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Review

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Regenerative Medicine

Toward a unified theory of aging and regeneration

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Growing evidence supports the antagonistic pleiotropy theory of mammalian aging. Accordingly, changes in gene expression following the pluripotency transition, and subsequent transitions such as the embryonic–fetal transition, while providing tumor suppressive and antiviral survival benefits also result in a loss of regenerative potential leading to age-related fibrosis and degenerative diseases. However, reprogramming somatic cells to pluripotency demonstrates the possibility of restoring telomerase and embryonic regeneration pathways and thus reversing the age-related decline in regenerative capacity. A unified model of aging and loss of regenerative potential is emerging that may ultimately be translated into new therapeutic approaches for establishing induced tissue regeneration and modulation of the embryo-onco phenotype of cancer.

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Aging is often defined as a progressive deterioration of an organism over time, wherein the risk of mortality increases exponentially with age in the postreproductive years. Although everyday environmental risks from predation or infectious disease (e.g., stochastic risks) necessarily lead to increased mortality over time, they are not considered core to the definition of the aging process *per se* [1,2]. Thus, an important criterion of aging is that it encompasses virtually every somatic tissue type, including the gonads (though not necessarily the germ-line cells themselves, given their role in potentially perpetuating the species) [3]. In order to distinguish the aging process from damage that occurs stochastically over time, Benjamin Gompertz described aging as a process leading to an exponential increase in mortality with time, that is, $R_m = R_0 e^{at}$ where 'R_m' represents the probability of mortality between ages 't' and 't + 1'. Accordingly, 'R_0' is a constant, while 'a' represents an exponential parameter corresponding to the rate of increase of mortality with age [1,4]. Diverse species exhibit wide variations in the rate of aging as high as 50-fold, and some species even show a negligible rate of aging as determined by Gompertz equation. However, the similarities in the aging process between species and within tissues of an individual support the conclusion that what we commonly call aging is the result of a series of developmentally timed processes occurring in somatic tissues [5].

The pathogenesis of aging, like the relatively complex etiology of cancer, is commonly viewed as being rooted in multifaceted processes intrinsic to somatic cells along with additional tissue-specific influences, inherited genetic predispositions and environmental factors. However, premature aging disorders such as Hutchinson–Gilford syndrome (progeria) and Werner syndrome showing the premature appearance of various age-related changes such as graying and loss of hair, coronary heart disease, stroke and osteoporosis [6] lend support to an intrinsic genome-based pacing mechanism underlying aging. In addition, these naturally occurring aging disorders provide evidence that certain molecular pathways may alter the time course of the aging in multiple organ systems, suggesting that similar pathways may underlie aging in numerous somatic tissue types.

Future Medicine

Additional insights into the molecular biology of aging can be gleaned from studies of segmental (tissue-specific) genetic predispositions as well as those comparing the genotypes of individuals with varied lifespan phenotypes. The use of genome-wide gene association studies has identified loci such as those near *APOE, CHRNA5, ANRIL, LPA* and *FTO* as being correlated with a long lifespan [7]. However, identical twin studies suggest that environmentally induced epigenetic effects potentially play an important role in the onset of age-related disease as well [8]. Therefore, despite recent progress in understanding the biology of aging, the field remains largely fragmented due to the lack of a central organizing hypothesis that could provide a framework for investigating how basic upstream biological processes regulate the timing of age-related changes in tissues and the influence of these changes on the onset of age-related degenerative disease.

The goal of this review is to assemble diverse observations about the etiology of aging into a more unified mechanistic model. Thus, we suggest that numerous genetic, environmental and metabolic perturbations alter the rate of aging by shifting the balance of euchromatin/heterochromatin in regulatory regions of the genome leading to either more permissive or more restrictive patterns of regenerative gene expression. Moreover, we propose that these changes in regenerative gene expression occur in a series of developmentally timed transitions that occur globally in many cell types and in various tissues of the body and that these changes impact multiple hallmarks of both aging and cancer. Using an historical perspective, we will review these developmentally timed transitions from the perspective of the loss of germ-line immortality in somatic cells and the changes in regenerative gene expression (antagonistic pleiotropy) leading to the progressive restriction of regenerative capacity and the subsequent effects on aging. Finally, we will discuss the epigenetic nature of these transitional changes that lead to aging and how their reversal during reprogramming suggests the possibility of restoring regenerative capacity to aging tissues and organs using an approach we have termed induced tissue regeneration (iTR).

Somatic restriction & antagonistic pleiotropy

In the 19th century, the German naturalist August Weismann described the elegant hypothesis that heredity is transmitted through an immortal lineage of cells commonly designated the 'germ line' [9]. This logically led to his proposal that since somatic cells do not carry their hereditary information forward to the next generation, they have no need for the capacity to replicate indefinitely. In other words, somatic cells are disposable from the viewpoint of evolution because there is no selective pressure for traits that would lead to their immortal replication. He therefore laid out the prediction that "death takes place because a worn-out (somatic) tissue cannot forever renew itself, and because a capacity for increase by means of cell division is not everlasting, but finite" [9].

Weismann proposed that the loss of immortal replication in somatic cells (one example of somatic restriction) associated with aging resulted from the loss of traits that were not evolutionarily advantageous (a passive process). In contrast, the evolutionary biologist George Williams proposed a model of antagonistic pleiotropy as a likely mechanism in which aging was under active selection. In his model, certain genetic traits have pleiotropic effects throughout the lifespan. Accordingly, natural selection necessarily increases the probability of those traits that lead to increased fecundity, therefore, once reproduction has occurred there is very little selective pressure to eliminate these same traits even if they exert deleterious effects on fitness or viability. As a result, the two outcomes are antagonistic, such that natural selection favored traits that led to the limited replication and regeneration of the soma despite potentially deleterious effects later in life (i.e., aging) because these same traits improved reproductive fitness early in life.

The Weismann and Williams hypotheses are not mutually exclusive. Indeed, both hypotheses have grown in acceptance since they were first published and as a result, it is now commonly accepted that mammalian aging is likely the result of a developmental program wherein somatic cells progressively lose the traits of immortal proliferation and regeneration (somatic restriction) and that such restriction is the result of active selection for increased reproductive fitness. Accordingly, selection for traits that decrease the risk of oncogenesis or increase resistance to genomic instability from endogenous retroviral elements would likely increase reproductive fitness but could also contribute to the decline of regenerative potential that is observed in later life.

The restriction of the twin traits of immortal proliferation and regeneration is designated as the 'Weismann barrier' herein as shown in Figure 1. Accordingly, Weismann's prediction that a worn-out tissue cannot forever renew itself was shown to be the case in many vertebrate species wherein scarless regenerative potential is repressed shortly after embryogenesis. In contrast, simple unicellular organisms such as *Tetrahymena* display replicative immortality, and a few primitive invertebrates such as hydra [10,11] and planaria show no evidence of aging as determined by

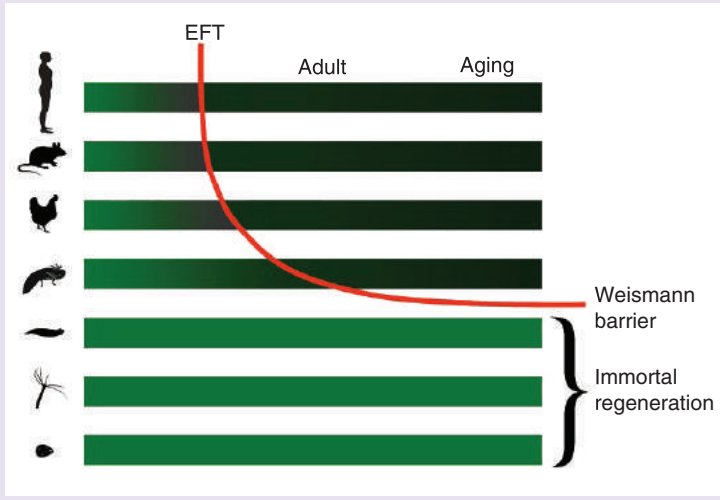

Figure 1. Evolution and the Weismann barrier. Primitive protozoa such as *Tetrahymena* appear to be capable of limitless reproduction in the absence of sexual recombination. Multicellular animals such as hydra and planaria likewise display profound regenerative potential with replicative immortality leading to an absence of aging as defined by Benjamin Gompertz. In vertebrates, repression of replicative immortality is commonly observed during early embryonic development and regeneration is repressed later usually around the embryonic–fetal transition defining the Weismann barrier (shown as a red line). For those examples where extensive regeneration is observed in the adult, it is common to see the adult arrested in an early developmental state as observed in axolotl pedomorphs. EFT: Embryonic–fetal transition.

an exponential rise in the risk of mortality with time. However, in general, advanced multicellularity without cancer required a phenotype that confined continual renewal to the germ line while restricting the regenerative capacity of somatic cells and tissues. As a result, as multicellular organisms evolved to even greater complexity their regenerative capacity became increasingly restricted, and as we propose herein, the restriction may occur globally in developmental stages and include numerous cell and tissue types. Thus, nearly all mammals, including humans, repress replicative immortality in somatic cells early in embryogenesis followed by a subsequent loss of regenerative potential after embryonic development is essentially complete. This loss of both immortality and regenerative potential defines the Weismann barrier (shown as red line in Figure 1).

The somatic restriction model predicts that organisms that retain juvenile properties throughout life would also retain enhanced regenerative capacity because they would never completely traverse the Weismann barrier. Accordingly, support for the model that the profound regenerative potential of primitive metazoans is retained from early ontogeny comes from studies in amphibians such as the urodeles. For example, axolotls live their adult lives in an arrested larval stage (pedomorphosis) due to the lack of appropriate thyroid hormone signaling [12,13]. These unusual animals show a profound regenerative potential in diverse tissues such as forebrain, jaw and heart [14–16]. In addition, they are even capable of regenerating amputated limbs through the formation of a limb bud-like blastema, a phenomenon designated as epimorphic regeneration [17]. Moreover, the observation that other anuran larvae (e.g., *Xenopus*) exhibit significant regenerative potential that is subsequently lost with metamorphosis supports the view that the retention of embryonic traits causes epimorphic regeneration in urodeles [18]. Furthermore, there are additional models provided by various species that retain regenerative potential into adulthood such as hemimetabolous insects that show an extended nymph-like state [19]. However, it is important to note that repeated injury to axolotls eventually leads to defective regeneration suggesting that while they may retain the profound regenerative potential of the larval state and appear to express telomerase, they may not have sufficient telomerase activity to regenerate indefinitely [20,21]. Therefore, urodeles, unlike certain more primitive organisms like planaria and hydra, do not completely escape the Weismann barrier (Figure 1). The lack of telomerase as well as other insults to the genome ultimately leads to cellular senescence, which profoundly affects regenerative capacity and tissue homeostasis later in life.

Somatic cell senescence

Weismann's prediction that somatic cells only possess a finite capacity for cell division was validated in the 1960s through the work of Leonard Hayflick who finally provided evidence for the mortality of cultured normal diploid human fibroblasts [22]. In the following years, virtually all normal human cell types capable of cell division *in vitro* were shown to have only a finite capacity for cell division. Importantly, this finite replicative lifespan was determined to be a result of the number cell doublings that a cell had undergone rather than simply the passage of metabolic time [23]. However, the precise nature of this replicative clock remained to be determined for several decades.

In 1973, Alexey Olovnikov proposed that the progressive shortening of terminal DNA sequences (telomeres) was the clocking mechanism for the Hayflick phenomenon, and that immortal cells such as germ line and cancer cells utilize a terminal transferase (now designated telomerase) that reverse transcribes the tandem telomeric repeat sequences utilizing an endogenous RNA template, thereby extending the telomere length [24]. However, Olovnikov's hypothesis could not be tested until the molecular cloning, in the 1990s, of the gene encoding the catalytic component of telomerase, known as telomerase reverse transcriptase (*TERT*). As predicted by the hypothesis, the exogenous expression of *TERT* extended the lifespan of normal cells that were grown *in vitro* [25]. Subsequent studies demonstrated the ability of the enzyme to immortalize various somatic cell types without inducing oncogenic transformation [26]. Additional studies using a sensitive PCR-based assay for telomerase activity, known as the telomere repeat amplification protocol (TRAP) assay, demonstrated that telomerase activity was present in approximately 98% of immortal cancer cell lines (but not in any normal cultured somatic cell types), approximately 90% of malignant tumors and 100% of normal gonads tested, but not in other normal somatic counterparts [27]. As shown in Figure 2A, the pattern of *TERT* expression fits the model of antagonistic pleiotropy. Embryonic stem (ES) cells have not repressed *TERT* and, therefore, they commonly maintain telomere length near that of germ-line cells such as sperm [28,29]. However, human ES (hES) cells that are differentiated to early embryoid bodies or partially differentiated to clonal embryonic progenitor cell lines [30] have passed the pluripotency transition (PT) and therefore do not express *TERT*. Additional support for the rapid repression of *TERT* following the PT is that primary cultures of dermal fibroblasts taken from late embryonic skin (Figure 2B) have already completely repressed *TERT* expression. Taken together, these data indicate that the *TERT* gene is repressed very early in development. Thus, repression of *TERT*, the enzyme proposed by Olovnikov as essential for immortal replication, occurs shortly after PT resulting in the initiation of somatic restriction with a shift from immortal pluripotency to mortal somatic cells.

As discussed earlier, the limited replication capacity of somatic cell types due to the absence of telomerase expression ultimately leads to significant telomere erosion beginning in embryonic and fetal development, and continuing with further erosion during normal aging (and accelerated aging in the case of chronic injury) resulting in chronic tissue damage. Accordingly, this continued telomere erosion eventually results in critically short telomeres that trigger cell senescence, which is thought to limit the capacity of adult tissues to repair damage. In addition, senescent cells contribute to a loss of tissue integrity and increased inflammation via the expression catabolic enzymes such as metalloproteinases [31] and plasminogen activators [32], a phenomenon now designated as the senescence-associated secretory phenotype [33]. Therefore, cellular senescence as seen in the context of somatic restriction and antagonistic pleiotropy is a consequence of the loss of indefinite somatic cell replication via the loss of telomerase, which may provide an evolutionary advantage to the species by, for example, reducing tumor formation, but eventually leads to detrimental effects later in life such as the loss of tissue regeneration associated with aging.

Inhibition of regeneration

Assuming the hypotheses of Weismann and Williams are correct, the same selective pressures that inhibit replicative immortality could also restrict regeneration following the completion of embryogenesis. Therefore, the associated molecular pathways would potentially be important for understanding aging and possibly even cancer. Indeed, regeneration research has attracted the interest of researchers since the time of Thomas Hunt Morgan at the beginning of the 20th century [17]. These early models included the study of planaria and hydra as well as other invertebrate species, but these early studies were largely descriptive. Current research has extended the number of animal models to include urodeles, hemimetabolous insects, *Xenopus* and even mammals such as the African spiny mouse [34,35] and embryonic marsupials [36].

Perhaps the first advancement in our understanding of the molecular basis of the restriction of regenerative potential during development began with the search for genes that alter the developmental timeline (heterochrony

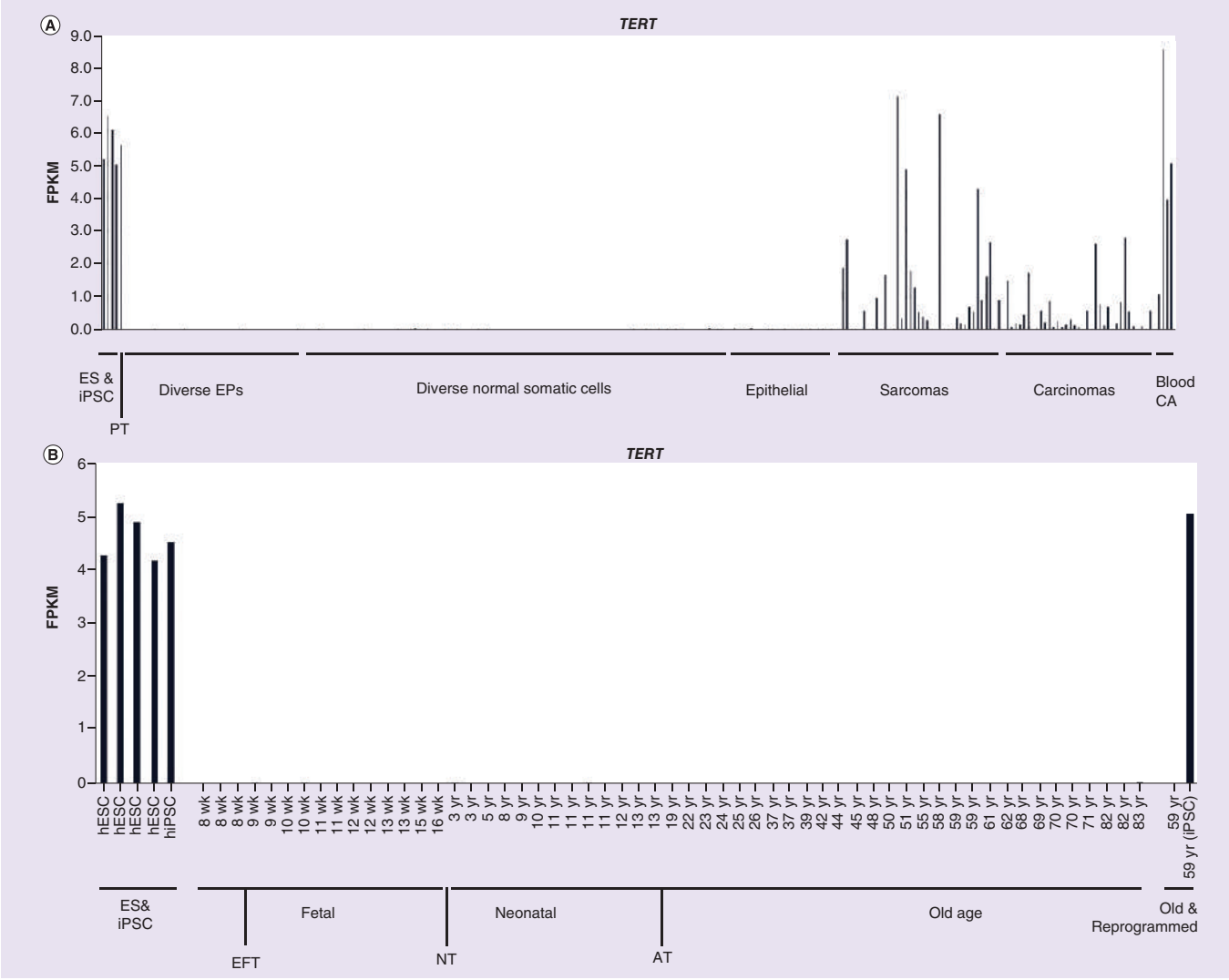

Figure 2. *TERT* expression during differentiation *in vitro* and *in vivo*. The catalytic component of human telomerase *(TERT)* is an archetypal example of a gene with antagonistic pleiotropic effects in aging and cancer. **(A)** *TERT* is abundantly expressed in naturally immortal hES and iPSCs and in the majority of carcinomas, sarcomas and blood cell cancers. It is not expressed after the pluripotency transition in diverse hESC-derived embryonic progenitor cells, diverse human somatic cell types including cultured mesenchymal stem cells, vascular endothelial cells, neurons and glia, hepatocytes, osteochondral cell types as well as others; nor is *TERT* expressed in cultured epithelial cells such as keratinocytes, respiratory, mammary, prostate or other epithelia. **(B)** *TERT* is expressed in pluripotent stem cells, but not in human dermal fibroblasts from late-stage embryonic upper arm skin-derived fibroblasts cultured *in vitro*, or in skin from fetal or adult sources throughout the human lifespan.
AT: Adult transition; CA: Cancer cells; EP: Embryonic progenitors; hESC: Human embryonic stem cell; NT; Newborn transition; iPSC; Induced pluripotent stem cells; EFT: Embryonic–fetal transition; FPKM: Fragments per kilobase of transcript per million mapped reads; PT: Pluripotency transition.

genes) in *Caenorhabditis elegans*. One example is the *lin41* (designated as *TRIM71* in humans), a gene that is implicated in regulating cell proliferation [37], that is expressed early in the *C. elegans* development but repressed in the larval stages. Another example is the noncoding gene, *let-7*, whose transcribed product is a miRNA [38]. The let-7 miRNA appears to control temporal developmental events such as the transition from larval to adult states in the worm. It operates through the silencing of numerous target transcripts during differentiation and appears to be largely conserved in metazoans from *C. elegans* to *Drosophila*, mouse and man. Let-7 is thus thought to restrict regeneration during the process of development and, therefore, would need to be repressed in the early pre-EFT (embryonic–fetal transition) stages of development.

Lin-28, which is expressed during embryogenesis and repressed following the EFT, is an RNA binding protein that regulates Let-7. Lin-28 appears to bind the primary and precursor forms of Let-7, thereby inhibiting its downstream processing. There are two homologs of Lin-28 present in the human species designated *LIN28A* and *LIN28B*. Both genes continue to be transcribed after *TERT* is repressed but they are eventually repressed at the EFT and, like *TERT*, they represent examples of genes that generate antagonistic pleiotropic effects in aging and cancer. Accordingly, *LIN28B* is abundantly expressed in naturally immortal hES cells and induced pluripotent stem cells (iPSC) but is generally repressed in a wide variety of hES cell-derived embryonic progenitor cell types except for embryonic vascular endothelial cells. *LIN28B* is not expressed in many different adult human somatic cell types including cultured mesenchymal stem cells, vascular endothelial cells, neurons and glia, hepatocytes and osteochondral cell types as well as various cultured epithelial cell types such as keratinocytes, respiratory, mammary, prostate or other epithelia (Figure 3A). In contrast, *LIN28B* is expressed at high levels in many carcinomas, sarcomas and blood cell cancers (Figure 3A) [39] but is expressed at much lower levels in normal cultured human skin fibroblasts than in cancer or pluripotent cells. These observations suggest a regenerative role for *LIN28* that is restricted upon its repression around the time of the EFT (completion of 8 weeks of development) (Figure 3B).

A considerable number of reports substantiate a role for genes in the LIN28/Let-7 pathway not only as markers of the developmental stage of mammalian cells, but also as having a direct role in regulating regeneration and cancer. For example, the expression of *LIN28A* and/or *LIN28B* has been reported to increase axon regeneration in both peripheral and CNS neurons [40]; to increase regeneration of cartilage, skin and digits [41]; and to increase hematopoiesis [42]. Moreover, the *LIN28*/*LET-7* target *IGF2BP1* can even reprogram adult cells to express fetal hemoglobins [43]. In the urodeles, thyroid hormone appears to play a role in triggering metamorphosis. As such, Lin28 has been reported to inhibit the expression of thyroid hormone target genes, delay development and prolong regenerative potential [44]. Consequently, given the importance of this pathway in developmental timing, it is interesting to ask whether activation of *LIN28A* and *LIN28B* or, alternatively, the abrogation of expression of the Let-7 family of miRNAs, can be used more broadly as a method of facilitating the induced tissue regeneration.

Metabolism also plays an essential role in development, regeneration and aging. Accordingly, developing embryonic tissues and presumably, regenerating tissues, have a greater need for anabolic precursors such as those originating from glycolysis as opposed to oxidative phosphorylation (OXPHOS). OXPHOS is necessarily minimized during embryogenesis by the rate-limiting lack of oxygen in the relatively anoxic environment of mammalian uterus. Interestingly, it has been recognized since the pioneering research of Otto Warburg, that a shift from glycolysis to OXPHOS occurs during early embryonic development and that a shift back to glycolysis, even in the presence of adequate oxygen, occurs in many cancers: a phenomenon known as the Warburg effect, or aerobic glycolysis [45]. These observations raise the question of what role the balance of glycolysis versus OXPHOS might play in the regenerative phenotype.

The embryonic–fetal transition

Placental mammals show a global loss of regenerative potential in numerous tissues during development *in utero*. Although the timing of the repression of scarless regeneration in humans depends on the tissue type, it is commonly associated with the EFT. In humans, this transition occurs at the completion of Carnegie Stage 23 (8 weeks of development). In the case of the mouse, this corresponds approximately with the close of Theiler Stage 23 (16 days post coitum [dpc]). Consequently, regeneration during the EFT can be easily studied in marsupial mammals because the animals emerge and enter the pouch while still in the embryonic prefetal state where they are readily accessible for experimentation. Thus it has been determined that scarring begins around pouch day 9 near the EFT [36].

The *in vitro* model of human cell aging discovered by Hayflick, facilitated the discovery of the role of telomere dynamics in aging. Accordingly, the field of regeneration research is likely to increasingly benefit from the initial discovery of culture conditions for isolating and growing human pluripotent stem cells *in vitro*. Indeed, ES cells and iPSCs [28,46] provide an *in vitro* model system to study human cells in the germ-line state as well as embryonic somatic cells of diverse types that can be compared with their adult counterparts for analyzing in the molecular biology of the EFT under rigorously controlled conditions. Moreover, there are opportunities to study naive pluripotent cells, which are thought to be capable of robust germ-line transmission and therefore more closely aligned with the classic definition of ES cells. Additionally, primed ES cells or 'ES-like' cells also retain telomerase expression and generally maintain telomeres at germ-line length as well as pluripotency, but unlike naive cells commonly do not show the ability to be incorporated into the germ line such as when they are introduced into

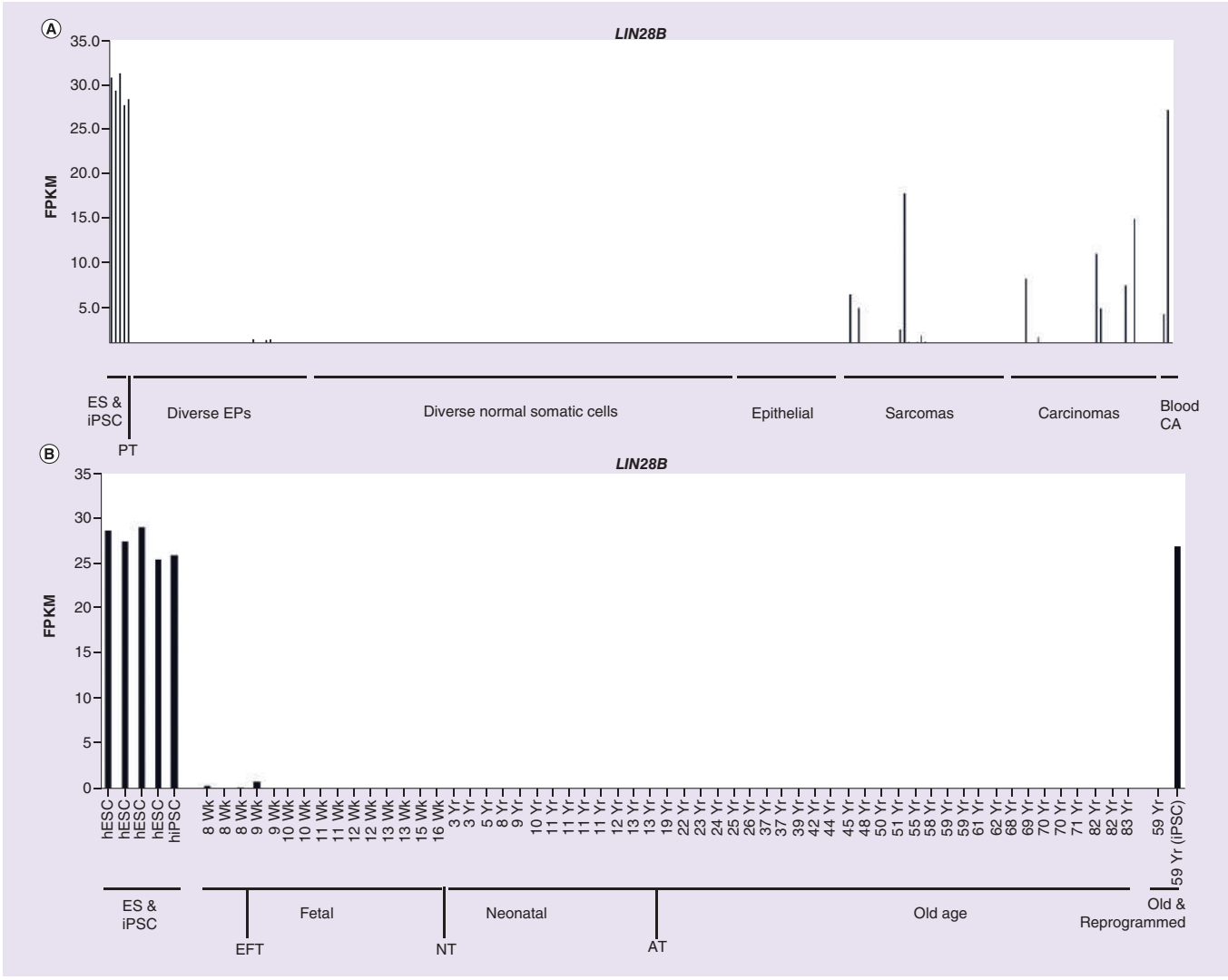

Figure 3. *LIN28B* expression during differentiation *in vitro* and *in vivo*. The gene encoding the RNA binding protein *LIN28B* is an example of a gene repressed subsequent to *TERT* that also generates antagonistic pleiotropic effects in aging and cancer. **(A)** *LIN28B* is abundantly expressed in naturally immortal hES and iPSCs, but not expressed in most diverse hESC-derived embryonic progenitor cells except for embryonic vascular endothelial cells. *LIN28B* is neither expressed in diverse adult human somatic cell types including cultured mesenchymal stem cells, vascular endothelial cells, neurons and glia, hepatocytes, osteochondral cell types as well as others, nor is *LIN28B* expressed in cultured epithelial cells such as keratinocytes, respiratory, mammary, prostate or other epithelia; however, it is expressed at high levels in many carcinomas, sarcomas and blood cell cancers. **(B)** *LIN28B* is highly expressed in human pluripotent stem cells; it is expressed at lower levels in human dermal fibroblasts from late-stage embryonic upper arm skin-derived fibroblasts cultured *in vitro*, but not in skin from later fetal or adult sources throughout the human lifespan.
CA: Cancer cells; EP: Embryonic progenitors; hESC: Human embryonic stem cell; NT; Newborn transition; iPSC; Induced pluripotent stem cells; EFT: Embryonic–fetal transition; FPKM: Fragments per kilobase of transcript per million mapped reads; PT: Pluripotency transition.

chimeric blastocysts. Therefore, both naive and primed ES cells have not yet initiated the process of differentiation into the classic three germ layers (endoderm, mesoderm or ectoderm) and thus have not passed the PT making them valuable *in vitro* models for PT and EFT studies.

The ability to grow hES cells and iPSC *in vitro* facilitates the exploration of the transitions occurring during human development. However, their potential to differentiate into a very large complexity of diverse cell types can complicate a rigorous exploration of these processes. One approach to this problem is the clonal derivation of a large diverse library of clonal progenitor cell lines from hES or iPSC [30]. These pure and scalable clonal lines can be characterized and compared with their adult counterparts. Moreover, the use of artificial intelligence algorithms to compare hES, embryonic progenitor and adult normal and tumor cell line transcriptome data has

facilitated the identification of robust markers of the PT and EFT stages [47]. One such marker, *COX7A1*, appears to begin expression in diverse stromal cell types at about the EFT. Accordingly, levels of expression of this gene are particularly high in adult skeletal and cardiac muscle, brown fat, and stromal cells, with a lower level in most epithelial cell types; however, there is no expression in many sarcomas and carcinomas [47]. Indeed, the repression of *COX7A1* expression in numerous malignant cell types is consistent with the model of antagonistic pleiotropy. The role of *COX7A1* in OXPHOS complex IV dimerization suggests that the onset of expression of the gene during development (post-EFT) plays a role in the repression of regeneration, whereas, the loss of expression in malignancy may play an important role in the Warburg effect and uncontrolled growth. Therefore, expression of *COX7A1* following EFT might contribute in part to the lack of regenerative potential in most somatic tissues (hepatocytes, blood and epithelial cells being exceptions). Indeed, an important hallmark of aging is the progressive accumulation of interstitial fibrosis [48,49] and the accumulation of fibrous connective tissue in many organs in old age may reflect this failed regenerative response to cumulative age-related injury. Further analysis of hES cells, iPSC cells and their derivatives in comparison with adult cells is likely to reveal additional molecular pathways involved in somatic restriction during developmental transitions such as PT and EFT.

The neonatal transition

The neonatal transition (NT) is designated here as the developmental transition resulting in profound global alterations in mammalian somatic gene expression that are associated with birth. This transition is marked by a dramatic decrease in growth rate. Indeed, body mass will increase dramatically by >100-fold during fetal development (EFT to NT) but growth will continue at a markedly slower rate following the NT. A survey of genes differentially expressed during the NT showed that many were associated with imprinted loci [50]. An example of this is the paternally imprinted fetal growth factor IGF2. Moreover, maternal and paternal imprints have been implicated in the regulation of not only placentation but fetal and perhaps postnatal growth as well [51]. Although endocrine factors such as thyroid hormone, IGF1 and IGF2, and growth hormone have been implicated in growth, the levels of these factors do not appreciably change with the cessation of growth in adulthood. Indeed, studies involving the transplantation of fetal rat tissues into adult rats suggest that factors such as growth hormone do not arrest the growth of the fetal rat tissue and indeed are necessary for differentiation [52]. These observations suggest that the regulation of growth at the NT results from an intrinsic developmental program as opposed to endocrine influences.

The mammalian heart appears to be under regulation by the NT and thus provides an important target organ model system to study the relationship between NT and regeneration. Unlike most organs, the heart retains an unusually high degree of regenerative potential after EFT, beyond NT and into the first postnatal week during which time cardiomyocytes begin to become binucleate [53,54]. Accordingly, ligation of the left anterior descending artery in 1 day old mice results in severe ischemic damage that is nevertheless completely regenerated scarlessly within 7 days. However, a similar injury induced in 7-day-old mice results in scarring instead of regeneration [55]. With regard to the mechanism, tracing studies argue in favor of the hypothesis that the regenerated myocardium in such perinatal mammals originates from the de-differentiation of mature myocardium as opposed to the competing theory that it originates from a mobilized pool of resident myocardial progenitors [56]. Therefore, in the case of the myocardium, the loss of regeneration occurring shortly after NT is likely not due to a loss or dysfunction of stem cells but rather a relative loss of plasticity in the differentiated cardiac cells (i.e., its ability to dedifferentiate and re-differentiate in response to injury). A better understanding of the NT and the mechanism of the regenerative capacity of the neonatal mammalian heart may enable new approaches to inducing regeneration in fully mature tissues and organs.

