diffusing capacity in respiratory function test was not measured in a number of cases. Therefore, diffusing capacity was excluded for scoring in this analysis. AKI was defined and classified into 3 categories according to AKIN criteria (Table 1). AKI within the first 100 days after allo HSCT was defined based on serum Cr. Urine output criteria included in the AKIN definition was not used as we were unable to obtain accurate records of urine output from all of the patients (9). eGFR was calculated by the Modification of Diet in Renal Disease formula, as modified by the Japanese Society of Nephrology: eGFR (mL/min/1.73 m²) = 194 × Cr$^{-1.094}$ × Age$^{-0.287}$ × 0.739 (if female) (12).

Statistics

Surviving patients were censored on October, 2010. Categorical variables were compared using the χ^2 test. Associations between several factors and incidence of AKI based on AKIN stage and OS were evaluated using Cox proportional hazard regression model. Age, sex, disease, donor, stem cell source, conditioning regimen were always included in the models. In addition to these variables, the following factors were considered as covariates; cycle of chemotherapies before HSCT, pre-transplant estimated GFR, HCT-CI, TBI dose, toxic level of calcineurin inhibitors (considered as a time-dependent covariate: TDC), acute GVHD, use of amphotericin B (TDC), episode of sepsis (TDC), episode of TMA (TDC), episode of SOS (TDC), and episode of hemorrhagic cystitis (TDC) in analysis of incidence of AKI, and HCT-CI, use of amphotericin B (TDC), acute GVHD (TDC), chronic GVHD (TDC), use of amphotericin B (TDC), episode of sepsis (TDC), disease recurrence (TDC), and episode of AKI (TDC) in the analysis of OS. Backward variable selection was used to construct models. Values of p <0.05 were considered statistically significant. Incidences of non-relapse death in each AKIN category were calculated according to the Kaplan-Meier method, and were compared using log-rank test. All analyses were performed using SAS version 9.2 (SAS institute Inc., Cary, NC, USA).

Table 2. Clinical Chracteristics of Patients with or without AKI

		Total (n=289)	No AKI (n=109)	AKI accoding to AKIN criteria		
				Stage ≥1 (n=180)	Stage ≥2 (n=92)	Stage ≥3 (n=46)
Age (years)						
	10-19	10	4	6	3	1
	20-29	47	20	27	9	4
	30-39	79	31	48	19	10
	40-49	67	27	40	24	9
	50-59	63	21	42	26	16
	≥60	23	6	17	11	6
Sex						
	M/ F	170/119	59/50	111/69	53/39	25/21
Diagnosis						
	ANLL	120	43	77	44	21
	ALL	58	19	39	24	13
	ATL	5	1	4	3	2
	CML	23	9	14	3	3
	MDS	42	19	23	9	3
	SAA	20	10	10	5	1
	HL	2	0	2	1	0
	NHL	9	5	4	1	1
	MM	5	3	2	1	1
	PMF	2	0	2	1	1
Cycles of chemotherapies						
	0	86	34	52	20	9
	1-5	135	53	82	51	28
	6-10	46	15	31	15	8
	≥11	22	7	15	6	1
Pretransplant eGFR						
	≥60/<60	266/23	101/8	165/15	85/7	42/4
HCT-CI						
	0/1-2/≥3	72/125/92	35/45/29	37/80/63	19/40/33	11/17/18
Donor type						
	RD/URD	82/207	33/76	49/131	21/71	7/39
Conditioning regimen						
	Myeloablative/non-myeloablative	233/56	88/21	145/35	73/19	39/7
TBI dose						
	noTBI/4 Gy/12 Gy	143/34/112	61/9/39	82/25/73	37/15/40	14/8/24
Stem cell source						
	BM/PB/CB	208/43/38	83/18/8	125/25/30	65/10/17	31/4/11
Episode of exposure of toxic dose of CNIs						
	Yes/No	198/91	79/30	119/61	59/33	26/20
Use of AMPH or L-AMPH						
	Yes/No	24/265	2/107	22/158	16/76	5/41
Acute GVHD (grade)						
	0-I/II-IV	189/100	69/40	120/60	59/33	26/20
Chronic GVHD						
	Yes/ No	68/221	32/77	36/144	14/78	4/42
Episode of sepsis						
	Yes/ No	60/229	19/90	41/139	26/66	19/27
Episode of SOS						
	Yes/ No	11/278	3/106	8/172	6/86	4/42
Episode of TMA						
	Yes/ No	18/271	8/101	10/170	7/85	5/41
Episode of hemorrhagic cystitis						
	Yes/ No	40/249	9/100	31/149	19/73	9/37

