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Human induced pluripotent stem cells for monogenic disease modelling and therapy

Paola Spitalieri, Valentina Rosa Talarico, Michela Murdocca, Giuseppe Novelli, Federica Sangiuolo

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Abstract

Recent and advanced protocols are now available to

derive human induced pluripotent stem cells (hiPSCs) from patients affected by genetic diseases. No curative treatments are available for many of these diseases; thus, hiPSCs represent a major impact on patient health. hiPSCs represent a valid model for the *in vitro* study of monogenic diseases, together with a better comprehension of the pathogenic mechanisms of the pathology, for both cell and gene therapy protocol applications. Moreover, these pluripotent cells represent a good opportunity to test innovative pharmacological treatments focused on evaluating the efficacy and toxicity of novel drugs. Today, innovative gene therapy protocols, especially gene editing-based, are being developed, allowing the use of these cells not only as *in vitro* disease models but also as an unlimited source of cells useful for tissue regeneration and regenerative medicine, eluding ethical and immune rejection problems. In this review, we will provide an up-to-date of modelling monogenic disease by using hiPSCs and the ultimate applications of these *in vitro* models for cell therapy. We consider and summarize some peculiar aspects such as the type of parental cells used for reprogramming, the methods currently used to induce the transcription of the reprogramming factors, and the type of iPSC-derived differentiated cells, relating them to the genetic basis of diseases and to their inheritance model.

Key words: Human induced pluripotent stem cells; Gene therapy; Monogenic diseases; Gene editing; Foetal cells; Reprogramming techniques; Differentiation

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Core tip: With the development of human induced pluripotent stem cells (hiPSCs) deriving from patients, we can begin to understand the molecular mechanisms underlying monogenic diseases and consequently identify new drugs for their treatment. hiPSCs can differentiate into many disease-relevant cell types, providing in this way to innovative applications in the field of cell replacement therapy, disease modelling, drug testing and

drug discovery.

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INTRODUCTION

For many years the research and experimentation on stem cells have been made, taking advantage of their extraordinary ability to divide and self-renew into undifferentiated cells. Pluripotent stem cells are able to differentiate into all cell types. These characteristics offer the possibility of different applications from the use of stem cells for model disease, for cell therapy and tissue regeneration and pharmacological and toxicological tests.

Researchers have always worked with both embryonic and adult stem cells for the study of disease and for gene therapy. Despite the unique characteristic of embryonic stem cells (ESCs), there are controversies moral, ethical and legal regarding their use.

Adult stem cells have limited differentiation potentiality, so this aspect reduces the options for their use.

Thus, induced pluripotent stem cells (iPSCs), derived from somatic cells, have equal characteristics of ESCs. It is possible reprogramming cells from patients with human diseases that reproduce a model of disease *in vitro* and summarize the pathological phenotypes and the etiopathology of the diseases. So, their use allows the development of innovative therapies, drug screening and toxicological testing^[1-3].

For some genetic diseases no therapeutic treatment is available and the animal model does not always fully possess the variability of the disease. In addition, the understanding of the pathogenetic mechanism at the base of the disease is slow.

The ultimate goal of reprogramming is the transplantation of progenitor cell, genetically corrected *in vitro* before transplantation, derived from a patient-specific human induced pluripotent stem cells (hiPSCs). These cells will not trigger an immune response, avoid tumour formation and recover the target-damaged tissue.

In 2007, iPSCs were obtained from human fibroblasts by manipulation and expression of genes involved in dedifferentiation and in the maintenance of "stemness"^[4,5]. Reprogramming somatic cells using the defined OCT4, SOX2, KLF4, and c-MYC (OSKM) factors led Yamanaka S and Gurdon JB to win the Nobel Prize in Physiology or Medicine in 2012. Thomson's group follow-up research produced iPSCs using NANOG and LIN28 instead of KLF4 and c-MYC^[5] and later, many other researchers developed alternative methods of reprogramming^[6].

The most commonly used method is the use of viral transduction of defined factors to somatic cells.

Lentiviral-based systems, for example, are the most efficient and reproducible, driving the integration of the reprogramming factors. Unfortunately, viral-based disease models still bear the risks of oncogene reactivation, insertional mutagenesis, immunogenicity, reactivation of reprogramming genes or their uncontrollable silencing, making them unacceptable for human clinical applications. In terms of the aspect of safety of reprogramming, various alternative approaches of gene delivery have been developed. Instead of integrating vectors^[1], plasmids^[7], Cre/loxP system^[8], piggy Bac vectors^[9], and minicircle vectors have been investigated^[10], in order to partially prevent transgene integrations and in the same time to simplify the methods to obtain cell reprogramming^[6].

Current studies have successfully reported the generation of transgene-free iPSCs using different approach, such as: Protein transduction^[11], non-integrating viral vectors such as: The Sendai virus^[12], episomal vectors^[13], transfection of modified mRNA transcripts^[14], and chemicals^[15]. Nevertheless, when using protein as inducing factor for reprogramming the efficiency is lower (approximately 0.001).

A modified mRNA-based strategy is currently being explored to produce transgene-free iPSCs^[16,17]. Other methods dealing with small molecules have also been reported to enhance the performance of iPSCs derivation^[18-25]. Similarly human telomerase reverse transcriptase (hTERT), P53 siRNA and Simian Vacuolating Virus 40 large T (SV40LT) Antigen successfully stimulate the reprogramming kinetics^[26,27]. Some others, like Estrogen related receptor β (Esrrb), Utf1, Lin28, and developmental pluripotency-associated 2 (Dppa2) generate iPSCs without of OSKM factors with single-cell level identification of reprogramming events^[28].

The typical yields of iPSCs production by the methods aforesaid range from 0.01%-5%, depending on the target cell and reprogramming system. Rais *et al.*^[29] reported the reprogramming efficiency of methyl-binding protein 3 deletion that reached up to nearly 100% within a few days, supporting that iPSCs reprogramming represents a deterministic process.

One factor strongly influencing the efficiency of reprogramming is the type of cell used as target. This choice can depend on the amount of DNA methylation, gene expression and stability of the pluripotent phenotype, as well as the epigenetic memory of the cell type. In addition, the gene delivery methods, and culture conditions as well as the transcription factors combination might also be the reason of the differences between the various iPS cell populations created. At least, several uncontrollable stochastic events can influence the success of the reprogramming^[30].

For all these reasons, researchers have studied the "best candidate parental population" to create iPSCs for *in vitro* investigations and eventual clinical trials^[31]. Fibroblasts are the main source of iPSCs, although other sources for iPSCs have been reported, like hepatocytes and mature B cells^[32]. To better understand

developments in personalized medicine, we will focus our review on the production and application of human iPSCs derived from foetal tissues, highlighting their higher responsiveness during the reprogramming.

Stem cells have been identified in several foetal tissues and from amniotic fluid, umbilical cord blood, and placenta at term^[33-35]. These cells have mesenchymal origin and they are capable of self-renewal and differentiation into multiple tissue types^[36-39]. According to their tissue derivation and to the gestational age, the heterogeneity of foetal stem cell populations is emblematic, in agreement to their phenotypic characteristic, properties and cell marker expression.

HUMAN FOETAL TISSUES

Human trophoblast stem cells

Trophoblast cells form the foetal part of the placenta. The placenta is an organ indispensable for the growth and survival of the developing embryo. The placenta is constituted by different trophoblast cell types aimed for embryo implantation, for vascular connection to the maternal circulation and nutrition of the fetus and immunological adaptation. Trophoblast cells derive from the trophoectoderm, which gives rise to both attachment and implantation of the embryo. The trophoectoderm is composed of floating and anchored villi and their specialized cell types, the syncytium and the cytotrophoblast. For the first 3 wk of pregnancy, it represents a continuously renewing epithelium^[40]. During gestation, the trophoblast changes morphologically and functionally. In particular, the cytotrophoblast, a self-renewing population located in the proliferation zone, divides continuously and fuses to form syncytiotrophoblasts, in which some authors report the presence of possible stem cells important for its renewal^[41,42]. Spitalieri *et al.*^[39] has isolated and characterized a subpopulation of multipotent cells, named human cytotrophoblastic-derived multipotent cells (hCTMCs) obtained from human chorionic villus sampling (hCVSs) with characteristics that are "intermediate" between mesenchymal and pluripotent stem cells. These cells express stem cell markers, such as ALP, SSEA4, OCT-4, CD117, NANOG, and SOX2. Also, these cells are capable of generate *in vitro* cells belonging to ectoderm, mesoderm and endoderm layers, but, if inoculated into Nod/SCID mice, they are unable to form teratomas. If injected into mouse blastocysts, hCTMCs are integrated and could be tracked into various tissues of the adult chimeric mice. These cells may be also a promising target for gene editing approaches, such as small fragment homologous recombination, as we report for an *in vitro* genetic modification of *SMN* gene in a fetus affected by spinal muscular atrophy (SMA). They can be genetically edited with high performance, allowing an innovative therapeutic approach to cure genetic defects^[39,43,44].

Human amniotic fluid stem cells

During amniocentesis, at the sixteenth week of gestation, small amount of fluid carry hAFSCs. Stem cells are

present also in amniotic fluid at term (from routine caesarean deliveries). Kaviani *et al.*^[45] collected up to 20000 cells from 2 mL of amniotic fluid, 80% of which are having the ability to grow. Membrane receptor c-kit (CD117), marker of stemness, is expressed on ESCs^[46], primordial germ cells and many others somatic stem cells, including a sort multipotent subpopulation of human amniotic fluid stem cells (hAFSCs) (round 1%)^[47]. Moreover, hAFSCs show a high telomerase activity with a self-renewal capacity, and display normal G1 and G2 cell cycle checkpoints. On top, at late passages they maintain a normal karyotype. Like ESCs, hAFSCs are capable of differentiating into all three germ layers, their efficiency depends on the gestational age^[33,48-50]. But, hAFSCs are unable to form teratoma, when inoculated into immunodeficient mice *in vivo*. It is possible, also, to use hAFSCs in a technique involving retrovirally tagged cells, because they have a high clonal capacity.

Amnion epithelial cells

From a single term amnion membrane it is possible isolate a multipotent epithelial cell population and obtain approximately 120 million viable epithelial cells^[51-53]. hAECs possess ability of multipotent differentiation^[54], low immunogenicity^[55] and anti-inflammatory functions^[56].

Therefore, as reported in the literature, both hCVSs and hAFSCs are heterogeneous populations composed by several stem/progenitor lineages^[57]. In response to external stimuli, they modulate gene and protein expression for their high plasticity, as already published^[58]. Moreover foetal stem cells have an higher proliferative capacity if compared to adult cells. Again, congenital malformations or genetic diseases in newborns could be treated thanks to the capacity to separate pluripotent autogenic progenitor cells during pregnancy^[59]. Only recently have these cells, traditionally used for prenatal diagnosis, been explored for their stemness and for reprogramming efficiency^[3,60].

HiPSCs vs hESCs

When compared with embryonic cells, iPSCs differentiate less efficiently into specialized cell lines, due to their "molecular identity"^[61]. Moreover, some iPSCs have a greater capacity to silence some genes demonstrated to be required for foetal development and differentiation^[62]. These differences constitute an active area of research that still requires a direct comparison of the pluripotency of hiPSCs vs hESCs. An explanatory situation is represented by the *FMR1* gene, involved in Fragile X syndrome (#300624), a genetic condition characterized by learning disabilities and cognitive impairment. The protein product is necessary for normal brain development. In Fragile X syndrome the *FMR1* gene acquires a silencing mutation. While this gene functions normally in human embryonic cells and becomes silenced as the cells differentiate, in hiPSCs remains inactive^[63].