The adult transition

The adult transition (AT) occurs at the termination of growth in the reproductively mature adult and represents another transition where the somatic restriction hypothesis predicts molecular changes connected to aging and the loss of regeneration. Accordingly, numerous metazoans with developmental programs of continued growth following reproductive maturity (no AT) show negligible aging [5]. These observations led to 'Bidder's hypothesis' [1], which states that the growth arrest of animals upon reaching reproductive maturity is causally connected to aging (perhaps due to a change in metabolic rate) [57,58]. Accordingly, George Parker Bidder summarized his theory as, "we die, therefore, as an alternative to becoming giants" [59].

The molecular mechanisms underlying the AT have not been fully elucidated. However, differentially methylated regions in the genome have been implicated in the NT as well as in the later adult growth arrest (AT) in most mammalian species. In addition, the identification of the Hippo signaling pathway including genes such as *MST4* and *TEAD4* has provided new insights into the molecular biology of growth arrest in the AT. For example, both *MST4* and *TEAD4* have been reported to play a role in cell proliferation and cancer [60,61]. However, only a few studies have attempted to identify alterations in mediators of Hippo signaling that could play a role in aging [62]. Accordingly, a better understanding of the molecular mechanisms involved in the AT with respect to tissue growth arrest may also inform mechanisms that regulate regeneration with aging. It will also be important to understand the role of environment has in developmental transitions, regeneration and aging.

Developmental plasticity in feast & famine

It seems likely that feast/famine cycles have had a profound effect on the evolution of metabolic regulation over evolutionary history. Consequently, many animals adapt to seasonal variations in caloric intake through their adipose tissue-based energy reserves. Additionally, they may catabolize somatic tissues such as muscle in severe conditions. Indeed, an extreme example is observed in certain animals such as planaria that dramatically regress in size in response to starvation [63]. However, when food becomes available again, their profound regenerative potential allows them to restore body mass. Remarkably, in the case of certain medusozoans, the adult can invert metamorphosis upon exposure to adverse conditions, wherein the pelagic medusa reverses the normal life cycle to become a juvenile benthic polyp [64]. Thus, in summary, numerous animal species have the potential to regress and regenerate to varying degrees in response to dietary restriction (DR). However, the molecular mechanisms controlling the induction of regenerative potential in DR are not fully understood.

Interestingly, DR is one of the most established means of modulating lifespan in a wide variety of species. Accordingly, one of the more reproducible observations about aging found in many laboratory animal models is that DR appears to forestall the onset of multiple markers of aging. Furthermore, DR decreases insulin/IGF-1 signaling (IIS), and increases activities of sirtuins and 5' AMPK, while decreasing mTOR activity, all of which are implicated in extending lifespan in model organisms [65,66]. The important role of metabolism in aging is consistent with the modulation of the activity of the nutrient-sensing kinases, AMPK and mTOR, by changes in the cell's nutritional state and the associated ATP levels even though cellular ATP concentration is generally well in excess of the Michaelis constant (K_m) of most signaling pathway kinases. Therefore, it is intriguing to consider that DR may induce, to some extent, tissue regeneration. Indeed, this may be the pathway through which DR, or targeted modifications in proteins such as AMPK, mTOR or IIS molecules, can modulate lifespan.

The somatic restriction theory of aging

The diverse facets of aging often appear difficult to harmonize. For example, aging could be the result of telomerase repression in somatic cells and consequent telomere shortening and cell senescence. However, there are also environmental factors such as metabolic stress associated with IIS signaling and AMPK or mTOR signaling or lack of sirtuin histone deacetylase activity. We propose that the diverse observations related to aging can be largely harmonized through what we designate as the 'somatic restriction theory of aging'. We suggest that during development, global programmed alterations in gene expression occur in many cells and tissues of the body. Salient examples of the above include those spanning the PT, EFT, NT and AT, though it is likely others exist as well. These global alterations result in a progressive restriction of the differentiated state of somatic cells that benefits the organism in the pre- and perireproductive period by reducing the risks of developmental plasticity and its associated oncogenesis [67] as predicted by Williams.

In addition, we propose that the narrowing of facultative plasticity of the differentiated state of cells in somatic tissues leads to a progressive failure to regenerate tissues in response to damage with regeneration being replaced with interstitial fibrosis. We further postulate that the developmental loss of regenerative potential leads to a switch toward a program whereby DNA damage leads to senescence as opposed to apoptosis. In other words, if tissues can robustly regenerate, then the cells can undergo apoptosis when they experience significant DNA damage. However, if tissues have lost regenerative potential, then damaged cells are more likely to institute DNA checkpoint arrest and subsequent senescence instead of apoptosis as a means of maintaining tissue function. Last, with regard to molecular mechanisms, we suggest that somatic restriction is mediated epigenetically by an altered distribution of heterochromatin as proposed theoretically [68,69] and empirically [70], and is therefore modifiable to some extent by feast/famine cycles, DR or potentially by partial reprogramming [71], and other novel interventions.

Early events associated with somatic restriction, such as developmental shifts in gene expression patterns, are likely attributable to altered chromatin structure. Accordingly, mammalian gene expression, although regulated on several levels, is perhaps most significantly regulated by transcription factors binding proximal and distal regulatory elements such as promoters and enhancers located in topologically associating domains that are constrained by insulating elements such as a transcriptional repressor CCCTC-binding factor (CTCF) binding sites. An example of the role of topologically associating domains in regulating the expression of developmentally timed gene expression is seen in the hemoglobin locus, where changes in chromatin structure define the progressive expression of genes from embryonic (ζ and ε) to fetal (γ) and adult (δ and β) globin genes [72]. Furthermore, an early determinative event in somatic restriction appears to be remodeling of the nuclear envelope and associated heterochromatin. Most eukaryotic cells are characterized by intranuclear euchromatin with heterochromatin being associated with the nuclear lamina on the inner surface of the nuclear membrane. Therefore, a shift in global gene expression may occur during embryonic development as the result of a shift in the composition of the protein components of the nuclear lamina from primarily lamin B receptor-dependent binding to a composite of lamin B receptor- and lamin A/C-dependent binding. Accordingly, *LMNA*, which is expressed at low levels in ES cells, is induced upon cell differentiation *in vitro* and is also induced on a protein level in a tissue-specific manner around the EFT *in vivo* [73]. These findings suggest possible role of chromatin structure and nuclear lamina structure in the somatic restriction of regenerative potential.

Epigenetic modifications are important mediators of chromatin binding to the nuclear envelope and subsequent alterations in gene expression patterns. Epigenetics was a term first introduced by Conrad Waddington in the mid-20th century. Robin Holliday defined epigenetics as 'the study of the mechanisms that impart temporal and spatial control on the activities of all those genes required for the development of a complex organism from the zygote to the adult' [74]. In addition, epigenetic modifications of chromatin and the resulting alterations in nuclear architecture in aging and cancer are of growing interest. Epigenetic modifications such as histone acetylation and methylation modulate chromatin binding and gene expression. Accordingly, it is now recognized that nuclear lamina-associated chromatin is silenced through lamina-associated histone–deacetylase activity [75]. Furthermore, the loss of acetylation and gain of methylation of histones such as trimethylation at lysines 9 and 27 of histone 3 (H3K9me3 and H3K27Me3) together with chromatin-binding proteins, such as HP1, are commonly associated with transcriptionally inactive heterochromatin [76]. In contrast, transcriptionally active euchromatin is commonly associated with the trimethylation at lysines 4 and 36 of histone 3 (H3K4me3 and H3K36me3) marks [76], or acetylation of histone 3 such as H3K27Ac.

Epigenetic modifications such as histone methylation and acetylation appear to play key roles in aging. Indeed, H3K9Me3 and H3K27Me3 marks are lost in numerous species with age supporting generalized loss of lamina-associated heterochromatin during aging [77,78]. Furthermore, regarding acetylation, silent information regulator SIR2, an nicotinamide adenine dinucleotide $(NAD)^+$-dependent histone deacetylase, extends budding lifespan in yeast and increases longevity in other species, in part by stabilizing DNA containing repetitive sequences [79]. These observations are consistent with a role of histone acetylation in aging, and the protective role of heterochromatin in maintaining the genomic integrity of repetitive sequences. Significantly, the segmental premature aging syndrome known as Hutchinson-Gilford Progeria Syndrome (HGPS) results from a dominant gain-of-function heterozygous mutation of codon 608 (G608G: GGC>GGT) in the lamin A/C (*LMNA*) gene, causing elevated expression of an isoform of lamin A, termed progerin. However, progerin may also play a role in normal aging as supported by the progerin-associated loss of nuclear H3K9Me3 staining in fibroblasts during the course of normal aging both *in vivo* and *in vitro* [78]. Moreover, blocking progerin formation has been shown to restore normal H3K9Me3 levels in cultured fibroblasts [80], suggesting a potential upstream causal role for lamin A changes in the loss of heterochromatic H3K9Me3. Thus, there is support for regulation of aging by a loss of epigenetic gene silencing that is mediated by lamin A and progerin.

Epigenetic regulation occurs at the beginning of early ES cell differentiation when lineage specificity is controlled at least in part by PRC2, containing the histone H3K27 methyltransferase EZH2. However, some 75–87% of genes appear to be regulated by H3K27 methylation of associated CpG islands [81]. Therefore, it seems unlikely that this modification by itself is specific for regulating the subtle effects of transitions such as the EFT. Nevertheless, there is considerable evidence for a role of the methyltransferase in the somatic restriction of regeneration as well as aging. For example, EZH2 is implicated, together with Let-7 microRNAs in the NT of hematopoietic cells [82]. In addition, knock-down of utx-1, a histone demethylase specific for the EZH2 product, H3K27me3, extends the lifespan of *C. elegans* [83]. Further, EZH2 is observed to decrease during human fibroblast senescence leading to

increased expression of cyclin-dependent kinase inhibitors such as p16 (CDKN2A) [84]. Finally, we have observed that EZH2 is down-regulated in fibroblasts at the NT [West *et al.* 2019, Unpublished Data].

The repressive histone modifications H3K9Me3 and H3K27Me3 also play an important role in the constitutive heterochromatin associated with repetitive DNA such as those sequences associated with telomeric repeats as well as subtelomeric DNA [85]. In addition to descriptive studies associating telomeres with heterochromatin, ectopically introduced telomeric repeats have been shown to generate relatively large regions (~225 kb) of facultative heterochromatin marked by H3K27Me3 [86]. It is important to note that telomeres are particularly vulnerable sites for DNA damage [87]. Indeed, they typically contain 2–15 kb of the double stranded tandemly-repeated sequence $(TTAGGG)_n$ with a terminal single-stranded overhang that is thought to circle back and integrate in a structure designated a t-loop [88]. Thus, telomeres contain a terminal single-stranded region that is sensitive to oxidative damage. However, they are shielded from some DNA repair enzymes by the shelterin complex [89], which normally would prevent them from being recognized as double-stranded breaks. Consequently, the relative loss of H3K9Me3 and H3K27Me3, and replacement of telomeric chromatin with acetylation marks with age may increase the likelihood of telomeric damage and/or the recognition of the single-stranded terminus as DNA damage leading to checkpoint signaling. Interestingly, decreased Sir2 expression in yeast results in increased acetylation of histones in the subtelomeric region and increased expression of the telomeric transcript called telomeric repeat-containing RNA (TERRA) that, in turn, can decrease telomere length [90]. Therefore, both histone acetylation and methylation may affect aging via their effects on telomeres. Indeed, the regulation of the heterochromatic state of telomeres may depend in part on H3K9Me3 and H3K27Me3. TERRA RNA has been reported to bind PRC2 facilitating the production of initially an H3K27Me3 mark followed by H3K9Me3 marks and heterochromatin production [91]. These findings suggest that the PRC2 complex plays a role in telomere maintenance.

Other epigenetic changes in addition to the aforementioned age-related changes in histone modification and chromatin structure are widespread changes in methylated DNA at CpG sites. Changes in DNA methylation patterns with age have been reduced to a classifier capable of accurately predicting chronological age in blood and other cells [92]. These DNA methylation changes may be regulated by histone modifications such as H3K9Me3, which can alter the methylation of associated CpGs. Conversely, nonmethylated CpGs can affect histone modifications through zinc finger CxxC (ZF-CxxC) domain-containing proteins such as KDM2A and KDM2B [93]. Therefore, age-related histone modifications and DNA methylation may not be entirely independent phenomenon.

Metabolism & epigenetics

As mentioned previously, mammalian homologs of yeast Sir2 such as the genes *SIRT1* – *SIRT7* act as NAD^+-dependent 'erasers' that remove the acetyl group from histones. The dependency of SIRT1 on NAD^+ and its upregulation under DR conditions [94] have generated significant interest in its role in aging. However, while overexpressing *Sirt1* in mice results in a healthier metabolic profile [95], only modest effects on lifespan have been reported to date [96]. In contrast to the 'erasers', histone 'writers' such as the lysine methyltransferases and lysine acetyltransferases also appear to be modulated to some extent by the metabolic state of cells. Accordingly, energy metabolites S-adenosyl-methionine and CoA provide the methyl groups and acetyl groups, respectively, for lysine methyltransferases and lysine acetyltransferases. Thus, the levels of both S-adenosyl-methionine and acetyl-CoA are examples of indicators of nutritional status that provide a mechanistic link between metabolism and the epigenetic modulation of gene expression [97,98]. Therefore, these links and others perhaps not yet identified may eventually explain the molecular basis of how fluctuations in caloric intake facilitate the adaptive response of regression and regeneration of body mass and the linkage, if any, of regeneration and lifespan extension in response to DR.

Prospects for reprogramming senescence

The demonstration of the feasibility of mammalian somatic cell de-differentiation by somatic cell nuclear transfer certainly shows that cell differentiation is entirely reprogrammable [99]. Moreover, while an initial analysis of telomere length in animals produced by somatic cell cloning suggested that telomere length was not restored in the animals [100], subsequent studies showed that somatic cell nuclear transfer could restore aged telomere lengths back to embryonic lengths or even longer [101]. Further, subsequent studies of telomere length dynamics in isogenic cells showed that induced pluripotency can also restore aged cell telomere restriction fragment (TRF) length back to that of cultured ES cells or even longer, though that is not always the case [102]. In addition, subsequent studies of the epigenetics of aging show that reprogramming by induced pluripotency can completely reset age-related DNA methylation changes [71].

The above findings logically lead to the question of whether reprogramming can transiently reverse the developmental age of cells only as far back as the Weismann barrier (pre-EFT) without inducing pluripotency and thus retaining the differentiated state of cells. Accordingly, it may be possible to use reprogramming approaches to unlock latent regenerative plasticity by transiently restoring cells to a 'pre-EFT' state. We have designated this strategy-iTR [47]. Given the importance of telomerase repression and telomere shortening with aging, we envision that iTR would also benefit from the transient induction of telomerase, therefore, whether or not telomere length is modulated in the course of iTR, we will refer to both strategies simply as iTR.

The challenge of iTR-related approaches is to reprogram the epigenetic age and other hallmarks of aging in adult cells in a manner that maintains cell identity while restoring the regenerative potential to pre-EFT levels but not increasing the risk of an embryo-onco phenotype and cancer. Accordingly, it is important to note that pluripotency-related pathways are increasingly recognized as playing an important role in oncogenesis [103]. Indeed, the expression of reprogramming factors such as *Klf4, Oct4, Sox2* and *Myc* in mice has been reported to generate pluripotent cells and teratomas *in vivo* [104]. While teratomas are benign counterparts of malignant teratocarcinomas, teratomas themselves can adversely affect health. Therefore, Ocampo *et al.* used a strategy of limiting the expression of reprogramming factors (i.e., cyclic expression) to achieve a partially reprogrammed state, which resulted in prolonging lifespan in a mouse progeria model and in increased regeneration following muscle and pancreatic injury in naturally aged wild-type mice [105]. Moreover, they reported that numerous markers of aging were reversed including the restoration of young H3K9Me3 levels. Similarly, another study demonstrated that partial reprogramming *in vivo* resulted in improved wound healing with reduced scar formation [106]. Additionally, alternative approaches such as chemical induction of reprogramming may also influence regeneration potential. For example, a chemical cocktail shown to promote reprogramming *in vitro* had a protective effect against liver damage *in vivo* [107]. Although in their early stages, these studies and other ongoing efforts to reverse aging without triggering oncogenesis indicate the potential for the eventual translation of iTR approaches into simple, safe and cost-effective therapies. Indeed, iTR could have important implications for aging, epimorphic regeneration, and cancer diagnosis and therapy.

The developmental repression of regeneration affects multiple organ systems and iTR could benefit aged patients afflicted with degenerative conditions of numerous types. Cardiovascular disease appears to be a prime candidate for iTR because it is the greatest single cause of mortality in the USA and Europe, accounting for about 17.6 million deaths annually [108]. Indeed, the prevalence of heart failure in Americans in the years 2013–16 was approximately 6.2 million [108]. Cardiac regeneration is often seen in animals with relatively high capacity for regeneration such as zebrafish and axolotls or mice in the first neonatal week. As discussed earlier, detailed tracing studies suggest that cardiac regeneration occurs not through the mobilization of resident stem cells, but rather from regression of the differentiated cardiomyocyte into a precursor capable of proliferation and re-differentiation [56]. Therefore, is not unreasonable to assume that de-differentiation and re-differentiation, as opposed to the recruitment of resident stem cells, may be the mechanism of regeneration in many of these models of epimorphic regeneration. Further study is required to determine how pervasive this mechanism is in other organ systems.

Discussion

While certain evolutionarily primitive metazoans, and perhaps some vertebrates show no evidence of aging, mammals typically show an exponential increase in the risk of mortality with age. Mammalian aging can be viewed as a global developmental program in many cells and tissues in the body wherein somatic cells are progressively restricted in their capacity for immortal regeneration (Figure 4). Accordingly, these steps begin with the repression of the expression of the catalytic component of telomerase *TERT* resulting in the antagonistic pleiotropic effect of decreased risk of cancer early in life but a finite replicative lifespan of somatic cells leading to cell and tissue aging later in life. Other genes such as *TRIM71* also appear to be repressed at or around the time of the PT, and the loss of expression may also play a role in restricting cell division. Moreover, subsequent developmental restrictions associated with the EFT, NT and AT may also play a role in the cadence of developmental changes that repress tissue regeneration following the completion of organogenesis and subsequent growth. In summary, widespread gene expression changes, like *TERT* repression, occur early in the life cycle, in many tissues within the soma and these early changes may have an antagonistic pleiotropic effect later in life leading to tissue disrepair associated with aging.

Conrad Waddington is credited with the model of cell differentiation and development pictured as a 3D landscape with increasingly restrictive canals representing the increasingly restrictive fate potential of somatic

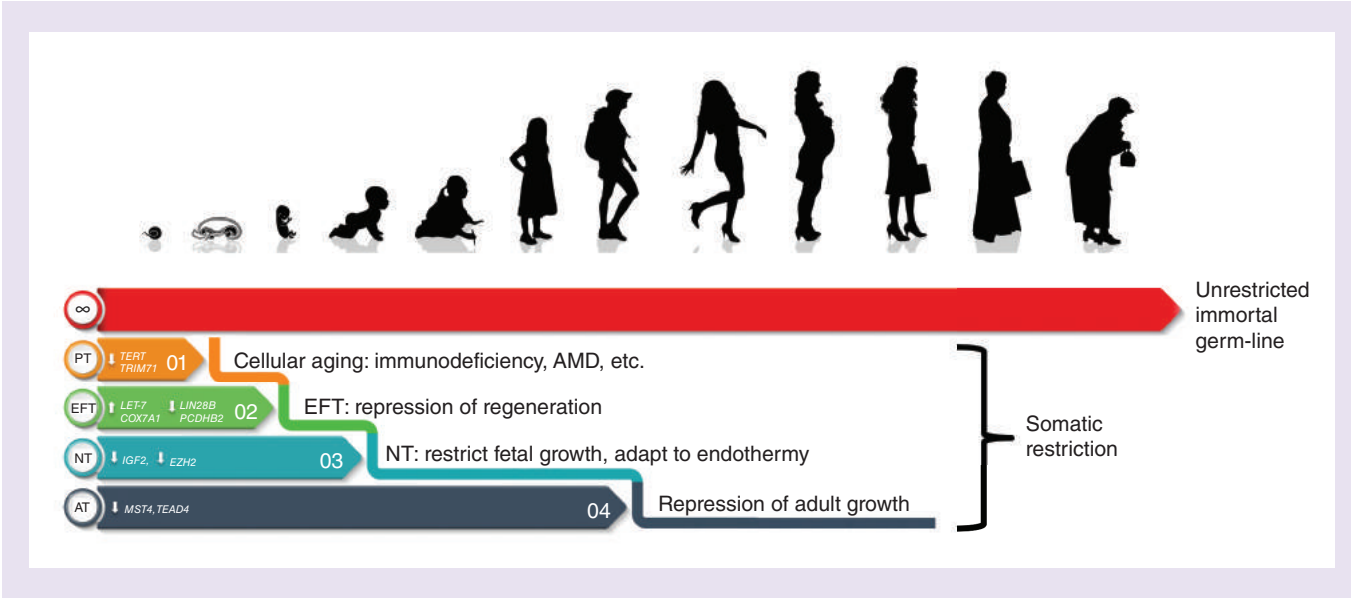

Figure 4. The stages of somatic restriction. While germ-line cells retain the potential to regenerate the species indefinitely, cells during the course of differentiation undergo progressive modifications that restrict either their capacity of indefinite replication (such as occurs at or around the time of the loss of pluripotency (pluripotency transition), or in subsequent steps that restrict their potential for regeneration and growth.
AMD: Age-related macular degeneration; AT: Adult transition; EFT: Embryonic–fetal transition; NT: Neonatal transition; PT: Pluripotency transition.

cells (Figure 5) [109]. The walls of the canals represent repressive mechanisms that constrain the plasticity of the differentiated state of the somatic cells wherein the topology and rigidity of cell fate is controlled by epigenetic influences tethered to gene expression (shown as cubes at the bottom of Figure 5). The staged developmental somatic restrictions (PT, EFT, NT and AT) are therefore seen as a narrowing and reinforcement of these walls to prevent cells from reversing their course or altering cell fate following, for example, the completion of embryonic development at the EFT. However, induced pluripotency can undo these restrictions, thus reversing the developmental age of cells to before the PT and thereby reactivating *TERT* expression, which restores the cells back to immortality. In contrast to induced pluripotency, iTR represents a novel strategy to revert somatic cells and tissues back only to the state of the cells before the EFT such that the plasticity of differentiated cells is restored as a means of promoting epimorphic regeneration.

The human body is thought to have considerable redundancy in homeostatic mechanisms. However, tissues within the body also have considerable interdependency. Accordingly, the time-dependent disrepair resulting in failure of just one tissue to maintain homeostasis can trigger cascades that throw other systems off balance. Indeed, this concept is supported by mathematical models of the resulting mortality curves that closely align with the empirical data. Furthermore, the models suggest that regenerative therapies that repair the initial degenerative triggering events will have a considerable impact on lifespan [110]. These theoretical predictions, combined with the power of technologies such as pluripotent stem cell-based cellular compositions, suggest that there may be a path not only to alleviate the high costs associated with the chronic degenerative diseases of aging [111] but also to extend human life expectancy. Since organisms with immortal somatic cells and full regenerative potential in the soma often show negligible senescence, the greatest advance may result from a strategy to implement transient induction of telomerase activity in combination with iTR.

Conclusion

Increasingly the theoretical framework underlying modern aging research is that progressive developmental transitions occur early in the life cycle that impact tissue regeneration and therefore aging in the soma. The theory of somatic restriction highlights the dichotomy of the immortal regenerative potential of the germ-line compared to the terminal mortal phenotype of most differentiated somatic cell types. The theory posits that somatic restriction occurs progressively in stages (pluripotency to differentiating embryo, embryo to fetal, fetal to neonate, and neonate

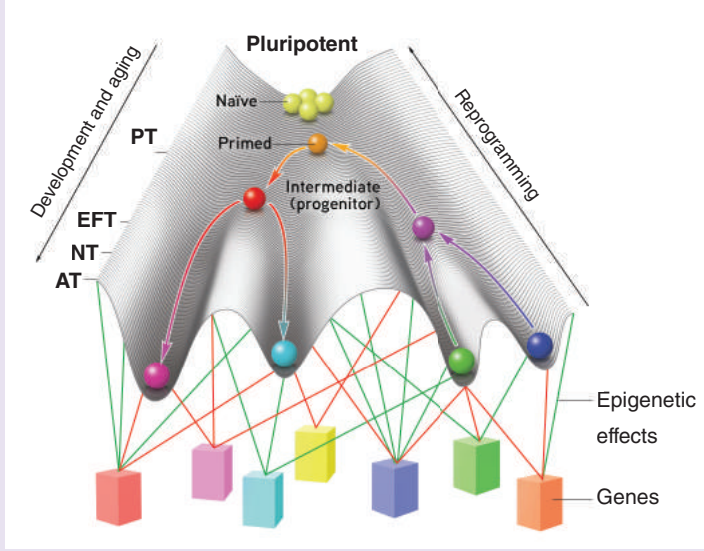

Figure 5. The Waddington epigenetic landscape of development, aging and reprogramming. Waddington proposed that genetic and epigenetic influences produced a developmental program of differentiation during development. This visual representation, based on Waddington's epigenetic landscape, illustrates somatic cell differentiation and somatic restriction at critical developmental transitions (PT, EFT, NT, AT) leading to loss of regenerative potential and aging. The transition from germ line-competent naive ES cells to primed ES cells leads to a reduced capacity for germ-line transmission but a retention of replicative immortality and the capacity to differentiate into all somatic cell types. The initiation of ES cell differentiation at the pluripotency transition leads to the production of diverse intermediate progenitors that generally lack telomerase expression. The subsequent differentiation of somatic cells and passage through the embryonic–fetal transition 'hardens' the pathways, reducing plasticity and thereby inhibiting regenerative potential. Induced pluripotent stem cell reprogramming is capable of restoring somatic cells back to either naive or primed immortality while iTR will likely be designed to partially reprogram cells back to a regenerative state while maintaining cell identity. The red line designates the Weismann barrier.
AT: Adult transition; EFT: Embryonic–fetal transition; ES: Embryonic stem; iTR: Induced tissue regeneration; NT: Neonatal transition; PT: Pluripotency transition.
Redrawn from [109].

to fully grown adult) and that many of these transitions occur globally in multiple organ systems. This conceptual framework provides a context for more detailed analytical studies of developmentally-regulated molecular pathways that were selected for reproductive fitness early in the life cycle, but result in homeostatic decline and failure of organ systems in aging adults (antagonistic pleiotropy). We conclude that modern molecular approaches to regenerative medicine such as reprogramming cells to pluripotency or partially reprogramming to induce tissue regeneration (iTR) effectively reverse most markers of aging and have significant potential for clinical application in aging.

Future perspective

The profound age-related demographic shift occurring in several of the largest industrialized countries of the world places an unprecedented strain on our healthcare systems. Fortunately, this tsunami of healthcare expenditures is contemporaneous with an equally unprecedented surge of advances in research into the biology of aging. This profound example of the confluence of an emerging supply and a rising demand presents the opportunity for innovative solutions to the healthcare crisis. Therefore, while many of the needs of the aged will be met on numerous fronts with diverse technologies, any therapeutic strategy aimed at the fundamental molecular biology of aging itself, that is also applicable to the diverse organ systems of the body, will almost certainly have the greatest impact on the healthcare industry and quality of life for the aging population. *In vitro* reprogramming is a promising technology, which can revert a donor cell of any age back to a state of pluripotency and immortality. Moreover, the fact that reprogramming also erases numerous markers of aging argues that cellular aging is reversable and thus opens the possibility of applying reprogramming as a therapeutic strategy to, for example, iTR in aging individuals.

However, the *in vivo* translation of reprogramming technologies to clinical applications will require significant preclinical testing to insure safety, particularly with regard to the potential to increase cancer risk.

Executive summary

Somatic restriction & antagonistic pleiotropy

- The advent of cell biology in the 19th century led to August Weismann's insightful hypothesis that heredity is transmitted by cells belonging to an immortal germ line, and that in most cases, the evolution of complex somatic cells and tissues is associated with a loss of immortal regeneration (somatic restriction) that results in aging. Thus, he correctly predicted the limited lifespan of cultured somatic cells due to cellular senescence.
- In 1957, George Williams hypothesized that aging evolved through a process of antagonistic pleiotropy, where traits benefitting fecundity early in life are selected for even though they simultaneously lead to age-related deterioration later in life.
- According to an emerging consensus view of the evolution of aging, primitive organisms showing negligible senescence have not traversed the Weismann barrier (loss of immortality and regeneration), while human somatic cell types cross the barrier early in development leading to downstream age-related change.

Somatic cell senescence

- The Weismann theory of somatic cell senescence was validated by Leonard Hayflick in the 1960s with the demonstration that normal diploid human somatic cell types display a finite replicative lifespan when cultured *in vitro*.
- Alexey Olovnikov first proposed that the Hayflick phenomenon was due to the absence of activity of a telomeric terminal transferase (telomerase) in somatic cells leading to progressive telomere shortening. In addition, he proposed that germ line and cancer cells can attain replicative immortality through the expression of an enzyme that can synthesize the telomeric DNA (the telomere hypothesis of cell aging and immortalization).
- The molecular cloning of the gene encoding the catalytic component of human telomerase (*TERT*) demonstrated that *TERT* is necessary and sufficient in most cell types to rescue cells from senescence.

Inhibition of regeneration

- Animals that retain replicative immortality and full regenerative potential as adults, such as hydra and planaria often show no evidence of aging.
- The search for genes that regulate developmental timing (heterochrony genes) in model organisms such as *Caenorhabditis elegans* led to the identification of miRNAs and RNA binding proteins such as let-7 and LIN28, which are highly conserved from worm to man.
- The LIN28/let-7 axis regulates diverse regenerative responses in tissues of all three germ layers; the decline of which is consistent with the theory of antagonistic pleiotropy. Notably, an embryonic pattern of gene expression in this axis appears to characterize numerous cancer cell types (the embryo-onco phenotype).

The embryonic–fetal transition

- Scarless regeneration appears to be markedly repressed in both placental and marsupial mammals at the embryonic–fetal transition, which occurs at about the ending of Carnegie Stage 23 (8 weeks of development) in human, and Theiler Stage 23 (16 dpc) in mice.
- Clonal human pluripotent cell-derived progenitor cell lines and corresponding fetal and adult counterparts enable an *in vitro* model for the molecular analysis of human cell developmental transitions.
- Transcriptional analysis has defined markers of the embryonic–fetal transition including the downregulation of *LIN28B* and *PCDHB2*, and the upregulation of *COX7A1*.

The neonatal transition

- The neonatal transition (NT) is characterized by alterations in numerous imprinted genes.
- The rapid pace of growth in the embryonic and fetal stages is markedly curtailed after parturition.
- The heart is an example of an organ profoundly altered during the NT. Mammalian cardiac muscle can regenerate scarlessly following trauma for the first week after the NT but not thereafter.

The adult transition

- The adult transition is defined as the cessation of growth in the adult at reproductive maturity. Some animals including vertebrates that show little evidence of aging also display the capacity for unlimited growth.
- Bidder's hypothesis proposes that the cessation of growth upon reaching adulthood is causative in aging.

Developmental plasticity in feast & famine

- Some animals such as planaria and jellyfish species can 'de-grow' in response to food scarcity.
- Humans have only a limited capacity to catabolize tissues in response to starvation and to regenerate upon the restoration of food supplies.
- Dietary restriction extends lifespan in numerous species through mechanisms that may include a limited induction of regenerative potential.

The somatic restriction theory of aging

- The somatic restriction theory proposes that aging is a global developmental program that progressively represses the capacity of most cells and tissues in the body for immortal regeneration.

- The regulation of chromatin structure, such as the regions of DNA compacted in heterochromatin versus euchromatin, is controlled in part by association with the nuclear lamina.
- Certain mutations in the nuclear lamin gene, *LMNA*, can lead to premature aging of diverse tissue types.
- Epigenetic modifications such as the acetylation of lysines 9 and 27 of histone 3 can compromise the stability of regions of the genome with repetitive sequences such as those in the telomere and subtelomere.

Metabolism & epigenetics

- The sirtuin family of histone deacetylases as well as the S-adenosyl-methionine-dependent histone methyltransferases and the acetyl-CoA-dependent histone acetyltransferases can translate metabolic states into epigenetic states that in turn alter gene expression states.

Prospects for reprogramming senescence

- The facile ability of somatic cell reprogramming to reset telomere length by reversing the repression of telomerase and its ability to reverse the epigenetic changes associated with aging suggest that it may be possible to induce tissue regeneration if the rejuvenation effects of reprogramming can be induced without reversion to pluripotency.
- Since pluripotent-specific gene expression is also observed in diverse types of cancer (the embryo-onco phenotype), *in vivo* reprogramming, if not tightly regulated, may increase the risk of teratomas or even malignancy.
- Induced tissue regeneration may have important applications in regenerative medicine, aging and cancer.

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Commentary

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Regenerative Medicine

What can we learn from California Institute for Regenerative Medicine's first 50 clinical trials?

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In 2004, California voters approved Proposition 71 [1], the California Stem Cell Research and Cures Initiative, which authorized US$3 billion to fund stem cell research. Proponents had advocated for the measure to have the resources to speed the delivery of stem cell treatments and cures to patients with unmet medical needs, especially to fund embryonic stem cell research, which was not receiving sufficient federal funding. The California Institute for Regenerative Medicine (CIRM) was established to administer the funds. CIRM has used the bond funds to make grants and loans to researchers, institutions and companies in California for basic research, training programs, the development of infrastructure and the support of clinical trials. This funding has launched California into the top ranks of stem cell science. CIRM currently has some 300 active stem cell programs in its portfolio and CIRM's funded researchers have published over 3000 articles in scientific journals [2].

