Stem cells, plasticity and cancer – uncomfortable bed fellows

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Summary

Stem cell research is a vibrant and rapidly moving field of science that investigates self-renewing cells in the adult and embryo. Two debates currently exist in the stem cell field concerning the transcriptional redirection of stem cell differentiation and fate, and the reorganization of cell commitment through nuclear reprogramming caused by cell fusion and nuclear transfer. The recent Keystone Symposium in Colorado on stem cells, organised by Fiona Watt and Leonard Zon, brought together both leading and upcoming researchers in the field to explore stem cell biology and these issues.

Stem cell research is one of the most controversial fields of science today. Controversies in the field include: access to early human embryos from infertility treatment clinics for the derivation of human embryonic stem (hES) cells (Reubinoff et al., 2000); the production of ES cells from embryos formed by nuclear transfer of somatic cells into oocytes (Munsie et al., 2000); the transdifferentiation or de-differentiation of adult stem (AS) cells of one tissue type to another; and the clinical applications of stem cell therapies.

The focus of many presentations at the Keystone Symposium earlier this year was the control of stem cell renewal, and the molecular and cellular mechanisms of stem cell differentiation, particularly the role of growth factors and extracellular inducers, the regulation of gene expression and the reoccurring activation of specific messenger pathways during differentiation.

The nature of pluripotential ES cells

Pluripotent stem cells produce all or most cell types in the body, whereas multipotential stem cells produce several cell types of a particular lineage. The important questions of how pluripotential ES cells are maintained and directed to differentiate into the primary embryonic lineages of endoderm, ectoderm, mesoderm and extraembryonic endoderm were addressed in several presentations. Austin Smith (University of Edinburgh, UK) demonstrated how important regulators of stem cell renewal and pluripotentiality can be identified in mouse (mES) cell cultures in the absence of serum or feeder cells. Leukaemia inhibitory factor (LIF) inhibits mES cell differentiation by activating the JAK-STAT pathway, but requires serum. However, LIF together with bone morphogenetic protein 4 (Bmp4) can block mES cell differentiation in serum-free medium, enabling the de novo derivation and maintenance of mES cells. Bmp4 works through the Smad pathway to increase the expression of the Id_1, Id_2 and Id_3 genes (which encode negative HLH proteins that inhibit differentiation), thus blocking mES cell differentiation into ectoderm, whereas activation of the LIF-Stat3 pathway blocks mesodermal differentiation (Smith, 2001).

There is considerable interest in the factors that govern pluripotentiality. If key regulators can be found, can these be manipulated to enable stem cells to be derived from a broader range of mouse strains and other species than is presently possible? The transcription factors Oct4 and Nanog are expressed in all mouse and human ES cells, and are characteristic of undifferentiated pluripotential cells. Decreasing *Oct4* expression levels in mouse and human ES cells generally results in the loss of pluripotentiality (Buehr et al., 2003). As Shawn Burgess (National Human Genome Research Institute, Bethesda, USA) discussed, microarray analysis has identified over 500 human, mouse and zebrafish genes that undergo significant changes in expression when *Oct4* levels are altered.

During mES cell formation, the blastocyst-stage pre-implantation embryo is allowed to attach to the culture dish, and the inner cell mass outgrowths form mES cell colonies. However, not all the cells of these outgrowths express *Oct4*. Joanna Maldonado-Saldivia (The Wellcome Trust/Cancer Research UK Gurdon Institute, Cambridge, UK) and colleagues in Azim Surani's laboratory have been examining the expression profiles of $Oct4^+$ and $Oct4^-$ cells. The *Oct4*-expressing ($Oct4^+$) cells inside the outgrowth retain the expression of other pluripotency genes, such as embryonal stem cell-specific gene 1 (*Esg1*). Preliminary experiments using RNAi suggest that transient loss of *Esg1* expression results in increased mES-cell differentiation and the promotion of ectodermal differentiation. Hence, there appears to be an organised microenvironment required for the generation of mES cells.

hES cells are derived similarly to mES cells, but their maintenance and renewal does not appear to involve LIF (Alan Trounson, Monash University, Australia). Sphingosine-1-phosphate (S1P) and platelet derived-growth factor (PDGF) maintain hES cells under serum-free conditions (A. Pebay, R. Wong, S. M. Pitson, E. J. Wolvetang, G. S.-L. Peh, A. Filipczyk, K. L. L. Koh, I. Tellis, L. T. V. Nguyen and M. F. Pera, unpublished). hES cell maintenance appears to involve signalling pathways that are activated by tyrosine kinase receptors acting synergistically with those that are downstream of lysophospholipid receptors.