Several studies suggest that cells undergoing reprogramming go through an intermediary state *via* resetting of the epigenetic landscape, whereby *c-MYC* and *KLF4*

are initially required to prime the cells that are then driven towards pluripotency by *OCT4* and *SOX2*. These observations open interesting scenarios for further investigations focused to discover methods to directly create progenitor therapeutic cell types from somatic cells^[6], bypassing the pluripotency step.

Thus, iPSCs that conserve genomic stability and free from any integrated agents represent an important aim for therapeutic uses. Recent studies suggest the use of a high-resolution method, such as the Affymetrix Cytoscan HD array (Affymetrix, Santa Clara, CA, United States), for monitoring genomic alterations throughout iPSC preparation to preserve clinical applications^[64].

Given the high level of manipulation and the lack of knowledge about their role *in vivo*, the use of hiPSCs in human trials is complex. Whether hiPSCs will prove an useful substitute for hESCs has yet to be determined, hESCs are still considered the gold standard for embryonic cell lines^[65].

At the same time, hiPSCs represent an ideal autologous cellular model for the study and the treatment of diseases, reaching the goal of personalized medicine. The field of personalized medicine is based on the idea that life is variable and that individuals behave differently from each other under disease conditions. By taking into account individual clinical, genetic and environmental information, personalized medicine optimizes medical care and outcomes for customized disease prevention, detection and treatment.

hiPSCs coincide perfectly with the concept of personalized medicine for disease modelling and further clinical application. In fact, hiPSCs bypass the limitation of immune rejection, being patient-specific cells, united to a "rejuvenation" of telomere length during reprogramming^[66], epigenetic memory and functional properties, offering enormous clinical potential.

The tumorigenic risk of hiPSCs arising from the use of integrating vectors for their derivation supports the use of integrating vectors that can be subsequently removed from the genome. Sommer *et al.*^[67], for example, reports the use of the human STEMCCA excisable polycistronic lentiviral vector^[3]. The delivery vectors are designed so that the ectopic genes are flanked by *loxP* sites, thus enabling their removal by transient Cre protein expression^[68,69]. This approach generates hiPSCs free of transgenic sequences that can improve and increase the safety of derivation methods.

HIPSCS-BASED MONOGENIC DISEASE MODELS

Due to ethical and technical challenges, human embryonic stem cells (hESCs) can represent unsuitable candidates for disease modelling. Therefore, hiPSCs, closely resembling the key features of hESCs such as self-renewal and pluripotent potentials, can be extensively exploited to study various inherited disorders.

Dimos *et al.*^[70] (2008) and Park *et al.*^[71] (2008) reported

for the first time disease-specific iPSC lines in 2008, mimicking human disease. This new strategy consists of screening patients for genetic mutations, isolating cell lines, returning them to iPSCs, and finally differentiating the iPSCs into one or more cell types phenotypically developing the disease.

The possibility of dedifferentiating patient-specific cells back to stem cells and again to differentiate them into cells representative of the disease organ or tissue, allows one to faithfully replicate the key aspects of the disease in a "petri dish" and to quantify disease phenotype in different tissues (Figure 1). Each of these points is difficult to obtain; however, many disorders have been successfully recapitulated *in vitro* contributing to research in the field of disease modelling. Most of these studies have focused on monogenic disorders that exhibit strong phenotypes *in vitro*. Moreover, obtaining pluripotent cells from patients with developmental or degenerative disorders also allows for new opportunities for drug discovery^[72]. When differentiated *in vitro* into relevant somatic lineages, hiPSCs are used both for assessing personalized pharmacological therapy and for *in vivo* cell therapy.

Therefore, iPSCs-disease models mimic human pathological development rather than trials utilizing conventional rodent and cell lines^[73]. Human cell culture assists the research using animal models of disease. Murine models of human inherited and acquired diseases are helpful systems but human pathophysiology cannot always be faithfully reproduced. When murine and human physiology are different, disease-specific pluripotent cells able of differentiation into the several cells affected establish disease pathophysiology, allowing *in vitro* investigation in a human tool monitored and proving a large number of genetically-modifiable cells in a specific manner for each genetic defects.

The main advantages of iPSCs-based model systems are: (1) iPSCs can be obtained from several sources (adult somatic cells, embryonic/foetal cells, adult stem cells and cancer cells); (2) iPSCs naturally maintain the genetic background; (3) iPSCs have the ability to differentiate into any desired cell types *in vitro*; (4) iPSCs can self-renew and maintain their undifferentiated state and pluripotent capacity; and (5) iPSCs resume early human embryo development during differentiation *in vitro*.

For all these reasons, iPSCs represent an available model system for studying the pathogenetic mechanisms of various diseases, particularly in those cases where animal models do not exactly reproduce human phenotype or when disease-target cells types are not available for research.

This review provides an overview of the current state of modelling monogenic disease by using hiPSCs and the ultimate applications of these *in vitro* models for cell therapy. We consider and summarize some peculiar aspects such as the type of parental cell used for reprogramming, the methods currently used to induce expression of the reprogramming factors, and the type of iPSC-derived differentiated cells, relating them to the

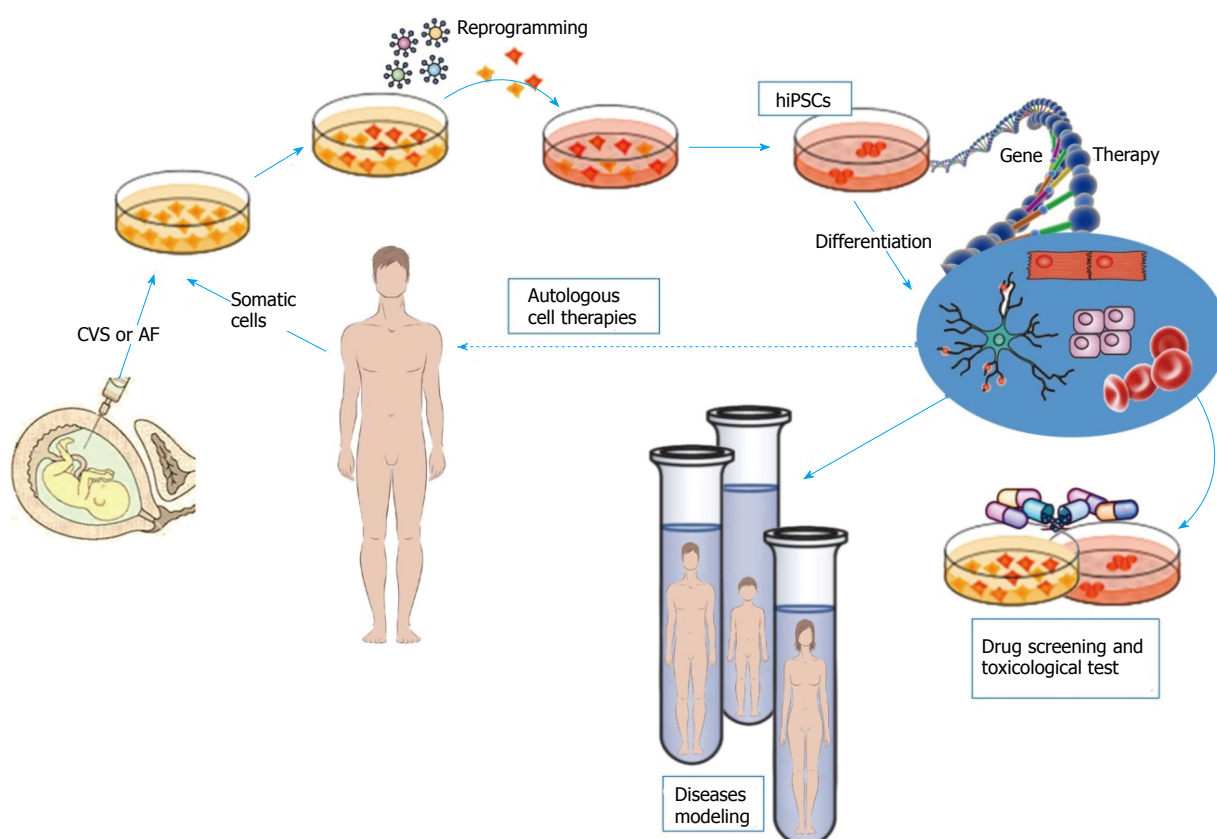


Figure 1 Representation of the workflow for the derivation of patient-specific induced stem cells from foetal or somatic cells and successively their use for the development of personalized therapy protocols. CVS: Chorionic villus sampling; AF: Amniotic fluid; hiPSCs: Human induced pluripotent stem cells.

genetic basis of diseases and to their inheritance model (Tables 1-3).

hiPSCs for modelling autosomal dominant disorders

Huntington's disease (HD; OMIM #143100) is an autosomal dominant neurodegenerative disorder that shows up later in life. It is caused by an expansion of the "CAG" triplets repeated in exon 1 of the *HTT* gene. The protein encoded by *HTT* gene is expressed in many tissues and organs, especially in the brain and testis^[74-77]. The normal function of *HTT* is still not fully identified as it differs from other known proteins.

HD is known as a neurological disease; however, peripheral HD-associated pathologies, such as cardiac defect and skeletal muscle malfunction, have also been described. The generation of HD-iPSC lines can facilitate the match of the affected phenotypes in "dish" with clinical discoveries in sick individual of their families, highlighting the genetic basis and molecular mechanism leading to the development of the HD disease^[78]. For this purpose, the HD-iPSC consortium recently promoted and defined an *in vitro* model of HD based on the creation of iPSCs and established by multiple lines, clones and repeat lengths, exhibiting that the clear association between the extension of the CAG repeats and the clinical pathology severity observed in HD patients could also be reproduced *in vitro* by HD-iPSC differentiated into neurons^[79].

Juopperi *et al.*^[80] generated an *in vitro* system deriving patient-specific iPSCs to study HD pathogenesis. Thanks to this model, they were able to describe a specific vacuolation phenotype in iPSCs-derived astrocytes. The same characteristics were previously observed in primary lymphocytes derived from HD patients^[80]. This study opens up new potential investigations using human iPSCs for model HD and for therapeutic drug screening. iPSC lines obtained from two homozygous individuals bringing 42/44 and 39/42 CAG repeats and from one heterozygote having 17/45 CAG repeats show a lysosomal activity increased when cultured *in vitro* and when differentiated in neurons^[81]. The sizes of the CAG repeats persisted constant during the period of culture. On the other hand, another HD-iPSC line carrying 72 CAG repeats had no phenotype when cultured *in vitro* both in undifferentiated state and in neural precursors. However, the HD-iPSCs display manifestation of HD disorder under condition of oxidative stress^[82], or proteasome inhibitors or if injected into neonatal brains for 33 wk^[83]. Increased caspase activation in HD-iPSCs is also observed in iPSCs-derived neurons^[84]. Thus, HD-iPSCs recapitulate the disease phenotype and represent an available tool to study HD and to develop novel therapeutics.