Around a fifth of CIRM's expenditures to date, US$647 million as of January 2019, has been devoted to supporting clinical trials, part of CIRM's rapidly expanding translational portfolio [3]. Most of these grants and loans were made in the past 3 years. A total of 33 trials were added between 2015 and 2018 and US$100.5 million invested in 2018 alone [4], as CIRM sought to accelerate the testing of candidate stem cell therapies in advance of returning to the voters in 2020 for a US$5 billion renewal of funding. The investment in the clinical trials necessary for the US FDA's authorization to market the therapy reflects CIRM's realization that testing cell-based therapies is far more complex than chemically based small molecule drugs and that the high costs associated with the trials are beyond the resources of the academic medical centers and small biopharmaceutical companies engaging in stem cell research [4]. Moreover, the complexity of testing candidate stem cell-based biologics increases the uncertainties and risks inherent in early clinical trials, especially since most of these therapies are first-in-human clinical trials.

In May 2011, CIRM funded its first clinical trial, which was also the first-in-human testing of a therapy developed from human embryonic stem cells. Some 7.5 years later, in December 2018, CIRM's Board approved funding for its 50th clinical trial [5]. This article analyses those 50 clinical trials to assess CIRM's priorities. In view of the central role of CIRM in the pluripotent stem cell field, the profile of the trials also affords an opportunity to assess progress in the stem cell field.

Profile of CIRM's first 50 clinical trials

CIRM's initial 50 clinical trials covered 10 different disease areas [3]. The greatest number of trials was for candidate therapies for blood cancers, other blood diseases and solid cancers. Together, these three categories represent slightly more than half of all of CIRM's assisted clinical trials. The distribution of the disease areas of the 50 clinical trials is as follows: blood cancers 19%, other blood diseases 19%, solid cancers 14%, neurological applications 12%, kidney disorders 8%, diabetes, heart diseases, eye disorders and HIV-AIDS each 6% and bone diseases 4% [2]. Table 1 provides an overview of CIRM's first 50 trials, detailing the institution to which the grant was made, the disease area, cell type of interest and the trial phase.

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Table 1. California Institute for Regenerative Medicines first 50 clinical trial grants.

Investigating organization	Disease area	Type of cell	Phase
University of Southern California (CA, USA)	AMD	hESC	I
University of California, San Francisco (CA, USA)	Alpha thalassemia major	HSCs	I
BrainStorm Cell Therapeutics (NY, USA)	ALS	MSCs	III
Cedars-Sinai Medical Center (CA, USA)	ALS	Genetically engineered adult stem cell	I/II
UCSD	B cell cancers, leukemia	Monoclonal antibodies	I/II
Stanford University (CA, USA)	B cell cancers, leukemia	Adult CAR-T cell therapy	I
Sangamo BioSciences (CA, USA)	Beta thalassemia	Engineered blood cells	I/II
University of California, San Diego (CA, USA)	Blood cancer	Antibody	I
Stanford University	Blood cancer, solid tumors	Antibody	I
Angoicrine Bioscience (CA, USA)	Blood cancer	Engineered T cells	I
ImmunoCellular Therapeutics (CA, USA)	Brain cancer	Immune cells	III
Beckman Research Institute at City of Hope (CA, USA)	Brain cancer	CAR T cells	I
Forty Seven, Inc. (CA, USA)	Colon cancer	Antibody	I/II
Capricor Therapeutics (CA, USA)	Heart disease	Donor heart cells	II
Capricor Therapeutics	Heart failure	Heart cells	II
University of California, Davis (CA, USA)	HIV-related lymphoma	HSCs	I/II
Calimmune, Inc. (CA, USA)	HIV/AIDS	Genetically modified bone marrow cells	I/II
Beckman Research Institute at City of Hope (CA, USA)	HIV/AIDS	Genetically modified blood cells	I
University of California, Davis	Huntington's disease	Engineered MSCs	I/II
Stanford University	Kidney failure	T cells	I
Humacyte (NC, USA)	Kidney failure	Donor adult cells	III
Stanford University	Kidney failure	Blood stem cells, T cells	I
Humacyte (NC, USA)	Kidney failure	Donor adult stem cells	III
Medeor Therpeutics, Inc. (CA, USA)	Kidney failure	Donor blood forming cells	III
Nohla Therapeutics, Inc. (WA, USA)	Leukemia	HSC stem cells	II
Forty Seven, Inc. (CA, USA)	Leukemia	Monoclonal antibodies	I
University of California, Los Angeles (CA, USA)	Lung cancer	Gene-modified dendritic cells	I
Caladrius Biosciences (NJ, USA)	Melanoma	Patients own tumor cells	III
University of California, Los Angeles	Melanoma skin cancer	Gene edited immune cells	I
Poseida Therapeutics (CA, USA)	Multiple myeloma	CAR-T modified T stem cells	I
Calibr (CA, USA)	Osteoarthritis	Adult KA34 stem cells	I
University of California, Davis	Osteonecrosis	MSCs	I/II
Cedars-Sinai Medical Center	Pulminary hypertension	Donor heart cells	I/II
University of California, Irvine (CA, USA)	Retinitis pigmentosa	Retinal progenitor cells	I/II
JCyte (CA, USA)	Retinitis pigmentosa	Retinal progenitor cells	I/II
University of California, San Francisco	ART-SCID	Genetically modified blood cells	I
St Jude Children's Research Hospital (TN, USA)	X-SCID	Gene therapy	I/II
Stanford University	X-SCID	Monoclonal antibody	I/II
University of California, Los Angeles	Sickle cell disease	Genetically modified blood stem cells	I
Beckman Research Institute at City of Hope (CA, USA)	Sickle cell disease	Donor blood cells	I
Geron Corp (CA, USA)	Spinal cord injury	hESCs	I
Asterias Biotherapeutics (CA, USA)	Spinal cord injury	hESCs	I/II
SanBio, Inc. (CA, USA)	Stroke	Modified MSCs	II
Caladrius Biosciences (NJ, USA)	Type 1 diabetes	Modified T cells	II
ViaCyte, Inc. (CA, USA)	Type 1 diabetes	hESCs	I/II
ViaCyte, Inc.	Type 1 diabetes	hESCs	I/II
University of California, Los Angeles	X-linked chronic granulomatous	Genetically modified blood cells	I/II
San Bio, Inc. (CA, USA)	Stroke	Modified MSCs	II

ALS: Amyotrophic lateral sclerosis; AMD: Age-related macular degeneration; ART-SCID: Artemis-deficient severe combined immunodeficiency; CAR: Chimeric antigen receptor; hESC: Human embryonic stem cell; MSC: Mesenchymal stem/stromal cell; X-SCID: X-linked severe combined immunodeficiency.
Data taken from [2,6].

As might be expected, the majority of these 50 clinical trials are early phase trials, primarily Phase I trials designed to evaluate tolerability and safety and not to assess efficacy as later stage trials do. Of the 50 CIRM supported trials, 36 are either Phase I or Phase I/II, which are trials that can integrate the planning and transition from Phase I to Phase II. Most of the CIRM-supported Phase I trials are designed to enroll small numbers of participants, ranging from 6 to 18 patients, but there are a few trials with as many as 57, 112 or even 156 patients targeted. A few of CIRM's supported trials have progressed to Phase II and four have begun Phase III recruiting. These latter trials are the BrainStorm Cell Therapeutics (NY, USA) trial for amyotrophic lateral sclerosis, Medeor Therapeutics (CA, USA) trial for kidney disease and one of Humacyte's (NC, USA) two candidate therapies for kidney failure.

Two Phase III trials have been closed. Caladrius Biosciences (NJ, USA) terminated testing during a Phase III trial for an experimental therapy for melanoma skin cancer, ostensibly for business reasons. Also, ImmunoCellular Therapeutics (CA, USA) suspended its Phase III trial of a therapeutic for glioblastoma brain cancer, reportedly for lack of financial reasons. Because Phase III trials have to enroll a greater number of patients than earlier phases, they are much more expensive. Many investigational products flounder at this stage, sometimes described as the 'valley of death', because of the financial expense and because of the difficulty of enrolling a sufficient number of patients. Even if CIRM receives a second round of funding by the voters, it will lack the resources to fully underwrite Phase III of the clinical trials it has previously supported and will need a partnership from other funders.

CIRM has invested in stem cell work conducted both by academic institutions and small biotechnology companies in California. Although 91% of all CIRM's funding to date has been allocated to academic institutions [7], a substantial number of the CIRM-supported clinical trials are being conducted by small biotechnology companies. CIRM has made grants or loans to 16 such companies to conduct stem cell clinical trials and six of these companies have received CIRM support to conduct two clinical trials.

Although the campaign for Proposition 71 stressed the need to have public funding to develop therapeutics from human embryonic stem cells, only five of the clinical trials CIRM has supported to date have tested candidate therapies derived from human embryonic stem cells. None of the CIRM supported clinical trials are as yet evaluating candidate therapies developed from human induced pluripotent stem cells which were first developed after the referendum.

The first clinical trial CIRM funded in 2011 was Geron Corporation's Phase I trial to assess GRNOPC1, a human embryonic stem cell-derived candidate therapy for treatment of severe spinal cord injuries. However, after five patients were injected, apparently without any suffering serious adverse effects or evidence of immune rejection of GRNOPC1 even after the withdrawal of the immunosuppresive drug, Geron halted the trial. In a statement, the company claimed its decision was motivated by capital scarcity and uncertain economic conditions and not the lack of promise of stem cell therapies. Geron's then recently appointed Chief Executive Officer, who apparently had a different set of priorities than his predecessor, indicated that the company had decided to focus on its novel cancer therapies which were further along in development [8]. Geron eventually sold its stem cell research and intellectual property to Asterias Biotherapeutics (CA, USA), another small California-based biotechnology company. Asterias has also received funding from CIRM, for its Phase I/IIa trial with an expanded number of patients and approval from the US FDA for testing on more types of spinal cord injuries. Asterias has now treated 25 patients with no serious side effects and some encouraging results [5].

Another CIRM grant for a human embryonic stem cell derived therapy went to researchers at the University of Southern California for a Phase I trial with a therapeutic being tested for age-related dry macular degeneration, a popular target for pluripotent stem cell researchers. Two other awards were given to Viacyte, Inc. (CA, USA) for its two trials of therapeutics for Type 1 diabetes. Viacyte is currently testing a human embryonic stem cell derived therapeutic to replace lost beta cells in persons with diabetes. The therapeutic is being administered by inserting it in a small pouch that is transplanted under the patient's skin to protect it from the immune system.

It might be asked why CIRM, which was ostensibly established to promote embryonic stem cell research, has supported so few clinical trials with pluripotent stem cells. My informal discussions with staff of CIRM in June 2018 affirmed that CIRM has been open, even enthusiastic, to funding research and clinical trials with therapies developed from human embryonic stem cells when given the opportunity to do so. Given its resources, CIRM has been able to fund all promising proposals submitted for consideration, and apparently the overwhelming majority of these proposals have been for candidate therapeutics developed from adult stem cells, many of them for cancer therapies. So, the simplest explanation is that CIRM was not given the option of funding additional pluripotent stem cell clinical trials.

Also Proposition 71 instructs CIRM to make maximum use of resources by giving priority to the stem cell research with the greatest potential for therapies and cures [1]. In the 15 years since Proposition 71 was adopted, there have been promising developments in cancer treatments, gene editing, immunotherapy and gene therapy that were not anticipated in 2004. Many of the 45 other clinical trials receiving CIRM's support incorporate these innovations. As well as using adult stem cells of various types from patients and donors, some of which have been genetically modified, CIRM funded clinical trials also involve antibodies, chimeric antigen receptors (CAR-T therapeutics), a type of cancer immunotherapy, proteins, zinc finger nucleases, an artificial restriction enzyme engineering to target-specific DNA sequences, monoclonal antibodies and drugs designed to either enhance or suppress the activity of cells.

To provide some examples of innovative therapies being evaluated in CIRM supported clinical trials, the University of San Francisco (CA, USA) has a Phase I clinical trial using hematopoietic stem cells from the mother's blood marrow to treat babies in the womb with alpha thalassemia major, a blood disorder that is almost always fatal. BrainStorm Cell Therapeutics (CA, USA) is beginning a Phase III trial using mesenchymal stem cells taken from patients' own bone marrow and modified in the laboratory to boost production of neurotropic factors to support and protect neurons in patients with ALS. The University of California in San Diego is testing the antibody cirmtuzumab to disable a protein and thereby slow the growth of leukemia and make it more vulnerable to anti-cancer drugs. Stanford University (CA, USA) is recruiting patients for a B-cell cancers leukemia trial with CAR-T cell therapy that works by isolating patient's own T-immune cells and then genetically engineering them to recognize a protein on the surface of cancer cells and thereby trigger their destruction. Capricor, Inc. (CA, USA) has completed a Phase II trial for patients with heart disease associated with Duchenne muscular dystrophy using donor cells derived from the heart. The City of Hope (CA, USA) is recruiting patients for a Phase I trial in which zinc finger nucleases modified autologous hematopoietic stem progenitor cells along with escalating doses of busalfan will be applied to patients with HIV/AIDS. Angiocrine Bioscience, Inc. (CA, USA), CIRM's 50th clinical trial award, plans to test genetically engineered cells derived from cord blood to see if it can help alleviate or accelerate recovery from the toxic side effects of chemotherapy in people undergoing treatment for lymphoma and other aggressive cancers of the blood and lymph systems.

Reflections

What does the profile of CIRM's first 50 clinical trials reveal about progress in the stem cell field? The profile of CIRM supported clinical trials indicates that the pluripotent stem cell field has developed more slowly than was anticipated by many supporters of Proposition 71 in 2004 who assumed that human embryonic stem cell research would quickly lead to the discovery of new therapies. Unfortunately, the publicity surrounding the pluripotent stem cells has often exaggerated positive claims and minimized the difficulties of translating pluripotent stem cells into therapies. The slow progress of gene therapy development should have provided a caution. Between 1989 and 2015, 2335 gene therapy clinical trials were completed, ongoing or being initiated worldwide, but it was not until 2012 that the first-gene therapy was approved in the EU and 2017 that the FDA approved a gene therapy for use in the USA [9,10]. Human embryonic stem cells were first derived in 1998 and human induced pluripotent stem cells initially discovered in 2007. The first clinical trial with human embryonic stem cells began in 2009. Therefore, the small number of pluripotent-based candidate therapies available in California is not surprising. However, this is likely to change in the near future.

The good news is that progress in bringing pluripotent stem cell-based therapeutics into clinical trials is being made both in California and elsewhere. A 2018 analysis of recent trends of clinical trials based on human pluripotent stem cells documented 29 such trials with therapeutics derived from human embryonic stem cells taking place in France, China, Brazil, Israel, Brazil, the UK, Canada, Korea and the USA. It also identified three clinical trials based on human induced pluripotent stem cells that were being conducted in Japan, Australia and the UK [11]. Additional trials with pluripotent stem cells have begun in the past year, several of which use induced pluripotent stem cells including trials in Japan for a variety of applications and a trial at UC San Diego Health utilizing induced pluripotent stem cells for colon cancer therapy [12]. Japan, which has a conditional approval scheme for regenerative medicine products, has accorded one induced stem cell treatment product conditional approval [13].

Analysis of all active CIRM grants as of June 2019 from the listing on its website [6] indicates that CIRM has many pluripotent stem cell research projects in its pipeline. CIRM divides its funding categories into several categories depending on the stage of development. Of the inception discovery stage projects CIRM funded in January 2019, nine were using adult stem cells, nine were employing induced pluripotent stem cells, three were

exploring therapies with human embryonic stem cells, one project involved direct programming, and the approach of one research team was to combine human embryonic stem cells and induced pluripotent stems. Added together, 13 of the 23 projects in the inception discovery stage were exploring potential therapies developed from pluripotent stem cells.

The next stage of research advancement, the quest discovery stage, had a similar profile. Eight of the grants utilized some form of adult stem cell, but there were a greater number of projects exploring applications of pluripotent stem cells. In this category there were eight proposed therapies derived from human embryonic stem cells. Another seven sponsors were exploring candidate therapies from induced pluripotent stem cells while three combined the two types of pluripotent stem cells. The other quest discovery stage initiatives were investigating the application of monoclonal antibodies, encapsulated enriched beta clusters, nanoparticles and direct programming.

CIRM-supported therapeutic translation research projects were equally divided between those using adult stem cells and pluripotent stem cells. The figures were four adult stem cell projects, two initiatives derived from human embryonic stem cell and two utilizing human induced pluripotent stem cells.

Late stage preclinical projects presumably on the threshold of seeking FDA approval were similarly equally divided between adult and pluripotent stem cell projects. Here, the figures were two applications of adult stem cells and one each of human embryonic stem cells and human induced pluripotent stem cells.

Conclusion

Now 15 years after the adoption of Proposition 71 CIRM is getting ready to ask the people of California for another US$5 billion to support the development of the stem cell field. If CIRM receives a second round of funding from the voters of California in 2020, it will be well placed to continue its leadership role in the stem cell field. Moreover, it will be able to play a central role in bringing a series of pluripotent-based stem cell therapies through the clinical trial process and hopefully for at least some of them to receive FDA approval.

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Commentary

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Regenerative Medicine

Engineering strategies for generating hypoimmunogenic cells with high clinical and commercial value

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The engineering of allogeneic cells to be 'hypoimmunogenic', also referred to as 'universal', so that they evade a host's immune system would be transformative for regenerative medicine. It would be one of the biggest advances toward the wide-spread translation of cell therapies from bench to bedside since the generation of human pluripotent stem cells (hPSCs) of embryonic origin in 1998 [1] and through cellular reprogramming in 2007 [2]. Importantly, human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) provide a cell source with unlimited proliferation capacity and a potential to generate any human cell type. This paper outlines the scientific principles underlying the generation of universal allogeneic cells, considers the main comparative strategies that have been explored to date, and discusses recently published experimental data.

The need for universal cells

Cell therapy can be autologous, where it is derived from a patient's own cells, or allogeneic, where it is produced from nonself donor cells and where large numbers of doses are generated from one donor to treat multiple patients. The allogeneic approach is superior on virtually all parameters [3], including high-quality source cells; industrially scalable and lower cost manufacturing; potential to treat genetic disorders with cells free of disease-causing mutations; commercialization using a simpler 'off-the-shelf' rather than a much more complicated 'service-based' business model; availability in acute medical situations; traditional pharma supply chain logistics; greater potential for reimbursement prices that are acceptable to payers; and ease of clinical adoption. The first-ever patient to have iPSC-derived therapy received autologous cells; however, due to the reasons noted above, the allogeneic approach is now favoured in iPSC-based human trials, including for Parkinson's disease, heart failure and spinal cord injury, which have recently been initiated in Japan, the world leader in iPSC research. The biggest challenge associated with allogeneic therapy is the powerful immunological response mounted by the host's immune system to nonself donor cells, leading to rejection.

Immune rejection of allogeneic cells is predominately due to donor and recipient mismatch of human leukocyte antigen (HLA) class Ia and class II proteins expressed on the cell surface. These proteins are highly polymorphic, meaning they show immense diversity between individuals in a population, and so provide an extremely effective mechanism to recognize 'self' from 'nonself'. Hence, HLA-mismatched allogeneic cells are rejected before permanent engraftment can take place to regenerate tissue or before a therapeutic impact can be achieved in cancer immunotherapy. As a result, to date, allogeneic therapies in human trials have largely used immunoprivileged mesenchymal stem cells for their potential transient regenerative effects through paracrine mechanisms, or administered hPSC-derived products at relatively immunoprivileged sites, such as the eye, together with short-term immunosuppression. The conventional approach to prevent immune rejection is to use strong immunosuppressant medication, but acute and chronic rejection remains a major problem. In addition, these drugs are associated with potent side effects, including opportunistic microbial infections and cancers, as well as kidney and liver toxicity.

Future Medicine

Since the advent of the field of regenerative medicine, its aspiration has always been: 'The ability to manufacture any human cell type, in any quantity desired, at a reasonable cost, for anyone needing it, without HLA matching or immunosuppression'. The clonal derivation of therapeutic cells from a hPSC source forms the basis to realizing the first part of this goal: 'The ability to manufacture any human cell type, in any quantity desired, at a reasonable cost'. To help achieve the second part of this aspiration, 'for anyone needing it, without HLA matching or immunosuppression', work is presently underway to engineer hESC and hiPSC lines to be hypoimmunogenic, from which universal nonpluripotent cells of desired cell lineages could be derived for therapeutic use.

Although, having a universal hPSC source would be ideal, the ability to directly engineer hypoimmunogenicity into nonpluripotent stem, progenitor or differentiated cell types would also be highly valuable for two reasons: first, our understanding of differentiating hPSCs to all desired cell types remains incomplete, and second, it would open up the possibility of making cell-based products currently in or approaching human trials today more broadly transplantable. In the perfect scenario, the need for immunosuppression would be completely eliminated with universal cells, meaning a broader usage of allogeneic cell therapeutics due to a much more favorable risk–benefit profile, although as a valuable first step, hypoimmunogenic cells may serve to significantly reduce immunosuppressive drug requirements in terms of dose and/or duration.

Engineering of HLA class Ia & II molecules

Strategies to ablate the highly polymorphic classical HLA class Ia (HLA-A, -B and -C) and class II molecules may play a key role in the engineering of hypoimmunogenic cells. HLA class Ia and class II mismatch results in a cytotoxic $CD8^+$ T cell and $CD4^+$ T helper cell response, respectively, with both ultimately leading to rejection. The advancement of genome-editing platforms, like CRISPR/Cas9 and others, means HLA class Ia and class II proteins can now relatively easily be eliminated (a discussion on the methods to do this is beyond the scope of this paper). The knockout of HLA class Ia is of much greater importance than that of HLA class II, given HLA class Ia proteins are expressed on all nucleated cells and platelets in the human body, whereas HLA class II molecules are only found on 'professional' antigen-presenting cells, such as macrophages, dendritic cells and B cells, but may be expressed under specific circumstances by a limited number of 'nonprofessional' antigen-presenting cells, such as fibroblasts or epithelial and endothelial cells.

Allogeneic HLA class Ia-negative cells are protected from cytotoxic $CD8^+$ T cells, but they face two significant problems. First, the absence of HLA class I proteins on cells means natural killer (NK) cells can no longer identify them as self and so kill them, in what is called a 'missing self' response [4]. To overcome this serious hurdle, groups engineering HLA class Ia-negative cells knock in one or more immunomodulatory molecules in an attempt to inhibit NK cell-mediated cytolysis. Second, by presenting cancer and virus-derived antigens to cytotoxic $CD8^+$ T cells, HLA class Ia plays a pivotal role in cancer immune surveillance as well as the removal of virally infected cells [5]. Hence, HLA class Ia-deficient cells may lose their predominant mechanism for doing this. As a worst case but presently only hypothetical scenario, if HLA class Ia-negative cells that express immunomodulatory molecules transformed to become cancerous, they may potentially be transmittable from one individual to another, given the absence of markers by which to be recognized as nonself and the presence of a shield to protect against a missing self response. It is not inconceivable that hypoimmunogenic allogeneic cells for human use in clinical trials and beyond may need to be engineered with HLA class Ia being left intact. A safety net in the form of a 'suicide switch' or another analogous mechanism might be applied to hypoimmunogenic cells, which could be activated in the event of complications to attempt to clear administered cells, although such approaches remain largely unproven in this context.

Abolition of HLA class II does not result in a 'missing self' response. Although, elimination of HLA class II in addition to HLA class Ia may be largely unnecessary, given only antigen-presenting cells express HLA class II, and if knocked out it would render these cells nonfunctional anyway.

Engineering of immunomodulatory HLA class Ib & immune checkpoint molecules

The expression of tolerance-promoting immunomodulatory molecules on the surface of allogeneic cells will be crucial in achieving a state of hypoimmunogenicity. First, they may be sufficient by themselves to shield HLA class Ia- and II-positive allogeneic cells from the host's immune system. Second, in the case of HLA class Ia-deficient cells, they will be required to protect against NK cell-mediated 'missing self' killing. Third, they may be needed to induce tolerance to allogeneic minor histocompatibility antigens (note, the major histocompatibility antigens, HLA class Ia and class II proteins, are the primary drivers of rejection, but mismatch of minor histocompatibility

antigens may also play a role). In a number of physiological and pathological states, immunomodulatory molecules occur naturally. Such molecules include HLA-G, HLA-E, CD47 and PD-L1, which are all deployed to a greater or lesser extent in fetal–maternal immune tolerance as well as by cancer and certain pathogens as an immune escape mechanism. Over the last few years, these four molecules have emerged as the top immunomodulatory contenders in the engineering of universal cells.

Experimental data published to date show that engineering approaches to express immunomodulatory molecules on hPSCs fall into two groups: 'HLA class Ib' and 'immune checkpoint' strategies. Other immunomodulatory molecules not neatly fitting into these two categories may also play a role in the future. HLA-G and HLA-E are nonclassical HLA class Ib molecules, which unlike HLA class Ia and class II proteins, display minimal polymorphisms, and are geared toward immune inhibition rather than activation. CD47 and PD-L1 are so-called 'immune checkpoints', which are inhibitory immune pathways critical for maintaining self-tolerance and controlling the extent of immune responses to minimize the killing of 'bystander' healthy cells.

HLA-G is a nonclassical HLA class Ib molecule, which is minimally polymorphic. Its chief role in nature is to protect a semi-allogeneic fetus from destruction by the mother's immune system during pregnancy, the only genuine physiological state of tolerance toward a semi-allograft [6]. HLA-G is strongly expressed on fetal-derived placental cells at the maternal–fetal interface, and is widely considered to be the major driver of tolerance in pregnancy [7]. Fetal-derived placental cells lack HLA-A and -B, but they do express HLA-C [8], meaning HLA-G must promote tolerance toward a highly polymorphic HLA class Ia molecule. Natural HLA-G polymorphisms that diminish HLA-G expression are associated with serious complications in pregnancy, including miscarriage, pre-eclampsia, preterm birth and recurrent spontaneous abortions [9]. HLA-G is unique among immunomodulatory molecules in that it has a potent immunosuppressive action on virtually all arms of the innate and adaptive immune systems, through inhibitory receptors such as ILT2, ILT4 and KIR2DL4, one or more of which is expressed on cytotoxic $CD8^+$ T cells, $CD4^+$ T helper cells, Treg cells, B cells, NK cells, macrophages, dendritic cells and monocytes [10,11].

Under normal physiological conditions, HLA-G is not expressed after birth, except in very few immune privileged tissues, such as the cornea; however, both cancer and pathogens utilize it as an immune escape mechanism [12]. Interestingly, growing evidence supports the role of HLA-G in allograft acceptance in solid organ transplantation. In 2000, it was demonstrated for the first time that HLA-G expression in an allograft or in serum following organ, in this case heart, transplantation was associated with a reduction in acute and chronic rejection events. Since then, in heart, lung, liver, kidney, liver–kidney and kidney–pancreas transplantation, expression of HLA-G in the allograft or in serum has always been associated with improved graft acceptance [6]. Note, HLA-G has seven different isoforms, four membrane-bound (HLA-G1, -G2, -G3 and -G4) and three soluble (HLA-G5, -G6 and -G7), with HLA-G1 and -G5 being the most potent [13] (HLA-G5 is the soluble equivalent of membrane-bound HLA-G1).

It has been reported that HLA class Ia-negative, HLA-G-positive iPSC-derived NK cells have reduced immunogenicity, leading to increased survival *in vitro* [14]. However, perhaps most importantly, it appears HLA-G is capable of promoting tolerance to allogeneic cells, in which HLA class Ia and class II has been left intact, such as in the following three reports. First, a study showed that allogeneic hPSCs and their epidermal derivatives engineered to express HLA-G1 maintained normal characteristics and had an inhibitory effect on NK cells and T cells [15]. Second, HLA-G1-expressing human dermal fibroblasts have been shown to have reduced alloreactivity [16]. Third, it has been demonstrated that hPSCs engineered to overexpress HLA-G1 maintained normal characteristics and produced differentiated HLA-G1-positive neural progenitor cells, which showed enhanced tolerance [17]. Hence, to date, HLA-G1 is the only immunomodulatory molecule that has been shown to single-handedly induce tolerance to allogeneic cells in which genetic engineering of HLA class Ia or class II molecules has not taken place.

HLA-E, like HLA-G, is a nonclassical HLA class Ib molecule; it is minimally polymorphic and expressed broadly in adult tissues at low levels [18]. The immunology of HLA-E is complex. A key function is to present peptides derived from the signal sequences of other HLA class I molecules (HLA-A, -B, -C and -G) to NK cells to prevent NK cell-mediated cytolysis [19]. It can also present self- and pathogen-derived antigens. At a simplistic level, HLA-E has a dual role, being an immune inhibitor via receptor CD94/NKG2A on NK and $CD8^+$ T cells, or an immune activator via receptor CD94/NKG2C on NK and $CD8^+$ T cells and via T-cell receptors on T cells [10,19]. Interestingly, HLA-G has been shown to enhance HLA-E surface expression [20]. While HLA-E is expressed on placental cells, its role is considered to be secondary to HLA-G in maternal–fetal tolerance [10]. Both cancers and viruses are known to exploit HLA-E for immune escape [21].

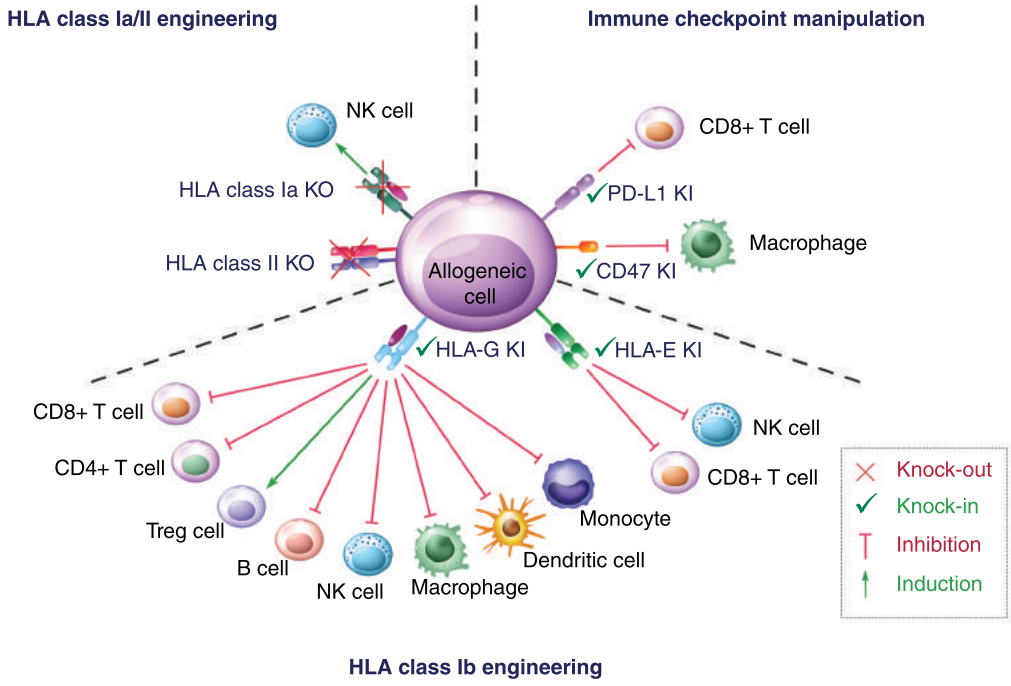

Figure 1. Engineering strategies to generate hypoimmunogenic human pluripotent stem cells.
HLA: Human leukocyte antigen; KO: Knock-out; KI: Knock-in; NK: Natural killer.

CD47 and PD-L1 are immune checkpoint proteins, which are being exploited in the generation of universal cells. CD47, which is expressed on all human cells [22], plays a key role in self-recognition by acting as a 'don't eat me' signal to macrophages to protect cells from phagocytosis [23]. This is achieved through interaction of CD47 with SIRPα/CD172a, an inhibitory receptor, found on macrophages. CD47 is extensively upregulated in solid and hematological malignancies for immune escape [24]. A number of anti-CD47 monoclonal antibodies are presently in human trials as a cancer immunotherapy. PD-L1, also known as CD274 or B7-H1, is expressed physiologically mainly on placental tissue and pathologically on cancer cells [25]. PD-L1 delivers a 'don't find me' signal to T cells, whereby it binds to the PD-1 receptor located on T cells to inhibit them. To date, several monoclonal antibodies blocking PD-L1 or PD-1 have been approved for the treatment of a range of cancers.

Bringing the key strategies together

It is clear that generating a universal hPSC line will deploy one or more of three key engineering strategies: deletion of HLA class Ia/II molecules, expression of HLA class Ib molecules, and manipulation of immune checkpoints (see Figure 1). These are the main strategies that have been explored in hPSCs to date, although others may also prove to be important as the field advances.

How close are we to universal cells?

Rapid progress has recently been made toward creating hypoimmunogenic cells. One group engineered HLA class Ia-negative hESCs with knock in of HLA-E [26]. Such hESCs and their differentiated derivatives ($CD45^+$ hematopoietic cells) escaped allogeneic $CD8^+$ T cell responses and were resistant to cytolysis by CD94/NKG2A-positive NK cells, both *in vitro* and in mice. However, a key issue with translating this approach to humans is that only around 50% of human NK cells express CD94/NKG2A [27], the receptor which binds to HLA-E to inhibit NK cells. Thus, HLA class Ia-negative, HLA-E-expressing allogeneic cells may still be eliminated by CD94/NKG2A-

negative NK cells. Another group engineered hiPSCs and their derivatives (endothelial cells and cardiomyocytes) to be HLA class I and II deficient, and to overexpress CD47 [28]. These hiPSCs and their derivatives were shown *in vitro* and *in vivo* to be protected from NK cell responses and immune rejection. Importantly, derivatives were able to survive long-term without immune suppression in mismatched, immune-competent, allogeneic, humanized mice. However, in this work, a fifth of endothelial cell allografts failed without explanation, raising important questions.