In the human, BMPs derived from hES cells drive the formation of extraembryonic endoderm in an autocrine manner that can be blocked by the BMP2 antagonist noggin (Pera et al., 2004). The apparently homogeneous colonies of noggin-treated hES cells are neuroectodermal in character, and form neurons and glia very efficiently under appropriate culture conditions. As Alan Trounson discussed, this is one of the first examples of the directed differentiation of hES cells. Treating hES cells with specific growth factors and embryonic tissues can also enrich for specific lineages. For example, cardiomyocytes are formed when hES cells are cultured with mouse visceral endoderm (Mummery et al., 2003), and respiratory precursors and prostate tissue form from hES cells

that are co-cultured with embryonic mesenchyme (R. Mollard, A.T., M. Denham, R. Jarred and G. Risbridger, unpublished).

Epithelial stem cells and cancer

There is increasing interest in the origins of cancer in adult stem cell populations and their derivatives. Mutations may arise in the slowly renewing stem cells that are retained in their niche; their oncogenic properties are then exposed when they are recruited for differentiation. Loss of regulatory control in early differentiation stages may also lead to tumours and could explain the heterogeneity of cell types encountered in cancers (see Fig. 1). Interesting observations from several stem cell types (particularly epithelial cells) that supported this idea were discussed.

The epidermis is continuously renewed throughout life by the precise regulation of a small population of stem cells. Epidermal stem cells do not express markers of differentiated cells, are relatively quiescent [and are called label-retaining cells (LRCs) as they retain BrdU label], and reside around hair follicles, sebaceous glands and interfollicular epidermis (Owens and Watt, 2003). These cells are responsive to Wnt signalling via the frizzled receptor, which increases β-catenin expression (Gat et al., 1998; Huelsken et al., 2001; Niemann et al., 2002). Fiona Watt and colleagues (Cancer Research UK, London, UK) have used a transgenic mouse with a β-catenin/oestrogen receptor construct that can be stimulated with the oestrogen analogue Tamoxifen (Celso et al., 2004) to investigate how different levels of β-catenin affect stem cell differentiation. High levels of β-catenin stimulate the hair follicle lineages to differentiate, medium levels stimulate the sebaceous gland lineage, and low levels stimulate the interfollicular epidermis. Lineage conversion also occurs between hair follicles, sebaceous glands and interfollicular epidermis. They also presented evidence that hair follicle tumours can be induced by activation of β-catenin, and sebaceous tumours form in epidermis in which β-catenin signalling is blocked with an N-terminally truncated form of Lef1. These data link epithelial stem cells with oncogenic progenitors.

Using fluorescence-activated cell sorting (FACS), it is clear that LRCs can also be isolated as a side-population (SP) of mammary cells. Jeffrey Rosen and colleagues (Baylor College of Medicine, Texas, USA) have been examining gene expression in normal and breast cancer cells that are derived from LRC stem cells. The SP and LRC cells represent ~0.5% of mammary epithelial cells, and 75% of the SP cells are positive for Sca1 (stem cell-associated antigen 1, which is present in many adult stem cell populations). $Sca1^{+}$ cells localize to the terminal end buds of growing ducts. When transplanted, these cells produce outgrowths, whereas $Sca1^{-}$ cells do not. Mammary hyperplasias and Wnt1-expressing tumours in MMTV-Wnt1 transgenic mice are $Sca1^{+}$ and express the transcription factor and master regulator of mammary epithelial cell fate, keratin 6 (K6). Mammary tumours in transgenic mice that express β-catenin and c-Myc, downstream components of the canonical Wnt signalling pathway, also have cells expressing K6. This indicates that breast tumour heterogeneity appears to derive from the activation of specific oncogene and/or tumour suppressor-regulated signalling pathways in specific mammary progenitors.