Abbreviations: AKIN: Acute Kidney Injury Network, AKI: acute kidney insuffciency, n: number, M: male, F: female, ANLL: acute non lymphoid leukemia, ALL: acute lymphoid leukemia, ATL: adult T cell leukemia and lymphoma, CML: chronic myeloid leukemia, MDS: myelodysplastic syndrome, SAA: severe aplastic anemia, HL: Hodgkin lymphoma, NHL: non Hodgkin lymphoma, MM: multiple myeloma, PMF: primary myelofibrosis, eGFR: estimated glomerular filtration rate, HCT-CI: haematopoietic cell transplantation-comorbidity index, RD: related donor, URD: unrelated donor, TBI: total body irradiation, BM: bone marrow, PB: peripheral blood stem cell, CB: cord blood stem cell, CNIs: calcineurin inhibitors, AMPH: amphotericin B, L-AMPH: liposomal amphotericin B, GVHD: graft-versus-host disease, SOS: sinusoidal obstruction syndrome, TMA: thrombotic microangiopathy

Table 3. Multivariate Analysis of Factors for AKI (Each AKIN Stage)

(1) AKIN ≥stage 1

Factors		HR	95% CI	p value
Age	20 years (vs 10 years)	0.90	0.36-2.25	0.820
	30 years	1.10	0.45-2.66	0.835
	40 years	1.06	0.44-2.55	0.905
	50 years	1.27	0.52-3.10	0.601
	≥60 years	1.29	0.54-4.08	0.628
Sex	Female (vs Male)	0.93	0.67-1.29	0.658
Disease	CML (vs AML)	0.75	0.50-1.13	0.164
	Lymphoma	0.80	0.50-1.42	0.446
	MDS or SAA	0.77	0.32-1.83	0.548
	Other disease	0.52	0.18-1.48	0.223
Donor	Unrelated (vs related)	1.12	0.70-1.81	0.629
Stem cell sources	CB (vs BM)	1.00	0.65-1.56	0.985
	PB	1.31	0.72-2.37	0.376
conditioning	NMA (vs MA)	0.98	0.63-1.53	0.922
Pretransplant eGFR	<60 (vs >60 mL/min/1.73m²)	3.01	1.61-5.63	0.001
AMPH-B	Yes (vs no use of AMPH-B)	1.80	1.01-3.21	0.047
SOS	Yes (vs no SOS)	2.99	1.38-6.48	0.006

(2) AKIN ≥stage 2

Factors		HR	95% CI	p value
Age	20 years (vs 10 years)	0.42	0.10-1.68	0.217
	30 years	0.60	0.16-2.19	0.434
	40 years	0.73	0.21-2.60	0.629
	50 years	1.71	0.49-5.99	0.400
	≥60 years	1.28	0.30-5.40	0.740
Sex	Female (vs male)	1.40	0.86-2.26	0.173
Disease	CML (vs AML)	0.24	0.07-0.75	0.014
	Lymphoma	0.17	0.04-0.64	0.010
	MDS or SAA	1.16	0.25-5.34	0.843
	Other disease	0.41	0.09-1.81	0.237
Donor	Unrelated (vs related)	0.95	0.44-2.05	0.896
Stem cell sources	CB (vs BM)	0.92	0.48-1.75	0.789
	PB	1.10	0.43-2.84	0.843
Conditioning	NMA (vs MA)	1.23	0.62-2.41	0.555
Cycles of chemotherapies	1-5 cycles (vs 0 cycle)	0.55	0.18-1.65	1.354
	6-10 cycles	0.40	0.12-1.35	0.141
	≥11 cycles	0.12	0.03-0.52	0.004
aGVHD (grade II-IV)	Yes (vs no aGVHD)	1.67	0.97-2.88	0.063
AMPH-B	Yes (vs no use of AMPH-B)	3.33	1.56-7.08	0.002
Sepsis	Yes (vs no sepseis)	2.36	1.21-4.60	0.012
SOS	Yes (vs no SOS)	5.59	2.23-14.0	<0.001
Hemorrhagic cystitis	Yes (vs no hemorrhagic cystitis)	2.36	1.35-4.13	0.003