Another group deleted HLA class Ia and II expression on hESCs and their derivatives (endothelial cells and vascular smooth muscle cells) and knocked in HLA-G, CD47 and PD-L1 [29]. *In vitro* and mice studies showed T-cell responses to only be blunted, but not abolished. Although, NK cell and macrophage responses were minimal. The residual T-cell activity observed remains a key concern for translation to man. Most recently, a group showed that deletion of HLA class Ia or class II is not required in mouse ESCs and their cell derivatives for immune escape in immunocompetent, allogeneic mice, if eight immunomodulatory genes, including *CCL21*, *PD-L1*, *FasL*, *SERPINB9*, *H2-M3*, *CD47*, *CD200* and *MFGE8*, are expressed [30], some of which code for cell surface molecules and others for intracellular or cell-secreted molecules. This work needs to be substantiated with hPSCs. Moreover, large numbers of gene insertions coupled with the incomplete precision of even the most sophisticated genome editing technologies, like CRISPR/Cas9 [31], may carry an unacceptable risk of oncogenic DNA transformations in cells.

In 2018, our group acquired patents to a proprietary, novel, modified form of the *HLA-G1* gene in order to engineer hypoimmunogenic cells (we term this immunotolerance technology platform UniverCyte™). Previously, *in vitro*, this modified *HLA-G1* gene had been shown to reduce the immunogenicity of hPSCs and their epidermal derivatives [15], as well as of human dermal fibroblasts, when inserted directly into this nonpluripotent cell type [16]. In both cases, no manipulation of HLA class Ia or class II was performed. Moreover, this modified *HLA-G1* gene was strongly resistant to the intense silencing pressure exerted on native HLA-G expression in virtually all human cell types after birth. Hence, the modification allowed for persistent, stable and high expression of HLA-G1, with up to 99% of engineered cells displaying cell surface positivity [16].

Discussion with the inventor of this modified *HLA-G1* gene highlights it was evaluated *in vivo* [Hantash BM (2019), pers. comm.]. hPSCs, in which HLA class Ia and II had been left intact, were engineered to express the modified HLA-G1 protein. In humanized mice, modified-HLA-G1-positive hPSCs formed larger and heavier teratomas compared to empty vector hPSCs. This observation provides support for the notion that teratomas not expressing modified HLA-G1 were rejected, hence shrinking tumor size; whereas modified-HLA-G1-positive teratomas were potentially hypoimmunogenic and hence may have escaped recognition by a functional human immune system, and so continued to grow and expand. Further work is needed to substantiate this *in vivo* observation.

What next?

It can be envisaged that in the not-too-distant future, it will be necessary for allogeneic cell therapies to be hypoimmunogenic to be clinically and commercially competitive, but perhaps even more importantly, many cell therapies that are presently being developed on an autologous basis for fear of immune rejection with the allogeneic approach may be able to shift over to the latter route.

To date, no approach has been shown to be conclusive in engineering universal cells and many issues remain unresolved. The most pertinent questions include: given their role in eliminating cancerous and pathogen-infected cells, will deletion of HLA class Ia proteins in humans be safe? This is particularly important given transplanted cells may remain engrafted for years or decades to come. Which immunomodulatory molecules will prove to be safe and effective? Will the expression of one highly potent immunotolerant agent, like HLA-G, acting on multiple arms of the immune system be the optimal strategy? Or will a better approach be to knock in several immunomodulatory molecules, with each one providing protection against a specific type of immune cell? Given the immune system is highly complex, cross regulated and cascade dependant, are multiple modifications more likely to produce unpredictable and unwanted downstream immunological effects? Moreover, will large numbers of gene insertions result in an unacceptable oncogenic risk? Will immunomodulatory molecules in their native form be powerful enough or will they need to be further engineered, for example, to ensure persistent and high cell surface expression? Most efforts to date have focused on creating universal hPSC lines, but should work also concentrate on directly engineering hypoimmunogenicity into nonpluripotent stem, progenitor or differentiated cells? For commercial enterprises, a crucial point will be whether robust IP can be generated around naturally-occurring molecules.

That being said, swift progress is now being made to develop platform technologies to engineer hypoimmunogenic cells. These technologies, in combination with the use of allogeneic hPSCs, will help regenerative medicine fulfill its true potential, as first aspired to: 'The ability to manufacture any human cell type, in any quantity desired, at a reasonable cost, for anyone needing it, without HLA matching or immunosuppression'.

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Review

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Regenerative Medicine

Dimethyl sulfoxide: a central player since the dawn of cryobiology, is efficacy balanced by toxicity?

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Dimethyl sulfoxide (DMSO) is the cryoprotectant of choice for most animal cell systems since the early history of cryopreservation. It has been used for decades in many thousands of cell transplants. These treatments would not have taken place without suitable sources of DMSO that enabled stable and safe storage of bone marrow and blood cells until needed for transfusion. Nevertheless, its effects on cell biology and apparent toxicity in patients have been an ongoing topic of debate, driving the search for less cytotoxic cryoprotectants. This review seeks to place the toxicity of DMSO in context of its effectiveness. It will also consider means of reducing its toxic effects, the alternatives to its use and their readiness for active use in clinical settings.

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Dimethyl sulfoxide (Me_2SO; commonly referred to as DMSO) has been a keystone agent in the development of applied cryobiology over the past 50 years. The use of DMSO as a cryoprotectant capable of mitigating freezing-related cell injuries was first documented by Lovelock and Bishop during slow cooling of bull sperm [1].

As a dipolar aprotic solvent (i.e., a solvent that cannot donate a hydrogen bond), DMSO has an array of properties that allow its participation in numerous chemical reactions. This chemical versatility has been studied for many decades [2–4]. It is beyond the scope of this review to detail these and further information can be found elsewhere [5]; but in brief, the agent has strong molecular stability, a high dielectric constant, properties of basicity, solvation of salts, particularly anions and a propensity to act as the acceptor atom in hydrogen bonding, in other words, aprotic. From earliest studies, it was estimated that a hydrogen bond between DMSO and water is stronger by about 30% than that between two water molecules [2]. It was also shown that DMSO could protect both against biological injuries sustained during cryopreservation and against some aspects of radiation injury [6]. Against this backdrop of intensive study of DMSO in the 1950's and 1960's, it is perhaps not so surprising that Lovelock and Bishop took

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forward studies on cryopreservation using the agent. Over the next 20 years, several comprehensive reviews on the chemistry and pharmacology of DMSO were produced [5,7]. Readers are directed to these for further insights.

Pure DMSO is a clear colorless liquid, which crystallizes at +18.5°C, and is hygroscopic and exothermic when mixed with aqueous solutions. The property of strong hydrogen bonding with water imparts its antifreeze characteristics. DMSO can readily pass through biological membranes largely with minimal evidence of injury, has widespread solvation properties [5] and has found utility as a vehicle for clinical drug administration and dissolving water-insoluble compounds for test systems. Metabolic products of DMSO (notably dimethyl sulfide) frequently impart a perceived bitter taste and a breath odor similar to garlic [8], which has often raised questions about personal acceptance of therapies involving DMSO, for example, when using the chemical as a solvent for topical applications of different drugs. Pharmacological effects such as nephrotoxicity and ocular toxicity have been reported in experiments where repeated high daily oral or dermal dosing was performed [5]. The impact of DMSO in multiple organisms and biological systems has also been reported by Shu *et al.* [9].

Table 1 shows highlights for some of these effects. However, the direct relevance of these observations for cryobiological applications of DMSO is questionable.

This current review will attempt to examine the evidence for and against the biological impacts of the use of DMSO as practiced in modern cryopreservation protocols, with particular reference to clinical applications.

DMSO into cryopreservation studies

Following the report by Lovelock and Bishop [1], other contemporaneous studies were made into the cryobiological applications of DMSO and the potential pharmacological changes of the agent under the same exposure conditions. For example, Farrant *et al.* studied cryopreservation of smooth muscle preparations, and reported absence of any specific pharmacological responses, but did note some nonspecific osmotic damage [28]. This was one of the earliest reports to highlight one of the issues which have confused discussions about DMSO toxicity in cryopreservation – the potential osmotic injuries incurred when loading and unloading cells and tissues with any cryoprotective agent (CPA) – not only DMSO – in the concentrations required for cryopreservation (frequently at levels of 10% w/v or around 1 M), versus any specific molecular alterations dependent on DMSO chemistry. In the same era, Ashwood-Smith *et al.* (1964) reported successful cryopreservation by slow cooling of murine lymphocytes and storage for 3 months at -196°C using DMSO concentrations up to 15% (w/v) [29]. In the following years, slow cooling cryopreservation using DMSO was applied to a range of cells (mostly blood cell subsets) and tissues [30–35]. Today hematopoietic stem cell (HSC) transplantation has become well-established procedure for treatment of many disorders of the hematopoietic system. According to the European society of Blood and Marrow Transplantation (EBMT) survey, in 2014 more than 40,000 transplants have been performed in European countries, with more than 55% of them being autologous and thus requiring cryopreservation [36]. The main indications for HSC transplantation are leukemias, lymphoid neoplasias including non-Hodgkin lymphoma (NHL), solid tumors and nonmalignant disorders. Many of these require either autologous stem cell transplantation or umbilical cord blood (UCB) HSC infusion after high-dose chemotherapy and require the inclusion of mandatory cryopreservation step into the treatment protocol.

Biological & clinical impact of DMSO

Having confirmed cryoprotective properties and ensuring high viability of cells post-thaw, the direct administration of DMSO-containing cellular products can be associated with a number of well-identified side effects and shortcomings [37]. According to the EBMT analysis, around 30–60% DMSO-containing transplants are associated with at least one side effect or complication [38,39]. The most common are nausea, vomiting, hypertension/hypotension, sedation or headache [38]. However, there is evidence of more serious complications such as cardiac arrhythmia, encephalopathy, acute renal failure, bronchospasm and neurologic disorders. The direct infusion of highly osmolar (around 1400 mOsmol for 10% DMSO, compared with ~290 mOsm for serum) solution into the isotonic environment such as blood, will result in the significant water uptake by the infused cells and subsequent detrimental changes in cell homeostasis, leading to apoptosis and cell death [40]. In this case, the efficiency of the transplantation will be significantly decreased or it will fail completely. Such observations have driven the clinical and research institutions to search for ways of minimizing these potentially hazardous effects by either reducing the concentration of DMSO, removing the cryoprotectant from the cryopreserved cell-containing grafts prior to application (as described in section 5 below) or replacing DMSO with an alternative cryoprotectant.

Table 1. Examples of reported biological effects of dimethyl sulfoxide across a range of biological systems.

Cell type/line	DMSO	T°C	Effect	Ref.
Microorganisms				
Saccharomyces cerevisiae BY4743		CryoT°	– Increasing expression of genes responsible for synthesis of proteins, ergosterol, cell wall biosynthesis, ribosomal biosynthesis, accumulation of low molecular weight substances (glycerol, arginine, proline) – Downregulation of genes involved in β-oxidation of fatty acids	[10]
Stem cells				
Human embryonic stem cells HUES-7, foreskin-derived mesenchymal stem cells	0.01, 0.1, 1.0% (v/v)	RT°	– Dose-dependent changes in cell viability, morphology, adhesion and gene expression: inhibition of embryoid bodies formation, decrease in adhesion, cell death – Low and medium DMSO doses upregulate mesodermal markers – Higher DMSO doses downregulate ectodermal differentiation	[11]
Human umbilical cord blood stem cells	40%, 10% and DMSO removal	RT° and post thaw	– 40% DMSO lethal. 10% DMSO no viability reduction after 1 h. DMSO washout improved viability Optimum 7.5–10%	[12]
Immune cells				
Undifferentiated pre-B and pre-T (RPMI-8402) lymphoid cells	1.5% (v/v) 24–72 h†		– B-cells are resistant to DMSO – Increased membrane fluidity, cytosolic Ca^{2+}, NOS activity – Reversible G1 growth arrest of T cells, increased apoptosis, changes in TdT expression – Alterations in CD external antigens, TdT, TCR, RAG-1 expression, involved in growth arrest, decrease in *c-myc* expression, ODC activity and intracellular polyamine content, inappropriate differentiation leading to apoptosis triggering	[13–16]
Neural cells				
Retinal neuronal cell line	1, 2, 4, 8% (v/v)		– Caspase-3 independent death of neurons – Inhibition of mitochondrial respiration, increase of cytosolic Ca^{2+}, phosphatidylserine externalization, translocation of apoptosis-inducing factor (AIF) from mitochondria to the nucleus, PARP activation, DNA fragmentation	[17]
Rat hippocampal cells	0.5, 1% (v/v)		Neurodegeneration, apoptosis	[18]
Astrocytes	1–5% 24 h†		– Decreased survival and viability of astrocytes (>1% DMSO) – Mitochondria swelling. Decrease in mitochondrial membrane potential. Cytochrome C release from mitochondria. Increased ROS formation – Apoptosis. Increased caspase-3 expression, decreased anti-apoptotic protein Bcl-2 and procaspase-3 expression	[19]
Hepatic cells				
Rat primary hepatocytes	1%		– Prolonged pre-apoptotic phase in hepatocytes due to 24-h post-isolation DMSO treatment. Redistribution of caspase-9 from the nucleus to cytosol – Delayed apoptosis triggering	[20]
Blood cells				
PBMC; MM6, RAW 264.7	0.25, 0.5, 1, 2, 4%	37°C	– DMSO concentration of 4% suppresses ROS and IL-6 production in all cell types (leukocytes and RAW 264.7 are the most sensitive). 0.25 and 0.5% DMSO stimulate ROS and IL-6 formation in MM6	[21]
Red blood cells	0.2, 0.4, 0.6% 0–72 h†	37°C	– Increased hemolysis at 0.2% DMSO after 6 h and at 0.4% after 0 h	[22]
Platelets	0.25–6%	CryoT°	– Inhibition of aggregation, decreased thrombin generation	[22]
EAhy926 cells‡	0.2, 0.4, 0.6% 0–72 h†	37°C	– Decreased proliferation, growth arrest at G1 phase, increased apoptosis	[22]
Cancer cells				
Monocytes and T leukemic cells	0.1, 0.2, 0.5, 1, 2, 5%	37°C	– Time- and concentration-dependent DMSO cytotoxicity. DMSO concentrations ≥2% cause decreased proliferation after 24-h incubation	[23]
Promyelocytic leukemic HL-60 cells	1.25% 6 d†		– Inhibited proliferation at the day 2 DNA degradation – Chromatin condensation and nuclear fragmentation – Apoptosis in terminal differentiated HL-60	[24]
HL-60 cells	0.25–1%	37°C	– Stimulation of ROS production	[21]
HeLa cells	0.1–15% (v/v) 72 h†	37°C	– Decrease in cell number, growth suppression, alteration of cell shape	[25]
Colon adenocarcinoma tumor cells Caco 2/TC7	2–100%	37°C	– LDH release from cells at DMSO concentration >10%	[26]
Human embryonal carcinoma NEC-8 and NEC-14 lines	0.2–1%		– Increased DNA methylation, aberrant differentiation, downregulation of stemness markers and resistance to cisplatin	[27]

†Incubation time.
‡Hybridoma cell lines from umbilical vascular endothelial cells and lung cancer cells exhibiting properties of vascular endothelial cells.
MM6: Mono Mac 6; PBMC: Peripheral blood mononuclear cells; ROS: Reactive oxygen species.

Direct *in vivo* inoculation of DMSO into rat pups has been shown to elicit significant dose-dependent neurotoxic effects evidenced by increased levels of apoptosis in neural cells, the severity of which was age related [17,18]. The toxicity of DMSO for T cells and monocytes seems to be responsible for the observed anti-inflammatory and anti-oxidant effects of DMSO [23] and this has been confirmed from other data showing the key role of DMSO to be reduction of IL-6 and reactive oxygen species (ROS) in leucocytes [21], rather than free radical scavenging. While levels of DMSO in the order of 0.1% v/v are capable of suppressing cell growth, short incubation periods are not believed to incur permanent significant effects [25]. In clinical use, exposure of hematopoietic stem cells (HSC) to DMSO at a level of 5–10% v/v for 1–2 h has been shown not to be toxic [41,42]. Reduction of DMSO to 5% has been reported to achieve improved engraftment recovery times for leucocytes, neutrophils and platelets [43], while in hematopoietic stem cell preparations this level of DMSO compared with 10% also promotes colony-forming capacity [44], reduces side effects in patients, promotes levels of $CD34^+$ cells and reduces cell death [45,46].

On exposure to DMSO a range of effects are observed, in a variety of cell types, specifically on the plasma membrane including changes in membrane fluidity in RPMI-8402 cells [13], induction of membrane dysfunction in colon tumor cells [26], leading to loss of lipid asymmetry and flipping of phosphatidylserine in retinal neuronal cells [13,17]. In colon cancer cells, DMSO at the concentration of 0.1% (v/v) can change secondary structure of proteins, decrease cholesteryl esters in membrane lipids and affect their oxidation status [47]. These membrane effects impact on important transport systems, resulting in changes of cytosolic Ca^{2+} levels [13,17] and translocation of specific messengers involved in mRNA transcription and cell differentiation [48]. Ultimate consequences of these effects are arrested cell growth and programmed cell death. However, the biological effects of DMSO are not limited to the plasma membrane and under specific conditions, can include induction of changes in cytoskeleton [11], oocyte microfilaments [49] microtubules [50] and chromatin changes [13,47] as well as induction of cellular differentiation [47,51].

The capacity of DMSO to elicit a wide range of effects on cell biology may point to potential concerns around its use in clinical practice. Numerous cell types have shown adverse biological impact of exposure to DMSO (Table 1). For example, a range of blood-borne cell types have shown increased expression of IL-6 and ROS at levels of DMSO, as low as 0.2% (v/v) [21]. Studies on early lineage B- and T-cells have proven to be more sensitive to DMSO (1.0% v/v for 24–72 h) showing increased apoptosis, reversible growth arrest and inappropriate differentiation. Adverse impact of DMSO on functionality of nominally nonleucocyte blood components have also been reported such as increased hemolysis of erythrocytes, inhibited disaggregation and decreased thrombin in platelets [22] at similarly low levels of DMSO (0.2% v/v). Human stem cells (human embryonic and tissue-specific stem cells [52] otherwise known as mesenchymal stem [or stromal] cells [MSCs)] show dose dependent (0.1–1.0% v/v DMSO) effects of DMSO at room temperature on viability, morphology, adhesion and differentiation [48]. Alteration in apoptotic responses has also been reported in rat primary hepatocytes [20] while in a range of neural cell types, exposure to DMSO (at 0.5–8.0% v/v) can either promote or decrease apoptosis [17–19]. Even potentially more robust cancer cell lines have shown a range of effects on ROS levels [21], growth rate and cell viability [22–26] or aberrant differentiation [27]. Microorganisms are also not immune to the effects of DMSO which is reported to influence expression of a range of genes relevant to cell metabolism and all cell integrity [10].

It should be noted that some of the effects of DMSO, other than for cryoprotection, have been put to good use in cell cultures. For example, DMSO has been commonly used as an inducer of cellular differentiation probably associated with its ability to cause chromatin condensation [13] and alter gene expression [48,51,53,54]. This property has been employed for *in vitro* assays to provide a mature cellular phenotype [51,54] and to establish continuous cell lines with irreversibly different characteristics [51].

The DMSO effects described above were observed in noncryopreserved cells. Similar results were obtained for cryopreserved fetal liver cells (reduction in stemness genes *NANOG, OCT4* and *SOX2* expression) [55], bone marrow cells (structural and functional changes in cells) [56].

Secondary cryoprotective agents reducing DMSO

One way to mitigate potential or perceived problems of toxicity when using DMSO in cryopreservation is to supplement the cryoprotective agents (CPA) mixture with other agents which themselves have cryoprotectant effects (all-be-it at often lower efficiency than DMSO itself), but which provide an opportunity to reduce the overall DMSO concentration. Cryobiologists have experimented with such mixtures for more than 40 years [57], and while combinations with other CPAs such as glycerol and polypropylene glycol have been used, their addition to DMSO has been addressed here and in particular in Section 3. Oligosaccharides used in association with DMSO.

Among a wide variety of molecules with cryoprotective properties, naturally occurring sugars merit special attention. Their addition to CPA formulations is intended to increase cell dehydration and lessen the required concentration and time of exposure to DMSO. Application of monosaccharides in cryopreservation is less common than disaccharides, due to the possibility of nonenzymatic glycosylation of cell proteins and lipids that may alter their functions. Nevertheless, numerous studies have reported the efficiency of glucose containing methanol solutions for cryopreservation of sperm [38,39].

Disaccharides, in contrast, are widely used as nonpermeating CPA supplements for freeze drying, vitrification and slow freezing of various cell types [40]. Nonreducing disaccharides, particularly trehalose and sucrose, are known to have higher glass transition temperatures compared with DMSO, ethylene glycol and 1,2-propandiol [58]. This property ensures the formation of a uniform glass structure with high viscosity and low motility, thus, restricting molecular interactions and lowering the cell damage during rapid freezing [59].

At slow cooling rates, the main causes of cell injury are prolonged exposure to hypertonic environment and the growth of extracellular ice crystals. Inclusion of disaccharides into the CPA composition, on the one hand, can significantly influence the morphology, shape and size of ice crystals, which, in turn, affects cell survival [60]. On the other hand, hydroxyl groups of disaccharides can interact with the hydrophilic part of proteins and lipid head groups, thus stabilizing membranes and preventing their damage upon dehydration [61,62]. Recently, it has been shown that trehalose does not directly interact with proteins but instead played a role in compaction and stabilization of molecules by decreasing their hydration [63,64]. However, the exact mechanism for improvement of post-thaw cell survival in the presence of sugars in CPAs remains uncertain.

Since there are no specific transporters for disaccharides in mammalian cells, when added into CPA they tend to remain in the extracellular space. In this case, their cryoprotective action is insufficient to ensure the preservation of structural and functional integrity of cells. Meanwhile, application of disaccharides with permeating cryoprotectant such as DMSO, even in low concentrations, can significantly improve the cryopreservation outcome.

Combination of 0.2 M trehalose and 0.5 M DMSO has been shown to be effective for long-term storage of adipose aspirates [65]. Cells isolated from lipo-aspirates immediately after thawing had slightly retained growth rate compared with fresh ones, however, this difference lessened during the following cell culture. Comparable results were obtained using 6% trehalose, 4% DMSO and 90% fetal bovine serum (FBS) for cryopreservation of adipose tissue-derived MSCs (tissue-specific progenitor cells [52]) [66]. MSCs frozen for 6 and 12 months showed the viability of 80 and 70%, correspondingly. After being thawed and plated, cells started to proliferate and were capable of differentiation into adipogenic and endothelial lineages.

Solutions with trehalose and a reduced concentration of DMSO have been successfully used for cryopreservation of hematopoietic stem cells [67,68]. Samples cryopreserved with 2.5% DMSO without disaccharide showed a significant reduction in viability and clonogenic potential compared with those cryopreserved with trehalose or with 10% DMSO (control group). Application of another nonreducing disaccharide, sucrose, as a cryoprotectant for hematopoietic stem cells of umbilical cord blood and fetal liver was also effective [67,69]. For instance, the inclusion of 0.3 M sucrose as an additive to the CPA containing 5% DMSO resulted in a significant reduction of human fetal liver cell loss during cryopreservation, increased metabolic activity and function (the number of the myeloid-macrophage colonies). The recovery rate of cells cryopreserved under protection of 5% DMSO and 0.3 M sucrose mixture was 20% higher than without sugar and did not significantly differ from 10% DMSO alone [69]. However, the efficiency of sucrose to reduce DMSO concentration seems to be lower than that of trehalose. Sucrose inclusion into CPA allowed reduction of the DMSO concentration to only 5% while for trehalose similar viability results were obtained with 2.5% DMSO [67]. Experiments carried out by the group of Mantri *et al.* [70]. have shown comparable data: CPA containing 0.2 M trehalose with 2% DMSO was superior that the one with sucrose and DMSO in the same concentrations based on post-thaw viability results and assessment of hematopoietic colony formation.

Saccharides have been proven to be excellent candidates for cryopreservation of hepatocytes. Yang *et al.* [71] tested three sugars (glucose, sucrose and trehalose) and three sugar alcohols (xylitol, maltol and sorbitol) for improvement of the metabolic function of human hepatocytes that were cryopreserved using DMSO reduced to 5% concentration. Cell viability, post-thaw attachment rate and metabolic activity (albumin, glucose, urea metabolism) of cryopreserved hepatocytes were enhanced by the addition of 0.4 M sorbitol into 5% DMSO solution. The authors concluded that the mixture of 5% DMSO and 0.4 M sorbitol can replace 10% DMSO in CPA for cryopreservation of human hepatocytes. Recent data obtained by Cardoso, *et al.* [72] have indicated the positive effect of saccharides (sucrose, glucose and trehalose) on hepatocytes post-thaw viability when they

were used in combination with 5% DMSO. Among the different tested sugars, trehalose showed the best overall outcome.

To increase cell viability after cryopreservation under the protection of saccharides and low nontoxic DMSO concentrations, CPA can be additionally supplemented with antioxidants such as, catalase, superoxide dismutase, glutathione, vitamin C and vitamin E. This approach has been tested on human HSCs and amniotic fluid-derived stem cells. It was demonstrated that a solution containing 60 mmol/l trehalose, 2.5% DMSO, and 100 μg/ml catalase produced results similar to those for 10% DMSO in terms of survival, proliferation, and expression of stem cell markers in cryopreserved amniotic fluid-derived stem cells [73].

Reduction of DMSO concentration to a nontoxic level might be reached not only by addition of saccharides into CPA but is also feasible by preliminary cell loading with sugars. In this case, saccharides can ensure cell preservation during freezing acting as permeating cryoprotectants.

In order to achieve loading of relatively large sugar molecules into cells, several approaches have been proposed. Earlier techniques using microinjection and electroporation were associated with increased risk of cell damage [74,75]. In the case of electroporation, the observed cell damage can be mitigated by optimizing the electric field characteristics as shown by Dovgan *et al.* [76]. In that instance, human adipose tissue-derived cells loaded with trehalose by reversible electroporation had high survival rates and maintained their functional characteristic after freezing. Recently, proposed methods for saccharide loading are mainly based on the use of genetic engineering to create modified cell membrane pores or to insert disaccharide biosynthetic genes into the cell genome, which can be time consuming and require additional manipulations [77,78].

An alternative protocol for sugar loading in which a critical step is cell culture in the presence of disaccharide prior to cryopreservation, was shown by Oliver *et al.* [79]. Their data demonstrated that MSCs were capable to upload trehalose from extracellular space by a clathrin-dependent fluid phase endocytotic mechanism. The same approach has been utilized for DMSO-free cryopreservation of endothelial cells and it allowed to achieve high post-thaw cell metabolic activity [80]. This protocol was considered to be potentially applicable not only for trehalose but for other disaccharides as well.

Recently, it has been confirmed that inclusion of mannitol, lactose, sucrose, trehalose and raffinose into the culture medium for 24 h prior to freezing resulted in a significant increase of human dermal MSCs (tissue-specific progenitor cells) survival [81]. The authors claimed that optimal concentration of disaccharides in extracellular space for cell pretreatment (loading phase) was 0.2 M. Nonetheless, culture of human adipose tissue-derived MSCs in the presence of sucrose in the same concentration led to cell lysis by osmotic shock [82]. Positive results were achieved when using a solution containing 0.1 M of sucrose. These observations point to the need for screening of optimal sugar concentration and exposure time for each specific cell type.

In conclusion, the cryoprotective effect of saccharides in a wide variety of biological systems has been confirmed in numerous studies and when combined with relatively low concentrations of DMSO, they are capable to preserve both structure and function of frozen cells.

Supplementation of DMSO with high molecular weight macromolecules

Mixtures of DMSO with high molecular weight macromolecules may have some advantages over solutions containing DMSO alone. During a relatively short time of equilibration required for penetration of DMSO into a cell, high molecular weight agents do not penetrate and act by extracellular mechanisms. The cryoprotection of cells is elicited by their ability to absorb water and confine osmotic gradients. As polymers are high molecular weight macromolecules which lower the freezing point (on the basis of kinetics of cooling), they permit avoidance of excess supercooling and reduce the cooling rate required for cell survival. These properties combined with high viscosities at low temperature, prohibit water molecules from forming ice crystals outside the cell during cooling and suppress recrystallization during thawing [83]. Due to different mechanisms of action of nonpenetrative high molecular weight agents added together with penetrative DMSO into a cryoprotective solution, there may be an additive effect resulting in reduction of DMSO concentration required for cell cryoprotection.

Mixtures of high molecular weight macromolecules in combination with DMSO and other penetrative CPAs, are frequently used for cryopreservation of the most biological objects by vitrification. Here we put attention on conventional slow freezing cryopreservation as an approach that allows to reveal a potential of individual high molecular weight macromolecules to enable the use of reduced DMSO concentration and, thus, minimize the toxic effect of this cryoprotectant.

Among a wide variety of high molecular weight agents for cryobiological projects, preference should be given to those that have proven biocompatibility in cell culture practice and/or approved for clinical application. FBS is a common supplement in culture media which contains proteins, growth factors and hormones and promotes cell adhesion and proliferation. FBS in concentrations from 10 to 90% in combination with 10% DMSO is usually included into CPA. Bovine serum albumin (BSA) is a major component of FBS that provides its cryoprotective efficiency due to stabilization of osmotic pressure and protection of cells during freezing and thawing. Thereby, FBS can contribute to reduction of DMSO concentration. Thus, Zhao *et al.* [84] have shown that the use of either 20% FBS or 70% FBS in combination with 5% DMSO produced similar results to combination with 10% DMSO for recovery of fetal human liver $CD34^+$ cells after conventional slow freezing cryopreservation.

Due to its xenogeneic origin, the US FDA does not permit the use of FBS in products intended for humans. This problem can be avoided by using autologous or allogeneic human serum, although obviously this must be screened for human pathogens. Mitchell *et al.* [85] compared viability, morphology, growth and capacity to multilineage differentiation of equine MSCs after cryopreservation with either FBS or autologous or allogeneic serum. It has been shown that an allogeneic or autologous serum can be used for animal component-free MSC cryopreservation. Moreover, independently of the source of serum (autologous, allogeneic or xenogeneic) their presence in freezing media in combination with DMSO reduced to 5% was sufficient to preserve viability and growth of MSCs at the level of 10% DMSO.

Many disadvantages associated with the use of serum, such as inconstant quality, variation in composition among serum lots, risk of pathogenic contamination, high cost etc., drive the development of serum-free (cryopreservation media suitable for clinical-grade cell banking. Though the roles of individual components of serum have not been fully determined, proteins seem to play a key role in its cryoprotective properties. For replacement of FBS in culture media, the protein sericin, derived from the silkworm cocoon, was proposed by Terada *et al.* [86]. It has been reported that addition of sericin in culture medium promoted cell attachment and proliferation, stimulated the synthesis of collagen and demonstrated antioxidant and mitogenic activities [87]. Sericin has been successfully used also for cryopreservation of various cell types [87–89]. Sericin at a concentration of 1% in the freezing medium containing 10% DMSO, may substitute FBS for cryopreservation of human MSCs, but is not effective with reduced DMSO concentration [90]. On the other hand, the combination of 1% sericin and 7% DMSO in CPA ensured similar survival rate and function (insulin secretion) of rat pancreatic islets compared with 10% DMSO with FBS [91]. After transplantation, islets cryopreserved with either 10% DMSO or 7% DMSO in combination with 1% sericin indicated similar results in blood glucose control and renal subcapsular engraftment. Given these results, it seems that supplementation of CPA with sericin could allow reduction in DMSO concentration.

Among synthetic high molecular weight macromolecules, there are advantageous examples that are able to biodegrade *in vivo* after cell transplantation or transfusion, thus avoiding the need to remove them after cryopreservation. Hydroxyethyl starch (HES) is one such molecule that exhibits colloidal properties similar to human albumin and is widely used as a plasma substitute in adults for the treatment of shock caused by hemorrhage, burns, sepsis, ischemia/reperfusion and other injuries [92]. HES is metabolized by amylase and HES fragments are subsequently eliminated from the circulation after infusion. HES is free of side effects and often has been used in cryopreservation protocols. Properties of HES as a cryoprotectant have been reviewed by Stolzing *et al.* [93]. In concentrations $\geq$10% HES alone has demonstrated highly protective effects for the cryopreservation of keratinocytes [94,95], bone marrow cells [96], Chinese hamster cells [97], red blood cells of human [98,99], domestic animal origin [100] as well as other cells. A comparative evaluation of HES and DMSO as cryoprotectants was performed and in two studies, Ashwood-Smith *et al.* have shown that HES alone is less effective than DMSO [97,101]. Many more papers have focused on the admixture of HES with other CPAs including DMSO. Naaldijk *et al.* [102] have confirmed that HES alone could not provide sufficient cryoprotection of rat MSCs, but in combination with DMSO was effective. Using several cooling protocols, it has been shown that the supplementation of CPA with 5% of HES permitted reduction of DMSO concentration to 5%, although the highest viability of MSCs after post-thaw recovery in culture was observed using the mixture of 8% DMSO and 2% HES. It is interesting that the cryopreservation with HES of different molecular weights in the range from 109 to 609 kDa had a minor effect on survival and differentiation capacity of MSCs. A more recent study of this team has confirmed the ability of HES to facilitate reduction of DMSO concentration for adherent skin tissue cells (fibroblasts and keratinocytes) [103]. Cell lines and primary fibroblasts in suspension and monolayer have been cryopreserved using controlled rate freezing in CPA containing HES, DMSO and FBS. Testing cell number and viability, maintenance of nuclei, actin and mitochondria markers

it has been shown that cryopreservation of fibroblasts and keratinocytes both in suspension and in monolayer was successful using mixture of 5% DMSO, 5% HES and 90% FBS.