Mutations in Wnt pathway genes represent the initiating mutation in nearly all colorectal cancers. *Tcf4* null mice have no proliferating cells in the intervillus region, where crypts that contain intestinal stem cells form. Hans Clevers and colleagues (Hubrecht Laboratory, Utrecht, The Netherlands) have shown how active transcription factor formed from β-catenin and TCF is found in low concentrations associated with APC (Roose and Clevers, 1999), which suppresses β-catenin/TCF signalling. Constitutively active β-catenin/TCF complexes are present in $APC^{-/-}$ colon carcinomas. The ephrin B2 (Ephb2) and Ephb3 receptor tyrosine kinases are β-catenin/TCF target genes that

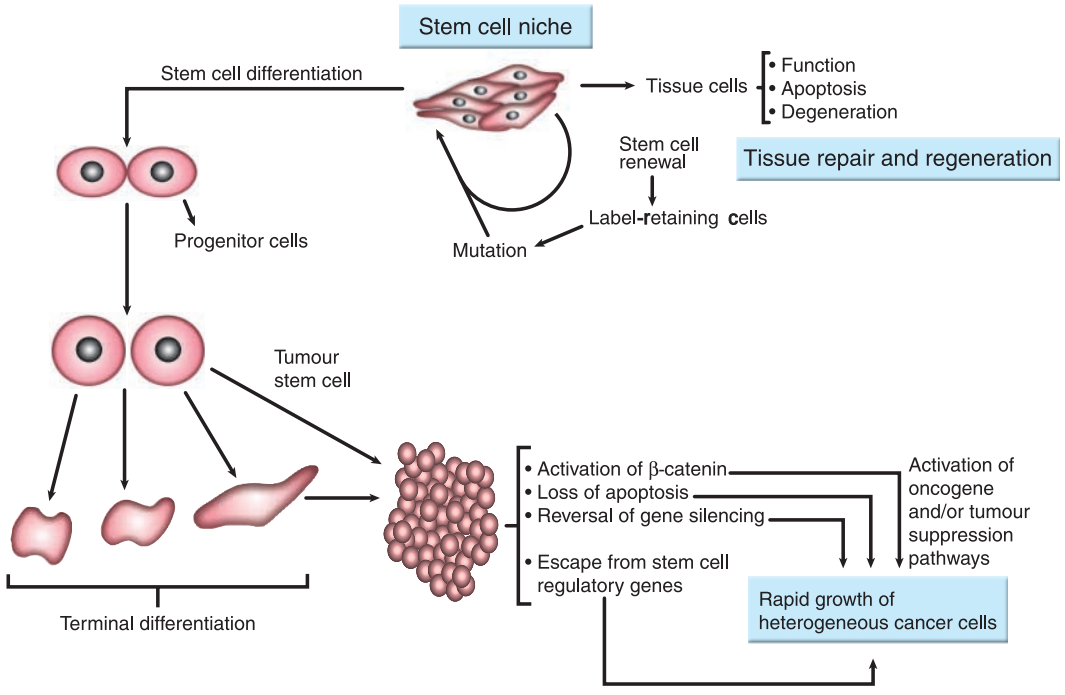

Fig. 1. Stem cells and cancer phenotype. Slowly renewing stem cells that exist within a niche may be subject to mutation, and to gene silencing or upregulated expression. This might be one reason for the delay between an effector stimulus and the cancer phenotype appearing, and might also explain the heterogeneity of cells in tumours. If stem cells or their progenitors escape from the regulation of specific genes, signalling pathways might activate genes, resulting in their escaping from apoptosis and their activating β-catenin, and/or oncogenes and tumour suppression pathways.

help direct localization of crypt cells and are associated with early neoplastic events in these cells. EphB expression is lost in malignant colorectal carcinomas, and very large adenocarcinomas form in Ephb2$^{-/-}$ mice. Therefore, epithelial crypt stem cells that escape from EphB regulation are associated with carcinoma formation.