(3) AKIN ≥stage 3

Factors		HR	95% CI	p value
Age	20 years (vs 10 years)	0.54	0.05-5.49	0.604
	30 years	0.76	0.09-6.52	0.806
	40 years	0.49	0.06-4.35	0.319
	50 years	2.90	0.36-23.48	0.994
	≥60 years	3.64	0.36-36.99	0.276
Sex	Female (vs male)	1.94	0.97-3.85	0.060
Disease	CML (vs AML)	0.06	0.01-0.30	<0.001
	Lymphoma	0.20	0.05-0.84	0.028
	MDS or SAA	1.47	0.15-14.00	0.739
	Other disease	1.76	0.34-9.17	0.500
Donor	Unrelated (vs related)	1.43	0.37-5.51	0.600
Stem cell sources	CB (vs BM)	1.19	0.49-2.87	0.894
	PB	1.12	0.22-5.73	0.700
Conditioning	NMA (vs MA)	0.78	0.26-2.38	0.663
Cycles of chemotherapies	1-5 cycles (vs 0 cycle)	0.40	0.11-1.48	0.171
	6-10 cycles	0.23	0.05-1.01	0.052
	≥11 cycles	0.01	0.00-0.19	0.013
aGVHD (grade II-IV)	Yes (vs no aGVHD)	3.04	1.43-6.44	0.038
Sepsis	Yes (vs no sepseis)	6.58	2.82-15.34	<0.001
SOS	Yes (vs no SOS)	12.56	3.79-41.64	<0.001
Hemorrhagic cystitis	Yes (vs no hemorrhagic cystitis)	2.44	1.12-5.35	0.025

Abbreviations: AKI: acute kidney injury, AKIN: Acute Kidney Injury Network, CML: chronic myelogeneous leukemia, AML: acute myeloid leukemia, MDS: myelodysplastic syndrome, SAA: severe aplastic anemia, CB: cord blood stem cell transplantation, BM: bone marrow transplantation, PB: peripheral blood stem cell transplantation, NMA: non myeloablative conditioning, MA: myeloablative conditioning, AMPH-B: amphotericin B, SOS: sinusoidal obstruction syndrome
*Backward variable selection was used to construct models, therefore, the variables whose p value had been less than 0.10 were dropped.

Discussion

We retrospectively reviewed 289 allo HSCT recipients, and 180 patients (62.2%) eventually developed AKI at a median time of 41 days (range, 1-100 days) after transplantation (Table 2). Median age at the time of AKI was 41.5 years (range, 17-66 years) and 111 patients were men (61%). Median follow-up was for 489 days (range, 2-2,256

days). AKI was classified as stage 1 in 88 patients (46.6%), stage 2 in 46 patients (24.3%) and stage 3 in 46 patients (24.3%) according to the AKIN classification. We initially sought to determine which variables were associated with the risk of AKI and we especially focused on the risk assessment based on each AKIN stage. As shown in Table 3, multivariate analyses of risk factors for each AKIN stage disclosed an episode of SOS as a major cause of any stage of AKI, which are in line with the recent report of risk

Table 4. Multivariate Analysis of Adverse Factors for Overall Survival

Table 4. Multivariate Analysis of Adverse Factors for Overall Survival

(1) AKIN >stage 1

		HR	95% CI	p value
HCT-CI	1 (vs 0)	1.48	0.87-2.52	0.147
	2	1.59	0.92-2.77	0.097
Age	20 years (vs 10 years)	0.32	0.12-0.90	0.030
	30 years	0.55	0.22-1.39	0.203
	40 years	0.44	0.17-1.12	0.084
	50 years	0.60	0.24-1.51	0.278
	≥60 years	1.00	0.36-2.76	1.000
Sex	Male (vs female)	1.01	0.69-1.48	0.948
Disease	CML (vs AML)	1.00	0.57-1.73	0.989
	Lymphoma	0.55	0.25-1.23	0.143
	MDS or SAA	2.94	1.09-7.96	0.033
	Other disease	4.46	1.44-13.79	0.009
Donor	Unrelated (vs related)	1.14	0.62-2.11	0.676
Stem cell sources	CB (vs BM)	1.37	0.80-2.35	0.250
	PB	0.97	0.48-1.94	0.930
Conditioning	NMA (vs MA)	0.59	0.34-1.03	0.063
AKIN	>Stage 1 (vs no AKI)	3.71	2.33-5.89	<0.001
aGVHD (grade II-IV)	Yes (vs no aGVHD)	1.90	1.22-2.95	0.005
cGVHD	Yes (vs no cGVHD)	0.58	0.31-1.10	0.096
Sepsis	Yes (vs no sepseis)	3.09	1.95-4.90	<0.001
AMPH-B	Yes (vs no use of AMPH-B)	5.00	2.80-8.93	<0.001
Disease recurrence	Yes (vs no recurrence)	5.98	3.92-9.13	<0.001