Due to a 6% HES of average MW 450,000 Da (Hespan®) is used for plasma expansion this hetastarch has been most widely studied in cryopreservation projects for reduction of DMSO toxicity. This is primarily related to cryopreservation of hematopoietic stem cells (HSCs). Mixing 5% DMSO with HES of MW 450,000 Da showed better recovery rates of $CD45^+$ and $CD34^+$ cells and colony forming unit (CFU) recovery after cryopreservation of HSCs derived from cord blood and peripheral blood than DMSO alone [104]. Cryopreservation of human bone marrow cells by conventional slow rate cooling protocol in CPA containing a combination of 5% DMSO, 6% HES and 4% human serum albumin, resulted in better survival and CFU recovery of HSCs in culture compared with 10% DMSO without serum [105]. Similar results have been obtained on canine bone marrow-derived HSCs cryopreserved using slow rate freezing in CPA containing either 10% DMSO and 90% FBS or 5% DMSO, 6% HES and 4% BSA [106]. Cryopreservation in the mixture HES, BSA and reduced concentration of DMSO provided higher post thaw cell viability and CFU recovery rates than 10% DMSO in fetal serum.

CPA containing HES with a lower average of MW (264,000 vs 450,000 Da) (Pentastarch) in combination with 25% human albumin and 5% DMSO has demonstrated high efficiency for cryopreservation of human cord blood HSCs [107]. A randomized Phase III clinical trial has shown that patients who received autologous peripheral blood HSCs cryopreserved with the combination of 5% DMSO, 6% HES and 4% human serum albumin, recovered their white blood cell level more quickly, than those who received peripheral blood (PB)-HSCs cryopreserved with 10% DMSO and 90% human serum [108]. This combination has demonstrated efficiency also on dog islets that after cryopreservation in CPA containing 6% HES and 4% FBS in composition with reduced to 5% DMSO maintained a high recovery rate.

In a number of countries, dextran is used clinically in plasma volume expansion, thrombosis prophylaxis and peripheral blood volume enhancement. Ashwood-Smith *et al.* [109] found that dextran of molecular weight (MW) 70,000 Da in concentration 9% has provided good protection of CHO cells cryopreserved with cooling rate approximately 20°C/min in CPA that contained 10% fetal calf serum. Addition of 1% DMSO to 9% dextran yielded survival rates as high as those obtained with 10% DMSO. In previous experiments of this group [109] performed on the same cell type (Chinese hamster cells) and using the same cryopreservation protocol in the absence of FBS showed no so significant improvement in cryoprotection when using a number of dextrans of average MW 10,000–500,000 Da. This data was corroborated by another report [110], where addition of 10% dextran of MW 100,000 Da into CPA without serum failed to improve viability of stem cells derived from human fetal liver after cryopreservation with DMSO in a range of concentrations from 2 to 10%.

Polyglucinum containing 6% of dextrans of MW 50,000–70,000 Da is also used for reduction of the volume of human UCB. Makashova *et al.* [111] have reported that dextrans in the concentration of 3% obtained after mixing of UCB with polyglucinum in the ratio 1:1 (v/v) is suitable both for collection and cryopreservation of nucleated cells. Cord blood nucleated cell concentrates containing 3% dextran were supplemented with DMSO in the range of concentration from 2.5 to 10% and cryopreserved using controlled rate freezing. Post thaw viability of $CD45^+$ cells did not differ in samples cryopreserved with the combination of 5%, 7.5% and 10% DMSO. Although this study did not compare post-thaw viability after cryopreservation with DMSO alone, the data indirectly demonstrate ability of dextran to reduce DMSO concentration in cryopreservation of hematopoietic cells.

In biomedical practice including cryobiological projects, synthetic high molecular weight polymers are widely used. These polymers demonstrate excellent biocompatibility, and are biodurable materials, because cells of animal and human tissues have no correspondent metabolizing enzymes and their biodegradation or resorption occurs by reticuloendothelial system. Among such agents, polyethylene glycol (PEG), methylcellulose (MC) and polyvinylpyrrolidone (PVP) are the most commonly used as nonpermeable cryoprotectants.

PEG is a polymer of ethylene glycol readily available in a wide range of molecular weights. It is nontoxic and has been applied in biomedical research as drug-delivery system, tissue engineering scaffolds, etc. PEG has been also included in solutions for hypothermic storage of kidney and liver [112,113]. There were several studies describing cryopreservation of red blood cells [114], pancreatic islets [115,116] and vein grafts with PEG alone [117]. However, more often it has been used in mixture with permeable CPAs, mostly DMSO to reduce its toxic effect.

Petrenko [110] has studied an influence of PEG of MW 1500, 3500 and 8000 Da in combination with different (2%, 5%, 10%) concentrations of DMSO on viability of human fetal liver cells after slow-rate cryopreservation. It has been shown that PEG with different molecular weight increased survival of cells after cryopreservation included three-step programmable freezing protocol (1°C/min down to -40°C, 10°C/min down to -80°C and storage in

liquid nitrogen). Survival rate determined after rapid thawing and washing out of CPAs increased with enhancement of molecular weight of PEG. The highest values for survival rates were obtained after cryopreservation of human fetal liver cells in CPA containing 6% PEG of MW 8000 Da in combination with DMSO. Supplementation of CPA with the PEG provided a high cell viability which enabled reduction of DMSO to 2% concentration.

Yang *et al.* [118] compared the effects of PEG and trehalose in CPA with a reduced concentration of DMSO on slow-freezing cryopreservation of bone marrow derived MSCs from mice, rats and calves. It has been shown that DMSO could be partially replaced by PEG and/or trehalose for MSCs cryopreservation from all three species. However, the post-thaw viability, metabolic activities, proliferative capacity and differentiation potential varied among the different species. CPA containing 2% PEG, 3% trehalose and 2% BSA in the combination with reduced to 5% concentration of DMSO demonstrated satisfactory values of viabilities and metabolic activities. In another study [119] these authors have shown the ability of PEG to reduce DMSO concentration for cryopreservation of human bone marrow-derived MSCs. The post-thawing viability of cells cryopreserved in CPA containing 7.5% DMSO, 2.5% PEG and 2% BSA was comparable with that obtained in conventional cryopreservation in CPA included 10% DMSO (82.9 ± 4.3% and 82.7 ± 3.7%, respectively). MSCs cryopreserved with reduced DMSO concentration were able to differentiate toward osteogenic, adipogenic and chondrogenic lineages. Unfortunately, the molecular weight of PEG in these reports has not been indicated. In the study [119] addition of 5 or 10% PEG of MW 8000 Da to CPA failed to reduce DMSO concentration in CPA after conventional slow-freezing cryopreservation of stallion germ cells, but gave better results than DMSO alone. At supplementation of CPA, containing 10% DMSO, enhanced viability of germ cells from post-pubertal stallions. Similarly, addition of 2.5% PEG with MW 1000 Da to CPA containing 10% DMSO and 10% FBS has significantly improved post-thaw recovery rate, culture potential and stem cell activity in cryopreservation of mouse testis cells enriched for spermatogonial stem cells [120]. In the frame of the current review it would be of interest to evaluate the ability of PEG to reduce DMSO concentration; however, it has not been done in currently available studies.

Methylcellulose (MC) is a macromolecular polymer that has been successfully used as a supplement in a serum-free cell cultures [121]. In such cultures, it has been shown that MC can replace animal serum in the standard CPA containing 10% FBS [122,123]. These data demonstrate that MC can be considered as an alternative to FBS in the cryopreservation of cells for their following clinical use. Thirumala *et al.* [124] have used methylcellulose either alone or in combination with reduced levels of DMSO for cryopreservation of short-term expanded (Passage 1) adult human adipose tissue derived stromal cells. It has been indicated that MC alone did not provide the protection of stromal cells after conventional slow-freezing cryopreservation protocol. After thawing high levels of necrotic and apoptotic cells have been observed. At the same time 1% MC produced similar satisfactory results with DMSO in the concentrations of 2, 4, 6, 8 or 10%. After cryopreservation in CPA containing the combination MC and reduced as low as 2% concentration of DMSO cells demonstrated high viability, low percentage of apoptotic cells and maintained their adipogenic and osteogenic potential.

PVP is a water soluble synthetic high molecular weight polymer, which has many desirable properties including low toxicity, chemical stability and good biocompatibility. Due to these properties, PVP has been used in biomedical applications such as binders in pharmaceutical tablets, hydrogels for wound dressings, disinfectants and as a blood plasma substitute. It has been reported that PVP with an average molecular weight of 40,000 can be used as alternative to permeable CPAs as DMSO for conventional slow-freezing cryopreservation of human adipose tissue-derived MSCs [125] and isolated rat islets [116]. Comparison of PVP and DMSO at concentrations of 10% after cryopreservation in CPA supplemented with 10% FBS using controlled rate freezing protocol has been studied on human Wharton's jelly-derived MSCs [126]. Although DMSO demonstrated a 20% higher post-thaw cell viability, after recovery in culture MSCs cryopreserved with both DMSO and PVP the cells maintained their morphological, immunophenotypic and differentiation characteristics. Poorer cryoprotective efficiency of 10% PVP compared with 10% DMSO has been also shown on human stromal vascular fraction of adipose tissue [127]. The combination of PVP and DMSO was tested for slow cooling cryopreservation of hepatocytes. Such combinations did not offer any advantage over DMSO alone for the cryopreservation of rat hepatocytes [128]. Gómez-Lechón *et al.* [129] reported that freshly isolated rat and dog hepatocytes, cryopreserved in CPA containing 10% DMSO and 2.3% PVP showed similar viability but significantly higher recovery (cell attachment to the plates and monolayer formation) than those frozen in CPA containing DMSO alone. Hepatocytes cryopreserved in CPA containing DMSO and mixture of DMSO and PVP similarly maintained their capacity of response to P450 inducers and drug-metabolizing activity. Thus, although there are no direct literature data that the supplementation of CPA with PVP reduces effective dose

Table 2. High molecular weight macromolecules able to reduce dimethyl sulfoxide concentration.

Agent	Cell type	Reduced DMSO concentration	Additional substitutes	Tests (activity, function)	Ref.
FBS 95%	Equine MSCs	5%	No	Viability, morphology, growth and capacity to multilineage differentiation	[85]
FBS 20%, 70%	Human fetal liver HSPCs	5%	No	Viability, CFU recovery	[84]
Sericin 1%	Rat pancreatic islets	7%	0.1 M trehalose	Survival, insulin secretion, blood glucose control and renal subcapsular engraftment	[91]
2–5% HES	Rat MSCs	5-8%	No	Viability, differentiation capacity	[102]
5% HES	Fibroblasts and keratinocytes	5%	90% FBS	Viability, maintenance of nuclei, actin and mitochondria markers	[103,132]
6% HES	Human BM-HSCs	5%	4% BSA	Survival, CFU recovery	[105]
6% HES	Canine-HSCs	5%	4% BSA	Survival, CFU recovery	[106]
6% HES (Pentastarch)	Human UCB-HSCs	5%	25% human albumin	Survival, CFU recovery, engraftment in SCID mice	[107]
6% HES	Human PB-HSCs	5%	4% human serum albumin	Viability of $CD34^+$ cells, engraftment	[108]
9% dextran (MW 70 kDa)	Chinese hamster ovary cells	1%	10% fetal calf serum	Survival	[109]
3% dextran (MW 50–70 kDa)	Human UCB nucleated cells	5%	No	Viability of $CD45^+$ cells	[111]
6% PEG (MW 8000 Da)	Human fetal liver HSPCs	2%	No	Survival rates	[110]
2% PEG (MW – NI)	Mice, rats and calves BM-MSCs	5%	3% trehalose and 2% BSA	Viability, proliferation and differentiation	[118]
2.5% PEG (MW – NI)	Human BM-MSCs	7.5%	2% BSA	Viability, differentiation	[119]
1% MC	Human AT-MSCs	2%	No	Viability, differentiation	[127]

BSA: Bovine serum albumin; CFU: Colony forming unit; FBS: Fetal bovine serum; HES: Hydroxyethyl starch; HSC: Hematopoietic stem cell; HSPC: Hematopoietic stem cell; MSC: Mesenchymal stem cell; UCB: Umbilical cord blood.

of DMSO, the mixture of PVP and DMSO manifests additive or synergistic enhancement of cell viability and function after cryopreservation.

As nonpermeable high molecular weight cryoprotectants special mention should be given to polyampholytes. Matsumura *et al.* [130,131] have reported that carboxylated poly-l-lysine of MW about 5000 Da at a concentration of 7.5% without any additional substitutes was as efficient for cryopreservation of multiple cell types as 10% of DMSO. The authors assume that cryoprotective mechanism of carboxylated poly-l-lysine differs from high molecular weight polymers and caused by membrane protection similar to antifreeze proteins.

Studies where supplementation of CPA with high molecular weight molecules resulted in reduction of DMSO concentration are summarized in the Table 2. Unfortunately, it is difficult to compare ability of different macromolecules to reduce DMSO concentration, because they were done on different cell types using different cryopreservation protocols. In many studies, additional substances such as trehalose, albumin or serum have also been used. The effect of the individual substances is difficult to assess. Anyway, these studies demonstrate that cryopreservation of different cell types using a mixture of DMSO and high molecular weight agents allows the reduction DMSO concentration resulted in avoiding it toxicity with simultaneous improvement of cell viability.

Removing DMSO from the cryopreserved cell-based grafts & preparations

In this section, we will discuss the current technologies for the removal of DMSO using the example of cryopreserved grafts, with the specific focus on clinical practices.

The common strategy of DMSO removal includes the gradual step-wise dilution of the cryoprotective agent-containing cell suspension with washing solution, at a rate that ensures the maintenance of cells within their osmotic tolerance limits, and thus, avoiding the extensive cell swelling [133,134]. For this approach, several parameters should be considered and adjusted in each specific case. The volume of the sample, concentration and osmolarity of the CPA solution applied during cryopreservation, the temperature of the cell preparation and finally the cell type are the most critical factors occurring during CPA removal. Considering these factors, it is possible to generate an optimized protocol for CPA removal by combining the washing solution(s) composition, addition/equilibration rate and the number of dilution steps needed to reach the best result. The composition of washing solution

would partly determine the overall rate of CPA removal. The larger the difference between osmolarities of washing and CPA solution, the slower the dilution rate used to maintain cells within osmotic tolerance limits. The supplementation of the washing solution with nonpermeable compounds (usually sugars) in quantities almost reaching the concentration of permeable CPA can moderate the influx of water into the cells and control the overall osmotic cell swelling [134]. After the washing medium is added, the cells are concentrated by centrifugation to remove the remaining CPA from the thawed sample, although that in turn, results in the additional stresses and damaging factors, associated with physical forces applied on the sensitized cells. Therefore, the development of the ideal protocol of CPA removal is challenging and requires good scientific understanding of many variables. Using mathematical modeling or computer simulation, significant efforts have been made to predict the outcomes and propose the best CPA addition/removal protocols for the specific applications [12,135,136]. However, our lack of ability to develop a unified protocol, due to the diversities in cellular characteristics, as well as the absence of easy to reach information sources (such as pre-adjusted software or open access on-line resources) to provide nonexpert researcher with the best options for the specific cell type, slow down the implementation of simulated data into routine cryopreservation practice.

The removal of DMSO in current clinical cryopreservation methods and banking of cell-based grafts is even more challenging, since additional highly regulated factors are introduced into the processing pipeline, but these are important to ensure patient safety and reproducibly effective application of the procedure. These issues have triggered the following multidisciplinary studies aimed to provide efficient options, combining cell biology/cryobiology and engineering approaches.

Removal of DMSO from cryopreserved hematopoietic stem cell grafts

The high clinical demand for hematopoietic stem cell transplants has driven the research toward the development of safe and efficient cryopreservation and cryobanking protocols for HSC grafts. In the majority of cases, the cryopreservation of HSC for clinical application is made under protection of 10% DMSO [36,137] that brings the problem of DMSO toxicity and optimization of the DMSO removal process to the forefront as a key issue to be resolved for sample processing.

Going back to the common steps of DMSO removal process, briefly discussed before, it is necessary to highlight at least several factors, which are critical in the case of clinical HSC processing.

1. Volume – typically a range of 25–100 ml of cryopreserved HSC graft is prepared in sealed cryogenic bags that require optimized protocols for DMSO removal. On the one hand, the rate of the dilution should be controlled to maintain the osmolarity of the total solution within the osmotic tolerance limits of processed cells, but on the other hand, it has to be fast enough to avoid the prolonged exposure of cells to the toxic DMSO.
2. Washing solution – research processes for DMSO removal, give the researcher wide flexibility in choosing the washing media components, however, the processing of cells for clinical use is more rigorous and consequently the choice is usually limited by the availability of suitable products that are currently registered pharmacopeia products permitted for direct infusion into a patient.
3. Closed environment – since the DMSO removal is considered as an additional open manipulation, the procedure of washing should be done either in the laboratory (i.e., clean room facilities suitable for GMP manufacturing) or prepared in closed systems, preserving sterility and thus, high safety of cell-based preparations.
4. Reproducibility and automation can be considered as a fourth major task in clinical DMSO removal.

Manual methods of DMSO removal based on dilution/centrifugation approach

The current standard methods for the DMSO removal from the cryopreserved HSC are based on the protocol proposed in 1995 by Rubinstein and colleagues from the New York Blood Center [138]. With or without small modifications, this method comprises two-step dilution of each stem cell unit (donor cell preparation with washing solution followed by centrifugation [133,139]. The precise step-by-step procedures of the current clinical-standard DMSO washing from HSC grafts are freely available from the FDA-approved product database (www.fda.gov). DMSO removal by washout methods has also been shown to improve viable recovery of cord blood cells [140]. Analyzing the information from different UCB banks, the common procedure of DMSO removal contains several steps, each at 4–10°C shown below:

- preparation of washing solutions in Luer-Lok syringes or washing bags;

- establishment of the closed system by connecting several bags/syringes by tubing and Luer-Lok connectors and clamps;
- unsealing and connecting the unit to the established closed system;
- first-step dilution of the unit with washing solution (5–25 ml) and transferring the unit into transfer/washing/transplant bag, using the established tubing;
- second-step dilution of the unit with larger volume of the washing solution;
- centrifugation at 400–880 $\times g$ for 10–20 min at 4°C;
- adjustment of the transplant volume using the washing solution or supernatant.

The compositions of the washing solution may vary in different institutions, but generally is limited to the mixtures of saline/electrolyte media (0.9% NaCl, Lactated Ringer's, Normosol-R®, Plasma-Lyte 148® or similar) with dextran-40 (5–10%), human serum albumin (1–5%) or HES (3–6%), Tetraspan® Volulyte®) or acid citrate dextrose anticoagulant [141–146].

Although the described procedure is considered as the standard, it is a very time- and labor-consuming process, which needs the involvement of trained personnel and special facilities assuring sterility of the graft. Moreover, the dilution process of the DMSO-containing grafts is not well controlled, since it is manual and affected by human factors and can result in lack of reproducibility. This drives the need of the development of automatic devices, providing a controlled, reproducible environment during the washing procedure.

Device assisted methods for DMSO removal based on dilution/centrifugation approach

Several devices have been proposed for automation of the centrifugation-based removal of cryoprotectant from cryopreserved hematopoietic grafts. The COBE® 2991 Cell Processor (Terumo BCT, Inc.) represents one of the solutions for fast automatic processing of blood or bone marrow units [139,142]. The key feature of the COBE 2991 cell processor is the presence of a flexible diaphragm. When the centrifuge is spinning, the flexible diaphragm located inside the centrifuge bowl, can be inflated with hydraulic fluid. As the flexible diaphragm inflates, it presses against the bottom of the cell processing bag. This gives the COBE 2991 cell processor the ability to express fluids and/or cells for removal or collection during centrifugation. Sepax S-100® (or Sepax 2 S-100), available from Biosafe SA®, GE Healthcare represents another option of automatic processing of hematopoietic grafts, including umbilical and peripheral blood or bone marrow [143,144]. The technology relies on the use of a separation chamber that provides both separation through rotation of the syringe chamber (centrifugation) and component transfer through displacement of the syringe piston. In both cases, manufacturers provide the possibility to prepare the washing procedure in a closed environment, significantly reducing the risks of contamination. Abonnenc *et al.,* showed that the Sepax-2 assisted washing of autologous HSC grafts resulted in 95% removal of DMSO (used as 10%) and post-wash viable $CD34^+$ cell recovery of around 80% [147].

Device-assisted methods of DMSO removal based on dilution/filtration approach

Another technologically different approach for CPA removal from the cryopreserved grafts is based on the filtration/dilution technique. The pioneering automation using this approach is based on the development of the spinning bowl for deglycerolizing of red blood cells, where cells initially sediment to the periphery of the bowl and the diluting solution is slowly introduced at the periphery [133,148]. Further progress in utilizing this technique resulted in the development of Haemonetics ACP215 Automated Cell Processor® (Haemonetics Corporation). However, this device is mainly employed for glycerolization and deglycerolization of red blood cell units and little is known about the use of this device for DMSO removal. Starting from the early 2000s several reports have been presented on the use of CytoMate® (Baxter/Nexell) closed fluid management system for automatic washing of hematopoietic transplants. Calmels *et al.* showed that the removal of DMSO from cryopreserved HSC grafts was associated with limited loss of $CD34^+$ cells, while 96% of DMSO was eliminated [149]. The successful application of this device was also confirmed for umbilical cord blood units [150] and peripheral blood products [151,152]. The authors showed that the degree of removal of DMSO is very high and therefore might ameliorate the transfusion-related side effects of cryopreserved HSC transplants. Recently, the new Lovo® filtration-based device has been launched by Fresenius Kabi for automatic washing and volume reduction of white blood cells [153]. Authors indicate that the Lovo device is an advantageous option for efficient washing and enriching of more than one thawed HSC graft simultaneously. The two- or three-cycle washing resulted in 96–98% elimination of DMSO and around 80% viable $CD34^+$ cell recovery from the cryopreserved peripheral blood HSC grafts [153].

Although, the presented automation solutions can significantly improve the reproducibility of processing of clinical HSC grafts, the high cost of these devices determines their adoption by large transfusion centers with high numbers of routine clinical applications of HSC transplantations. Adaptation of a hollow fiber dialysis approach for CPA removal can represent an alternative cost-efficient option for the controlled and safe washing of DMSO from cryopreserved tissue products [154,155], however, the more wide-spread use of this method is limited by the large volumes of the processed samples and requires adaptation for smaller size tissue products.

Removal of DMSO from small volume cell-based & tissue-engineered grafts

In the previous sections, we have reviewed the possibilities of clinical-grade DMSO removal from large-volume HSC grafts. However, currently the field of regenerative medicine has stepped far beyond HSC transplantation toward the development of effective treatments for nonhematopoietic disorders, specifying so-called multipotent MSCs as the most frequently applied cell type [155]. The accurate nomenclature and biological identity of these cell preparations is the subject of current debate and any differences within this cell type in response to DMSO is not yet clear [52,156,157].

The common practice of nonsystemic local administration of MSCs includes the application of more than 0.5×10^6 cells per kg of bodyweight in a limited volume of vehicle solution. In this case, the final administration dose can comprise up to 10^8 cells in only a few milliliters of the solution (<10 ml). Cryopreservation may represent the only choice to provide fast availability of cellular graft for application as well as assure the safety and quality of the product in repeatable application. However, the presence of high concentrations of DMSO in the vehicle solution may negatively act locally on the patient's cells and tissue within the site of injection as discussed above. For example, Galvao *et al.* showed that the DMSO induces retinal apoptosis *in vivo* at low concentrations (>1%) [17]. In an *in vitro* rat hippocampal culture preparation, DMSO produced neuronal loss at concentrations of 0.5 and 1.0% [18] and other reports are explained in Section 1. These reports confirm the need for reducing DMSO concentration in cryoprotective medium, especially when the cellular product is supposed to be used for the restoration of neural tissue. Moreover, safety guidance remains similar to large volume grafts (e.g., HSC transplants). Thus, the development of controllable engineering approaches of DMSO removal from the small volume cryopreserved grafts seems highly desirable to achieve the necessary therapeutic outcomes and minimize vehicle solution-associated side effects.

Recently, Tostoes *et al.* demonstrated the use of a novel filtration system for the removal of DMSO from cryopreserved small volume samples, containing 10^7 fetal lung fibroblasts or bone marrow MSCs [158]. The system resulted in the elimination of 85–95% of DMSO with more than 70% cell recovery. Memon *et al.* introduced the device for controllable extraction of DMSO from the frozen-thawed samples, based on sandwich structure, composed of two channels, separated by polytetrafluoroethylene (PTFE) membrane and syringe pumps [159].

Microfluidic devices have emerged as a promising technology for manipulating cell populations in small scaled microchannel systems [160]. The removal of CPA in such devices is achieved mainly through diffusion, reducing osmotic stresses and thus, damaging factors, which may occur in cells subjected to other techniques [161]. Moreover, microfluidics systems provide the user with highest degree of process control and reproducibility, which are important aspects of clinical processing of cell-based grafts. Park *et al.* applied an electro-wetting-on-dielectric-based microfluidic platform to screen DMSO mixtures on cell toxicity and cryoprotection efficiency [162]. Song *et al.* applied a microfluidic device to control loading and unloading of CPA by diffusion and laminar flow [160]. The group of Hubel also studied in some depth, the possibilities of using microfluidic devices based on diffusion-based extraction of DMSO from cell suspensions [161,163,164]. Starting from a two-channel flow system, where the DMSO-containing cell suspension is simultaneously flowing in parallel with the washing solution [163], the process has been optimized by introducing either several stage devices [164] or three-channel systems [161]. As a result, more than 95% of DMSO extraction can be achieved, while preserving the viability of cells. Authors assumed, that the transport of CPA from the cell-laden stream to the wash streams occurs solely by cross-stream diffusion, driven by the concentration gradient [161].

The administration of stem cells within biomaterial scaffolds may provide several advantages over the classical cell therapy in particular applications. 3D organization, more physiological elastic properties and cell–cell, cell–matrix interactions provide additional cell support and enhance the survival and therapeutic properties of stem cells in localized delivery. To provide wide availability of tissue-engineered grafts for further applications, efficient cryopreservation protocols are needed, including the efficient loading of CPA into 3D construct, finding the optimal freezing and thawing protocols and finally washing off the CPA from the thawed sample. In this case unusually, the

cell–cell and cell–matrix interactions may negatively affect cell viability and function after cryopreservation [165–167]. It has been shown that cells attached to gelatin- and Matrigel-based substrates can largely survive cryopreservation, although the cell viability of adherent MSCs is significantly lower (up to 35%) than that in suspension [165,166]. There are several reports on the cryopreservation of 3D tissue-engineered grafts, employing DMSO as a cryoprotectant [167–172]. However, the analysis of the functional properties of cryopreserved grafts has been currently limited to the *in vitro* studies. The DMSO unloading is usually prepared in static conditions (diffusion), by continuous changes of the CPA with washing solution (usually culture medium) [168,170]. In turn, the clinical cryobanking of tissue-engineered grafts would likely bring similar challenges, presented for cell-based transplants and thus, the development of reliable, automation devices, assuring reproducible cryopreservation outcomes and avoiding the possible toxicity associated with DMSO residues is an important aspect. Recently, several reports have shown the application of bioreactor-based devices for cryopreservation of MSCs within 3D scaffolds [167,169]. The application of oscillating perfusion systems for the controlled loading and unloading of DMSO resulted in a significant increase in MSC recovery within collagen scaffolds [169]. Applying a microfluidic bioreactor system Bissoyi *et al.* concluded that MSCs attached to a particular substrate can be effectively cryopreserved, if subjected to lower shear stress [167]. Altogether, the precise control over the environment could assist in the establishment of standardized cryopreservation protocols for complex tissue-engineered grafts for clinical applications.

Concluding remarks on post-thaw DMSO dilution to reduce negative effects

Much effort has been made toward the development of safe and reproducible systems and protocols of DMSO removal from the cryopreserved grafts in clinical scale, especially in the field of HSC transplantation. However, it remains the case, that DMSO elimination is not provided in the majority of HSC transplants [137] associated with negative effects. Therefore, the development of low-cost, automatic devices to remove DMSO, assuring minimal contamination probability and high control and reproducibility of the process may be considered critical in translating stem cell/tissue engineering research into a wider range of clinical practice.

Alternatives to DMSO

In recent years, the concept that it will be possible to replace DMSO in cryopreservation media while still maintaining good post-thaw recoveries has been discussed [173]. However, a wide application for such an approach has yet to be achieved. This section gives an objective assessment of the current status for this approach.

Theoretical alternatives based on equivalent mechanism of action

In the following, we discuss the potential substitution of the specific mechanisms that have been attributed to DMSO in generating a cryoprotective effect.

Perhaps the most significant aspect of DMSO-related cryoprotectant activity is linked to its capability to enter cells easily both through osmosis and potentially via aquaporin 3-independent channel diffusion [174]. One consequence of this is intracellular water displacement, which is a putative cryoprotective mechanism limiting intracellular ice crystal formation. Water displacement with limited osmotic stresses is also associated with other 'penetrating' cryoprotectants such as sugar alcohols (e.g., ethylene glycol, propylene glycol, glycerol) and other compounds. Although findings differ significantly for different cell types, in general terms, DMSO seems to exhibit the lowest membrane permeability coefficient times off all established penetrating cryoprotectants, with the possible exception of glycerol [175].

In connection with its ability to transition membranes, DMSO has further modulating effects on cell membrane permeability. In contrast to other cryoprotectants, DMSO is unique in dehydrating the phospholipid headgroups while raising the membrane phase transition temperature [176]. Even the quickly permeating glycerol seems to exert a very different hydrogen-bonding capability by increasing the repulsive forces between bilayers [177].

In turn, much of the damage related to cryopreservation is linked to membrane integrity. DMSO reduces membrane rigidity and, at high concentrations, induces pore formation, a mechanism presumed to contribute to reduction of mechanical and osmotic stresses during cryo-related cellular swelling and shrinking, tissue perturbation and ice formation [178]. To modulate membrane rigidity by other means, the amount of unsaturated fatty acids can be altered in pre-treatment [174] or lipid molecules can be added to the cryo-protective solution [179] (although the unique action of DMSO on lipid bilayers, for example on liposomes, needs to be considered in comparative studies) [180]. To induce membrane transition channels or pores, transfection agents like polyethylene glycol and

PVP have sometimes been added to cryo-protective solutions and have proven effective in comparison with DMSO [181–183].

Other chilling injuries have been attributed to lipid peroxidation and protein denaturation. Some antioxidant properties have been attributed to DMSO itself [184,185], specifically its reaction with hydroxyl radicals, producing methyl radicals, in turn producing ethane, and also undercutting the formation of carbonyl groups [186]. Addition of a wide variety of antioxidants has been attempted in the formulation of cryoprotectant solutions including mitragynine, glutathione, curcumin [187,188] including natural compounds [189] and mitochondria-targeted antioxidants [190,191]. It has been observed that other antioxidants may have at least equivalent antioxidative potency compared with DMSO [29]. However, in the specific pursuit of a cryoprotectant solution that emulates the molecular interactions of DMSO as closely as possible, it should be borne in mind that some of these alternatives (e.g., vitamin E) [192,193] are not known as particular scavengers of hydroxyl radicals.

Further innovations that have been considered in counteracting the results of oxidative damage and other cryoinjuries include the use of apoptosis inhibitors [194], with a caspase I inhibitor having been adopted into a commercially available medium (CryoStor CS5N - STEMCELL Technologies), and the use of anti-freeze proteins (AFP) as a DMSO alternative [195]. Incorporating AFP qualities into polymer design has shown some promise [196]; although cryoprotectant success with polymers goes well beyond the emulation of AFP macroscopic properties. Nonpenetrating polymers of high molecular weight have long been investigated, not for their capability to replace DMSO, but to supplement cryoprotection from the extracellular vantage point, by inhibiting ice crystallization and reducing mechanical damage. For example, for HES, a 6% admixture with 5% DMSO was generally favored for partially historical reasons [102]. Other polymers derived from natural compounds include gelatin, chitosan or alginate, all in themselves employed as an addition to, not a substitute for DMSO. However, as more recently developed polymers have entered the field and it has been suggested that they are a viable substitute for DMSO as discussed in Section 4 and in the following section.

Known DMSO-free solutions

In the preceding section, we have discussed the theoretical considerations and challenges of replacing DMSO with other compounds mainly from the perspective of the putative mechanism of action of DMSO as a cryoprotectant.

However, it may be that for any given purpose the specific effects of DMSO are not deemed pertinent or important enough to attempt their emulation. Replacement strategies may instead begin by first looking at evidence whether the alternative is at least as effective as DMSO.

Table 3 lists such examples of 'successful' DMSO-free cryopreservation, irrespective of whether these were aimed at addressing specific mechanisms of action. In consideration are only those studies that compared a DMSO-free cryo-protective solution directly to a DMSO-containing solution. Not included are studies that aimed at a reduction rather than total elimination of DMSO, or studies where the efficacy of the proposed cryo-protective solution was not experimentally compared with a DMSO-containing solution. Table 3 also excludes the various alternative solutions advanced only for gametes – it is a long-established tenet of the field that results on cryoprotectant efficacy and toxicity vary greatly between different cell types and situations, but we can at least summarize that for a variety of cells which are relevant in regenerative medicine and tissue engineering DMSO-free cryoprotection has been demonstrated using a number of cryo-protective solutions from 'traditional and basic' CPAs like glycerol, to different sugar combinations and novel polymer gels. Equivalence or superiority was established by a variety of assays, but often not going much beyond cell survival (which is not always the most conclusive measure) [102]. Further analysis of such solutions will be required in the context of advanced development. However, the ability to fully assess DMSO-free solutions depends on understanding their chemical compositions. As indicated in Table 4, a range of DMSO-free CPA are now commercially available, but solution composition is frequently subject to proprietary information.

Commercial development & regulatory considerations

Commercial preparations of DMSO containing cryoprotectants are now widely available and in use for final product formulation in more than 20 cell therapy clinical studies and trials (e.g., NCT00672542 [205], NCT02467387 [206], NCT02166177 [207], NCT01671072 [208], NCT02500849 [209], NCT02208362 [210], NCT02028455 [211], NCT02387723 [212], NCT0212988 [213], (Personal Communication from manufacturers and suppliers of CryStor® [Biolife, STEMCELL Technologies] and CellBanker® [Zenoaq, ASMBio]). However, improved cryoprotectant solutions are urgently needed, in part to address the ever-increasing complexity and di-

Table 3. Reported successful comparisons of 'dimethyl sulfoxide-free' cryopreservation solutions with dimethyl sulfoxide-based cryoprotective solutions.