The origins of leukaemia in the stem cell cascade are also being investigated by Irvine Weissman (Stanford University, CA, USA). Leukaemia cells avoid apoptosis, and in haematopoietic cells isolated by FACS from leukaemic bone marrow, high levels of CD47 expression are observed (high levels of CD47 are indicative of immunity to macrophage attack). In the chronic phase of leukaemia, Wnt signalling is not increased, but in a blast crisis, β-catenin is activated. Weissman's laboratory are now studying the expression profile of the tumour stem cell, and where it appears in the haematopoietic stem cell cascade.

Stem cell differentiation and commitment

There has been considerable debate about the transdifferentiation of adult stem cells, particularly of haematopoietic stem cells (HSCs), into other tissue types. If stem cells can repopulate a variety of tissue types (that is, be multipotential), or if tissue repair can be induced by the mobilisation or delivery of harvested stem cells, the clinical options for repairing damaged tissues are increased. However, there is considerable controversy about the contributions that HSCs can make to non-haematopoietic tissues, except in rare cases where cell fusion has occurred (Balsam et al., 2004; Wagers et al., 2002). Diane Krause (Yale University, NY, USA) believes that bone marrow-derived cells might be short-term proliferating cells that contribute to the lung and other tissues, without cell fusion, as seen in mice with irradiated bone marrow. However, Markus Grompe (Oregon Health and Science University, OR, USA) discussed how adult bone marrow HSCs (defined as c-Kit^{+}, Lin^{-}, Thy^{lo}, Sca1^{+}) can give rise to cells that express hepatocellular markers when transplanted into lethally irradiated hosts, and can reverse hepatic dysfunction in a mouse model of hereditary tyrosinaemia type 1 (HT1) liver disease. Transplants of female bone marrow expressing the *lacZ* reporter gene (*Fah*$^{+/+}$×*Rosa26*$^{+/-}$) into HT1 affected (*Fah*$^{-/-}$) male recipients, and then to *Fah*$^{-/-}$ female recipients, resulted in hepatocytes with fusion karyotypes (see Fig. 2). However, the fusion of putative transdifferentiated hepatocytes with recipient hepatocytes does not occur. Transplanted granulocyte-macrophage progenitors themselves were also able to generate hepatocytes by fusion. Margaret Goodell (Baylor College of Medicine, TX, USA) also postulated that muscle repopulation by HSC in acute muscle injury is by cells of the macrophage lineage. Grompe also noted that failed cytokinesis can result in mono- and bi-nucleated (2n), 4n and 8n cells in the liver, and that fused cells can undergo tetraploid reduction division to diploidy (e.g. 80 XXXY to 40XY and 40XX). Therefore diploidy in donor-derived cells cannot be used to demonstrate transdifferentiation and to rule out cell fusion as the underlying mechanism.

However, studies from Helen Blau (Stanford University, CA, USA) show that bone marrow-derived cells can replace muscle fibre satellite cells in the empty niche of irradiated mice (LaBarge and Blau, 2002). Bone marrow derived satellite cells are activated to contribute to muscle fibers by exercise or toxin-induced damage. As Blau discussed, bone marrow cells can also contribute directly to muscle fibers in parabiotic mice that have a permanently shared circulation system. Bone marrow cells with a reporter gene of one mouse can be identified in the muscle of the other mouse of a parabiotic pair. Moreover, in human brains, Y chromosomes are detected in the Purkinje cells of adult women who have been transplanted with male bone marrow (Weimann et al., 2003a), and, as presented by Blau, stable binucleate Purkinje neurones can be identified in the cerebellums of mice one year after bone marrow transplantation (Alvarez-Dolado et al., 2003). In these mouse Purkinje neurones, the bone marrow-derived nuclei enlarge with chromatin dispersal, and the Purkinje-specific transgene, L7-GFP is activated whereas haematopoietic markers are downregulated (Weimann et al., 2003b). Thus, the contribution of HSCs to tissues appears now to be irrefutable, although the underlying mechanism (transdifferentiation versus fusion) remains unclear.

