CHAPTER 16

Regenerative Medicine and Ageing: Is Senescence Reprogrammable?

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INTRODUCTION

Few biological phenomena have proved as intractable to scientific investigation and yet remained as fundamental to the human condition as the process of ageing. Despite this prolonged resistance to analysis, there is mounting evidence that the molecular pathways regulating senescence, like much other complex pathology, can be identified using current molecular and biochemical methodologies. In this brief review, we will describe some recent research trends, with a particular focus on those relating to regenerative biology, and suggest possible paths for future inquiry and therapeutic development.

Alex Comfort proposed that ageing may be defined as 'a failure to maintain homoeostasis under conditions of physiological stress, and that this failure is associated with a decrease in viability and an increase in vulnerability of the individual (Comfort, 1979)'. While potentially circumscribing the bulk of the observations relating to ageing, Comfort's definition is so general to be of little use in current scientific investigation. How does one, for instance, perform genome-wide association studies that correlate genotype with 'a failure to maintain homeostasis' or 'increased vulnerability' of an individual?

Benjamin Gompertz proposed another commonly used definition where ageing is characterised as biology that leads to an exponential increase in mortality with time ($R_m = R_0 e^{at}$) where R_m is the chance of dying at time t, R_0 is a constant and a is the exponential parameter, which gives the rate of mortality increase with age (Comfort, 1979; Kowald, 2002). This criterion is potentially more useful than Comfort's as it may differentiate stochastic mortality risk from programmed ageing. However, recent analysis of mortality risk in humans suggests that there may be subtle exceptions. Studies of individuals over 100 years of age suggest that the risk of mortality is not strictly exponential in advanced old age (Barbi et al., 2018).

Another fundamental observation to consider when laying an empirically based foundation for research in ageing is that the process appears to affect every tissue type, including reproductive organs (Hayflick, 1994; Strehler, 1999). However, despite this, the reproductive lineage of cells themselves must necessarily retain a potential for

Second Generation Cell and Gene-based Therapies
ISBN 978-0-12-812034-7
https://doi.org/10.1016/B978-0-12-812034-7.00016-9

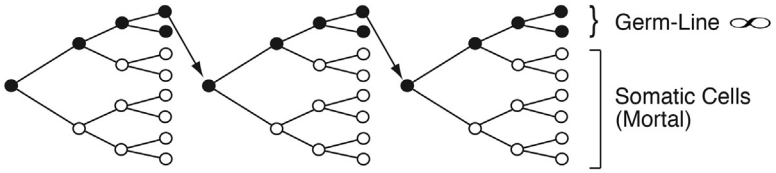

Figure 16.1 ***The germ line/soma dichotomy.*** In addition, he is commonly credited with the hypothesis that ageing, or the mortality of the somatic cell lineages, is a reflection of a fundamental difference in the germ line lineage of cells that are capable of regenerating new life cycles and replicating indefinitely (replicative immortality), while somatic cell types are deprived of indefinite regeneration and replication. (Figure does not include representation of sexual reproduction and meiosis.)

replicative immortality. After all, the senescence of the germ line would abrogate the continual reproduction of the species in question (McLaren, 1992; McLaren, 2001). The 19th century naturalist August Weismann is generally credited with describing the dichotomy of cell fates of the immortal lineage of reproductive cells (potentially interrupted by meiosis and sexual recombination in most species) designated the 'germ-line' lineage of cells and the diverse mortal types of cells in the body (somatic cells, Fig. 16.1).

In an Essay entitled *The Duration of Life* Weismann postulated 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 (Weismann, 1891)'. Put another way, he hypothesised that there were at least two differences in immortal germ line cells compared with their mortal somatic counterparts:

1. Mortal somatic cells cannot *regenerate* forever, and;
2. Mortal somatic cells have lost the capacity to *replicate* indefinitely.

The lack of senescence in the germ line does not appear to be merely an epiphenomenon of sexual reproduction because even vertebrates are capable of reversion to parthenogenesis without compromising fecundity or the immortality of the species (Newton et al., 2016).