DMSO-free CPA composition	DMSO as sole CPA	Additives present in both DMSO-free and DMSO CPAs	Assay	Cell type	Ref.
FCS+ 0.5 M sucrose+ 20% glycerol	10%	No carrier solution information	Cell survival	Human umbilical cord tissue	[197]
7.5% PLL, 5% ectoin, 5% dextran	10%	DMEM (?)	– Cell survival – Apoptosis – Proliferation	NK cells	[173]
1) 300 mM trehalose, 10% glycerol, 0.01% ectoin 2) 300 mM ethylene glycol, 1 mM taurine, 1% ectoin	10%	Normasol-R™	– Cell attachment – Differentiation	Jurkat cells MSC	[198]
32% Pentaisomaltose	20%	4% human serum albumin, 2 IE/ml heparin carrier solution (?)	– Cell survival – Colony growth – Engraftment	HSC	[199]
20% Trehalose	20%	10% FBS, RPMI-1640	Cell survival	GLC-82	[200]
6,8,10% l-carnitine	6%	10% FBS, RPMI-1640 DMEM- depending on cell type	– Cell survival – Proliferation	MCF-7 NIH-3T3	[200]
1) 10% PVP40, 2) 0.05 M glucose, 0.05 M sucrose, 1.5 M ethylene glycol	10%	10% FBS, ADMEM	– Apoptosis – Cell survival – Differentiation – Phenotyping	Human MSC	[126]
1-10% Betaine	1-20% +Trehalose	10% FBS, medium (?)	Cell survival	Hela GLC-82 MCF-10	[201]
Nil	10%	1-20% Trehalose, 10% FBS, medium (?)	Cell survival	Hela GLC-82 MCF-10	[201]
200 mmol/l Trehalose, 200 mg/l cholesterol, 30% PVP40, Lecithin	10%	10% serum, RPMI-1640	– Morphology – Apoptosis	Sheep ovar	[182]
5% HES	10%	10% serum, DMEM-medium	– Cell survival – Proliferation – Differentiation	Human MSC	[102]
25% PLL	10%	10% PVA–gelatin (ratio 9:1, w:w), 10% serum, HEPES-buffered RPMI-medium	Cell survival	Vascular smooth muscle cells Endothelial cells	[202]
1) 1% proline, 10% ectoin, 2) 5% proline, 5% ectoin, 3) 10% proline, 10% ectoin, 4) Biofreeze (Biochrom)	10%	1% methycellulose in PBS	– Cell survival – Differentiation	Human MSC	[203]
1) 10 uM ROCK inhibitor, 2) 2.5% PEG, 3) 10 uM ROCK inhibitor, 2.5% PEG	10%	No carrier solution information	– Cell survival – Colony Formation – Apoptosis	hESC	[181]
5-20% PLL	10%	DMEM-medium	Cell survival	L929	[204]
7.5% PLL	10%	DMEM-medium	– Cell survival – Differentiation	MSC	[204]

FBS: Fetal bovine serum; hESC: Human embryonic stem cells; MSC: Mesenchymal stem cell; NK: Natural killer; PBS: Phosphate-buffered saline; PLL: ε-poly-L-lysine.

versity of cell-based products, but also other aspects of regenerative medicine including the preservation of complex tissues and organs. Publication of preservation media compositions and results in clinical trials (as in the examples above) will be crucial to promoting efficient and safe development of cell therapies. However, the main driver of the field remains the fertility sector and innovation by trial and error during in-house protocol development.

While there has long been talk in the field of employing classical drug screening, discovery and design methodologies to cryoprotectant development, these methods are still uncommon and in their infancy. Cell cryopreservation in 96-well tissue culture trays has been demonstrated for several cell types [132,174,214], but not yet scaled to 384-well plates more commonly used in high-throughput screening. Differential evolution algorithms [198] and quantitative

Table 4. Commercially available dimethyl sulfoxide-free cryoprotective reagents, with indications of GMP status and availability for clinical application.

Name	Company	Replacement CPA	Reported GMP status	Description
PRIME-XV® FreezIS DMSO-Free	Irvine Scientific	Not disclosed	'cGMP' Drug Master File registration	– For MSC only – Animal-free, – Chemically defined
FREEZEstem™	BioLamina	Not disclosed	'GMP-compliant' ISO13485	– Serum free – Xeno free
STEM-CELLBANKER® 'GMP grade' (NB DMSO free 'GMP-grade' version manufactured since 2015)	Zenoaq Resource (supplier Amsbio)	1,2-dihydroxypropane serum Glucose Polymer	Drug Master File (US) 'GMP-grade'	– Chemical defined – Animal free – Xeno free
ReproCryo RM	Reprocell	Not disclosed	R&D only (NB the DMSO free version is not manufactured in a GMP-compliant facility) Drug Master File (JP)	– Xeno free – Chemically defined
CryoNovo™ P24	Akron Biotech	Not disclosed	R&D only Raw material to Pharmacopeia (US, EP, JP)	– Serum free – Xeno free
CryoSOfree™ DMSO-free Cryopreservation Medium	Sigma	ε-poly-L-lysine	R&D only	– Chemically defined – Animal free
pZerve™ Cryopreservation solution	Sigma	Not disclosed	R&D only	– Chemically defined – Animal free
CryoNovo™ X12	Akron Biotech	Not disclosed	R&D only	– Serum free – Xenofree
StemCell Keep	Funakoshi	Not disclosed	R&D only	– Serum free – Xenofree
CryoScarless DMSO-free	Funakoshi	Not disclosed	R&D only	– Protein free – Serum free
ReproCryo DMSO FreeTM	Reprocell	Not disclosed	R&D only	– Xenofree – Chemically defined
Ibidi Freezing Medium DMSO-free	Ibidi	Not disclosed	R&D only	– Serum free
Biofreeze	Biochrom GmbH	Not disclosed	R&D only	– Animal free – Serum free
Bambanker DMSO Free	Nippon Genetics	Not disclosed	R&D only	– Serum free

GMP: Good manufacturing practice; R&D: Research & development.

structure–activity relationship (QSAR) models have been employed [174], but use of functional relationship models has not yet been widely reported.

Recently, there has been a marked influx of several novel commercial cryoprotectants, many specifically aimed at the regenerative medicine market, that are marketed 'DMSO-free' (Table 4). These products are now beginning to be used in clinical studies and trials for cell therapy. It is important to be careful in the process of qualification for such materials to ensure they are suitable for use in manufacture of products for use in humans: a label of 'GMP-grade' will not be sufficient to assure adequate quality and safety for new applications. In fact, the term 'GMP-grade' is not recognized by regulators and raw materials typically cannot be certified by a regulator as made to GMP. Any raw material publicized as 'GMP-grade' will require considerable interrogation of the supplier to establish what their statement actually means (n.b. DMSO can be manufactured as a GMP therapeutic product in its own right, it is not then necessarily intended to be used as a raw material). The construction of a dossier of information, including manufacturing processes, testing and risk assessment, is an important first step in checking the suitability of a new CPA for use in humans. Where such dossiers have already been evaluated by regulatory authorities (such as in a drug master file) this adds confidence that the CPA will be acceptable but does not necessarily preclude the need for a case-by-case evaluation for each new product. Currently, reagent suppliers are utilizing a number of approaches including CE Marking and ISO13485 compliance to demonstrate suitability, although these are more typically used for medical devices. Useful guidance has been published in the United States Pharmacopeia (USP) (USP 1043, Ancillary materials for cell, gene and tissue-engineered products) and the European Pharmacopeia (EP) 5.2.12 for raw materials of biological origin. Manufacturers will need to understand how best to utilize such approaches for their own cell therapy products. There are significant challenges on the path

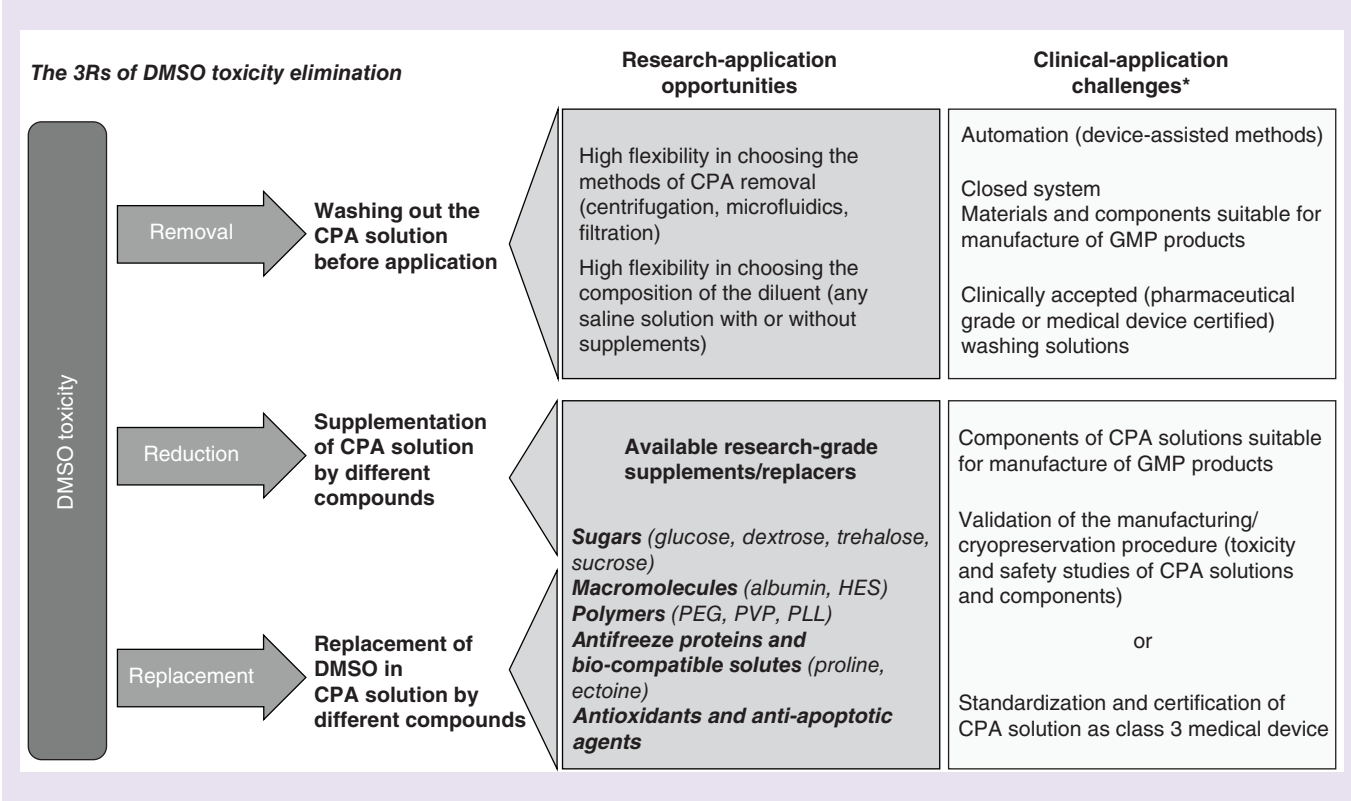

Figure 1. Options to consider when developing cryoprotective agent strategies to minimize concerns around including dimethyl sulfoxide as an additive. These comprise '3 Rs' – removal (by dilution or washout in the thawed cell product); reduction (by lowering the DMSO concentration in the CPA mixture and adding other secondary CPA such as sugars – often an achievable strategy as long as efficacy of the thawed product can be maintained); replacement (by developing CPA mixes using a range of solutes avoiding DMSO altogether – which also raises questions about the efficacy of any new mixture, and whether these solutes have existing regulatory approval for human application).
*Before actual clinical application there may be extensive work on product development which will require a good understanding of the specific regulatory requirements for the final cell therapy product and the most applicable standards [215].
CPA: Cryoprotective agent; DMSO: Dimethyl sulfoxide; GMP: Good manufacturing practice; HES: Hydroxyethyl starch; PEG: Polyethylene glycol; PLL: ε-poly-L-lysine; PVP: Polyvinylpyrrolidone.

from research product to clinical application as outlined in Figure 1. This transition will involve considerable work on product development which will require a good understanding of the specific regulatory requirements for the final cell therapy product and the most applicable standards [215].

Such innovation is facilitated because regulatory barriers to marketing a cryoprotectant even as 'clinical grade' (n.b. This term is not recognized by regulators) are comparatively low compared with the medical products that they are aimed to preserve. From a regulatory perspective, cryopreservation is required to 'not alter the relevant biological characteristics' of tissues and cells (see below). This implies that product developers must mitigate for the challenge of incorporating cryoprotectant effects – as illustrated by the fact that in spite of decades of widespread use, DMSO is acknowledged to elicit cytotoxicity and other long-term effects on cellular behaviors and these are still being explored. The consideration that cryoprotectant-related changes to membrane integrity, epigenetics, cyto-metabolism and proteomics might affect therapeutic mechanisms of action (particularly in those cell therapies that rely on complex cellular interactivity, 'paracrine' or 'bystander' effects) is unlikely to be limited to DMSO. The current Industry practice of not disclosing the exact composition of cryo-protective solutions is a hinderance to protocol improvement and development of greater understanding of preservation of the complex and diverse cell-based products.

Recognition of the crucial effect that preservation may have on the quality and safety of cell therapy products is acknowledged and accommodated within existing regulatory considerations for manufactured biological products. These considerations have been captured as part of guidance documents dealing with consistency and comparability.

Consistency of a product from batch to batch is crucial and would also be expected for a preservation stage within a licensed manufacturing process such as cell banking, preservation of 'intermediates' (such as preserved progenitor cells derived from the original pluripotent stem cell line) and the final product, as well as storage and transportation. Comparability is the regulatory term used for the requirement to demonstrate that when a process has changed, such as the type or composition of a CPA, the product has not been affected adversely by that change from quality and safety perspectives. The manufacturer should consider the need to demonstrate 'comparability' between the product made by the original process and the new methods/materials. Relevant information on performance of a comparability study is given in the guidance document ICH Q5E [216] which is mutually recognized in EU, US and Japanese regulation.

For consideration of both consistency batch–batch and comparability after process change, it is also important for the manufacturer to develop adequate knowledge of the cell preparations they are using and ensure use of suitable critical quality attributes [217]. This may demand multiple parameters for viability and a panel of biomarkers and functional assays for the cells involved. This will be important and expected by regulators to give confidence that the chosen bioanalytics regime will detect changes of significance for safety and efficacy of the final product.

Overall process verification requirements will have implications for how a manufacturer should assess their cryopreservation methods and is captured in document ICH Q8 [218]. More specifically, the manufacturer should also consider the composition of CPA solutions and assess the risks from additives or excipients in CPAs. These risk elements might include, in particular, those of biological origin (notably animal origin with attendant risk of viral contamination), toxic compounds, allergens, mitogens and particulates. In addition, cryovial material should be assessed for potentially harmful leachates and particle release. It is also important not to forget the potential influence of cell culture growth media components included with the CPA during cryopreservation, both in terms of their direct effect on the cells 'solutions effects' (see Mazur *et al.* 1972) [219] as the unfrozen solutes are concentrated around the cells, and also in terms of what chemical interactions may occur between CPA, media or cryovial components.

Cryostorage and the potential long-term exposure of cells to CPAs, is also a relevant part of manufacturing considerations reviewed by regulators. In particular, it is worth noting that EU GMP guidance now includes a new annex (Annex 15, section 6, 'Verification of transportation') which came into effect in 2015 and provides further guidance useful for cryopreserved products.

General conclusion

The use of DMSO has been important in the successful treatment of many thousands of patients worldwide with serious conditions such as leukemia, and continues to be present in drug preparations and to provide the CPA of first choice for the diverse and complex range of novel cellular therapies now being developed. However, the wide-ranging biological impact of DMSO we have described above, combined with the known adverse reactions which may occur in patients subjected to DMSO containing preparations, means that a greater selection of CPA solutions may well be in demand for the future [220]. We have described the commercially available DMSO-free reagents already on the market and in some cases, already in use in clinical trials of new cell-based medicines. The possible toxicities and impact on cell behavior will need to be investigated in different therapeutic cell types to avoid early abandonment of DMSO, only to discover unexpected adverse events arising from the premature uptake of new lesser-studied and unproven CPAs. The rapid development of novel and combination therapies means that the search for new CPA molecules will and must continue, as should careful evaluation of their biological impact in diverse cell types. Knowledge of the nature and diversity of cell populations within cell-based clinical products is vital if the results of cryopreservation investigations are to be compared and this is particularly true of the so-called 'MSC' group, which clearly represents a number of biologically different cell types that may respond differently to a particular CPA. As a generalization, concerns surrounding DMSO toxicity can also be addressed by considering the '3R's of DMSO toxicity mitigation' (Figure 1); removal (after cryopreservation); reduction (using additional secondary CPA) or replacement (using other CPA to completely avoid DMSO). These strategies should be given due consideration by anybody developing cryopreservation protocols. In particular, the current challenges being experienced by developers of combined antibody receptor (CAR) T-cell products may benefit from the use of the 'DMSO 3Rs' or alternative CPAs outlined in this review.

The development of new CPAs will require more sophisticated high-throughput screening systems and possibly establishment of model cell systems for a range of cell types as proposed by Stacey *et al.* [220]. However, few drugs or clinical interventions are risk free and it is highly likely, that with the availability of refined combination CPAs

and automated DMSO removal devices, DMSO will continue for the foreseeable future, to contribute to the roll out of life saving interventions for an increasing range of cell-based medicines.

Future perspective

Over the past decade, there have been many comments recorded about possible negative implications of the use of DMSO as a cryoprotectant, and exhortations made to find replacements. We fully support continued objective research into these topics and robust audit of current cryopreservation practices where DMSO is used. However, in parallel it must be recognized that at the present time, the vast majority of applied cryopreservation practices depend upon the efficacy provided by DMSO for high functional capacities after thawing. The development of new CPAs will require more sophisticated high-throughput screening systems and possibly establishment of model cell systems for a range of cell types as proposed by Stacey *et al.* [220]. Many of the novel cryoprotectants appearing in research publications have not progressed to widespread uptake or regulatory approval at the current time but they will provide invaluable signposts to how to better assess both efficacy and biological impacts of all cryoprotectants (including DMSO) moving forward. However, few drugs or clinical interventions are risk free and it is highly likely, that with the availability of refined combination CPAs to lower DMSO concentrations, and automated DMSO removal devices, DMSO will continue for the foreseeable future to contribute to the roll out of life saving interventions for an increasing range of cell-based medicines, and improved cell cryo-banking in high-value biotechnologies.

Executive summary

A key limiting step in the development of novel advanced cell therapies is having the ability to store & ship cell-based medicines in a viable yet stable state

- Shipment of nonfrozen materials is challenging, and local manufacture of a cell therapy requires demonstration of product comparability at different manufacturing sites.
- The diversity and complexity of cell therapies therefore demands new cryopreservation methodologies and cryoprotectants.

Dimethyl sulfoxide has been the cryoprotectant of choice for most animal cell cryopreservation protocols since the early history of cryopreservation technology

- Clinical experience in transplantation and transfusion over more than 40 years has shown that dimethyl sulfoxide (DMSO) can be used safely and effectively.
- Nevertheless, it can affect the phenotype and genotype for both mammalian and nonmammalian cells and exhibit apparent toxicity in patients, which is driving the search for less cytotoxic cryoprotectants.

In this review, we evaluate the history of clinical use of DMSO & seeks to explain the 3Rs for use of DMSO: reduction, removal & replacement

- We consider the adverse events that may be associated with the toxicity of DMSO in the context of its effectiveness, reviewing the real incidence of adverse effects in patients and the probable causes.
- We review the key approaches already in use as a means of reducing its toxic effects by reduction of DMSO concentration, removal of DMSO from cell preparation post-thaw and alternative cryoprotectants are considered and assessed in terms of their progression to suitability for clinical use.

Conclusion

- DMSO has long served to enable cells to be stored for future use and continues to be the cryoprotectant of first choice.
- Replacement cryoprotectants are being developed but are yet to make significant impact in cell therapies.

Author contributions

I Buriak: drafting, reviewing and approving manuscript. R Fleck: drafting, reviewing, approving manuscript. J Kerby: reviewing, editing and approving manuscript. M Awan: reviewing, editing and approving manuscript. P Mericka: drafting, reviewing and approving manuscript. A Petrenko drafting, reviewing and approving manuscript. Y Petrenko: drafting, reviewing manuscript, approving manuscript. O Rogulska: drafting, reviewing and approving manuscript. G Stacey: drafting text and compiling author input. A Stolzing: drafting, reviewing, approving manuscript.

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Research Article

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Regenerative Medicine

Characterization of an umbilical cord blood sourced product suitable for allogeneic applications

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Aim: Umbilical cord blood (UCB) sourced allografts are promising interventions for tissue regeneration. As applications of these allografts and regulations governing them continue to evolve, we were prompted to identify parameters determining their quality, safety and regenerative potential. **Materials & methods:** Flow-cytometry, mass-spectrometry, protein multiplexing, nanoparticle tracking analysis and standard biological techniques were employed. **Results:** Quality attributes of a uniquely processed UCB-allograft (UCBr) were enumerated based on identity (cell viability, immunophenotyping, proteomic profiling, and quantification of relevant cytokines); safety (bioburden and microbiological screening), purity (endotoxin levels) and potency (effect of UCBr on chondrocytes and mesenchymal stem cells derived exosomes). These attributes were stable up to 24 months in cryopreserved UCBr. **Conclusion:** We identified a comprehensive panel of tests to establish the clinical efficacy and quality control attributes of a UCB-sourced allograft.

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Keywords: endotoxin • exosomes • graft versus host disease • quality control • tissue regeneration

The field of regenerative medicine holds abundant promise for the treatment of diseases afflicting millions worldwide. Thousands of clinical trials are underway for trauma and degenerative conditions in the USA alone (www.clinicaltrials.gov). The interest in the field is further amplified by the aging population's need to find alternatives to surgery, effectively offloading the financial burden from the healthcare system as well as patients [1]. An in-depth understanding of stem cell biology and therapeutic benefits of cytokines, along with improved techniques for optimizing these factors are helping regenerative medicine to grow at an unprecedented pace.

Recent research has demonstrated that UCB is a convenient source of adult stem cells containing hematopoietic (HSC) as well as mesenchymal stem cells (MSC) [2,3]. HSC in cord blood prompted its application in certain hematological diseases [4], whereas the presence of multipotent MSC that can differentiate into other structural tissues like bone and cartilage has further expanded the potential applications of UCB [5–7]. While the cellular characteristics of UCB have been extensively studied for the purposes of clinical applications, not enough has been reported on regenerative potential of the associated biomolecules. This is partly because regenerative medicine has long been associated with stem cell therapy. However, it has been shown that regenerative potential is often derived from the paracrine effect of the interventions and the activated host stem cells, rather than the direct involvement of exogenous cells administered at the site of injury [8]. We have previously demonstrated that a uniquely processed UCB product contains cytokines and growth factors, which are integral to bone regeneration [9]. These factors initiate signaling crosstalk with target cells that positively impacts the molecular milieu of the tissue microenvironment [10].

Exosomes are extracellular vesicles (EVs) comprising of several factors including cytokines. MSC release EVs packed with proteins and genomic materials which induce biological changes in the target cells to accomplish tissue regeneration [11]. Translational research on exosomes derived from the MSC have demonstrated that they can mimic the therapeutic attributes of the MSC themselves [12–14]. Clinical trials on rheumatic pathologies, cartilage

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development, brain injury and cardiovascular diseases are also currently testing this hypothesis [15]. Based on these findings, clinical suitability of cord blood allografts must account for the noncellular components while evaluating its promising role in tissue regeneration.

As UCB allografts are increasingly used in the clinics for tissue regeneration, the regulatory framework continues to evolve. Such products may be classified and regulated differently from cell and gene-based therapies. The quality control guidelines for cyto/genetic therapy includes cell identity, viability, purity, sterility and potency to minimize lot-to-lot variability [16]. Since UCB allografts are a complex embodiment of cellular and noncellular components, a quality control approach recommended for such products should be appropriately modified to address suitability and release criteria. Claims to regenerative function of these allografts should be based on characterization of identity and consistency of the noncellular components. UCB allografts have heterogeneous cellular and molecular composition due to inherent donor variability and alterations in the processing methodologies will add to this factor [17]. Variability can however be largely reduced, if not completely eliminated, by careful optimization of the manufacturing process and ensuring a stringent quality control mechanism.

The present study is aimed to address the unmet need for a uniform quality control framework to determine clinical suitability and safety of cord blood allografts. A uniquely processed UCB sourced allograft (UCBr) reportedly used for soft tissue and ligament repair application was investigated for identity, purity, safety and stability. Additionally, it is important to determine the biological potency of UCBr to establish a comprehensive quality control regime. The potency assays provide the mechanistic insight into how noncellular components of UCBr support biological processes, which may positively impact cartilage repair. Finally, the long-term stability of UCBr was over a period of 24 months. Taken together, to the best of our knowledge, this is the first study that identifies a comprehensive panel of tests to adequately address the quality attributes, clinical suitability and efficacy of UCB-sourced allografts.

Materials & methods

UCB-sourced allograft

UCB obtained from consenting donors was processed by the patent pending method following US FDA's regulatory guidelines. UCB-sourced allografts, UCBr (BioBurst Rejuv, Burst Biologics, ID, USA) were stored at -80°C until further use.

Preparation of UCBr lysate

UCBr was sonicated on ice (three pulses of 10 s each), centrifuged at 12,000 g for 15 min at 4°C and supernatant was collected and used for downstream experiments. Supernatant was not subjected to more than one freeze thaw cycle. When appropriate, supernatant was diluted in serum free media (SFM) at 1:5 or 1:10 dilution.

Cells & tissue culture media

Human Knee Articular Chondrocytes (HC, Lonza CC-2550) were cultured in the basal media supplemented with growth kit (cell applications; 410PR-500 and 411-GS). Bone marrow derived mesenchymal stem cells (MSC, ATCC, PCS-500-012) were also cultured in the respective basal media supplemented with growth kit (ATCC; PCS-500-030 and PCS-500-041). Cells were maintained in a humidified incubator at 37°C with 5% CO_2. Cells between passages 3 and 5 were used for all experiments.

Cell viability measurement

UCBr was passively thawed and mixed in equal volumes with acridine orange/propidium iodide (AO/PI) staining solution. The mixture was loaded onto the SD100 cell counting chamber slides, and cells were visualized and counted using Cellometer K2 (Nexcelom, MA, USA). Data were analyzed using the K2 software optimized for frozen peripheral blood mononuclear cells (PBMC) using 15-micron cutoff for nonviable cells.

Fluorescence activated cell sorting

UCBr was thawed and added dropwise into RPMI supplemented with 10% FBS and 400 units of DNAse. The cell suspension was centrifuged at 300 g for 5 min, and the cell pellet was resuspended in 10% FBS/RPMI containing 50 units of DNAse. Cells were sequentially washed with PBS and FACS Buffer (2% FBS/HBSS, 2 mM EDTA). Cells were stained with antibodies (listed in Supplementary Material). Cells stained with all the fluorophores minus one fluorophore (FMO) were used as controls to determine the gating boundaries of positive and negative

populations. Unstained and single-stained beads were used to calculate compensation values using the automated calculation function. Flow cytometry was performed on an ACEA NovoCyte with blue and red lasers and detectors for six color analysis. 10,000 events at 14 ml/min were collected for compensation controls and 100,000 events at 35 ml/min were collected for UCBr cells.

Mass spectrophotometry & proteomic analysis

LC-MS/MS was performed using methods established previously with modifications [18]. Briefly, UCBr lysates were run for approximately 1 cm in 10% Bis-Tris gel and excised gel pieces were reduced, alkylated and digested with trypsin overnight at 37°C (Thermo Fisher Scientific, MA, USA). LC–MS/MS analysis of the resulting tryptic peptides was conducted on a Velos Pro Dual-Pressure Linear Ion Trap mass spectrometer equipped with a nano electrospray ionization source and coupled with an Easy-nLC II nano LC system (Thermo Fisher Scientific). Raw spectrum data were searched against the UniProtKB/Swiss-Prot protein database for Humans (acquired from www.uniprot.org on 12 February 2019). Further analysis was done using Database for Annotation, Visualization and Integrated Discovery web tool to analyze candidate genes and pathways [19].

Cytokine measurement

Cytokine concentration was measured in UCBr lysate (prepared from 31–64 donors) using multiplex ProcartaPlex Panel (Invitrogen, EPX450-12171-901). Luminex xMAP magnetic-bead fluorescent immunoassays (Invitrogen) were run on MAGPIX™ and measurements were done as per the manufacturer's protocol. Universal assay buffer provided baseline values for the assay. For each standard, percent recovery values outside 90–110% were invalidated using xPonent Analysis software and concentration of cytokines were calculated using a standard curve with $R^2 \geq 0.9$.

Maternal peripheral blood serology & UCB bioburden testing

Maternal peripheral blood samples were tested for infectious diseases using antibody-based, nucleic acid based and PCR-based detection methods. Serology testing was performed at VRL (TX, USA), a Clinical Laboratory Improvement Amendments certified laboratory. UCB samples were tested for anerobic and aerobic bacterial contamination prior to processing as per current Good Tissue Practice (cGTP) guidelines. To test possible contamination of UCBr, it was passively thawed and inoculated in either TSB, Sabdex or FTH broth and corresponding agar plates. For all tests appropriate positive and negative controls were included. Growth was monitored every 72 h up to 14 days.

Endotoxin test

Endotoxin levels were determined in UCBr directly using EndoSafe® nexgen PTS™ and US FDA licensed cartridges (PTS2005F or PTS5505F, sensitivity – 0.05 EU/ml, Charles River, MA, USA) following manufacture's protocol.

Mixed lymphocyte reaction

PBMCs were isolated from the buffy coat of healthy donors (PBMC Donor #36, male, age 46 years, White; Donor #50, male, age 25 years, White; Donor #42, female, age 24years, African American) by Ficoll density gradient centrifugation and cryopreserved. Prior to use, the cryopreserved samples were thawed and rested at 37°C overnight in RPMI-1640 medium supplemented with 10% fetal bovine serum. Cryopreserved UCBr were thawed and plated at 10^5 cells/well in a 96-well plate. Rested PBMC cells were co-cultured with 1×10^5 UCBr cells or PBMCs from unrelated donor at a density of 2×10^5 cells/well in the mixture of 200 µl AIM-V. For mitogenic stimulation, the PBMC cells were stimulated with 2 µg/well phytohemagglutinin. Background controls were performed for all UCBr samples and each institutional review board donor. Cells were incubated at 37°C, in a 5% CO_2 incubator for 4 days. Bromo-deoxy uridine (BrdU) was added on day 3, and proliferative responses were determined by absorbance based BrdU ELISA assay on day 4 (Roche, Basel, Switzerland). BrdU optical density was recorded at 450 nm. The assay was performed by Xeno Diagnostics, LLC (IN, USA) and PBMC used were approved through their institutional review board program.

Cell proliferation

HC and MSC were seeded at density of 5000 cells/well in a 96-well plate and incubated overnight. Cells were starved for 2 h and then grown in SFM, complete media or diluted UCBr lysates (1:10 or 1:5) for additional

24 h. Cell proliferation was measured by CyQUANT (Thermo Fisher Scientific) assay according to manufacturer's protocol.

Transwell cell migration

Transwell inserts were coated with 5 μg/ml of fibronectin (Sigma–Aldrich, MO, USA) and left overnight for surface adsorption. HC were serum-starved for 2 h, suspended in SFM and 4×10^4 cells were added to the upper chamber of a 24-well, 8 μm pore membrane transwell insert. Complete media, SFM or UCBr lysate (1:10) were added to the lower chamber as a chemoattractant. After 24 h, cells on the upper surface were removed, cells on the lower surface were fixed in 5% glutaraldehyde (Sigma–Aldrich, MO, USA), stained with 1% (w/v) crystal violet (Sigma–Aldrich) and visualized at 20× magnification under stereoscope (Leica, NJ, USA).

RNA isolation & reverse transcriptase PCR

Cells were treated with UCBr lysates (1:5) for 18–20 h, total RNA was extracted using Quick RNA kit (Zymo Research, CA, USA) and 1 μg was reverse transcribed into cDNA and subsequently amplified with appropriate primers (listed in Supplementary Material) using SuperScript™ VILO™ cDNA Synthesis Kit and Power SYBR™ Green PCR Master Mix (Thermo Fisher Scientific) respectively. All reactions were run in QuantStudio 3 (Thermo Fisher Scientific), and data are represented as relative mRNA expression with 18 s rRNA as internal control.

Western blot analysis

Proteins were extracted in RIPA buffer (Thermo Fisher Scientific) from cells treated with UCBr lysates (1:5) for 24 h and estimated by BCA assay. Total 20–60 μg of proteins were separated in 10% Bis-Tris gels by electrophoresis, transferred onto nitrocellulose membrane and immunoprobed with antibodies to detect HMOX1, Cu/ZnSOD, MnSOD, Catalase, Thioredoxin 1 (Cell Signaling Technologies, MA, USA) and Hsp60 (SCBT, TX, USA). Chemiluminescence was captured with iBright FL1000 (Thermo Fisher Scientific). Hsp60 was used as loading control and was used for normalization of band intensities using ImageJ software.

Exosome isolation & characterization

MSC in 100 mm culture plates with 80–90% confluency were briefly subjected to 2 h of serum starvation. Cells were subsequently treated with UCBr lysate (pooled from 6 or more donors) for 18 h. Exosomes were extracted by Total Exosome Isolation (TEI) kit according to manufacturer's protocol (Thermo Fisher Scientific) or Exo-Quick kit (SBI, CA, USA). Isolated exosomes were first characterized by fluorescent nanoparticle tracking analysis (performed by System Biosciences, CA, USA). For downstream proliferation assay on HC and MSC, cells were treated with isolated exosomes diluted 1:2 for 24 h. Cytokine level was measured as described previously. All experiments, including the exosome analysis, were repeated twice independently, each time at least in triplicates.

Data analysis & statistics

All experiments, unless otherwise stated, were repeated independently three-times. All cell biological assays were performed with UCBr lysates obtained from at least three donors. Data are expressed as mean ± standard deviation or standard error of the mean as indicated. Student's t-test and ANOVA were used for statistical analysis between two groups, and among three or more groups respectively. Statistical significance was set at $p < 0.05$ (*).