Marfan syndrome (MFS; OMIM #154700) is an autosomal dominant hereditary disorder of connective tissue strongly involving skeletal, ocular, and cardiovascular systems^[85,86]. The mutated gene responsible for MFS is

Table 1 Models of monogenic dominant diseases

Diseases	Genetic defects	Target cells for reprogramming	Delivery methods	Disease relevant cells	Ref.
Huntington's disease (OMIM #143100)	Expanded CAG repeat in <i>HTT</i> gene	HF	Lentiviral vectors (O, S, K, M, NANOG and LIN28)	Neurons	[79]
		HF	Retroviral vectors (O, S, K, M)	Astrocytes	[80]
		HF	Lentiviral vectors (O, S, K, M)	Neurons	[81]
		HF	Retroviral vectors (O, S, K, M)	Neurons	[82]
		HF	Retroviral vectors (O, S, K, M)	GABAergic neurons	[83]
		HF	Retroviral vectors (O, S, K, M)	Striatal neurons	[84]
Marfan syndrome (OMIM #154700)	<i>FBN1</i> mutations	HF	Retroviral vectors (O, S, K, M)	Osteogenic cells	[87]
Myotonic dystrophy type 1 (OMIM #160900)	Expanded CTG repeat in <i>DMPK</i> gene	HF	Retroviral vectors (O, S, K, M)	NS	[92]
		HF	Retroviral vectors (O, S, K, M)	NSC	[94]
Achondroplasia (OMIM #100800)	<i>FGFR3</i> mutations	HF	Episomal plasmid vectors (O, S, K, M, LIN28 and p53 shRNA)	Chondrocytes	[97]
Familial hypercholesterolemia (OMIM #143890)	<i>LDLR</i> mutations	HF	Retroviral vectors (O, S, K, M)	HLC	[99]
		HF	Lentiviral vectors (O, S, NANOG, LIN28)	Hepatocytes	[100]
Timothy syndrome (OMIM #601005)	<i>CACNA1C</i> mutations	HF	Retroviral vectors (O, S, K, M)	HLC	[101]
		HF	Retroviral vectors (O, S, K, M)	Cardiac myocytes	[102]
		HF	Retroviral vectors (O, S, K, M)	Cortical neuronal precursor cells and neurons	[103]
		HF	Retroviral vectors (O, S, K, M)	Cortical neuronal precursor cells and neurons	[104]

NS: Neurosphere; NSC: Neural stem cell; HLC: Hepatocyte-like cells; HF: Human fibroblasts; O: OCT4; S: SOX2; K: KLF4; M: C-MYC.

located on chromosome 15 and encodes for fibrillin-1 (*FBN1*).

Originally, the fibrous connective tissue disorders of MFS were attributed to structural weakness of the fibrillin-rich extracellular matrix. Increased bioavailability of TGF β is associated with the pathological signs, indicating the resemblance to MFS-related disorders. In fact, mesenchymal cells, derived from both MFS iPSCs and ESCs differentiate spontaneously into chondrogenic cells contrary to wild type iPSC/ESC-derived mesenchymal cells that need exogenous TGF β chondrocytes, demonstrating an alteration of TGF β signaling in MFS cells^[87]. This model is in agreement with the skeletal manifestations of MFS and increases the knowledge of molecular mechanisms underlying the pathogenesis of abnormal skeletogenesis in human diseases caused by mutations in *FBN1*.

The use of the iPSC technology permits the reprogramming of MFS adult fibroblasts containing different *FBN1* mutations, allowing the clearance of the mechanisms underlying the pathological variability, and shows the benefit of personalized therapeutic interventions.

Myotonic dystrophy type 1 (DM1; or Steinert's disease, OMIM #160900) is the most common muscular dystrophy in adults^[88]. DM segregates as an autosomal dominant pathology and is caused by the expansion of a CTG repeat located within the 3'-untranslated region of the dystrophin myotonia protein kinase (*DMPK*) gene on chromosome 19q13.3^[89]. In the classic form, the major features include myotonia, muscle weakness and wasting, cardiomyopathy with conduction defects, insulin-resistance, frontal balding, cataracts and disease-specific serological abnormalities^[90]. hiPSCs offer the possibility to study unstable repeat expansions by generating a model

disease and disease-impaired cells in culture. These aspects are helpful in order to investigate unstable repeat pathologies^[91].

In particular, DM1 patient-derived iPSCs could be an ideal model to study triplet-repeat instability. Du *et al.*^[92] first generated iPSCs from DM1 patient fibroblasts and detected CTG.CAG triplet repeats in each iPSC clone. Homogeneous lengths of CTG.CAG triplet repeats in each iPSC clone allows for the study of the mechanisms of repeat expansion, and offers knowledge of a general mechanism of triplet-repeat expansion in iPSCs^[92].

Recently, Xia *et al.*^[93,94] reported neural stem cells (NSCs) derived from iPSCs of DM1 patients, a helpful device for the study of DM1-NSCs neuropathogenesis. Both DM1 iPSCs and iPSC-derived NSCs show the presence of nuclear RNA foci, representing a molecular hallmark of disease, allowing them to be used as cellular models to understand the dynamic changes of RNA foci during the cell cycles^[93,94].

Achondroplasia (ACH; OMIM #100800) is the most common skeletal dysplasia, with disproportionate short-limb dwarfism. The mutated gene encodes for fibroblast growth factor receptor 3 (*FGFR3*)^[95,96]. The study of skeletal dysplasia, as well as many other diseases, exploits the development of iPSCs technology.

Yamashita *et al.*^[97] demonstrated that the chondrogenically differentiated ACH-hiPSCs adequately recapitulate the primary abnormalities found in *FGFR3*-related disease patients. These cells manifested lower proliferation and higher apoptosis when differentiated into chondrocytes^[97]. Thus, hiPSCs technology is instrumental in investigating the effects of several therapeutic molecules, including statins, on ACH iPSCs-derived chondrocytes^[98] (Table 1).

Table 2 Models of monogenic recessive diseases

Diseases	Genetic defects	Target cells for reprogramming	Delivery methods	Disease relevant cells	Ref.
Spinal muscular atrophy (OMIM #253300)	<i>SMN1</i> mutations	HF	Lentiviral vectors (O, S, NANOG, LIN28)	Neurons/astrocytes/motor neurons	[109]
		HF	Retroviral vectors (O, S, K, M)	Motor neurons	[110]
		HF	Lentiviral vectors (O, S, NANOG, LIN28)	Motor neurons	[112]
		HF	Retroviral vectors (O, S, K, M)	GABAergic neurons	[113]
β -thalassaemia (OMIM #613985)	Point mutations or deletions in the β -globin (<i>HBB</i>) gene	HF/AF/CVS	Retroviral vectors (O, S, K, M)	Hematopoietic cells	[116]
		AF	Lentiviral vectors (O, S, K, M)	Hematopoietic cells	[118]
		HF/MSCs	Lentiviral vectors (O, S, K, M)	Erythroid cells	[119]
		HF	PiggyBac transposon	Hematopoietic cells	[120]
Cystic fibrosis (OMIM #219700)	<i>CFTR</i> mutations	HF	Lentiviral vectors (O, S, K, M)	Mature airway epithelial cells	[126]
		HF	Modified RNAs (iPSC)	Mature airway epithelial cells	[127]
		HF	Retroviral vectors (O, S, K, M)	Mature airway epithelial cells	[128]
		HF	Retroviral vectors (O, S, K, M)	CLCs	[130]
Sickle cell disease (OMIM #603903)	<i>HBB</i> mutations	HF	Retroviral vectors (O, S, K, M)	Erythrocytes	[135]
		HF	Lentiviral vectors (O, S, K, M)	None	[136]
Hutchinson-gilford progeria syndrome (OMIM #176670)	<i>LMNA</i> mutations	HF	Retroviral vectors (O, S, K, M)	Neural progenitors, endothelial cells, fibroblasts, VSMCs, and MSCs	[137]
		HF	Retroviral vectors (O, S, K, M)	Vascular SMCs	[138]
		HF	Retroviral vectors (O, S, K, M)	MSCs and osteogenic cells	[139]
Niemann-pick disease type C1 (OMIM #257220)	<i>NPC1</i> mutations	HF	Retroviral vectors (O, S, K, M)	Neurons	[140]
		HF	Lentiviral vectors (O, S, K, M)	Neurons	[141]
		HF	SeV vectors (O, S, K, M)	HLCs and neural progenitors	[142]

CLCs: Cholangiocytes; VSMCs: Vascular smooth muscular cells; MSCs: Mesenchymal stem cells; SMCs: Smooth muscle cells; MSCs: Mesenchymal stem cells; HLCs: Hepatocyte-like cells; SeV: Sendai virus; AF: Amniotic fluid cells; CVS: Chorionic villus; HF: Human fibroblasts; O: OCT4; S: SOX2; K: KLF4; M: C-MYC.

hiPSCs for modelling autosomal recessive disorders

SMA (OMIM #253300) is an autosomal recessive neuro-degenerative disorder. SMA is caused by mutation or deletion of the survival motor neuron-1 (*SMN1*) gene^[105,106]. The clinical phenotype is typically characterized by the degeneration of α -motor neurons in the spinal cord, leading to muscle weakness, atrophy and premature death^[107,108].

All SMA patients have also a highly homologous gene copy (*SMN2*) in different copy number. *SMN2* is not able to produce sufficient levels of SMN protein, due to its defective splicing pattern. However *SMN2* copy number is inversely correlated with the severity of the SMA phenotype.

One study describes human iPSCs derived from skin fibroblasts to model SMA^[109]. The main characteristic is the degeneration of motor neurons caused by a loss of SMN1 protein in all cells of the body. The use of SMA-iPSCs-derived motor neurons may help to elucidate the role of *SMN1* in disease initiation and progression, but also to screen new drug useful in future pharmacological therapies for SMA.

Later studies reported the establishment of five iPSC lines from type 1 SMA fibroblasts. iPSCs-derived neurons with a decreased ability to generate motor neurons and an altered neurite outgrowth. Exogenously induced expression of *SMN* in these iPSC lines determined a normal motor neuron differentiation and rescued the aberrant neurite outgrowth, confirming the role of the *SMN* defect in the disease^[110]. Successively, several reports have been published using these cells to test novel compounds for efficacy prior to administration to patients, increasing the possibility of success in the

treatment of this serious disorder^[111-113].

β -thalassemia (β -Thal; OMIM #613985) is an inherited autosomal recessive blood disorder, caused by either point mutations or deletions of nucleotides in the β -globin gene, provoking a reduced/abnormal or absent synthesis of β -globin chains that make up hemoglobin. Affected patients have severe anemia and an shortened life span^[114].

The generation of patient-specific iPSCs and the subsequent editing of the disease-causing mutations provide an ideal therapeutic solution to β -thalassemia and other haemoglobinopathies^[115]. Disease-specific autologous iPSCs have been generated from somatic cells and differentiated into haematopoietic cells, both *in vitro* and *in vivo* in SCID mice^[116,117]. Fan *et al.*^[118] used cultured β -thalassemia-amniotic fluid cells as target cells for an efficient reprogramming by using a single polycistronic lentiviral vector. iPSCs producing insufficient amounts of β -globin can be induced to increase β -globin product by infecting them with a viral vector carrying an exogenous copy of the β -globin gene and successively to differentiate into "restored" erythrocytes^[119]. Recently, other approaches have been used for targeting the *HBB* gene in β -thalassemia-derived iPSCs, demonstrating how TALENs was able to mediate a higher homologous recombination efficiency than that obtained by CRISPR/Cas9^[120]. The development of innovative gene editing protocols opens new promising prospects for the use of iPSCs as a target of gene therapy for monogenic diseases.