In the 20th century, numerous studies provided support to the Weismann hypothesis. The life span of phylogenetically diverse animals was examined and compared with Weismann's twin properties of regenerative potential and cell life span. As shown in Fig. 16.2, protozoa, such as those of the genus *Tetrahymena,* appear to have not passed the Weismann barrier in that they display the primordial characteristic of replicative immortality even in the absence of sexual reproduction (Nanney, 1974). Multicellular organisms such as hydra (Martinez, 1998) and the sponges (Funayama, 2013) were shown to display complete regenerative potential as well as an absence of observable senescence. In the case of the sponge *Monorhaphis chuni*, one animal was reported to be approximately 11,000 years of age (Jochum et al., 2012). Even some primitive bilaterians such as the planaria species *Schmidtea mediterranea* have been shown to display complete regenerative potential and an absence of observable senescence (Dalyell, 1814; Sahu et al., 2017).

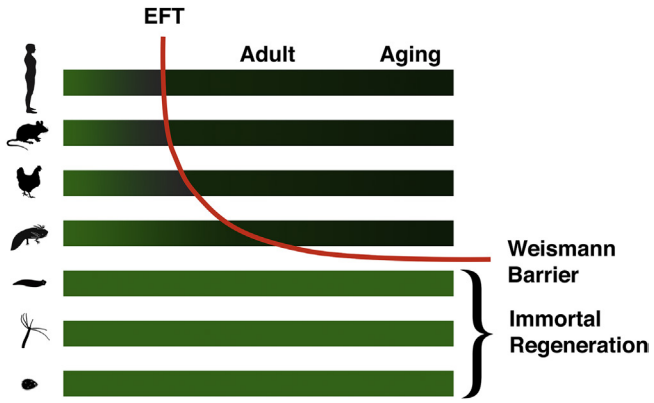

Figure 16.2 ***The Weismann barrier.*** The evolution of increasing somatic cell complexity is displayed on the Y-axis and time on the X-axis. Shown are representative organisms that appear to display indefinite regenerative potential over time such as members of the genus Tetrahymena, Hydra or family Planariidae. Organisms of increasing complexity, such as the Axolotls, display a relatively long life span with profound regenerative potential, but they do not show a phenotype of indefinite regenerative potential. Finally, more complex vertebrates such as members of the genus Gallas, Mus or Homo show regenerative potential only in the early stages of embryonic development.

It has been recognised that some species of a comparable complexity to planarians, such as the adult roundworm *Caenorhabditis elegans,* live only a couple of weeks. It has been suggested that the profound difference in longevity between species such as these reflects the complete or nearly complete differentiated nature of the somatic cells in *C. elegans*. There appears to be a lack of pluripotent or even multipotent stem cells in the soma of *C. elegans,* while planarians have reserves of up to 25% of their total cellular mass comprising such stem cells designated 'neoblasts'. Neoblasts appear to be capable of regenerating indefinitely all somatic and germ line cells (Reddien and Sanchez Alvarado, 2004). As a result, the planaria species have not functionally traversed the Weismann barrier because the large reservoir of totipotent cells and their unlimited replicative capacity can be drawn on to repair or replace damaged somatic cell components indefinitely.

If replicative immortality and complete regenerative capacity of somatic cells is a property of some primitive multicellular organisms and confers longevity benefits, an interesting question to ask is why these properties are not frequently present in more complex species. In 1957, George C. Williams proffered the theory of antagonistic pleiotropy to explain the origin of ageing (Williams, 1957). According to his hypothesis, there is a subset of genes with pleiotropic effects, in this case, differential effects on the organism when it is young as compared with when it is old and has passed the reproductive period. Williams proposed that ageing is caused by just such an evolutionary selection of phenotypes and associated genotypes and that the selected traits are antagonistic. That is, they increase the likelihood of survival and reproductive fecundity in the young, while decreasing viability in the postreproductive years and old age. Today, it is

commonly asserted that William's antagonistic pleiotropy may have largely centred on a selection of traits that function in tumour suppression, reducing the risk of cancer early in life but negatively impacting cell proliferation and tissue maintenance late in life.

To test the Weismann and Williams hypotheses, it is interesting to ask whether human germ line cells can be compared with mortal somatic cell types cultured in vitro to provide a model system for studying the molecular biology and biochemistry of human ageing and whether, indeed, such processes function in tumour suppression.