Another adult cell type with multipotential capacity is the mesoangioblast; mesoangioblasts are bone marrow progenitor cells that can enter the circulation and contribute to skeletal muscle regeneration (De Angelis et al., 1999; Ferrari et al., 1998; Minasi et al., 2002). As Giulio Cossu (University La Sapienza, Italy) presented, clones derived from a single cell taken from a mouse embryonic aorta express early endothelial

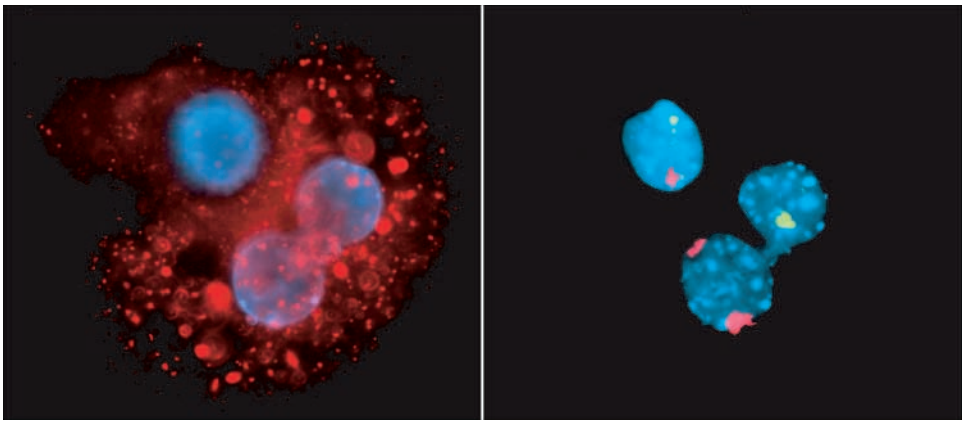

Fig. 2. Detection of a male fumarylacetoacetate hydrolase (Fah)-expressing hepatocyte after transplantation of female GMP (granulocyte/macrophage progenitors) into a male *Fah*$^{-/-}$ mouse. The presence of donor-specific Fah protein (red, left panel) and host-derived Y chromosome signals (pink, right panel) in the same cell indicate that this trinucleated hepatocyte originates from fusion with a transplanted female (X chromosomes are stained green) myelomonocytic cell. Sequential Fah immunocytochemistry and X/Y FISH were provided by Holger Willenbring and Yassmine Akkari (Department of Molecular and Medical Genetics, Oregon Health and Science University).

markers and differentiate into endothelium and skeletal muscle. These cells are also able to differentiate into endothelium, smooth, cardiac and skeletal muscle, and cartilage and bone. The mesoangioblasts express various cell markers, including Sca1, Thy1 and transient Flk1, but are Oct4^{-}. Research by Cossu and colleagues suggests that Flk1^{+} progenitor cells may differentiate into haemoangioblasts or mesoangioblasts (Cossu and Bianco, 2003; Motoike et al., 2003). Injecting wild-type mesoangioblasts into α-sarcoglycan knock-out mice also corrects their dystrophic phenotype (Sampaolesi et al., 2003).

Haematopoiesis

Identifying the factors that direct pluripotential stem cells into becoming HSCs, and that maintain their renewal and control their multipotential differentiation is a priority for several research groups, who are making important contributions towards establishing the key regulators and pathways.

Len Zon (Harvard Medical School, Boston, USA) has shown that zebrafish are an excellent model for studying embryonic haematopoiesis and stem cell differentiation. He discussed the *kgg* mutant, which has a defect in the caudal-like homeobox gene *cdx4* and a deficit in *scl*-expressing haematopoietic precursors. Erythropoiesis in *kgg* mutants is rescued by the overexpression of *hoxb7a* and *hoxa9a*, but not by *hoxb4* or *hoxb8a* (*hoxb6b* rescued poorly), whereas *scl* overexpression induces ectopic haematopoiesis but does not rescue erythropoiesis. In addition, *cdx4* overexpression induces ectopic erythroid cells in the midline of embryos and increases *hoxb4* expression dramatically. These data, together with the finding that mouse *Cdx4* expressed in mES cells expands the multipotential haematopoietic progenitor cell compartment, show that the Cdx-Hox pathway is necessary and sufficient for haematopoietic stem cell formation (Davidson et al., 2003).