Cystic fibrosis (CF; OMIM #219700) is an autosomal recessive disorder. The primary defect is the regulation of

Table 3 Models of monogenic X-linked recessive diseases

Diseases	Genetic defects	Target cells for reprogramming	Delivery methods	Disease relevant cells	Ref.
Fragile X syndrome (OMIM #300624)	<i>FMR1</i> silencing	HF	Retroviral vectors (O, S, K, M)	Neurons	[146]
		HF	Retroviral vectors (O, S, K, M)	Forebrain Neurons	[147]
		HF	Sendai virus	NPC	[148]
Duchenne muscular dystrophy (OMIM #310200)	Dystrophin gene mutations	HF	Retroviral vectors (O, S, K, M)	Myogenic cells	[154]
		HF	Retroviral vectors (O, S, K, M)	CMs	[155]
		HF	Retroviral vectors (O, S, K, M)	Neurons	[156]
Wiskott-aldrich syndrome (OMIM #301000)	<i>WASP</i> mutations	HF	Retroviral vectors/sendai virus vectors (O, S, K, M)	Megakaryocytes	[157]
Rett syndrome (OMIM #312750)	<i>MeCP2/CDKL5</i> mutations	HF	Retroviral vectors (O, S, K, M)	Neurons	[158]
		HF	Retroviral vectors (O, S, K, M)	NPCs/mature neurons	[159]
		HF	Retroviral vectors (O, S, K, M)	NPCs/mature neurons	[160]
Hemophilia A (OMIM #306700)	Deficiency of factor VIII	Urine cells	Episomal vectors (O, S, K, SV40LT)	Hepatocytes	[161]

NPC: Neural progenitor cells; CMs: Cardiomyocytes; HF: Human fibroblasts; O: OCT4; S: SOX2; K: KLF4; M: C-MYC.

epithelial chloride transport by a chloride channel protein, encoded by the CF transmembrane conductance regulator (*CFTR*) gene^[121]. CF is a multisystem disorder characterized by loss of function in the *CFTR* in organs with secretory function^[122,123]. Recurrent pulmonary infections are responsible for 80%-90% of the deaths in CF patients^[124].

Fibroblasts from patients with CF can be reprogrammed to iPSCs and differentiated into lung airway epithelium^[125]. From the point of view of translational medicine, patient-specific iPSC-derived airway epithelial cells open the way to personalize therapeutic interventions for the treatment of serious lung diseases. Different groups report the generation of iPSCs from CF patients and their differentiation into pulmonary cells, creating a platform for dissecting human lung disease^[126-129]. Unfortunately, the development of iPSCs-based models of human lung disease is hampered by the inability to differentiate hiPSCs into lung progenitors and subsequently into mature pulmonary epithelial cell types.

Moreover mutations in the *CFTR* gene are also responsible for CF-associated pathologies, such as cholangiopathy, resulting in reduced intraluminal chloride secretion, increased bile viscosity and focal biliary cirrhosis. Sampaziotis *et al.*^[130] generated hiPSCs from skin fibroblasts of a CF patient and differentiated them into cholangiocytes (CLCs), showing that CF-hiPSC-derived CLCs (CF-CLCs) represent a good model CF biliary disease *in vitro*. In fact, their use for treatment with the experimental CF drug VX809 has demonstrated the *in vitro* rescue of the disease phenotype (phase 2a clinical trials)^[131,132]. The use of gene targeting specific nucleases to correct *CFTR* gene sequences has been reported^[129,133,134].

Crane *et al.*^[124] designed zinc-finger nucleases to target endogenous *CFTR* for editing the inherited genetic mutation in patient-derived iPSCs *via* homology-directed repair (HDR). When induced to differentiate *in vitro*, modified hiPSCs demonstrated a restored expression of the *CFTR* gene, recovering the expression of the mature *CFTR* glycoprotein and of the chloride channel functions^[124] (Table 2).

hiPSCs for modelling X-linked disorders

Fragile X syndrome (FXS, OMIM #300624) is an inherited disorders due to CGG triplet expansion located within the 5' untranslated region of the Fragile X mental retardation gene (*FMR1*). The expansion causes the epigenetic silencing and the consequent loss of the Fragile X mental retardation protein (FMRP), a cytoplasmic mRNA transport factor^[143-145]. Species-specific differences in molecular and neurodevelopmental aspects of FXS require a human FXS model and hiPSCs enable disease modelling.

It has been reported that FXS hESCs and hiPSCs differ in the epigenetic state of the *FMR1* gene. In fact, the *FMR1* gene is unmethylated and expressed in hESCs, presenting full-mutation repeats, converting to methylated and silenced in the differentiated state^[64]. In contrast, FXS hiPSCs do not return to the naive epigenetic state because the *FMR1* gene remains methylated and silenced during reprogramming^[64,146]. Although FX-hiPSCs do not reproduce the methylation state of the *FMR1* gene, they represent a useful model for studying the role of *FMR1* in neural cells. The distinction between FX-hES and FX-hiPSCs at the *FMR1* locus suggests a more general epigenetic phenomenon in human pluripotent stem cells, which highlights the need for more studies to clarify the similarity and differences between ESCs and iPSCs.

Different hiPSCs cell lines have been generated from multiple patients with FXS and successively induced to differentiate into post-mitotic neurons and glia^[146]. In these cells, an aberrant neuronal differentiation of FXS hiPSCs is observed, directly associated to the epigenetic modification of the *FMR1* gene and to a loss of FMR protein expression, evidencing a key role for the FMR protein early in human neurodevelopment prior to synaptogenesis. iPSCs-derived neurons represent disease-associated cellular phenotypes, very useful for discovering novel therapies for FXS and other diseases sharing common pathophysiology.

Another paper reports the reprogramming of hiPSC lines from FXS fibroblasts. FXS forebrain neurons have been differentiated from these iPSCs, displaying both

defective neurite initiation and extension^[147]. iPSCs constitute a platform to examine potential neuronal deficits caused by FXS and develop assays for drug discovery^[148].

Because the main consequence of the lack of FMRP in FXS is the synaptic defect, another group has reported a cellular model focused on neuronal cells that well represent the disease and express a transcriptional and proteomic pattern similar to that present in the neurons of the brain^[148]. This aspect is important for identification of the target that modulates FMRP expression because its study in other cellular models could be erroneous. Current therapy for FXS is only at the behavioral level.

Duchenne muscular dystrophy (DMD; OMIM #310200) is the most prevalent congenital pediatric muscular dystrophy. It is an X-linked genetic degenerative myopathy and multisystem disease characterized by disease-specific serological abnormalities, dilated cardiomyopathy, cataracts, insulin-resistance, cardiac conduction defect, myotonia and muscular dystrophy, which can lead to the loss of motor function in puberty^[149].

The disease is a myopathy that affects in approximately 1 in 5000 male births and is caused by mutations within the dystrophin gene (locus Xp21.2)^[150,151]. The disease is characterized by a reduction in dystrophin, a protein assembles with the dystrophin glycoprotein complex (DGC), associating the cytoskeleton to the extracellular matrix in skeletal and cardiac muscles^[152]. Consequences of DGC inefficiency are severe muscle wasting, contraction-induced damage, necrosis and inflammation^[153].

Cell transplantation and hiPSCs offers an encouraging way for cell-based therapy, in fact myogenic cells derived from hiPSCs are an unlimited source for cell-based therapy of DMD. Goudenege *et al.*^[154] have established the usefulness of genetically corrected human multipotent cells for muscle repair. Transplantation studies in hiPSCs highlight the advantages to correct the patient own cells, avoiding the immune response against the donor myoblast or mesoangioblast. Lin *et al.*^[155] have utilized DMD iPSCs to replicate and analyze the major phenotypes of dilated cardiomyopathy (CMs) found in DMD-affected individuals, and thus to reveal the disease mechanism. Their study has identified a pathway determining increased apoptosis in DMD-CMs that can be regulated by drug therapy. Thus, these cells might represent an *in vitro* system for preclinical testing of future therapy^[155] (Table 3).

GENE THERAPY APPROACHES IN PATIENT-SPECIFIC HIPSCS FOR THE TREATMENT OF MONOGENIC DISEASES

The opportunity to derive patient-specific iPSCs in combination with the current development of gene modification protocols surely represents a good opportunity for cell therapy of several inherited genetic diseases.

Different gene editing methods have been demonstrated to modify defective genes in hiPSCs. Their choice depends on the gene correction approach and on the mutation type.

In the last few years, substantial progress has

been made by using BACs (bacterial artificial chromosomes)^[162-164], viral vectors^[165-167], and other relatively new methods, such as zinc finger nucleases (ZFNs)^[168-171], transcription activator-like effector nucleases (TALENs)^[172,173] and especially the clusters of regularly interspaced palindromic repeats (CRISPR) /Cas-derived RNA-guided endonucleases^[174,175].

BAC-based targeting vectors can obtain a high efficiency of homologous recombination in iPSCs of different genetic backgrounds, but this approach has the difficulty of confirming the homologous recombination event. Adenoviral and retroviral vector-mediated gene targeting appear to be effective considering the efficiency of transduction and the homologous recombination. The preparation of these viral vectors needs expertise and an enough biosafety facility. Moreover, their use is confined to insertional mutagenesis, although, self-inactivating (SIN) lentiviral vectors have now become almost safe clinical strategies^[167,176-178].

The correction of disease mutations by nucleases in iPSCs has been described for different diseases. These nucleases cleave chromosomal DNA, generating DNA double strand breaks (DSBs), whose repair *via* endogenous mechanisms, such as homologous recombination (HR) or non-homologous end-joining (NHEJ), leads to targeted mutagenesis and chromosomal rearrangements^[179-181]. Hence, before delivery to patients, gene-corrected-iPSCs are differentiated into the appropriate somatic cells, to evaluate the expression of the corrected gene and to avoid teratoma formation in patients. In particular, different research groups have created nucleases for genome engineering in hiPSCs by linking the cleavage domain of the *FokI* restriction enzyme to a designed zinc finger protein (ZFP). The ZFN, which works as a dimer, is mediated by its linked ZFP domain. In particular, the ZFNs are designed to bind to a genomic sequence of sufficient length (18-36 bp)^[134,137,170,173,182,183]. The ZFN method is rapid and suitable but has a poor targeting density and lack of targetable sites for genome editing in small DNA sequences.

A recent and interesting approach for engineering DNA binding specificities is based upon TALEs from *Xanthomonas* plant pathogens. TALEs are a different way to the creation of site-specific nucleases^[184-187]. TALEs are transcription factors that specifically bind and regulate plant genes during pathogenesis^[188,189]. The DNA binding domain of TALEs is composed of multiple 34 amino acid units (TALE repeats) that are organized in tandem. Their sequence is almost equivalent, but presents two highly variable amino acids that set up the base recognition specificity for each unit. Each single domain allows the specificity of binding to one of the four possible nucleotides in the TALE recognition sequence, so any desired genomic sequence can be recognized as a DNA-binding domain. An example of TALENs application to correct a defective gene in iPSCs is the correction of the Niemann-Pick type C (*NPC1*) mutations in iPSCs-derived hepatic and neuronal cells of patients affected by NPC disease, a lipid storage disorder causing severe neurodegeneration and liver dysfunction. This approach allows for the rescue of the

phenotype, including the dysfunctional autophagic flux directly linked to loss of NPC1 protein function^[190]. It is important to keep in mind that the DNA methylation and histone acetylation in inactive chromatin could influence the efficiency of genome editing *via* TALENs.

The latest and the greatest mechanism for gene editing is represented by the bacterial cluster and regularly interspaced short palindromic repeats/CRISPR-associate nuclease 9 (CRISPR-Cas9). CRISPR is yet another example for how scientists have learned from nature's inventions helping them to discover new gene functions with high sensitivity and precision. This technology was created from type II CRISPR-Cas systems, by which bacteria degrade targeted nucleic acids. CRISPRs are components of the genomes of most bacteriophage-resistant *Bacteria* and *Archaea*. Cas9, a CRISPR-associated endonuclease, can be confined to specific DNA loci to induce double-strand breaks beneath the guidance of the trans-activating CRISPR RNA (tracrRNA): CRISPR RNA (crRNA) duplex. The dual tracrRNA:crRNA was additionally evolved as a single guide RNA (sgRNA) for genome engineering and consists of the 5' end 20-nucleotide sequence disposing the DNA target site conforming to Watson-Crick base pairing and 3' end double-stranded structure binding Cas9. The sgRNA could lead CRISPR-Cas 9 to any target DNA sequence with a protospacer-adjacent motif (PAM) by modifying the guide RNA sequences^[174,175]. In a genome editing system, the right choice of delivery system is crucial for the targeted cells.