Results

Characterization of the cellular components of UCBr

Our previous publication shows that intact cells in a cord blood allograft are not directly involved in the regeneration process, and possibly act as reservoirs of the intracellular and membrane bound cytokines that are released by autolysis or passive secretion. We specifically demonstrated that the cytokine concentration in the conditioned media (CM) derived from intact UCB allografts was not significantly different from the CM derived from lysed UCB allografts [9]. However, it is critical that we evaluate the cellular components in UCBr from a quality control perspective, as the reproducibility of cell viability and characteristics will indicate a consistent processing technique.

Cell viability assay

Total 21 random lots of UCBr, stored at -80°C for a period of at least 30 days post processing were passively thawed and viability of the primary cell population was measured. Cell count, viability and diameter were determined by

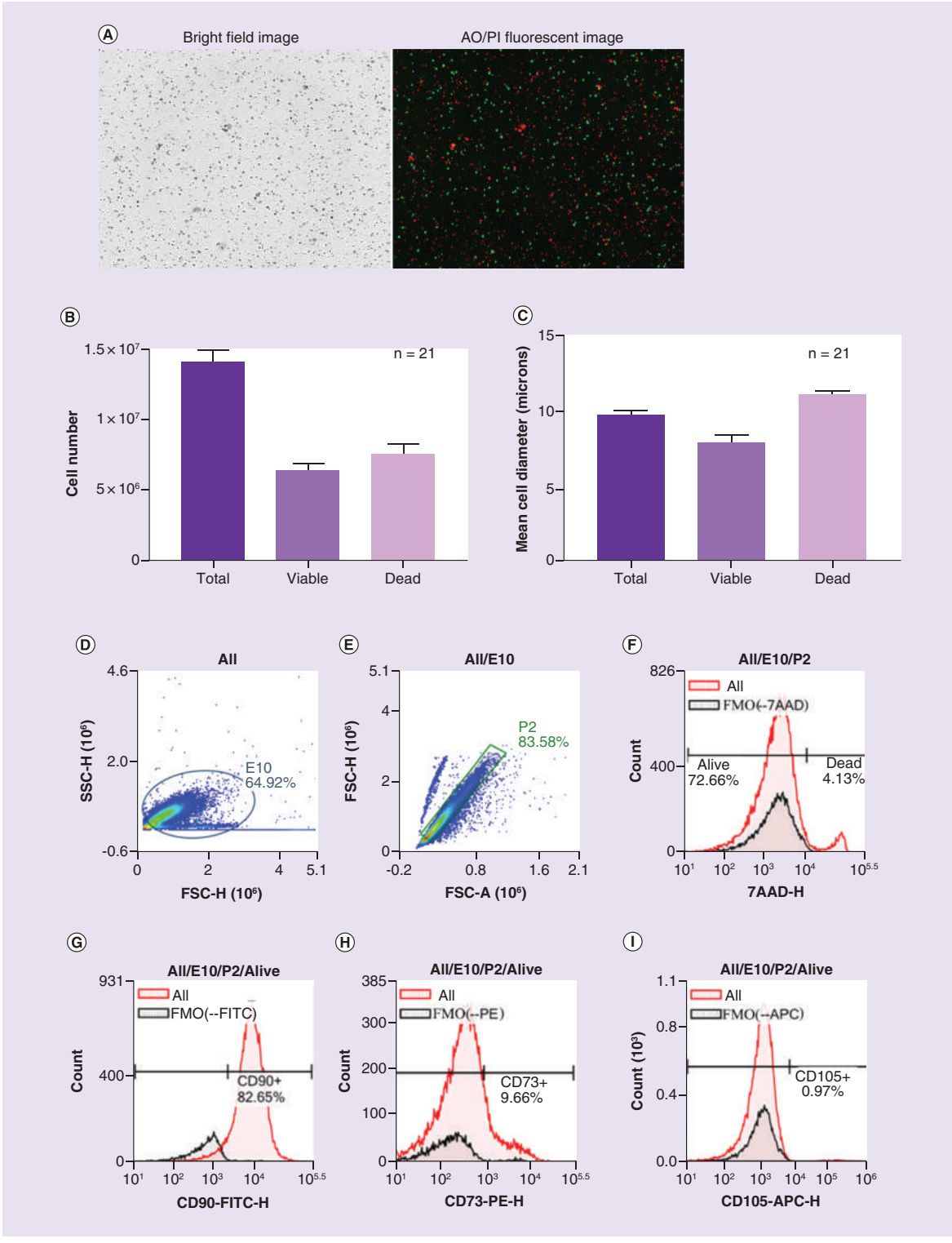


Figure 1. Characterization of cellular components of umbilical cord blood allograft by cell viability measurement and immunophenotyping using flow cytometry. UCBr from 21 donors was passively thawed and mixed with AO/PI dye solution and visualized under Nexcelom K2. **(A)** Representative photomicrographs of a random field with bright field (left panel) and fluorescence (right panel) were shown, AO-green/viable and PI-red/dead. **(B)** Cell number and **(C)** cell diameter were quantified. Values are expressed as mean ± standard deviation. **(D–N)** Expression of surface markers of UCBr cells from a representative donor. Cells were appropriately gated **(D, E & K)**. In figures **(F–J)**, 'all' indicates cells stained with CD90-FITC, CD73-PE, CD105-APC, CD45-PerCp-Cy5.5 and 7-AAD monoclonal antibodies and 'fluorophore' indicates cells stained with all the fluorophores minus the one as labeled in respective figures. Viability determined by 7-AAD exclusion **(L)** and stained with CD34 and CD45 monoclonal antibodies to determine $CD34^+$ and $CD45^+$ cells **(M & N)**. **(O)** The average expression percentage of cells stained with individual or combination of antibodies in UCBr.
AO: Acridine orange; PI: Propidium iodide; UCBr: Umbilical cord blood allograft.

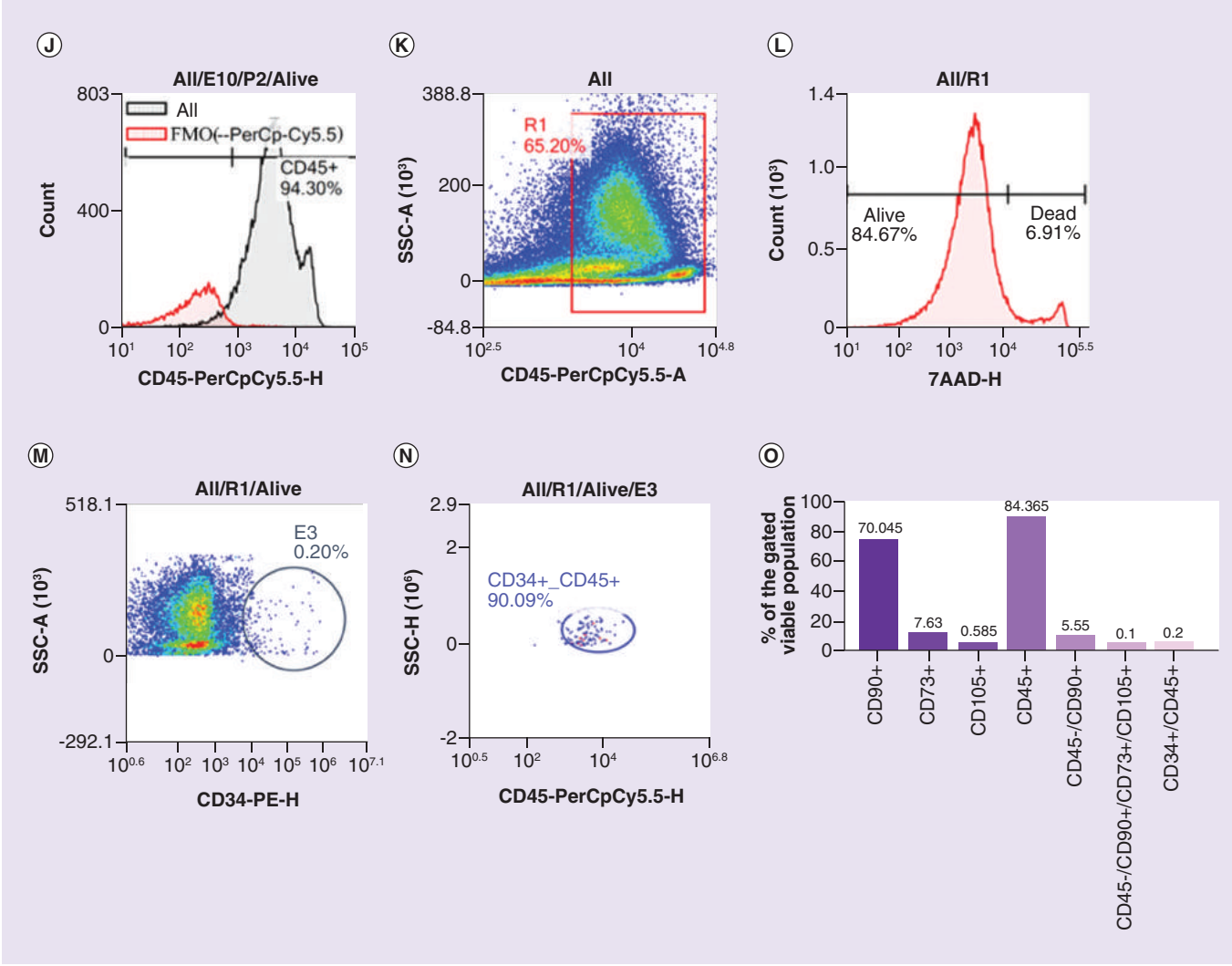


Figure 1. Characterization of cellular components of umbilical cord blood allograft by cell viability measurement and immunophenotyping using flow cytometry (cont.). UCBr from 21 donors was passively thawed and mixed with AO/PI dye solution and visualized under Nexcelom K2. **(A)** Representative photomicrographs of a random field with bright field (left panel) and fluorescence (right panel) were shown, AO-green/viable and PI-red/dead. **(B)** Cell number and **(C)** cell diameter were quantified. Values are expressed as mean ± standard deviation. **(D–N)** Expression of surface markers of UCBr cells from a representative donor. Cells were appropriately gated **(D, E & K)**. In figures **(F–J)**, 'all' indicates cells stained with CD90-FITC, CD73-PE, CD105-APC, CD45-PerCp-Cy5.5 and 7-AAD monoclonal antibodies and 'fluorophore' indicates cells stained with all the fluorophores minus the one as labeled in respective figures. Viability determined by 7-AAD exclusion **(L)** and stained with CD34 and CD45 monoclonal antibodies to determine $CD34^+$ and $CD45^+$ cells **(M & N)**. **(O)** The average expression percentage of cells stained with individual or combination of antibodies in UCBr.
AO: Acridine orange; PI: Propidium iodide; UCBr: Umbilical cord blood allograft.

bright field and dual-fluorescence imaging using acridine orange/propidium iodide staining (Figure 1A). The mean total cell count was 14.1 ± 3.4 million and the viability was close to 50%. The mean diameter was 8 ± 1.4 µm for viable and 11.12 ± 0.85 µm for the nonviable cells (Figure 1B & C).

Characterization of UCBr cells by flow cytometry

All studies reporting isolation of MSC from UCB have allowed the cord blood cells to adhere to culture dishes and expand for a few generations before performing flow-cytometry to characterize MSC based on cell surface markers [20–22]. Other methods like fluorescence activated cell sorting (FACS) have been used to enrich and purify the small but significant fraction of stem cells present in UCB [23]. Without such downstream manipulations, the sensitivity of the current assay procedures makes it extremely difficult to identify the stem cell population

directly in the UCB cells [24]. Therefore, instead of identifying a particular subpopulation of cells in UCBr through lineage specific enrichment, we chose to characterize the heterogeneous population by immunophenotyping. Two UCBr samples were thawed, washed and stained with respective antibodies. Dead cells were identified using 7-Aminoactinomycin D (7AAD). Of the viable cells in UCBr, the average expression of CD90, CD73, CD105, CD45 were 70.04 ± 17.8%, 7.63 ± 2.87%, 0.585 ± 0.54%, 84.37 ± 14.1% respectively, indicating a major population of the cells is lymphocytes (Figure 1D–O). CD45 cells that stained positive for CD90 were 5.55 ± 0.48%. As expected in such heterogeneous population of frozen UCB cells, less than 1% of the cells in UCBr stained for all the MSC markers ($CD45^-/CD90^+/CD73^+/CD105^+$). Albeit very low, expression of HSC progenitor marker CD34 was also detected.

Characterization of the noncellular components of UCBr

Mass spectrometric analysis of UCBr

In addition to cytokines, signaling molecules such as protein kinases and redox enzymes also impact tissue regeneration. To identify such signaling proteins, we performed proteomic analysis of UCBr lysates by mass spectrometry. Only those proteins identified with two or more peptides, an expected p-value of <0.05 and a false discovery rate of $\leq 1\%$ were reported. Proteomic analysis of two UCBr samples using Uniprot human protein database identified 1801 and 2335 proteins respectively, including serine threonine kinase, mitogen activated protein kinase, tyrosine kinases, cyclin dependent kinases and von Willebrand factor in addition to growth factors like PDGF, TGFβ1 and FGF2 (data not shown).

We identified 784 proteins that were common between the samples (Figure 2A). The high overlap of proteins between the donors suggests that the UCBr processing technique is consistent and the methods for protein identification are reliable. Bioinformatic analysis of the overlapping proteins using Database for Annotation, Visualization and Integrated Discovery 6.8 and gene ontology annotation indicated that proteins common between the donors were mainly involved in wound healing, cell proliferation and migration, cell cycle, redox metabolism, glucose metabolism and signaling pathways (Figure 2B). Pathways that yielded a Fishers exact p-value <0.05 and involved at least eight genes were reported. All the molecular functions and biological processes identified by this bioinformatic analysis are physiologically relevant and clearly established the role of the noncellular component of the UCBr in tissue regeneration.

Cytokine profiling of UCBr

Mass spectrometry analysis identified several signaling molecules including PDGF and VEGF in UCBr that play a critical role in wound healing and tissue repair (data not shown). We have previously reported 44 different cytokines in the cord blood allograft derived CM [9]. In this study, we quantified the cytokines present in the UCBr lysates (Figure 2C). This enabled a better understanding of the molecular composition of the product with a direct clinical relevance. Total 31 cytokines including several growth factors (PDGF and EGF), neurotrophic factors (BDNF), angiogenic factors (VEGF) and interleukins (IL-1, IL-2, IL-5, IL-8, IL-10) were identified in samples prepared from 31–64 donors. The mean concentration of the cytokines ranged from 7.83 ± 0.3 pg/ml (SCF) to 30933 ± 1901.2 pg/ml (IL-1RA). IL-1RA is a major anti-inflammatory cytokine and several clinical trials with IL-1RA have reported pain reduction in patients following acute injury [25]. Presence of important cytokines like EGF (323.16 ± 19.02 pg/ml); PDGF-BB (3256.8 ± 495.9 pg/ml) and VEGF (1600.1 ± 91 pg/ml) in UCBr may be the regulatory factors for inducing cell proliferation, migration and angiogenesis and hence, constitute an important criterion to determine product quality and donor suitability.

Pre- and postprocessing microbiological safety testing on UCBr

Absence of microbial contamination in the product is critical from clinical safety perspective and must be an integral part of the quality control process. UCBr is manufactured under controlled, clean room conditions with minimal environmental exposure which must be subsequently verified by an array of safety testing to ensure the aseptic nature of the final allograft.

Infectious disease testing on maternal peripheral blood samples

To begin with, a well-defined inclusion–exclusion criterion based on personal lifestyle, medical history, etc. determined donor initial qualification. Serology testing was performed by Clinical Laboratory Improvement Amendment certified lab as per US FDA's guidelines for human cells, tissues, and cellular and tissue-based product (HCT/Ps).

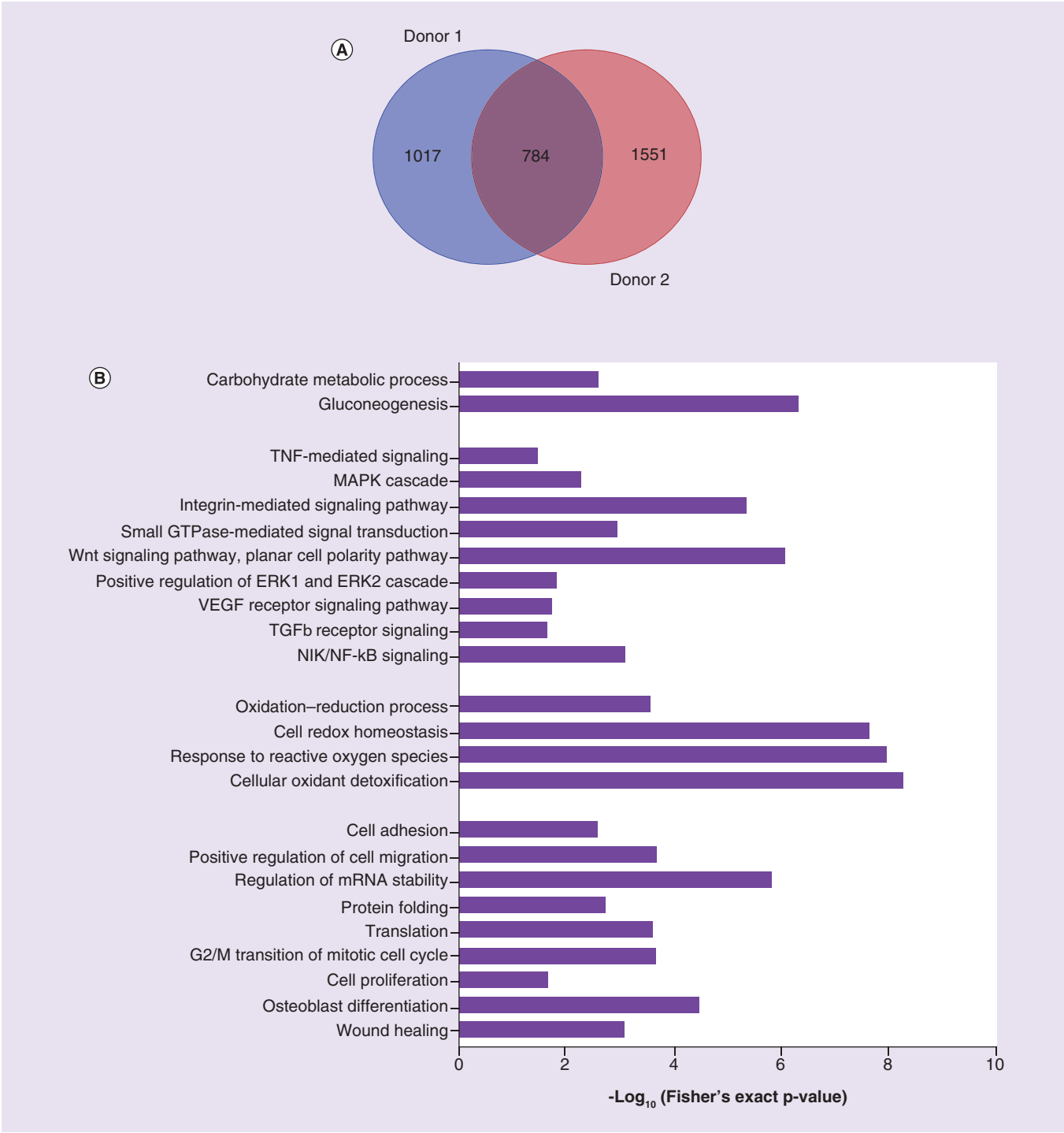


Figure 2. Characterization of noncellular components of umbilical cord blood allograft by mass spectrometry and protein multiplexing. **(A)** Venn diagram showing the number of unique and shared proteins in donor samples obtained by mass spectrometry. **(B)** Enrichment analysis of the 784 common proteins in donor samples using DAVID. **(C)** Protein multiplexing analysis for 31 cytokines in UCBr samples. Cytokine concentration was measured by Luminex xMAP technology on MAGPIX™ platform and expressed as mean values in picograms/ml (± standard error of the mean) from 31–64 donors.
UCBr: Umbilical cord blood allograft; DAVID: Database for Annotation, Visualization and Integrated Discovery.

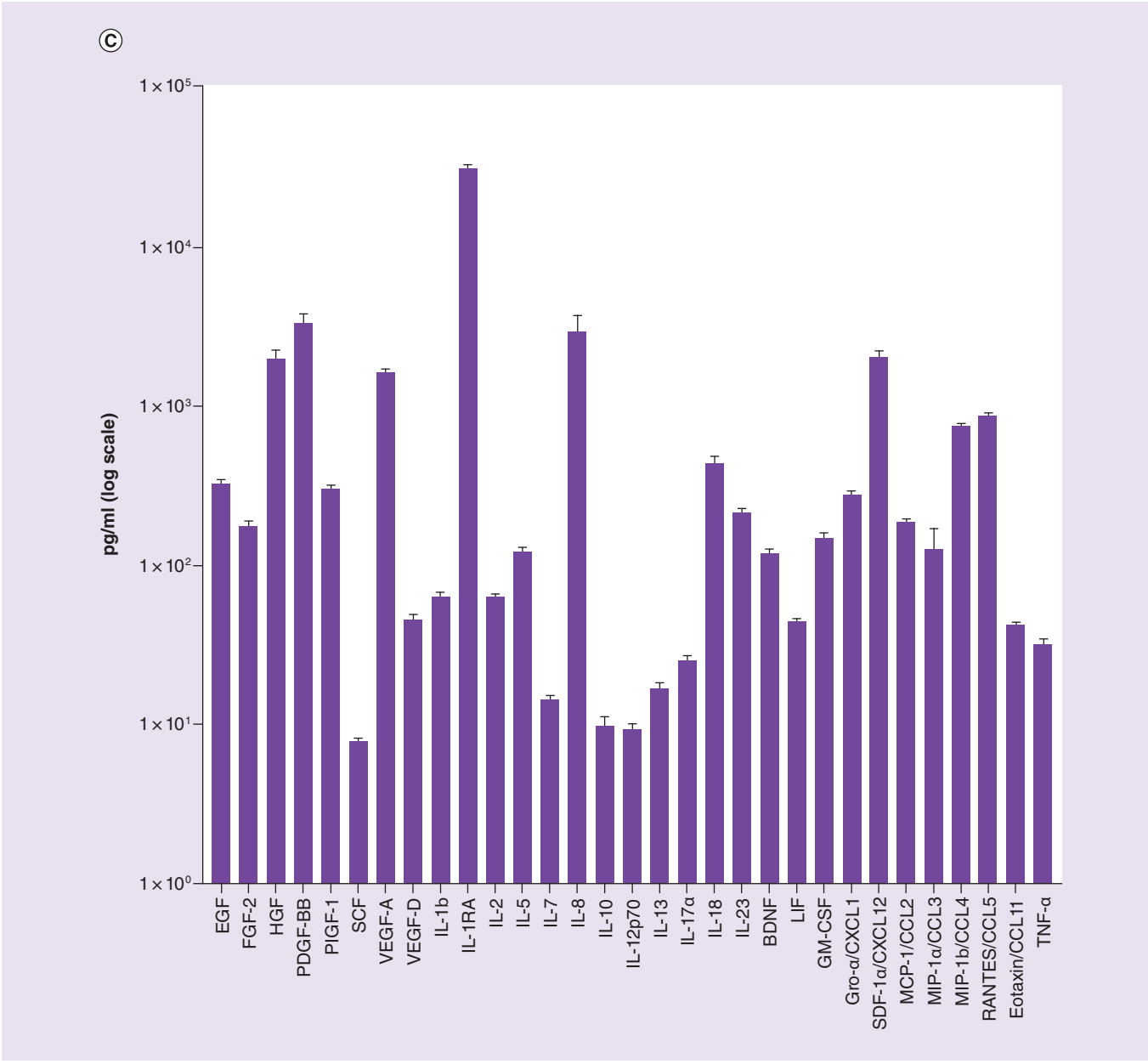


Figure 2. Characterization of noncellular components of umbilical cord blood allograft by mass spectrometry and protein multiplexing (cont.). (A) Venn diagram showing the number of unique and shared proteins in donor samples obtained by mass spectrometry. **(B)** Enrichment analysis of the 784 common proteins in donor samples using DAVID. **(C)** Protein multiplexing analysis for 31 cytokines in UCBr samples. Cytokine concentration was measured by Luminex xMAP technology on MAGPIX™ platform and expressed as mean values in picograms/ml (± standard error of the mean) from 31–64 donors.
UCBr: Umbilical cord blood allograft; DAVID: Database for Annotation, Visualization and Integrated Discovery.

Testing included detection of antibodies to HTLV-1 and 2, syphilis and hepatitis B surface antigen. Nucleic acid testing (NAT) was performed for hepatitis B, hepatitis C, HIV and West Nile virus. Peripheral blood samples were also tested for total antibody of CMV, while Zika and other flavivirus were identified by PCR amplification, to eliminate possibility of past or recent exposure to the virus because of traveling to certain geographical zones. In a typical 24 months period, 5.2% of the total donors were rejected due to positive antibody test, 1.2% were rejected due to positive NAT outcomes and 1.0% of the donors were rejected based upon PCR-based identification. Overall, 92.5% donors were negative for any infectious disease serology and the cord blood collected from these donors underwent final processing (Table 1A).

Table 1. Determination of product safety using pre- and postprocessing microbiological testing.

(A) Maternal peripheral blood infectious disease serology testing		
Serology test		**Percentage positive**
Antibody-based detection		5.209
Nucleic acid test		1.195
PCR-based identification		1.0247
		7.42 (Total)
(B) Umbilical cord blood microbial cultures		
Incubation temperature	**Culture media**	**Percentage positive**
22.5°C	Tryptic soy broth	0
32.5°C	Fluid thioglycolate broth	0.028
	Tryptic soy broth	0.008
		0.036 (Total)
(C) Umbilical cord blood product microbiological testing		
Incubation temperature	**Culture media**	**Percentage positive**
22.5°C	Tryptic soy broth	0
	Sabdex broth	0.001
	Tryptic soy agar	0
	Sabdex agar	0
32.5°C	Fluid thioglycolate broth	0.007
	Tryptic soy broth	0.006
	Tryptic soy agar	0.007
		0.021 (Total)

(A) Percentage of maternal peripheral blood samples that tested positive for the presence of infectious serology using antibody, nucleic acid and PCR based testing in a period of 24 months. **(B)** Percentage of cord blood samples that tested positive in aerobic and/or anerobic microbial cultures over a period of 24 months. **(C)** Percentage of UCBr that were positive in either one or more growth-based microbiological culture tests in 24 months.
UCBr: Umbilical cord blood allograft.

Microbial cultures on cord blood

In order to determine if collection, storage and shipment of cord blood samples introduced any microbial contamination, it is important to test bioburden in cord blood. Each cord blood sample was tested for rapid growth of aerobic and anerobic organisms using tryptic-soy and fluid thioglycolate broth culture methods. In a period of 24 months, less than 0.04% of the total donors were rejected for positive bioburden outcomes (Table 1B).

Quarantine & microbiological quality control testing on final product post-thaw

To ensure that none of the processing step(s) and/or reagent(s) introduced microbial contamination, one vial from each donor lot of the final UCBr product stored at -80°C was thawed and tested. Broth and plate-based cultures were monitored every 3–5 days, up to 2 weeks. Temperature and growth media were suitable to determine presence of fungi and aerobic, anerobic as well as facultative bacteria. In a period of 24 months, approximately 0.02% of the total donor lots tested were found to be positive for one or more types of contamination and summarily discarded (Table 1C).

Purity of UCBr

Endotoxin Assay

Presence of bacterial endotoxin, 'pyrogens' may induce fever and other adverse reactions caused by inflammatory mediators. Commercially available products marketed as HCT/Ps are regulated solely as section 361 of PHS Act and regulation 21 C.F.R 1271. Such products do not require endotoxin testing under cGTP. Nevertheless, reported use of such allografts often involves different routes of administration which may require information on the endotoxin levels. So, we identified criteria set forth under different regulatory frameworks while determining the allowable limit of endotoxin in UCBr. Therefore, endotoxin testing of UCBr is included in our quality control regimen. Current allowable limits for endotoxin range from 5 EU/kg bodyweight (non-IV drugs) to 0.2 EU/kg bodyweight (intrathecal application). Based on the most stringent criteria, we chose to qualify UCBr at the limit set for intrathecal applications. Considering a subject of 70 kg, 0.2 EU/kg limit allows up to 14 EU in a single application. Endotoxin levels in UCBr were determined using a kinetic chromogenic test in which the reaction

Table 2. **Determination of product purity using endotoxin assay.**

(A) Inhibition/enhancement test and detection of MVD						
Sample dilution	**S1**	**S2**	**S3**	**S4**	**S5**	**S6**
1:10	1298%	80%	139%	81%	166%	186%
1:50	98%	81%	81%	125%	110%	122%
1:100	89%	76%	98%	130%	94%	127%
1:250	76%	72%	125%	115%	145%	106%

(B) Endotoxin values of seven representative UCBr by kinetic chromogenic LAL test							
UCBr	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Dilution	1:50	1:50	1:50	1:50	1:50	1:50	1:50
Sample reaction time CV	0% Pass	0% Pass	0% Pass	0% Pass	0% Pass	0% Pass	0% Pass
Spike value (recovered)	0.325 EU/ml	0.570 EU/ml	0.460 EU/ml	0.376 EU/ml	0.456 EU/ml	0.786 EU/ml	0.614 EU/ml
Spike reaction time CV	2.4% Pass	1.8% Pass	13.1% Pass	4.5% Pass	0.5% Pass	5.4% Pass	6.1% Pass
Spike recovery	54% Pass	95% Pass	77% Pass	63% Pass	76% Pass	131% Pass	102% Pass
Test suitability	Pass	Pass	Pass	Pass	Pass	Pass	Pass
Sample value	<2.5 EU/ml	<2.5 EU/ml	<2.5 EU/ml	<2.5 EU/ml	<2.5 EU/ml	<2.5 EU/ml	<2.5 EU/ml

(A) Percentage spike recovery for UCBr from six donors using inhibition/enhancement test cartridge. Each sample was tested at four different dilution levels (1:10, 1:50, 1:100 and 1:250) with 1:50 dilution showing the most consistent spike recovery across the samples. Dilution of 1:50 was designated as MVD and used in the endotoxin testing. **(B)** Endotoxin testing parameters (sample value, spike recovery, % spike recovery and spike recovery time CV) reported for UCBr from seven random donors using EndoSafe-NexGen-PTS platform.
CV: Coefficient of variation; MVD: Maximum valid dilution; UCBr: Umbilical cord blood allograft.

time of the sample is compared with that of control standard endotoxins (CSE). We tested four dilutions of UCBr ranging from 1:10 to 1:250 (Table 2A) to determine a maximum valid dilution that will not interfere with the biochemical reaction. We concluded that dilution of 1:50 was noninterfering and consistently yielded a spike recovery within 50–200%. The exdotoxin limit for allograft release was set at <2.5 EU/ml based on sensitivity of the cartridge (0.05 EU/ml) and MVD. All UCBr samples tested were <2.5 EU/ml. Representative values of seven samples are provided in Table 2B.

Presence of undesirable components

UCB based allografts are often processed using reagents that may have proteins of nonhuman origin and could potentially end up as residues in final product. Our processing technique for UCBr does not involve any such reagents and thus contaminating molecules are not expected. In order to confirm this, the proteins identified in UCBr samples by mass spectrometry were cross-checked against the Repository of Adventitious Proteins database that identifies contaminating proteins from dust, physical contact or common laboratory reagents like bovine serum albumin. No such contaminants were detected in any of the UCBr samples tested (data not shown).

Immunogenicity of UCBr

UCB sourced allografts for homologous use are considered non-immunogenic as UCB cells are MHC class I (MHCI) dull and negative for MHC class II (MHCII) [26]. However, as applications of such allografts continue to evolve there is a need for direct measurement of the immunogenicity of the allograft itself as a part of the quality control regimen. Immune responsiveness of each of the three allogeneic human PBMC donors was evaluated in a mixed lymphocyte reaction (MLR) format with 30 UCBr donors. Proliferative response was determined using a BrdU ELISA assay on day 4 (Figure 3). Allogeneic PBMC proliferative responses to all UCBr were negligible, being equal to or below mean baseline PBMC proliferative responses with stimulatory index (SI) <1.2. In contrast, the mean two-way control PBMC allogeneic responses for the three donors had SI values between 3.2 and 28.5. In 4-day MLR reaction, all UCBr samples displayed negligible SI compared with the positive control indicating that these cells were not metabolically active. In summary, UCBr failed to stimulate human PBMCs and were nonimmunogenic.