Neural stem cells

The application of cell therapies in the treatment of neurodegenerative disease depends on neural stem cell researchers discovering how to recapitulate the functions of neuronal and glial cells in the central and peripheral nervous systems. Progress in this field is providing a range of cell types that are giving encouraging results in various animal models.

Small populations of neurones are formed in the adult olfactory bulb and hippocampus of vertebrates from putative stem cells. Stem cells can be harvested from brain and spinal cord, and will differentiate into mature glia and neurones depending on their local environment. Fred Gage and colleagues (Salk Institute, CA, USA) are studying the cellular, molecular and environmental influences that regulate neurogenesis in the adult mouse brain. The gene *Tlx* (*Nr2e1* – Mouse Genome Informatics) appears to be involved in the maintenance and renewal of adult-derived neural stem cells. Mutant *Tlx* cells (which are Gfap^{+}) do not propagate, and the mutant can be rescued by lentivirus-transfected *Tlx*. Tlx maintains the nestin^{+} state of neural stem cells and inhibits Gfap and differentiation into the glial lineage. Hence, single factors, such as Tlx, can have multiple functions in neurogenesis.

The remarkable capacity of ES cells to generate functional tissues was described by Ron McKay (National Institute of Neurological Disorders and Stroke, Bethesda, USA). His studies focus on cell cycle control, choice of cell fate, and the differentiation of stem cells into electrophysiologically functional neurones (Vicario-Abejon et al., 2000). Mouse ES cells can be efficiently directed into neuronal and glial fates, and can integrate into the neonatal and adult brain (Brustle et al., 1999; Kim et al., 2002; Lee et al., 2000; Studer et al., 1998). These studies show that mES cells generate functional midbrain dopamine neurones that function in vivo. Similar strategies have been used to generate neurones that secrete dopamine from nestin^{+} cells, which have been selected from spontaneously differentiated hES cell-derived embryoid bodies. Neurones generated from hES cells have synaptic and action potentials that can be measured in vitro. Embryoid bodies also form endoderm precursors and endodermal organ rudiments that can form many hepatocytes.

Yoshiki Sasai and colleagues (RIKEN Center for Developmental Biology, Japan) have identified a stromal cell-derived inducing activity (SDIA) that directs mES cells to differentiate into neural cells, including midbrain TH^{+} dopaminergic neurones. The identification of bioactive neural-differentiating factors is crucial for producing sufficient numbers of cells for therapeutic purposes. SDIA also induces TH^{+} neurones in partially disassociated primate ES cell colonies that will colonise the substantia niagra in non-human primate models of Parkinson's disease. This induction requires E-cadherin-mediated cell-cell contact. SDIA also induces co-cultured ES cells to differentiate in CNS tissues (both ventral and dorsal cells). Early exposure of SDIA co-cultured ES cells to Bmp4 suppresses neural differentiation and promotes epidermogenesis, whereas late Bmp4 exposure (>fourth day) induces differentiation into neural crest cells and dorsal-most CNS cells. Shh suppresses the differentiation of the neural crest lineages and promotes motor neurone formation.

Plasticity and multilineage potential

It is apparent that some adult stem cells, such as mesenchymal stem cells, have a greater plasticity than others and are able to contribute to a range of different tissues (Pittenger and Marshak, 2001). The identification of a pluripotential adult stem cell with the properties of ES cells is of particular biological and clinical interest.