Different delivery protocols have been adopted to vehicle *in vitro* plasmid DNA encoding Cas9-gRNA complexes through cell membranes in cell culture including electroporation, nucleofection and lipofectamine-mediated transfection^[177,191,192].

An important characteristic of the CRISPR/gRNA is its easy design and preparation. In addition, the system recognizes approximately 23 bp of the target site, which is a relatively shorter sequence than that recognized by TALENs. Recent papers have also corroborated that the RNA-mediated CRISPR/CAS9 system has a high tolerance to a few base pair mismatches towards the 5' half of the target site and a high potential for off-target risk in human cell lines.

A lot of studies have successfully modified genes in monogenic disorders using patient-specific iPSCs.

An interesting use of CRISPR-Cas9 is the correction of the *HBB* gene in human iPSCs generated from patients carrying a homozygous missense point mutation in the *HBB* gene. Using a specific guide RNA and Cas9, Huang *et al.*^[193] corrected, without off-targeting mutations, one allele of the *HBB* gene by homologous recombination with a donor DNA template containing the wild-type *HBB* DNA. Erythrocytes were then generated by differentiating hiPSCs in which normal expression of the β -globin protein (due to CRISPR-Cas9 modified *HBB* allele) was detected^[193].

Recently, another group has demonstrated the restoration of the dystrophin protein in DMD patient-

derived iPSCs by TALEN or CRISPR-Cas9. They used three correction methods (exon skipping, frameshifting and exon knock-in) to restore protein levels. After investigating the genome integrity, they differentiated the corrected iPSCs towards skeletal muscle cells and detected the expression of the full-length dystrophin protein^[194].

Haemophilia A is an X-linked genetic disorder caused by mutations in the *F8* gene encoding the blood coagulation factor VIII. The CRISPR-Cas9 protocol has been used by Park *et al.*^[195] to repair two large inverted regions back to the normal orientation in Haemophilia A patient-derived iPSCs. They demonstrated the expression of the *F8* gene *in vitro* in endothelial cells after their differentiation. Targeted deep sequencing and whole genome sequencing analyses showed no off-target effects, as they chose a target sequence that differs from any other site in the human genome by three nucleotides^[195].

The CRISPR protocol has been recently adopted for correcting the $\Delta F508$ mutation in CFTR- mutated hiPSCs. An excisable selection system is used to improve the efficiency of the correction. The possibility of any genomic footprint at the target site allowed Firth *et al.*^[196] to edit a point mutation in the genome, while leaving no genomic scar in patient cells. Modified hiPSCs were then differentiated to mature airway epithelial cells in which the functional recovery of the *CFTR* channel protein was demonstrated. Half of the CRISPR-corrected epithelial cells stably responded to stimulation by the whole-cell patch clamp method^[196].

The number of disease-specific hiPSC lines is increasing and the technology of gene editing using engineered nucleases holds considerable promise for progressing science and enhancing human health with the potential to create a variety of novel therapeutics for a range of diseases, many of which are untreatable today. Through the use of guiding RNA and nuclease activity, genes can be located and modified in a less expensive way. These technologies allow researchers to make changes to human DNA (human germline editing) in the nuclei of cells, in eggs, in sperm or in human embryos. In this way replacing or eliminating disease-causing genes could reverse disease symptoms.

However, these technologies also induce several social considerations and have not yet carried out an ethical experimental protocol useful for current medicinal practice. As a result, the *National Academy of Sciences* and the *National Academy of Medicine* are promoting an important initiative to lead decision making about research activities involving human gene editing. Many researchers report problems linked to mutations in other genes or off-target effects of the Cas9 nuclease whose consequence is largely unknown. These problems are most likely caused by the high level of functionality of the Cas9 protein, posing a dilemma in research goals requiring both powerful methods and efficacy. Several methods may minimize these problems, selecting for example unique target sequences and changing the

structure of the crRNA or sgRNA, strengthening nuclease expression levels, making use of truncated sgRNAs or recombinant proteins instead of plasmids.

Nonetheless, more detailed and comprehensive studies will be needed to determine the relative merits of these technologies in different experimental procedures to open the next era of gene therapy.

CONCLUSION

Human iPSCs are central in modelling and studying monogenic diseases. They represent an affordable and applicable tool to investigate the pathogenesis and the progression of human disease and, at the same time, can be used for the *in vitro* screening of new therapeutic compounds.

The advantages of induced pluripotent stem cells are represented by the generation of patient-derived cells and the maintenance of a versatile differentiation potential, opening new perspectives for the development of cell-based therapy and personalized medicine.

The possibility of obtaining a wide variety of disease relevant cells would allow investigators to design an efficient test system that permits large-scale screening of drugs for the targeted treatment of specific human diseases *in vitro*.

Moreover, this technology offers the opportunity to develop cell therapy protocols for several serious diseases requiring the restoration of cells or organs damaged in the pathological process. The possibility of using powerful methods for genome engineering, such as ZFNs, TALENs, and the CRISPR/Cas9 system, allows the correction of mutated genes *in vitro* and thus safe transplantation back to the patients to assess their therapeutic efficacy.

When concerning iPSC-derived models, it is necessary to examine the inherited genetic and epigenetic variations among patients in the phenotypic analysis. Regardless their potential, iPSCs-based clinical trials cannot be used except if all obstacles discussed in this review are overcome. For example, the residual pluripotent stem cells form teratomas after cells transplantation. Any aberrations regarding tumour formation or malfunctions of epigenetic memory, as well as the genomic instability, the delivery and expression of the reprogramming factors, and the growth of cells in culture, should also be closely monitored.

However, promising results have already been obtained in pre-clinical studies in different disease models, and also in the first clinical study currently on-going in Japan^[197,198]. In summary, only 8 years after their first report, iPSC cell technologies provide a promising prospect for clinical uses.

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Migration of bone marrow progenitor cells in the adult brain of rats and rabbits

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Abstract

Neurogenesis takes place in the adult mammalian brain in three areas: Subgranular zone of the dentate gyrus (DG); subventricular zone of the lateral ventricle; olfactory bulb. Different molecular markers can be used to characterize

the cells involved in adult neurogenesis. It has been recently suggested that a population of bone marrow (BM) progenitor cells may migrate to the brain and differentiate into neuronal lineage. To explore this hypothesis, we injected recombinant SV40-derived vectors into the BM and followed the potential migration of the transduced cells. Long-term BM-directed gene transfer using recombinant SV40-derived vectors leads to expression of the genes delivered to the BM firstly in circulating cells, then after several months in mature neurons and microglial cells, and thus without central nervous system (CNS) lesion. Most of transgene-expressing cells expressed NeuN, a marker of mature neurons. Thus, BM-derived cells may function as progenitors of CNS cells in adult animals. The mechanism by which the cells from the BM come to be neurons remains to be determined. Although the observed gradual increase in transgene-expressing neurons over 16 mo suggests that the pathway involved differentiation of BM-resident cells into neurons, cell fusion as the principal route cannot be totally ruled out. Additional studies using similar viral vectors showed that BM-derived progenitor cells migrating in the CNS express markers of neuronal precursors or immature neurons. Transgene-positive cells were found in the subgranular zone of the DG of the hippocampus 16 mo after intramarrow injection of the vector. In addition to cells expressing markers of mature neurons, transgene-positive cells were also positive for nestin and doublecortin, molecules expressed by developing neuronal cells. These cells were actively proliferating, as shown by short term BrdU incorporation studies. Inducing seizures by using kainic acid increased the number of BM progenitor cells transduced by SV40 vectors migrating to the hippocampus, and these cells were seen at earlier time points in the DG. We show that the cell membrane chemokine receptor, CCR5, and its ligands, enhance CNS inflammation and seizure activity in a model of neuronal excitotoxicity. SV40-based gene delivery of RNAi targeting CCR5 to the BM results in downregulating CCR5 in circulating cells, suggesting that CCR5 plays an important role in regulating traffic of BM-derived cells into the CNS, both in the basal state and in response to injury. Furthermore, reduction in CCR5 expression in

circulating cells provides profound neuroprotection from excitotoxic neuronal injury, reduces neuroinflammation, and increases neuronal regeneration following this type of insult. These results suggest that BM-derived, transgene-expressing, cells can migrate to the brain and that they become neurons, at least in part, by differentiating into neuron precursors and subsequently developing into mature neurons.

Key words: Stem cells; Bone marrow; Hippocampus; Cell therapy; SV40; Brain; Nestin; Doublecortin; Neurons; Development; Epilepsy; Seizures

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Core tip: It was previously thought that the development of new neurons did not take place in the adult brain of higher vertebrates. There has been substantial progress in understanding neurogenesis in the adult brain during the last decade, showing that neural progenitor cells can induce neurogenesis, mainly in three areas: Subventricular zone, subgranular zone of the hippocampal dentate gyrus, and olfactory bulb. More recently, it has been shown that bone marrow progenitor cells can participate in neurogenesis in the adult brain. In this review, we discuss the mechanisms of the migration, differentiation, and maturation of bone marrow progenitor cells in the adult brain. We also consider the increase of adult neurogenesis following experimental seizures, provided that neuroinflammation is decreased by reducing the expression of chemokines, and consequently the related migration of inflammatory cells into the brain parenchyma.

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ROLE OF BONE MARROW PROGENITOR CELLS IN ADULT NEUROGENESIS

Neurogenesis in the adult brain is a relatively new concept. There are three regions of the adult brain in Mammals where neurogenesis can take place in the adult mammal: Subgranular zone (SGZ) of the dentate gyrus (DG); subventricular zone (SVZ) of the lateral ventricle; and olfactory bulb (OB). The cells participating in neuronal development in adults have been characterized using molecular markers. It has been more recently suggested that a population of bone marrow (BM) progenitor cells could also contribute to adult neurogenesis. One way to verify if this hypothesis is correct would be to stain BM stem cells *in situ* and to track them in the body. In this review, we report that injecting the BM of rats and rabbits with SV40 vectors

results in the transduction of BM precursor cells that are migrating, among other organs, to the brain where they differentiate in neurons and microglial cells. It has also been previously shown that neuroinflammation can hamper the process of neuroregeneration following insult in the DG. We show here that reducing the levels of certain chemokine receptors in circulating cells by gene transfer of siRNA against these receptors in a context of a rat model of neurotoxicity leads to a decrease in inflammation and an increase of BM-derived cells migrating to the brain.