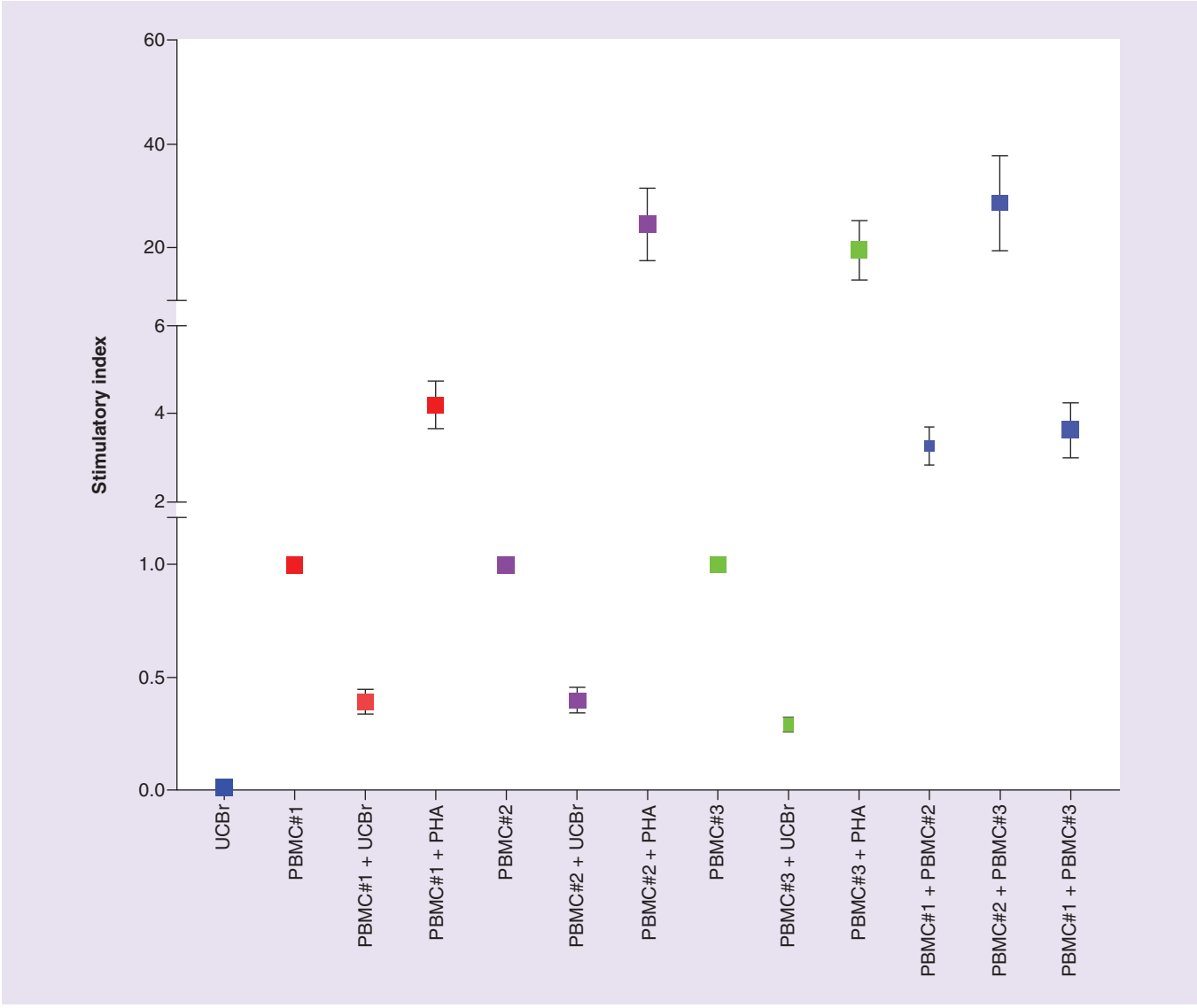


Figure 3. Immunological characterization of umbilical cord blood allograft *in vitro*. (A) Evaluation of UCBr immunogenicity in a two-way mixed lymphocyte reaction assay. Stimulatory index of 30 donors and three PBMCs is expressed as mean ± standard deviation. Data were analyzed by Student's t-test and $p \leq 0.05$ was considered statistically significant.
PBMC: Peripheral blood mononuclear cell; UCBr: Umbilical cord blood allograft.

Determining the biological effects of UCBr

One of the reported applications of UCBr is for articular cartilage injuries. Articular cartilage has inherent limited healing potential, and its damage poses a challenging problem for orthopedic surgeons. The structure and function of this cartilage is dependent on the proliferation, migration and homeostasis of articular chondrocytes that are present in the cartilage. To determine potency, effects of UCBr were investigated on articular chondrocytes isolated from human knee.

UCBr induces chondrocyte proliferation

Articular chondrocytes proliferate and secrete extracellular matrix to maintain and sustain the cartilage, and constitutes a promising strategy for cartilage repair [27]. UCBr lysate at 1:10 and 1:5 dilution induced higher proliferation in HC after 24 h of treatment (Figure 4A) as compared with cells cultured in SFM with no other exogenous factors. HC treated with UCBr at 1:10 dilution showed approximately fourfold increase in proliferation, whereas 1:5 dilution showed about fivefold higher proliferation. Cells that were cultured in the complete growth

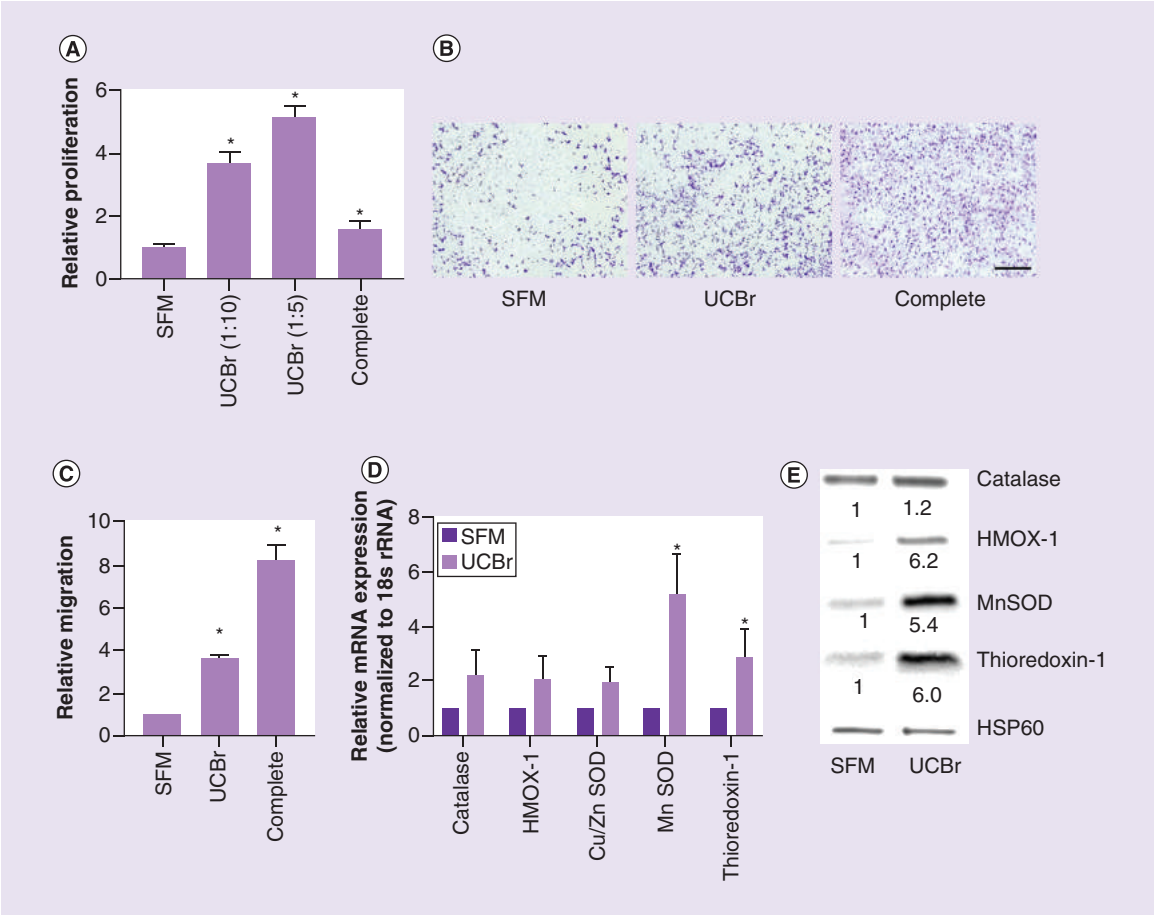


Figure 4. Biological effects of umbilical cord blood allograft. (A) Proliferation rate was measured by fluorescence quantification of total cellular DNA in HC treated with either SFM, UCBr lysates or complete media (10% FBS) and data presented as mean ± standard deviation relative to SFM treatment. **(B)** Cell migration of HC was evaluated using transwell system and representative photomicrographs of the migrated cells in response to SFM, UCBr lysates or complete media are provided. **(C)** Cells migrated per visual field were quantified using ImageJ, normalized first to SFM, then to the rate of proliferation and expressed as mean of relative migration ± standard deviation. Experiments were repeated three-times, each time with 3–5 donors pooled together. **(D)** qPCR was performed on RNA extracted from HC treated with SFM or UCBr lysates pooled from at least three donors. The 18S rRNA transcript level was used to normalize the data, which are presented as mean values for fold change ± standard deviation. **(E)** Protein levels of redox markers in HC treated as in **(D)** were determined by western blot. Densitometry was performed taking signals from Hsp60 as the internal control. Values are expressed relative to SFM treated control and indicated in the panels below the immunoblots. Data were analyzed by ANOVA and $p \leq 0.05$ was considered statistically significant.
*$p \leq 0.05$; Scale bar: 50 μm.
FBS: Fetal bovine serum; HC: Human knee articular chondrocytes; Hsp60: Heat Shock Protein Family D; qPCR: Quantitative reverse transcription PCR; Redox: Reduction–oxidation; SFM: Serum-free media; UCBr: Umbilical cord blood allograft.

medium served as an internal reference of the normal cell proliferation rate. The proliferation rate of the UCBr treated cells was higher than the cells cultured in complete medium. Combination of cytokines and growth factors present in UCBr promotes chondrocyte proliferation.

UCBr induces chondrocyte migration

Chondrocyte migration is challenging as the cells need to overcome the density and pressure of the surrounding matrix to migrate to other sites. Research has shown that chondrocyte migration can help in restoring osteochondral defects [28]. The potential of the UCBr as a chemo-attractant was therefore specifically tested by transwell migration assay using complete growth medium (10% FBS), SFM or UCBr lysates (1:10 dilution, Figure 4B). When normalized to their respective proliferation rate, the net migratory index of the HC cells treated with UCBr was

3.6 ± 0.1 ($p < 0.05$) fold higher when compared with that of the control cells (Figure 4C). These data indicate that the UCBr may aid in osteochondral repair by stimulating chondrocyte migration.

Impact of UCBr on redox metabolism

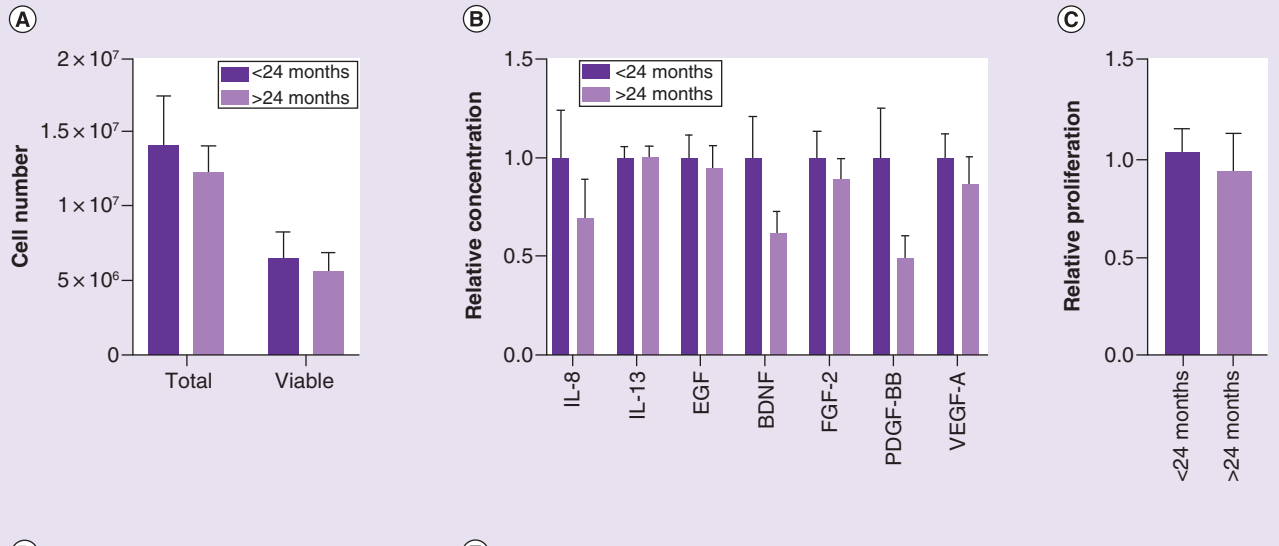
There is ample evidence that cartilage degradation is often due to impaired ROS homeostasis. Strategies to target antioxidant systems may reduce degradation and aid in repair of damaged cartilage [29]. Bioinformatic analysis of the proteins identified in UCBr lysates by mass-spectrometry indicated that several of these proteins are involved in redox metabolism. Therefore, the effect of UCBr on antioxidant enzymes was evaluated both at the transcriptional and translational levels. mRNA expression of several redox marker genes was quantified in chondrocytes treated with UCBr lysates. MnSOD and Thioredoxin 1 were significantly bolstered by 5.2-fold ($p < 0.0001$) and 2.9-fold, respectively ($p < 0.05$), when compared with untreated controls (Figure 4D). Immunoblotting of these markers further confirmed that Thioredoxin 1 and MnSOD were also upregulated at the protein levels (Figure 4E) by similar treatment. Interestingly, mRNA level of *HMOX1* was not significantly upregulated at the transcriptional level but at the protein level HMOX1 expression increased (~6.2-fold) and was similar to Thioredoxin 1 (~6.0-fold) and MnSOD (~5.4-fold). This might indicate a possible post-transcriptional modification of HMOX1 in HC. We were unable to detect Cu/ZnSOD at appreciable levels by immunoblotting of control and treated HC.

Determining the stability of UCBr

Based on literature, the current expiry period of UCBr was set at 24 months [30]. To establish that this criterion is valid, we compared certain aforesaid quality parameters between samples that were less than 24 months (N = 26, median of 3 months) and those above 24 months (N = 23, median 25 months). No significant difference in cell viability was observed between these groups (Figure 5A). As cytokines in UCBr are the key factors influencing tissue regeneration, concentration of seven cytokines (IL-8, IL-13, EGF, BDNF, FGF-2, PDGF-BB and VEGF-A) was measured in these two groups. compared with the samples grouped under <24 months, there was a slight but not statistically significant drop in the relative cytokine concentration (Figure 5B). The biological potency of the products was tested based on its effect on proliferation of HC. Proliferative rate of the samples less than 24 months and greater than 24 months were similar without any statistically significant difference (Figure 5C). Four additional samples between 24 and 30 months were tested for their aseptic nature and no growth was detected (Figure 5D). The endotoxin values of five representative samples between 24 and 30 months were also tested for their endotoxin limits and were found to be <2.5 EU/ml (Figure 5E) same as the samples with median age of 3 months as shown in Table 2B.

UCBr increases the yield of MSC derived exosomes

Current dogma in regenerative medicine indicates that the EVs including exosomes released by the MSC have therapeutic benefits. Altering the conditions of culturing MSC have a significant influence not only on the yield but also on the efficacy of exosomes in terms of the proteomic and genomic complexities [14]. Here, we investigated if exosomes derived from MSC treated with UCBr lysates differ in yield and biological functions when compared with the ones derived from untreated MSC. EVs were characterized based on particle concentration and size distribution using fluorescence-based Nanoparticle Tracking Analysis which specifically identifies microparticles with intact membranes (Figure 6A & B). The concentration of the EVs in the samples obtained from UCBr treated MSC was significantly higher (~twofold) compared with the untreated counterpart (Figure 6C). Nano Tracking Analysis also confirmed a more homogeneous population of EVs obtained from untreated MSC (mean 104.4 ± 20.7 nm) compared with samples obtained from treated MSC which ranged from 91 to 698 nm, with a mean of 288.7 ± 4.1 nm (Figure 6A & B). The heterogeneity of the particle size identified in samples derived from treated MSC may be attributed to the yield of a higher ratio of EVs of varying size in response to the UCBr treatment. The proliferative effect of these EVs was further tested on MSC and HC. EVs derived from UCBr lysate treated MSC showed approximately 2.1-fold (MSC) to approximately fivefold (HC) increase in proliferative rate when compared with the respective controls (Figure 6 D & E). Proteomic analysis of MSC-derived EVs has shown that they contain the factors influencing angiogenesis such as FGF, VEGF, HGF, EGF and IL-8 [13]. We measured the concentration of EGF and VEGF present in the exosomes derived from treated or nontreated MSC. In unit volume, EGF was approximately 2.14-fold higher and VEGF was approximately 2.8-fold higher in the exosomes released by the MSC pretreated with UCBr lysate (Figure 6C).



D

No. of samples >24 months	4
Growth detected under following conditions	
Tryptic soy broth (22.5°C)	0/4
Sabdex broth (22.5°C)	0/4
Tryptic soy broth (32.5°C)	0/4
Fluid thioglycolate (32.5°C)	0/4
Tryptic soy agar plate (22.5°C)	0/4
Sabdex agar plate (22.5°C)	0/4
Tryptic soy agar plate (32.5°C)	0/4

E

>24 months	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
Slope:	-0.402	-0.402	-0.402	-0.371	-0.371
Intercept:	+2.319	+2.319	+2.319	+2.402	+2.402
Spike value (recovered):	0.833 EU/ml	1.22 EU/ml	0.863 EU/ml	0.699 EU/ml	0.632 EU/ml
Spike reaction time CV:	13.6% pass	9.7% pass	21% pass	5.5% pass	3.4% pass
Spike recovery:	98% pass	144% pass	102% pass	91% pass	82% pass
Sample value:	<2.5 EU/ml	<2.5 EU/ml	<2.5 EU/ml	<2.5 EU/ml	<2.5 EU/ml

Figure 5. Determination of umbilical cord blood allograft stability. UCBr were divided into two groups based on their manufacturing date, less than 24 months (N = 9–26) or greater (N = 4–23). Cells were stained with AO/PI and counted with Nexcelom K2. Total and viable cell count **(A)** was determined. **(B)** MAGPIX protein multiplexing assay was performed to determine cytokine concentration in UCBr lysates grouped as in **(A)** with N = 20 donors in each group. Cytokine concentration of samples grouped as >24 months was expressed relative to the mean concentration of the UCBr samples grouped as <24 months. **(C)** Proliferation of HC treated with UCBr lysates was determined by CyQUANT assay. Data are normalized to samples grouped as <24 months and represented as mean of relative proliferation rate ± standard deviation. **(D)** Data summarize growth-based microbiological testing to confirm aseptic nature for UCBr from four donors grouped as >24 months. **(E)** Endotoxin levels for UCBr from five donors grouped as >24 months was tested using EndoSafe-NexGen-PTS platform. Data were analyzed by Student's t-test and $p \leq 0.05$ was considered statistically significant.
AO: Acridine orange; HC: Human knee articular chondrocytes; PI: Propidium iodide; UCBr: Umbilical cord blood allograft.

Discussion

As the clinical application of UCB allografts gains prominence, the mechanistic link between these products and tissue regeneration must be fully explored. Research on such allografts has mainly focused on the stem cell content due to their origin from birth tissues. However, studies in preclinical models make it evident that rather than stem cells, it is the trophic factors that have actual therapeutic impact *in vivo* [8]. We have previously shown that UCB-sourced allografts can induce proliferation, migration and angiogenesis by virtue of the cytokines present in UCB. The knowledge being gained from ongoing research on such allografts has prompted efforts to identify new quality control parameters that may be useful to define suitability release criteria. As the regulatory guidelines continue to evolve, parameters addressing regenerative mechanisms will also be likely included in the developing quality control framework. This is a first-of-its-kind study to put forth a set of quality control attributes which can be used to determine the clinical suitability of UCB-sourced allografts.

We enumerated the cellular component in addition to quantifying cytokines and identifying novel signaling proteins to have a complete picture of the allograft. The total cell count and percentage of cell viability was

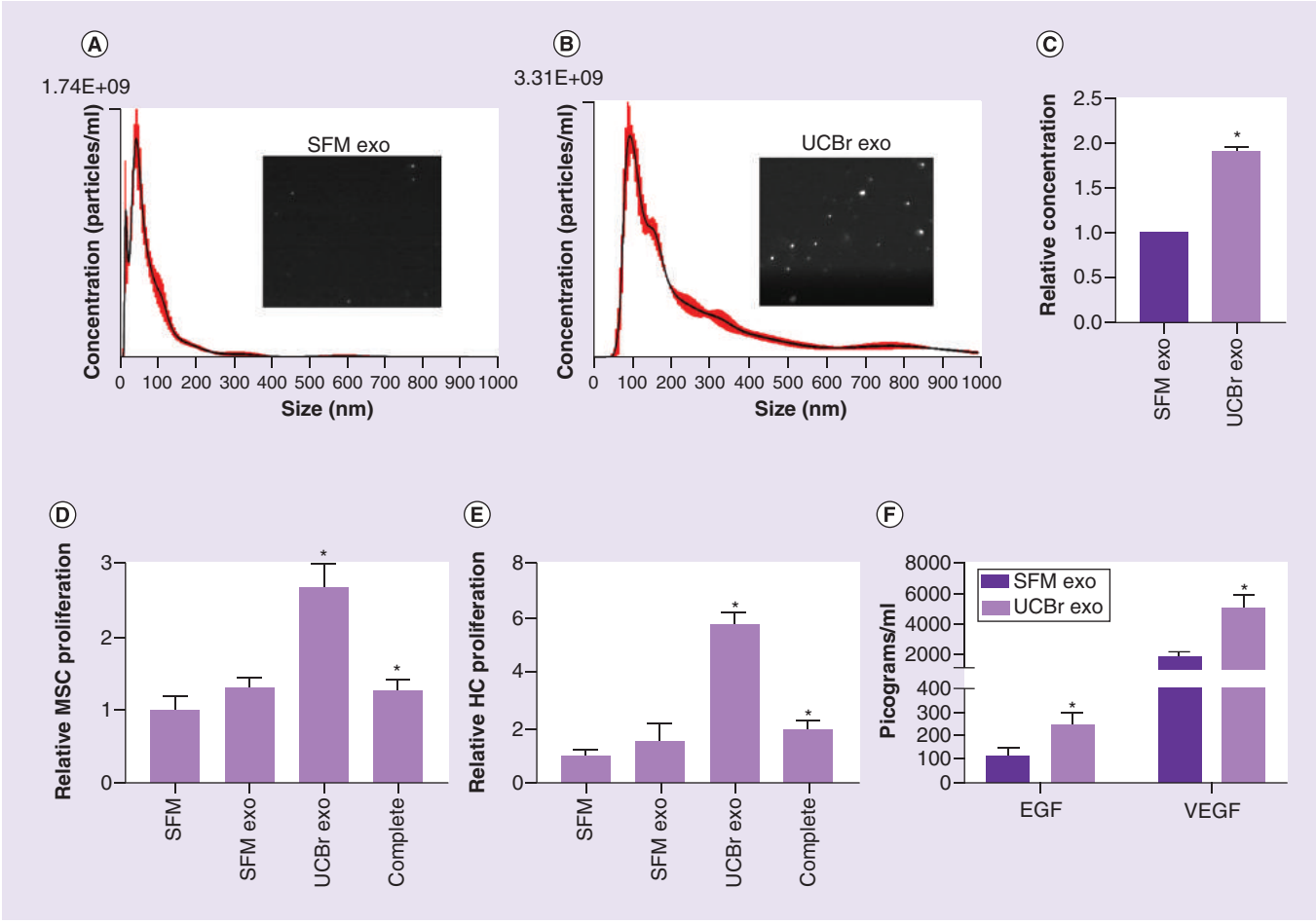


Figure 6. Effect of umbilical cord blood allograft on mesenchymal stem cell derived exosome. MSC were treated with SFM or UCBr lysate for 18 h and exosomes were collected from the respective conditioned media using protocol described in materials and method section. **(A & B)** Quality/size distribution and representative screenshots of the isolated exosome as determined by fluorescence based nanoparticle tracking assay. **(C)** Relative yield of the microparticles by MSC treated with SFM or UCBr lysate is provided as mean ± standard deviation. **(D & E)** Proliferation of MSC **(D)** and HC **(E)** treated with either SFM, SFM-Exo, UCBr-Exo or complete media was measured by CyQUANT assay and values are expressed as mean fold change ± standard deviation. **(F)** EGF and VEGF levels were measured using Luminex xMAP assay on MAGPIX™ platform in exosomes isolated from MSC treated with SFM or UCBr lysates.
Data were analyzed by Student's t-test or ANOVA and $p \leq 0.05$ was considered statistically significant.
*$p \leq 0.05$.
Exo/UCBr-Exo: Exosomes derived from MSC treated with SFM/UCBr; HC: Human knee articular chondrocyte; MSC: Mesenchymal stem cell; SFM: Serum free media; UCBr: Umbilical cord blood allograft.

consistent among the samples tested. Although cell viability and its metabolic activity cannot be correlated to the efficacy of the product, it is indicative of the uniformity of the manufacturing process. We identified a significant percentage of viable cells in UCBr; however, a very low fraction stained positive for stem cell markers according to the International Society for Cellular Therapy guidelines [31]. These data do not preclude the possibility of stem cell presence in the cord blood sourced allograft cells. Isolation of stem cells from UCB has been successfully accomplished previously using specific tissue culture approaches [20,32]. It is interesting to see if UCBr cells cultured under such *in vitro* conditions over extended period of time enables better immunostaining of the stem cell surface markers. However, it is beyond the scope of this quality assessment study and may be a focus of future research. While we do not foresee dependence of UCBr on the viable cell population as a function of its clinical benefit, it demonstrates that the DMSO free cryopreservation techniques are gentle but effective to sustain these delicate cells. The two-way mixed lymphocyte reaction indicated that the cells present in UCBr do not proliferate, and the biological effects are independent of the metabolic function of these cells. Proteomic profiling of the samples revealed several proteins that can influence wound healing, ROS metabolism etc. To our knowledge, this is the first

report identifying global proteins associated with UCB allografts. Random sampling for proteomic profiling as a part of routine quality control may identify possible deviation in the manufacturing process. About 31 cytokines that have a direct or indirect role in tissue regeneration were quantified from a large cohort of donors and found to be consistent. While donor variability is inevitable, strict inclusion–exclusion criteria based on quantitative identification of critical trophic factors can limit product variability and be a part of the quality control parameter.

cGTP guidelines were followed for determining the bioburden of the donated cord blood and the aseptic nature of UCBr. Less than 10% of the donated blood samples were rejected due to serology outcomes highlighting the importance of effective donor screening with well-defined donor inclusion–exclusion criteria. Processing in a constantly monitored, controlled environment can drastically reduce microbial contamination. In a span of 24 months, 99.98% processed products were aseptic. Allografts regulated as HCT/Ps are not required to be tested for endotoxin levels or immunogenicity. But based on the analysis of the reported use of these allografts, these tests may bear clinical relevance. Endotoxin levels of all UCBr products tested were <2.5 EU/ml using the most stringent criteria of allowable endotoxin limit for pharmaceuticals. Testing endotoxin levels on each donor lot may be incorporated as a routine quality control measure to ensure clinical safety on certain instances. UCBr was nonimmunogenic as determined by MLR, on the contrary it suppressed the proliferation of the allogeneic PBMC. This can partially be attributed to the anti-inflammatory cytokines like IL-6, -10 and -13. Concentration of another anti-inflammatory cytokine IL-1RA was approximately 30,000 pg/ml, the highest among all the cytokines tested. Clinical trials on inhibition of IL-1 activity with IL-1RA have concluded that it is a safe and efficient way of alleviating GvHD that failed to respond to conventional treatment [33]. In future, it would be interesting to study the effects of UCBr in mitigating GvHD using preclinical models. Assessing the immunogenicity of the product at routine intervals indicates that the minimal processing requirement is effectively met such that the inherent nonimmunogenic property of the cord blood has not been compromised.

Apart from safety, another critical part of controlling product quality is determining consistency and lack of major variability from lot to lot. Reported use of UCB allografts in cartilage and soft tissue repair prompted us to perform experiments using articular chondrocytes as the model system. HC shows a continuous age-related decline in the proliferative response but can be activated by the mitogenic factors and chemokines like PDGF, SDF1α and VEGF [34–36]. Cell free extracts of UCBr induced both proliferation and migration of chondrocytes by several folds. Results observed with a series of individual growth factors and cytokines identified PDGF as the most potent chemotactic factor for human articular chondrocytes [28]. Cytokine profiling of UCBr has confirmed presence of PDGF and several other factors. ROS generation is another age dependent factor leading to cartilage degradation [37,38]. Lack of defense toward the elevated levels of ROS generated from dysfunctional mitochondria has been shown to be mitigated by elevating the expression of antioxidant proteins [39,40]. Proteomic profiling of UCBr identified proteins that demonstrated involvement in ROS homeostasis. Therefore, we evaluated the effect of UCBr on expression of antioxidants. UCBr specifically upregulated MnSOD, Thioredoxin 1 and HMOX-1 but not Catalase, indicating that the antioxidant effect may be specific than indiscriminate. HMOX-1 is known to play a protective role against tissue injury in human cartilage by upregulating MnSOD, and is a therapeutic target in several pathological conditions related to elevated ROS generation [41]. Our data indicate potential involvement of several cytokines, enzymes and signaling molecules of UCBr in activating proliferation and migration of chondrocytes coupled with building antioxidant defense mechanisms to help in cartilage repair.

Long-term stability is another integral aspect of quality assessment. Volume reduced cord blood stored up to 15 years showed no alteration in post thaw quality [30,42,43]. However, there are no established guidelines to determine the maximum storage period for cryopreserved UCB sourced allografts. Initial storage conditions of UCBr at less than -65°C was set for a period of 2 years. All post-thaw quality attributes were met by samples stored between 1 month and 25 months with no significant differences. Given these results, it can be concluded that UCBr is stable up to 2 years, but ongoing analysis as required by regulatory agencies will determine the actual expiration period.

With the advancement of our knowledge on secretome based therapy, we have started to appreciate the role of EVs/exosomes and consider it to be the next quantum leap in regenerative medicine [44–47]. While MSC derived exosomes are known to contain bioactive molecules that can alter the genetic and proteomic landscapes of the target cells, we were particularly interested to know if the yield and function of exosomes can be modulated by application of UCBr. This will be clinically relevant because host MSC activated by UCBr can release exosomes acting as 'enriched capsules' to trigger a more sustained regenerative outcome. Fluorescence-based nanoparticle tracking analysis identified intact EV-specific particle size and concentration. Current EV isolation methods may

include protein aggregates, membrane fractions and other background particles, which have confounding effects. The structural integrity of the prepared EVs needs to be further characterized by cryo-electron microscopy. While most of these ideas are speculative, initial experiments indicate that exosomes derived from UCBr primed MSC have a higher cytokine content and proliferative effect, along with a higher yield. These data argue favorably for a potential liaison among UCBr, host MSC and their derived exosomes to influence tissue regeneration *in vivo*. We are actively pursuing this line of research to elucidate the mechanistic role of EVs in tissue regeneration in greater detail.

Conclusion

As the field of regenerative medicine continues to evolve, the parameters required to establish its quality must be reviewed periodically and updated accordingly. Here, we put forward a set of experimental approaches to access the identity, safety, purity, potency, and stability of this UCB-based allograft.

Translational perspective

Procuring birth tissues to manufacturing allografts for regenerative therapy has been one of the most remarkable accomplishments in medical practice. However, leveraging the full benefit of these allografts depend on three major factors. First, there is a need to have a complete understanding of the molecular mechanism of action of the allograft; second, to exercise end-to-end control in the processing of the allograft from the birth tissues and at last to have adequate checks and balances in the quality control regime to maintain the highest standard of clinical safety. As the clinical demand of the allografts increases, the regulatory oversight on their manufacturing, marketing and clinical use is parallelly evolving to ascertain that patient safety and interest are not compromised. Considering these factors currently concerning the clinicians practicing regenerative medicine, we performed an array of experiments to develop a quality control regime suitable to clinically qualify a UCB allograft, based on its molecular/cellular component, purity, safety, efficacy and stability. Lessons from both translational research and ongoing clinical trials have led to a paradigm change in identifying the role of exogenous stem cells in tissue repair with concomitant appreciation of the effects of trophic factors in activating the endogenous host stem cells to execute the regenerative process. Therefore, when accounting for the clinical efficacy of UCB allografts, quality control measures should focus more on its ability to initiate signaling pathways to efficiently activate the host's MSC and other healing cells at the site of injury. In the future more clinical studies are required to explore the role of cytokines and growth factors associated with these allografts to fully harness their healing potential.

Summary Points

- Umbilical cord blood (UCB) sourced allografts are widely used in clinics for trauma and degenerative conditions. Since many products are registered as human cells, tissues, and cellular and tissue-based products, a defined regulatory framework is still evolving to address quality control attributes for such allografts.
- In this study, we identified tests to address critical quality attributes of UCB-allograft (UCBr) from a safety and potency perspective. In practice, identity, purity and safety tests can be adapted from existing guidelines, but potency assays/end points will vary based on reported application of the product.
- Here, we characterized the 'identity' of UCBr-cellular components, based on cell count, diameter and viability. Flow cytometric analysis of the mixed cell population using stem cell surface markers was used to effectively categorize cellular population in UCBr. Mass spectrometry and protein multiplexing in decellularized UCBr quantified several different proteins, cytokines, growth factors and signaling molecules which constitutes the noncellular portion of this allograft. These tests may be routinely employed to monitor the product and assess the consistency of the manufacturing techniques.
- Endotoxin assay was used to determine 'purity' of the allograft. Aseptic clinical products derived from biological materials have an inherent risk of microbial contamination in the final product. Stringent donor inclusion–exclusion criteria were established based on peripheral blood serology and cord blood microbial testing. Additionally, cryopreserved final product underwent 'safety' testing based on growth-based microbiological cultures.
- Based on the reported application UCBr in cartilage repair and regeneration, biological efficacy of UCBr was tested *in vitro* using human chondrocytes.
- UCBr stored at temperature less than -65°C was stable up to 24 months after production. Stability was established with protein multiplexing (stable chemical composition), endotoxin assay, potency assays (intact biological efficacy) and microbiological testing (maintaining aseptic nature).
- Identifying the tests that will characterize a biologics product is often challenging. To the best of our knowledge, this is the first report enumerating the tests to address identity, purity, safety and potency for a UCB allograft which may be developed into qualified and validated assays.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/full/10.2217/rme-2019-0058

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Financial & competing interests disclosure

MS Sane, H Tang, S Banerjee Mustafi, CD Jones and S Malara are employees of Burst Biologics. N Misra was previously employed at Burst Biologics. Burst Biologics has filed a patent pertaining to the processing method of the UCB sourced allograft described in the manuscript. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

UCB was procured from consenting adults and manufactured in a US FDA-registered facility following all necessary regulatory guidelines as per the FDA's current Good Tissue Practice.

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