HUMAN CELL SENESCENCE AND IMMORTALISATION

In the 1960s, Leonard Hayflick published support of one of the predictions of Weismann; namely, that normal diploid human fibroblasts when cultured in vitro displayed a finite replicative capacity, today commonly referred to as the 'Hayflick Limit' (Hayflick and Moorhead, 1961; Hayflick, 1968). Subsequently, numerous other human somatic cell types were reported to display a mortal phenotype. In the following years, it came to be recognised that all foetal and adult human somatic cell types capable of proliferation in vitro displayed the mortal phenotype, even multipotent stem cells such as mesenchymal stem cells (Bruder et al., 1997). In contrast, consistent with the model of antagonistic pleiotropy, cells cultivated from malignant tumours commonly showed an indefinite replicative capacity or 'immortality' to borrow Weismann's terminology (Holliday, 1975). However, for most of the 20th century, it was not known whether the immortalisation of somatic cells associated with oncogenesis occurred in vivo or in vitro, as a result of cell culture, as opposed to the formation of malignancies in vivo as predicted by Williams.

In the 1970s, Olovnikov proposed a clocking mechanism for somatic cell replicative senescence (Olovnikov, 1971, 1973). He suggested that somatic cells progressively lose telomeric DNA due to the lack of expression of an enzyme capable of fully replicating the chromosome ends leading to senescence. In contrast, he proposed that germ line and cancer cells express an enzyme capable of extending telomeres allowing for replicative immortality. That enzyme came to be known as telomere terminal transferase, or, more succinctly, telomerase. The cloning of the telomerase RNA and catalytic components demonstrated that the catalytic component alone was limiting in mortal somatic cells (Feng et al., 1995; Nakamura et al., 1997) and allowed for the first time a test of the Olovnikov hypothesis. It was subsequently reported that expression of the catalytic component of human telomerase (*TERT*) was capable of immortalising diverse types of human somatic cells (Bodnar et al., 1998) and that telomerase was expressed in nearly 90% of cancer cell types (Kim et al., 1994). The latter strongly supported Williams' model of antagonistic pleiotropy. In this case, it can reasonably be proposed that the repression of telomerase early in life is selected for in some organisms as an effective barrier to the extended proliferation required for cells to undergo multiple genetic hits and clonal expansion in the course of oncogenesis, while later in life, the absence of telomerase leads to the deleterious effects of cell senescence.

THE REGULATION OF HUMAN TISSUE REGENERATION

As discussed previously, in addition to the loss of replicative immortality, Weismann described a repression of *regeneration* in the soma that prevented a tissue from renewing itself following injury. It is well established that in mammals, the primordial capacity for profound regeneration is present early in development and that it is lost as early as the embryonic-foetal transition (EFT). In some tissues, such as the heart, this potential for scarless regeneration extends even to the postnatal period, detectable for approximately a week past the prenatal-postnatal transition period for cardiac tissue (Porrello et al., 2011). Given the importance of understanding and modulating tissue regeneration and tissue growth for the fields of regenerative medicine and oncology, improved methods for modelling and modulating the biology in vitro and in vivo have significant potential utility in research and clinical practice.

In the case of the telomere hypothesis of cell ageing and immortalisation, the in vitro model of human cell ageing provided an invaluable model system for deciphering the mechanisms of cell ageing and immortalisation by allowing the comparison of mortal cells with their immortal counterparts. This model eventually led to the discovery of the upstream mechanisms of telomerase regulation of the transition from the immortality of the germ line to the mortality of somatic cell types. Is it possible to generate a similar human in vitro model to analyse the second factor in the Weismann barrier? In this case, instead of the model being one of comparing mortal and immortal cells, the goal would be to provide a human in vitro model of diverse cell types such as those of vascular, osteochondral, muscular lineages, in both a pre-EFT and post-EFT phenotype so that the EFT can be studied analytically across the spectrum of somatic cell types.

Human embryonic and human pluripotent stem cell lines in the late 1990s were derived from discarded IVF embryos (human embryonic stem (hES) cells) (Thomson et al., 1998) and embryonic gonads (human embryonic germ (hEG) cells) (Shamblott et al., 1998). As a result, they provided a source of diverse somatic cell types and their respective embryonic anlagen. Evidence that the cells differentiated from hES and hEG cells possessed a pre-EFT regenerative phenotype came from the observation that derivative cells, such as those from embryoid bodies, displayed a capacity for self-assembly into three-dimensional organoids, a field that has generated interest as a potential pathway for both obtaining tissue for transplantation (Singh et al., 2015) and modelling human embryonic development. In contrast to embryonic cells, foetal- and adult-derived cells often show reduced potential for organogenesis in vitro and epimorphic regeneration in vivo. Epimorphic regeneration, sometimes referred to as 'epimorphosis', refers to a type of tissue regeneration wherein a blastema of relatively undifferentiated mesenchyme proliferates at the site of injury with the result that the cells differentiate restoring the original tissue histology (Morgan, 1901).