GENE DELIVERY TO BONE MARROW PROGENITOR CELLS

Ex vivo gene delivery is the most utilized procedure for transducing hematopoietic stem cell (HSC). However, in order to replace the *ex vivo* approach of transduction and reimplantation HSC, direct delivery of viral vectors into the BM has been proposed^[1-3]. This procedure has been suggested because *ex vivo* gene transduction and reimplantation may modify the homing properties and can change the functions of progenitor cells and HSC^[4-6]. Furthermore, HSC transduced by *ex vivo* gene delivery procedures may become exposed to infectious agents^[4]. We tried here to assess the efficiency of intramarrow injection in the femoral cavity of rats using rSV40 vectors. Levels of transgene expression were evaluated in peripheral blood population during several months^[7]. Transgene expression was observed during several months in multiple BM and peripheral blood lineages by using this method^[7]. Long term expression of transgene in platelets and the correction of haemophilia phenotype for at 5 mo were observed in other studies^[8]. Sustained gene expression was also found present in neuronal cell after *in vivo* gene transfer^[9]. The direct injection of viral gene delivery in the bone marrow can take full advantage of the stem cells that are present within the bone marrow including non hematopoietic cells^[8,10]. The targeting of HSC within their niche may be advantageous in the treatment of Fanconi anemia (FA) by ensuring that they maintain their function and by enabling the correction of the remaining stem cells^[6]. This approach also eliminates challenges, such as the requirement for preconditioning^[8,10], thus making it very promising in the treatment of FA^[6] and haemophilia^[8]. Moreover, gene transfer based on direct intramarrow injection should prevent difficulties seen during *ex vivo* approach such as stimulation by cytokines, putative loss of engraftment, and keeping HSC properties overtime^[8,10]. Gene transfer to the BM improves the efficiency of HSC viral vector transduction and strengthens the supportive microenvironment by opposition to intravenous inoculation that delivers vector to blood^[8,10]. Consequently, this procedure leads to better preservation of stem cell viability and capacity^[10]. We used the intrafemoral gene delivery approach to BM progenitor cells to study their fate in the body, and more particularly in the brain. We will first review the cells

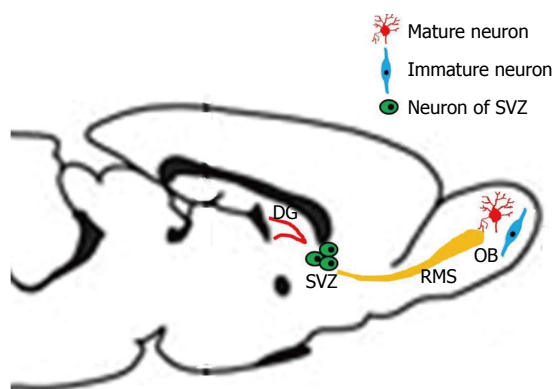


Figure 1 Different areas of neurogenesis in the adult rodent. The three areas of neurogenesis in the adult are the dentate gyrus of the hippocampus, the subventricular zone, and the olfactory bulb. Some progenitor cells are migrating from the SVZ to the OB, along a rostral migratory stream. DG: Dentate gyrus; SVZ: Subventricular zone; OB: Olfactory bulb; RMS: Rostral migratory stream.

involved in adult neurogenesis, their molecular markers, the different areas involved in adult neurogenesis, and the factors influencing neuronal development. Then, by using gene delivery into the femoral cavity, we will show the migration and differentiation of transduced BM progenitors to the brain. Finally, we will demonstrate that reducing neuroinflammation in an animal model of experimental seizure leads to an increase of BM progenitor cells migrating towards the brain.

NEUROGENESIS IN THE ADULT BRAIN

The traditional concept was that the development and growth of new neurons from neuronal stem precursor cells does not take place in the adult brain of higher vertebrates. In 1962, Altman^[11] demonstrated neurogenesis in the brain of an adult rat. There was no further report until Goldman and Nottebohm^[12] reported evidence of neurogenesis in canaries. This was further substantiated in 1992 when studies reveal similar evidence of adult neurogenesis^[13]. There has been substantial progress in understanding neurogenesis in the adult brain during the last decade. However, molecular events leading to the increase number, migration, and differentiation of progenitor cells in the brain need to be better characterized. The participation of BM progenitor cells to adult neurogenesis has also been suggested more recently.

Adult neurogenesis occurring in mammalian brain is now a well accepted idea, essentially in three regions: SGZ of the DG, SVZ of the lateral ventricle, and olfactory bulb^[14]. Dividing cells see their number reduced after birth, except in the SGZ of the DG and the SVZ (Figure 1)^[15].

Neuroblasts and progenitor cells from the SVZ also migrate through the rostral migratory stream (RMS) to the OB maturing into new neurons (Figure 1). The same process has been documented in primates, including humans^[16-18]. Neurons, produced in the DG and the SVZ are primarily granular neurons^[17] and to a lesser extent

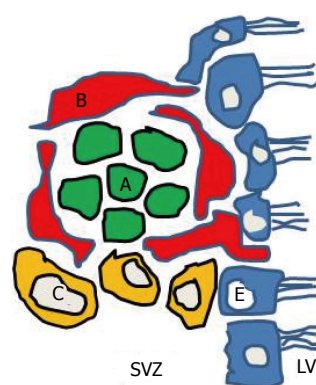


Figure 2 Four different types of progenitor cells in the subventricular zone. Type-A cells are migrating towards the olfactory bulb along the rostral migratory stream. Type B cells are believed to be the neural precursor cells and will give rise to both type C and type A cells. Type C cells are rapidly dividing immature precursor cells arranged in clusters along the migrating chains, and they are found only in the SVZ. E cells belong to ependymocytes lineage. LV: Lateral ventricle; SVZ: Subventricular zone.

periglomerular neuronal cells for the OB^[19].

The SVZ of the lateral ventricle is mainly located in the lateral wall of the lateral ventricle which is facing the striatum^[20]. It contains various neural progenitor cells along its wall. Four different types of cells have been identified in the SVZ of the lateral ventricle of mice^[20,21]. They are described as types A, B, C and E cells (Figure 2). These cells differ from each other based on the ultrastructure, morphology and molecular markers expressed by the cells. Type A cells, the most common ones, are darker than B cells in electron microscopy. Their cell bodies are elongated and contain up to two processes; they have abundant lax chromatin with two to four nucleoli; the cytoplasm is dark and contained free ribosomes. The rough endoplasmic reticulum has only a few short cisternae, the Golgi apparatus is small, and many microtubules are arranged along the long axis of the cell^[20]. These type A cells were described as being connected to each other by junctional complexes^[20]. Type A cells are neuroblasts expressing polysialated form of the neural cell adhesion molecule (PSA-NCAM). Type A cells are migratory in nature and course tangentially to the walls of the lateral ventricle. Type B cells are slow dividing astrocytes that enclose the migrating neuroblasts. These cells have different characteristics compared to type A cells^[14]. Their nuclei is irregular and the cytoplasm is lighter stained. They are characterized by their abundant intermediate filaments and the dense bodies within their cytoplasm. A further subdivision of type B cells into type B1 and a type B2 has been reported^[20]. Type B1 cells are larger, and contain more cytoplasm. It is believed that type B cells are the neural precursor cells and that they give rise to both type C and type A cells^[22]. Type C cells are found only in the SVZ and they are rapidly dividing immature precursor cells arranged in clusters along the migrating chains. Type C cells are larger, more spherical, contain larger golgi apparatus than type B cells; however their size is similar to type B1 but they have fewer processes^[20].

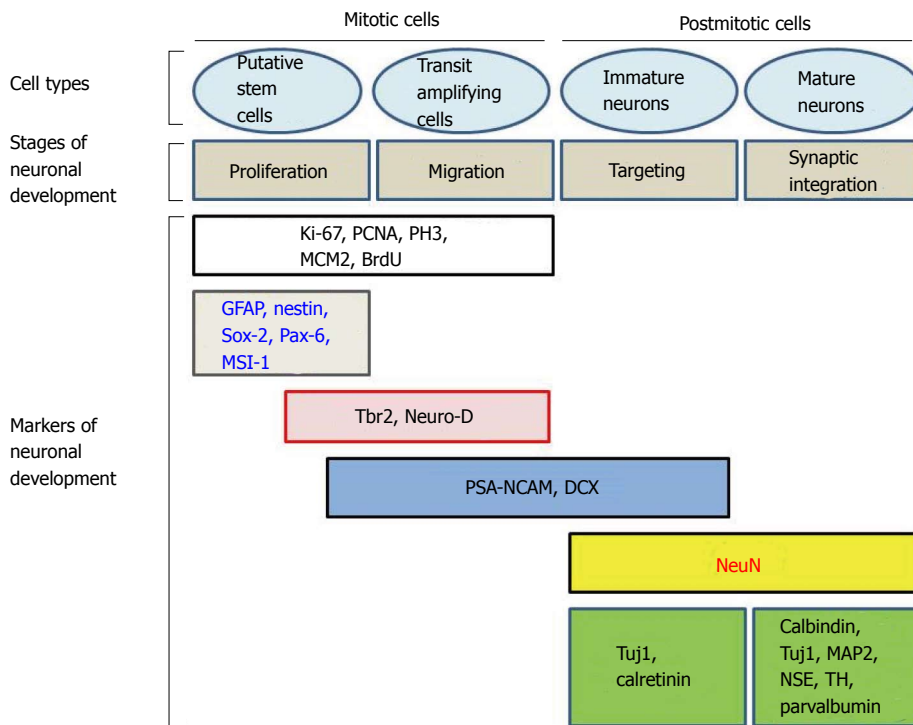


Figure 3 Different markers of neuronal development in mitotic and postmitotic cells (modified from Ref. [21,35]). PSA-NCAM: Polysialated form of the neural cell adhesion molecule; DCX: Doublecortin; GFAP: Glial fibrillary acidic protein; PCNA: Proliferating cell nuclear antigen; PH3: Phosphorylated form of histone 3; MCM2: Minichromosome maintenance protein 2; MSI-1: Musashi-1; MAP2: Microtubule-associated protein 2; NSE: Neuron specific enolase; TH: Tyrosine hydroxylase.

Type E cells belong to ependymocytes lineage (Figure 2).

Cells in the SVZ that are originating from the lateral wall of the lateral ventricle migrate along the RMS pathway to be incorporated into the OB^[14,23]. The rostral migratory stream pathway is constituted mainly of type A and type B cells^[20]. There has been no evidence so far of type C cells within the RMS and studies suggest that this region is devoid of these cells. Type A cells are arranged in chains surrounded by type B cells within the RMS, similarly as within the SVZ. These cells reach the olfactory bulb and become interneurons. In addition, microglial cells and endothelial cells are also present in the RMS^[24]. The division and migration of the neuroblasts within and to the olfactory bulb is independent of the process that takes place within the olfactory bulb^[25].

SGZ of the DG is situated between the hilus and the granular cell layer of the DG in the hippocampus. This is one of the main areas where neurogenesis takes place^[17,26] with an estimated 9000 new cells generated each day^[27]. Not all of the neurons generated in the hippocampus will survive and become incorporated into the neuronal circuit of the brain. Approximately 50% of the neurons born in the SGZ will die after birth without being incorporated in the neuronal circuit of the brain. Within the hippocampus, three types of cells are identified: (1) Type 1, radial cells, which give rise to type 2 cells; (2) Type 2, non-radial intermediate, neural progenitor cells; and (3) Neuroblasts, derived from type 2 cells^[28]. Type 1 cells are found to be progenitor cells similar to type B cells found in SVZ and are slow dividing cells, whereas type 2 cells are rapidly dividing ones^[29].

MOLECULAR MARKERS OF NEUROGENESIS

Adult neurogenesis involves a succession of events occurring in a specific order^[30-32]. For example, hippocampal neurogenesis is thought to begin with stem cells located in the SGZ of the DG. There, these cells proliferate, differentiate and give rise to new neurons. Neurons at various stages of neurogenesis express different markers. Consequently, the fate, and differentiation, of cells during neurogenesis can be followed^[33,34] (Figure 3 and Table 1).

Different markers help to determine the cells in the stage of neurogenesis and their role during particular stages. Some of the most commonly used markers include glial fibrillary acidic protein (GFAP), nestin, Neuro D, Hu, Tuj1, doublecortin (DCX), PSA-NCAM, neuron specific enolase (NSE) and neuronal specific nuclear protein (NeuN). Markers are useful to distinguish cellular proliferation, early adult neurogenesis, later steps of adult neurogenesis, and mature stage.