(2) AKIN >stage 2

		HR	95% CI	p value
HCT-CI	1 (vs 0)	1.62	0.95-2.76	0.080
	2	2.01	1.16-3.48	0.013
Age	20 years (vs 10 years)	0.34	0.12-0.95	0.040
	30 years	0.65	0.26-1.66	0.370
	40 years	0.40	0.15-1.01	0.053
	50 years	0.54	0.21-1.34	0.183
	≥60 years	0.77	0.27-2.16	0.620
Sex	Male (vs female)	0.96	0.66-1.41	0.845
Disease	CML (vs AML)	1.20	0.68-2.12	0.525
	Lymphoma	0.87	0.40-1.90	0.721
	MDS or SAA	2.84	1.02-7.90	0.045
	Other disease	4.39	1.37-14.12	0.013
Donor	Unrelated (vs related)	1.24	0.66-2.32	0.502
Stem cell sources	CB (vs BM)	1.54	0.91-2.61	0.106
	PB	1.16	0.55-2.44	0.701
Conditioning	NMA (vs MA)	0.69	0.40-1.20	0.188
AKIN	>Stage 2 (vs no AKI or AKIN stage 1)	4.38	2.84-6.74	<0.001
aGVHD (grade II-IV)	Yes (vs no aGVHD)	1.91	1.23-2.97	0.004
cGVHD	Yes (vs no cGVHD)	0.60	0.32-1.12	0.108
Sepsis	Yes (vs no sepseis)	3.06	1.91-4.91	<0.001
AMPH-B	Yes (vs no use of AMPH-B)	4.76	2.66-8.52	<0.001
Disease recurrence	Yes (vs no recurrence)	5.06	3.28-7.80	<0.001

(3) AKIN >stage 3

		HR	95% CI	p value
HCT-CI	1 (vs 0)	1.66	0.96-2.87	0.071
	2	1.98	1.14-3.43	0.016
Age	20 years (vs 10 years)	0.25	0.09-0.70	0.009
	30 years	0.61	0.25-1.53	0.292
	40 years	0.45	0.18-1.14	0.092
	50 years	0.52	0.21-1.32	0.170
	≥60 years	0.57	0.20-1.65	0.302
Sex	Male (vs female)	0.83	0.56-1.23	0.351
Disease	CML (vs AML)	1.12	0.64-1.97	0.688
	Lymphoma	0.70	0.32-1.52	0.365
	MDS or SAA	3.05	1.11-8.40	0.031
	Other disease	2.80	0.89-8.86	0.080
Donor	Unrelated (vs related)	1.37	0.73-2.57	0.327
Stem cell sources	CB (vs BM)	1.60	0.94-2.73	0.084
	PB	1.21	0.58-2.52	0.618
Conditioning	NMA (vs MA)	0.78	0.45-1.34	0.359
AKIN	>Stage 3 (vs no AKI or AKIN stage 1 or 2)	5.49	3.38-8.94	<0.001
aGVHD (grade II-IV)	Yes (vs no aGVHD)	1.75	1.12-2.74	0.015
cGVHD	Yes (vs no cGVHD)	0.63	0.33-1.18	0.150
Sepsis	Yes (vs no sepseis)	2.13	1.30-3.50	0.003
AMPH-B	Yes (vs no use of AMPH-B)	5.40	2.99-9.75	<0.001
Disease recurrence	Yes (vs no recurrence)	5.03	3.25-7.80	<0.001

Abbreviations: AKIN: Acute Kidney Injury Network, HCT-CI: hematopoietic cell transplantation comorbidity index, AKI: acute kidney injury, CML: chronic myelogeneous leukemia, AML: acute myeloid leukemia, MDS: myelodysplastic syndrome, SAA: severe aplastic anemia, CB: cord blood stem cell transplantation, BM: bone marrow transplantation, PB: peripheral blood stem cell transplantation, NMA: non myeloablative conditioning, MA: myeloablative conditioning, AKI: acute kidney injury, aGVHD: acute graft-versus-host disease, cGVHD: chronic graft-versus-host disease, AMPH-B: amphotericin B

analysis of AKI (5). Sepsis, hemorrhagic cystitis, and acute GVHD were associated with more severe AKI. While, the use of amphotericin B and pre-transplant GFR were dropped in models of severe stage of AKI. Risk analysis was also performed to estimate overall survival (Table 4). Multivariate analysis revealed that severity of AKI was significantly associated worse OS; AKIN >stage 1 (HR, 3.71; 95%CI, 2.33-5.89; p<0.001), AKIN >stage 2 (HR, 4.38; 95%CI, 2.84-6.74; p<0.001) and AKIN >stage 3 (HR, 5.49; 95%CI, 3.38-8.94; p<0.001). In addition to severity of AKI, sepsis and use of amphotericin B were also significantly associated worse OS. In view of the cumulative incidence of non-