As discussed above, the developmental timing of the loss of epimorphic potential cannot be fixed precisely, and likely varies with tissue type. Nevertheless, the EFT which

occurs at about the end of 8 weeks of human development (Carnegie Stage 23) is commonly associated with a significant loss or scarless regeneration in numerous tissues. In the mouse, the corresponding loss of skin regeneration is reported to occur between E16.5 (scarless) to E18.5 (scarring) (Walmsley et al., 2015), and similar timing is observed in marsupial mammals which are born at Carnegie Stage 15–17, and lose the potential for scarless repair at a comparable stage as placental mammals (Armstrong and Ferguson, 1995).

Therefore, one approach in identifying the pathways regulating the loss of regenerative potential utilises human pluripotent stem cell–derived cells and compares them with their adult counterparts to identify markers of the transition. Once such markers are identified, they can be validated by determining whether they show a parallel pattern of expression during human and mouse tissue development in vivo. The isolation of many diverse hES cell-derived clonal embryonic progenitor cell lines (West et al., 2008) allows a robust model for comparing a multiplicity of human embryonic (prefoetal) somatic cell types to their adult counterparts.

The transcriptome of diverse hES cell-derived clonal embryonic progenitor cells were compared with their adult counterparts facilitated by deep neural network machine learning algorithms (West et al., 2018). In a search for pan-EFT markers, transcripts such as that for *COX7A1* were identified that are not expressed in diverse embryonic tissues but are seen to be increasingly expressed following the EFT in many stromal and to a lesser extent epithelial cell types. In the case of *COX7A1*, a similar upregulation was observed at about the predicted EFT of the developing mouse and human dermal fibroblast samples. Lastly, it was observed the majority of sarcoma and carcinoma cell lines express a pre-EFT pattern of gene expression (an embryonic phenotype) consistent with Williams' model of antagonistic pleiotropy. This and other genes regulated at the EFT may provide useful markers for studying the basis of the transcriptional regulation of the EFT in normal development and providing insights into modulating the pathways for therapeutic effect.

Medawar (Medawar, 2008) and Leopold (Medawar, 2008) distinguished the use of the terms 'ageing' and 'senescence'. The term senescence was used for the progressive degenerative processes that lead to age-related degenerative disease and death. In contrast, the term 'ageing' refers to a broader category of time-related processes, including those related to growth and development. An interesting possibility for future investigation is to test more broadly the unified hypotheses of Weismann and Williams that a repression of immortality and regeneration occur early in development, both potentially selected for as a means of tumour suppression, and the consequence is age-related disease and death. Williams may have perceived this synthesis when he commented concerning ageing that 'It is indeed remarkable that after a seemingly miraculous feat of morphogenesis a complex metazoan should be unable to perform the much simpler task of merely maintaining what is already formed (Williams, 1957)'. In this model of ageing, the critical upstream mechanisms regulating senescence are not as much alterations that

occur late in life as they are developmental ageing alterations that can occur early in life, even as early as embryogenesis to suppress oncogenesis later in life. The rare emergence of cancer would then be expected to reflect a reversion of a mortal/nonregenerative adult cell to an immortal/embryo-onco phenotype that contributes to tissue growth. Both the role of cellular ageing and repression of regeneration need to be tested by stimulating and inhibiting key regulators of the processes to determine their effects in animal models of ageing.

This model of ageing is not inconsistent with many of the published reports implicating nutrient sensing and associated growth signalling pathways in regulating life span. Pathways such as AMPK (Kahn et al., 2005; Salminen and Kaarniranta, 2012), FOXO (Kenyon, 2010), mTOR (Sabatini, 2017), NAD-dependent deacetylases such as SIRT1 (Guarente, 2013) and GH/IGF-1 axis (Milman et al., 2016) have all been suggested to play important roles in linking nutrition, metabolism and ageing. However, *how* they impact ageing has never been clearly described. Because animals commonly experience alternating periods of feast and famine and have adapted not only by drawing on with fat reserves in times of famine but also by catabolising other tissues such as skeletal muscle to provide energy, it is reasonable to propose that energy sensing pathways may also regulate the ability to regenerate those tissues in times of energy surplus. Dietary restriction is well documented to modulate life span in numerous species. Is it therefore possible that nutrient sensing pathways effect ageing and life span by modulating regenerative potential?