Different markers of proliferation can be used. The most utilized markers are proliferating cell nuclear antigen, and Ki-67, respectfully characterized by a long half-life, and a short half-life of about one hour. Ki-67 is rarely used in this application. Other markers include minichromosome maintenance protein 2 and phosphorylated form of histone 3^[35]. Bromodeoxyuridine (BrdU) incorporates into DNA during S phase and is also used as a marker of proliferating cells. To conclude that BrdU-positive cells are of neuronal lineage, and to characterize the cell lineages involved, BrdU-labeling needs to be combined with markers of neurons at different stages of neurogenesis^[33,35].

Table 1 Markers of cells involved in neurogenesis with corresponding stages of neurogenesis

Marker	Cells	Stage of neurogenesis
Nestin	Neuronal stem cells, radial glia cells, transit amplifying cells	Proliferation, differentiation
GFAP	Neuronal stem cell, mature astrocytes	Proliferation, differentiation
PSA-NCAM	Migrating neuroblast, immature neuron	Differentiation, migration, targeting
Tuj1	Migrating neuroblast, immature neuron, mature neuron	Differentiation, migration, targeting
Doublecortin	Migrating neuroblast, immature neuron	Differentiation, migration, targeting
NeuN	Mature neuron	Targeting, synaptic integration

GFAP: Glial fibrillary acidic protein; PSA-NCAM: Polysialated form of the neural cell adhesion molecule.

Markers of early stages of adult neurogenesis include GFAP, which is an intermediate filament protein expressed by mature astrocytes within the adult brain^[33,36,37]. Its expression has been described in type B progenitor cells present in the SVZ and type 1 cells described in the SGZ^[21,36-38]. Nestin is a structural analog to intermediate filament protein, found to be positive in type B neural progenitor cells and transit amplifying type C cells in the SVZ^[21], in type 1 (radial glial cells) and 2 cells in the SGZ^[29,39]. Nestin is thought to be a marker of precursor cells. Consequently, nestin synthesis decreases, as differentiation of nervous tissue progresses. Neurofilament and glial (e.g., GFAP) proteins begin to be expressed during the differentiation of neurons and astrocytes respectively when nestin expression starts to be reduced. Nestin expression declines during the postnatal period. However, rare cells expressing nestin are present in the adult DG and the SVZ^[40]. Expression of nestin is abruptly terminated. Other early markers of adult neurogenesis are Sox-2, a SRY-related HMG-box gene 2, paired box gene 6 (*Pax-6*), and musashi-1 (*MSI-1*), a RNA-binding protein preferentially expressed in the CNS. However, neuronal specificity of some of these markers is not absolute^[35].

The markers of later stages of adult neurogenesis include DCX and PSA-NCAM, both markers of immature neurons. DCX is a protein positive in cells within the RMS and is present in migrating progenitor of neurons^[41,42]. DCX induces polymerization of microtubules. DCX is a marker expressed by neuroblasts during their migration and immature neurons of the granular layer of the DG. It is also present in newly generated cells located at the border of the granular layer of the DG and in the SGZ. DCX is expressed in newly generated olfactory, hippocampal, and striatal neurons, but not in the cortex. DCX is a marker of late mitotic neuronal precursors and early postmitotic neurons. There is no overlap between the expression of DCX and nestin^[33,34]. As such, DCX is a good marker for adult neurogenesis. Expression of the (PSA-NCAM) is observed at the same stage. Thus, both mitotic neuronal precursors and early postmitotic new neurons are positive for PSA-NCAM and DCX^[35]. The basic helix-loop-helix protein NeuroD and T-box brain protein 2 (*Tbr2*) are also expressed during later steps of adult neurogenesis, and show some overlap with the expression of Pax-6. Neuro D is a protein inducing microtubule polymerization. NeuroD is expressed in proliferating neurons and in migrating neuroblasts^[33].

Both *Tbr2* and *NeuroD* are coexpressed with DCX and PSA-NCAM. Moreover, *Tbr2* is downregulated when cells are committed to neuronal lineage^[35].

Finally, NeuN is a soluble protein that is found in the nucleus and cytoplasm of postmitotic neurons. NeuN is expressed by mature neurons^[33]. In the hippocampus (HC), postmitotic cells are immunopositive for NeuN. NeuN is a marker of both newly generated postmitotic neurons and "normal" postmitotic neurons. There is a correlation between the decreased expression of DCX and the beginning of NeuN expression^[33]. Additional markers can be used to characterize mature neurons, and some of them can be specific of certain areas of the brain. Hu is a RNA binding protein from the elav family expressed from the early stages of neurogenesis to the end^[43]. Tyrosine hydroxylase is the enzyme that catalyzes the formation of L-DOPA, precursor of dopamine. Several calcium-binding proteins can be expressed in mature neurons: Calbindin-D28k is particularly abundant in the cerebellum; calretinin is a 29 k protein with 58% homologies to calbindin-D28k; parvalbumin is a small calcium-binding albumin protein involved in calcium signaling. Microtubule-associated protein 2 induces assembly of microtubules, an essential stage in neuritogenesis. NSE is often used as a marker of mature neurons. Neuron-specific class III β -tubulin (*Tuj1*) contributes to axonal transport and provides stability to microtubules in axons and somas. However, the list of the markers for mature neurons is not exhaustive (Figure 3).

Markers expressed in the SVZ and the subgranular zone of the DG during adult neurogenesis are presented in Tables 2 and 3 respectively.

MIGRATION OF BONE MARROW PROGENITORS TO THE BRAIN AND THEIR DIFFERENTIATION INTO CELLS OF DIFFERENT CNS LINEAGES

rSV40-transduced bone marrow progenitor cells migrate to the brain

Gene delivery to the brain has focused mainly on transducing neurons directly. However, an alternative approach may be to consider those areas of the brain where neurogenesis continues well into adult life: The DG of the HC, and the SVZ. New neurons are generated throughout life in the DG. These new neurons are involved

Table 2 Markers expressed during neurogenesis originating in the subventricular zone

Neuronal lineage	Type B cells	Type C cells	Type A cells	Neuronal cells
Cell identity	SVZ Astrocytes Putative stem cells	Transit amplifying progenitor cell	Migrating neuroblast	Mature and immature neuron
Identifying marker	GFAP Nestin	Nestin	PSA-NCAM Tuj1 Hu	DCX NeuN

SVZ: Subventricular zone; GFAP: Glial fibrillary acidic protein; PSA-NCAM: Polysialated form of the neural cell adhesion molecule; DCX: Doublecortin; NeuN: Neuronal specific nuclear protein.

in the repair of brain insults. Targeting endogenous brain cell progenitors *in situ* might be attempted in order to genetically engineer them. For example, proliferation of such engineered cells after injury or during disorder of the brain would lead to functional brain cells expressing a transgene of potential interest. However, this approach is limited by the low number of endogenous progenitors in the adult brain. Furthermore, the life span of endogenous stem cells might be not as long compared to pluripotent stem cells with different properties as well.

Migration of BM stem cells to the brain and their differentiation into different types of brain cells has been reported in rodents^[44,45] as well as in humans^[46]. When they are injected intravascularly or intraperitoneally in rodents, adult BM progenitor cells are able to migrate to the adult CNS where they differentiate into neuronal^[45,47], or non-neuronal cells^[48]. In patients with transplantation of BM, the autopsy of brains demonstrated that human HSC can trans-differentiate into neurons, astrocytes, and microglia following long term marrow engraftment. These results were observed without fusion between cells. They suggest that human HSC coming from BM-transplantation could be used as a potential therapeutic source not only for long term regenerative neurogenesis^[46], but also for gene delivery in the brain.

The potential of direct transduction of HSC in the BM has been raised. However, *in situ in vivo* gene delivery to HSC/progenitors by direct injection of viral vectors in the BM has been rarely described, despite the putative interest of this method. The fate of cells positive for the transgene in the body has never been studied in this context. Our group previously reported that the inoculation of a Tag-deleted recombinant SV40 vector carrying a marker gene (FLAG epitope appended to HIV-1 Nef as a carrier protein) in the femoral BM of rats caused positive results^[7]. Expression of the transgene in the blood lasted throughout the 16 mo of the study. Twenty-five percent of femoral marrow cells and between 4%-12% (average, 5%) of blood nucleated cells of all lineages were positive for the transgene FLAG throughout the whole study. However, it remained to be determined if HSC could migrate to the brain in this experimental paradigm. The

Table 3 Markers expressed during neurogenesis in the subgranular zone of the dentate gyrus

	Mitotic cells		Post mitotic cells	
Stages of neuronal development	Putative stem cell	Transit amplifying progenitors	Immature neurons	Mature neurons
Markers for neuronal development	GFAP Nestin	Nestin Doublecortin	Doublecortin Tuj1	Tuj1 NeuN NSE

GFAP: Glial fibrillary acidic protein; NeuN: Neuronal specific nuclear protein; NSE: Neuron specific enolase.

aim of the study was to determine the localization and the type of transgene-positive cells in the DG and in the SVZ. DG is composed of the hilus area surrounded by the granule cell layer (GCL), formed by an inner (upper) and outer (lower) blades. We therefore investigated by immunohistochemistry^[49,50] if some BM progenitor cells, transduced *in vivo* by intramarrow injection of a rSV40 vector, could migrate to the adult CNS and differentiate into different brain cell lineages^[51,52].

Transgene expression was seen in cells with shape of neurons in the DG 16 mo after intramarrow injection of the vector. Transgene expression was not seen in the DG of control animals whose BM was either inoculated with a control vector [SB(BUGT)], or saline. Transgene-positive cells were mostly observed in the DG and SVZ (Figure 4A). Transgene-positive cells were not seen before 1 mo and few of them were detected at 4 mo. Numerous transgene-positive cells were observed in the DG and SVZ 16 mo after intramarrow injection of the vector. Some of the transgene-expressing cells were also positive for NeuN, a marker of mature neurons, not only in the GCL but also in the hilus. Transgene-positive cells expressing NeuN had the shape of neurons of the DG. There were also some transgene-expressing cells that were not NeuN positive in the same areas, and some of these cells had the morphology of microglial cells and were immunopositive for CD11b-C3bi, and CD68, both markers of microglial cells. Very rare transgene-positive were astrocytes. Sixteen months after intramarrow injection of the vector, approximately 5% of DG cells were positive for the transgene. Forty-eight point six percent, 49.7% and 1.6% of these transgene-positive cells were respectively expressing markers of neurons, microglia, and astrocytes, as assessed by double immunocytochemistry for the transgene and lineage markers. We also determined transgene expression in the SVZ. The pattern of FLAG expression in the SVZ was similar as in the DG. One and 4 mo after intramarrow injection of the vector, no or few cells were expressing FLAG; numerous transgene-positive cells were observed in the SVZ 16 mo after intramarrow injection of SV(Nef-FLAG). About 50% of transgene-positive cells were of neuronal lineage because stained by neurotrace (NT), a neuronal marker. This percentage is close to the one observed in the DG. Thus, transgene-positive cells can be observed in areas of the CNS other