Multipotential adult cells (MAPCs) that appear after the long-term culture of bone extracts were identified by Catherine Verfaillie (University of Minnesota, MN, USA), who believes that they are a mesenchymal stem cell but with a greater potency (Jiang et al., 2002). These cells have been isolated in mouse, rat, monkey, pig and human. In mouse, they can be grown for >200 population doublings, and in human the for >80 population doublings. Single MAPCs differentiate in vitro into most mesodermal cell types, and engraft haematopoietic and epithelial tissues in response to local cues after postnatal transplantation. These cells may be closely related to the CD45^{-}, Sca1^{lo}, c-Kit^{-} and Thy1^{lo} quiescent cells of bone marrow, and could be culture induced or a remnant of a pluripotential population. They express ES cell specific genes, such as *Oct4* and *Nanog*, and contribute to all germ layers when injected into mouse blastocysts.

A skin-derived precursor (SKP) cell has been derived from adult mammalian dermis, which can differentiate into neural and mesodermal progeny (Freda Miller, University of Toronto, Canada). These cells share many characteristics with neural

crest stem cells, and occupy a distinct niche within developing and adult dermis. SKP spheres grown from neonatal and adult mouse skin express some neural precursor markers. They generate cells with smooth muscle morphology and express nestin, fibronectin and vimentin, but not P75, GAD, tyrosinase and c-Kit. SKPs differentiate into peripheral nerves and glia (Schwann) cells. They are found in the dermal papillae of hair follicles, and appear to be neural crest stem cell-derived cells.

Lineage restriction during the differentiation of pluripotential cells is probably determined by the availability of specific transcription factors and by changes to chromatin structure, which presumably affects the accessibility of particular genes to transcription factors. Veronique Azuara (Medical Research Council, Clinical Sciences Centre, Hammersmith Hospital, UK) has shown that DNA replication timing can be used as an indicator of chromatin accessibility (Azuara et al., 2003). Genes that encode neural commitment factors frequently switch from being early replicating (as in ES cells) to being late replicating (as in HSCs or mature lymphocytes). Interestingly, Azuara proposed that this 'chromatin profiling' could be a way of discriminating stem cells from progenitors, or of predicting the developmental potential of stem cell populations isolated from different sources.

ES cell applications

Clinical applications of hES cells may depend on their compatibility for transplantation. As they are derived from embryos with their own genomic and histocompatibility profile, they may need to be tissue matched to avoid rejection. One approach to this problem is to form 'patient specific' ES cells by nuclear transfer (see Munsie et al., 2000). This technique involves the substitution of the oocyte nucleus with that of an adult somatic cell. The method is used to produce cloned offspring in animals. Unfortunately, many embryos, foetuses and offspring generated by nuclear transfer are abnormal (Rhind et al., 2003) because of abnormal genomic reprogramming in the early embryo. Ian Wilmut (Roslin Institute, Edinburgh, UK) described cloned offspring as, ironically, being more variable than siblings. However, ES cells produced from nuclear-transfer embryos do not show the abnormal phenotypes of cloned animals, indicating that stem cells with normal embryonic epigenetic characteristics have been selected, or that they undergo further reprogramming as stem cells in vitro or in vivo when incorporated as tissue progenitors. However, the success rate of producing hES cells by nuclear transfer is very low (Hwang et al., 2004).

The inherent difficulty of reprogramming cells of a committed lineage by nuclear transfer will potentially limit the application of ES cell technologies. Rudi Jaenisch (Whitehead Institute, Boston, USA) posed the question: can a postmitotic cell be reprogrammed for development? Olfactory neurones express specific receptors (1 of 1000) and specific receptor genes, which can be labelled with GFP. mES cells formed from nuclear transfer show a low proportion (~1%) of GFP expression, and cloned mice showed no difference in olfactory receptor repertoire. Hence, the choice of receptor in this population remained epigenetic, and terminally differentiated cells can be reprogrammed to a pluripotential state. The implications of this work for stem cells is that even end-differentiated adult stem cells can be manipulated into pluripotentiality, and that future research will provide a means of doing this successfully for tissue repair and regeneration.

Conclusion

The key issues for stem cell research are the determination of the inducers and directors of their differentiation. The degree of plasticity of adult stem cells and the demonstration of function in their tissue of final residence is very important for clinical application. The function of ES cell-derived tissue progenitors needs to be confirmed, and strategies are needed to enable compatibility for transplantation. All of these areas are actively being researched.

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