Another area of current research interest is the effect of therapeutically targeting senescent cells for apoptosis, a strategy designated 'senolysis (Baar et al., 2017)'. As tissues capable of regeneration can simply replace cells targeted for removal due to genotoxic events, while there may have been selection of a repression of apoptosis in terminally differentiated cells that cannot regenerate, it is tempting to speculate that induction of regeneration in famine-feasting cycles may also facilitate senolysis as those tissues may have increased potential to regenerate. All these hypotheses need more serious investigation to determine the role of regeneration in ageing.

THERAPEUTIC STRATEGIES FOR AGEING

The feasibility of modulating the nonregenerative and mortal state of adult somatic cells was clearly demonstrated with the advent of somatic cell nuclear transfer and induced pluripotent stem (iPS) cell technologies. Both technologies were shown to reverse both the developmental aspects of ageing and cellular ageing (the activation of telomerase and extension of telomere length) (Lanza et al., 2000;Vaziri et al., 2010). These experiments suggest that ageing is epigenetic in nature and profoundly reprogrammable. Therapeutic cloning (Lanza et al., 1999) and iPS cell technology (Takahashi et al., 2007) therefore enable methods for the production of functionally young cells from old individuals potentially useful in treating the degenerative diseases associated with ageing.

Analytical reprogramming (the use of individual or defined modulators of induced pluripotency) include not only the expression of genes such as *KLF4, OCT4, SOX2, MYC* or *LIN28* but also small molecule effectors. These tools for reprogramming adult somatic cells to pluripotency may provide reagents for understanding the reprogramming cells only back to a regenerative state (i.e., back before the EFT) while otherwise leaving their differentiated status intact, a field of research called 'induced tissue regeneration' (iTR).

Evidence that iTR may impact regeneration and even ageing in a mammal results from the production of transgenic mice carrying constructs for the expression of *KLF4, OCT4, SOX2, MYC* or *LIN28*. In one report, the leaky expression of *Lin28a* was reported to lead to increased regenerative potential compared with wild type (Shyh-Chang et al., 2013). In addition, the induction of *KLF4, OCT4, SOX2* and *MYC* in such a manner as to result in 'partial reprogramming' has been reported to activate telomerase and extend telomere length in animal models and reverse markers of ageing (Ocampo et al., 2016).

Perhaps nearer to clinical utility is the use of pluripotent stem cells to manufacture on an industrial scale a wide-array of cell types (Fig. 16.3) for use in transplantation. Because many pluripotent stem cell-derived cell types derived in vitro appear to express markers of a pre-EFT state, such cell-based therapies offer considerable promise to restore functionality in the context of age-related degenerative disease. One salient example currently in clinical trials is the transplantation of pluripotent stem cell–derived RPE cells into the subretinal space as a therapeutic strategy for age-related macular degeneration, which the leading cause of blindness in an ageing population (Singh et al., 2018).

PERSPECTIVES

The biology of ageing is increasingly the focus of biomedical research due to the rapid growth of new insights into the molecular pathways involved as well as the enormous economic challenges ageing presents to numerous industrialised countries around the world. Up to 80% of the elderly experience chronic degenerative disease and 63% have been reported to have two or more such conditions. The biology of cellular ageing and regenerative biology may provide deeper insights into the mechanisms of ageing and provide a conceptual framework for testable hypotheses in what would otherwise be an unintelligible maze of interacting pathways. Regardless of the role of epimorphic tissue regeneration in the biology of ageing, a means of restoring functionality to discrete tissues through the use of pluripotent stem cells provides a means of potentially improving the quality of life in individuals afflicted with age-related chronic degenerative disease.

Planaria appear to escape ageing by carrying totipotential and immortal stem cells accounting for up to 25% of their total cellular mass that can be drawn on to repair even severe traumatic tissue damage. The question remains as to the extent the human species

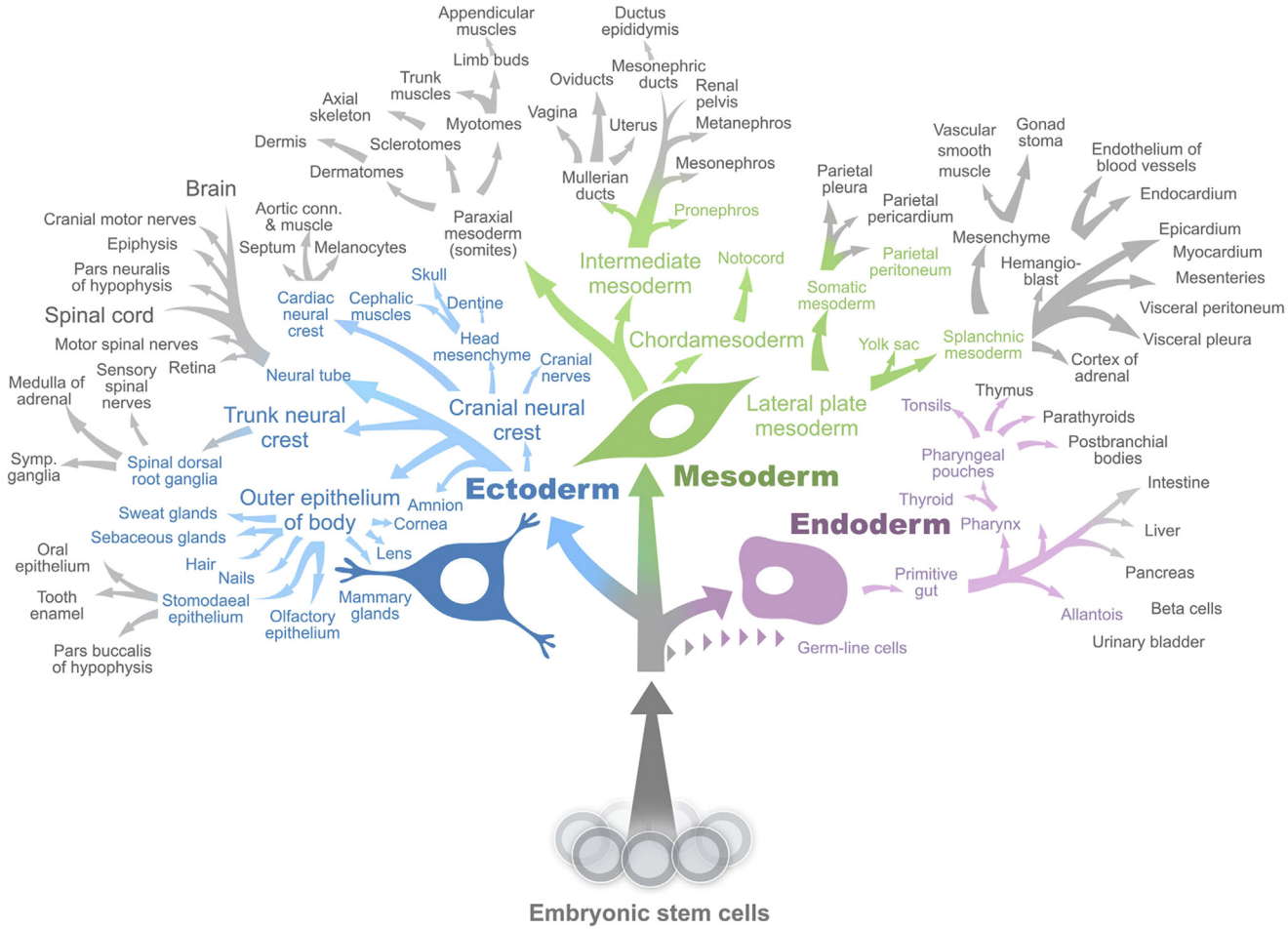

Figure 16.3 ***The pluripotency of human embryonic stem cells.*** Human embryonic stem and induced pluripotent stem cells, like their murine counterparts, show the capacity of differentiating into derivatives of all three embryonic germ layers: endoderm, mesoderm and ectoderm. However, unlike many murine cell lines, human cells are commonly 'primed' wherein they have reduced capacity for germ line differentiation.

will be able to match the performance of the lowly flatworm in not only coming into existence through a 'miraculous feat of morphogenesis' but also performing 'the much simpler task of merely maintaining what is already formed'.

COMPETING INTEREST STATEMENT

The author is an employee and shareholder of BioTime, Inc. (Alameda, CA) and AgeX Therapeutics, Inc (Alameda, CA) both of which are developing therapeutics in the field of regenerative medicine and ageing.

ABBREVIATIONS

EFT Embryonic-foetal transition
hEG cells Human embryonic germ cells
hES cells Human embryonic stem cells
iPS cells Induced pluripotent stem cells
iTR Induced tissue regeneration
RPE Retinal pigment epithelium
SCNT Somatic cell nuclear transfer

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