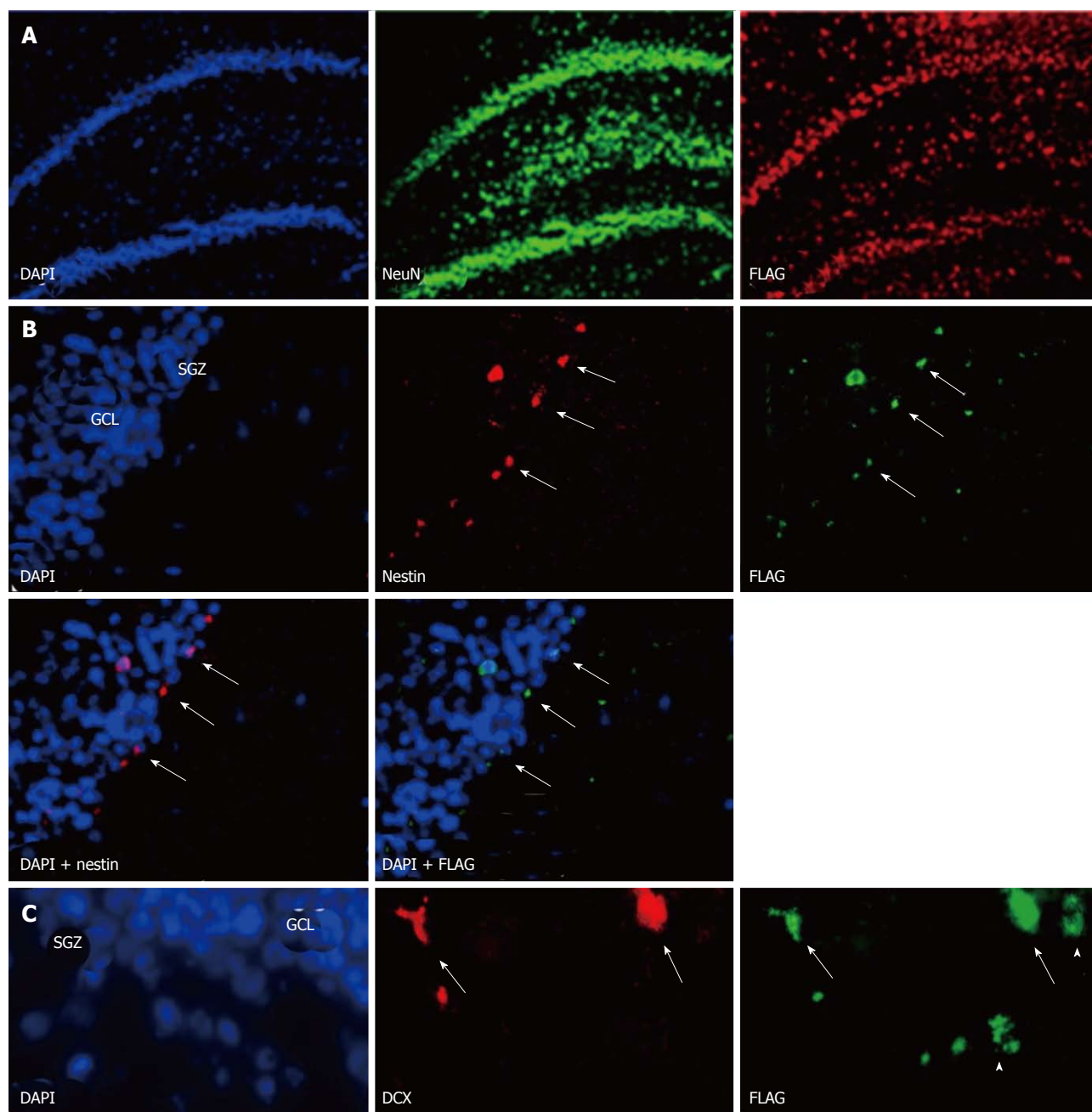


Figure 4 Bone marrow derived cells can migrate to the rat normal hippocampus. Sixteen months after injection of SV (Nef-FLAG) into the rat bone marrow (BM), transgene expressing cells were detected in the dentate gyrus (DG). A: FLAG-positive cells colocalized with NeuN, a marker of mature neurons. FLAG+/NeuN+ cells were located in the granular cell layer (GCL), as well as in the subgranular zone (SGZ) and the hilus. No FLAG-expressing cells were detected in the brain after injection of SV (BUGT), a control vector, in the BM; B: Nestin+/FLAG+ cells were detected mainly at the border SGZ/GCL, and more rarely in the GCL (arrows); C: DCX+ positive cells expressing FLAG were seen at the border SGZ/GCL and more rarely in the GCL (arrows). Note that some FLAG+ cells were DCX-, and were probably mature neurons (arrowheads). In all experiments, nuclei were stained in blue by Vectashield containing DAPI.

than the DG. Our results show that cells present in the adult BM can migrate to the CNS where they differentiate into cells of CNS lineages.

Consequently, it appears, that gene delivery to the BM induces transgene expression in brain cells of different lineages, in the DG and the SVZ. These results show that adult neurogenesis continues in the DG and SVZ during adult life, and that at least some of the cells generated that way derive from one or more populations of resident BM cells at the time of the administration.

Other hypotheses can explain these observations. rSV40 vectors can transduce CNS cells when directly administered into the brain^[53]. However, DG cells would unlikely be transduced at the time of the injection of the vector in the BM. Only 16 mo after the injection were transgene-positive cells seen in the DG. Transgene-positive cells were absent the first weeks after injection of the vector and were rare 4 mo after intramarrow injection of the vector. Moreover, transgene-positive cells were rarely detected in epithelial cells of other

organs usually transduced by intravenous rSV40 administration (e.g., liver, kidney), at all times after injection, suggesting that the vector did not diffuse significantly from the site of injection^[54,55].

Others also suggested that BM progenitor cells might differentiate into mature brain cells. It has been shown that endogenous neural stem cells are present in the DG and SVZ, and can generate different types of brain cells^[56]. These endogenous progenitor cells may be responsible for the constant remodeling taking place in the HC and OB^[15,16]. The apparent maturation and differentiation of BM progenitors seen in the present study is likely a physiological process because no insult was caused to the brain. Various types of injury can lead the adult rodent brain to repair itself by neurogenesis^[57-59]. Neurogenesis taking place in the HC and SVZ following such insults is based on the generation of new neurons that are able to migrate a considerable distance from their origin^[57]. Moreover, these regenerated neurons can replace dying cortical ones^[60,61].

It is still unclear if such process takes place in humans during physiologic or pathologic conditions. However, our results were observed without injury given to the CNS. Both experimental and human data showed that transplantation of male HSC coming from the BM into female recipients resulted in CNS neurons that were bearing Y chromosome^[45,46]. Cogle *et al.*^[46] detected neurons in the HC of women recipients coming from male BM transplants. However, this process took several months to occur. These data are coherent with our results^[46,62,63]. Similarly, BM progenitor cells from male mice, transplanted into immunocompromised female recipients, generated cells positive for Y-chromosome, that were detected in the brain and were positive for neuronal markers^[45,46].

Studies using gene-marked BM cells have shown that BM-derived cells may transdifferentiate into CNS cells of one or more lineages^[47]. Cell-cell fusion might support some of the reports^[62-64]. However, when they are exposed to certain growth factors (*i.e.*, brain-derived neurotrophic factor, epidermal growth factor), or when they are grown together with fetal mesencephalic or striatal cells, cultured BM stromal cells can express some glial and neuronal antigens^[65]. Similarly, when they are administered into brain ventricles of neonates, BM stromal cells can differentiate into cells immunopositive for astrocytic and neuronal markers^[66]. It has been suggested that BM progenitor cells can trans-differentiate into brain cells of different lineages^[45]. However, other reasons can explain these observations as well, and the mechanism(s) of such trans-differentiation are not clear as yet.

The first step in the migration of BM progenitor cells to the brain would be the homing of the BM-derived population(s) to their target. The mobilization pathway of HSC towards the brain involves a complex and intimate collaboration between adhesion molecules, cytokines, proteolytic enzymes, stromal cells, and HSC. This process can explain the regulation of HSC release, migration, and homing from the BM to the brain^[67].

Vascular and extracellular matrix molecules have an important role as well^[22,45,68-70]. Numerous reports involve mechanisms and factors inducing or promoting mobilization of HSC from BM into peripheral blood (PB). Several cell membrane proteins, including CXCR4 and its ligand, α -chemokine stromal-derived growth factor-1 (SDF-1), are involved in the mobilization of HSC to the PB^[71-74]. Different factors can increase the number of HSC migrating to the PB. Among these factors, some are related to tissue or organ injury, strenuous exercise and stress, local or systemic inflammation, and finally pharmacological agents such as CXCR4 small-molecule antagonist AMD3100 and granulocyte colony-stimulating factor (G-CSF)^[74-77]. Among these factors affecting HSC mobilization, G-CSF and SDF-1 are the best known. It must be noted that the activation of the complement cascade is activated by all these processes^[74,76].

BM niches are retaining HSC through the interaction between the chemokine CXCR4 receptor and $\alpha 4 \beta 1$ integrin. The respective ligands of CXCR4 and $\alpha 4 \beta 1$ integrin are α -chemokine SDF-1 and vascular adhesion molecule-1 (VCAM-1, also known as CD106), and they are present on cells in the BM niches (e.g., fibroblasts and osteoblasts)^[74-77]. One of the main factors affecting HSC mobilization, G-CSF, operates in two ways: First, it disrupts the anchoring relationship by decreasing the expression of SDF-1, thus reducing the binding of SDF-1 to CXCR4. Secondly, G-CSF enhances serum levels of other cytokines and growth factors^[67,78,79].

SDF-1 (chemokine CXCL12) is highly expressed in the BM where it is generated by osteoblasts in the endosteal region, as well as by endothelial cells and reticular cells located in the BM stroma. SDF-1 is a potent chemoattractant for HSC and it controls cell adhesion and survival as well. Synthesis of SDF-1 obeys to a circadian rhythm regulated by the sympathetic nervous system. Noradrenaline operates *via* $\beta 2$ -adrenoreceptors present on osteoblasts and *via* $\beta 3$ adrenoreceptors expressed in nestin-positive stem cells in order to decrease their production of SDF-1^[80,81].

CXCR4 and CXCR7 have been described as two chemokine receptors for SDF-1. The relationship between SDF-1 and CXCR4 in HSC is believed a key factor in the control of the traffic of HSC in the BM^[82,83]. AMD3100 is a powerful bicyclam CXCR4 antagonist that acts synergistically with G-CSF in humans. AMD3100 enhances mobilization of HSC in the BM^[84]. Once mobilized, HSC express decreased levels of CXCR4. CXCR7 is a second high-affinity receptor for SDF-1. However, CXCR7 is not linked to signaling pathways for migration of HSC. SDF-1 is internalized then degraded once bound to CXCR7; consequently, CXCR7 appears to act as a SDF-1 sink^[67,78]. CXCR4 and CXCR7 interact, but CXCR4 inhibition does not seem to modify the role of CXCR7^[67].

There are several factors influencing the interactions SDF-1-CXCR4 and $\alpha 4 \beta 1$ integrin-VCAM-1. For example, the sensitivity to SDF-1 depends on the incorporation of the CXCR4 receptor into membrane lipid rafts^[67,85,86]. As a consequence, the migration of HSC is influenced

by gradients of the bioactive lipids sphingosine-1 phosphate (S1P) and ceramide-1 phosphate (CP1). S1P and CP1 result from membrane lipid metabolism and are involved in stem cell trafficking.

Other important molecules involved in HSC mobilization are proteolytic enzymes released from activated granulocytes and monocytes that are present in the BM. These enzymes operate by attenuating SDF-1-CXCR4 and $\alpha 4\beta 1$ integrin-VCAM-1 interactions in the BM microenvironment^[74-77]. Another example of molecular intervention is the role of innate immunity in this process. One of the molecules involved in innate immunity, $\beta 2$ -defensin ($\beta 2$ -D), influences the sensitivity of HSC to SDF-1^[76]. Different proteolytic cascades such as the complement cascade, coagulation cascade, and fibrinolytic cascade, as well as several other proteolytic enzymes secreted by cells present in the BM might have a role as well^[87]. Different stress situations, local or systemic inflammation, and administration of pharmacological mobilizing agents (*e.g.*, G-CSF and AMD3100) can influence these proteolytic cascades^[76].

Activated complement stimulates oxidative stress, activation of platelets, and injury of erythrocyte membranes, interacts with different proteolytic cascades in the BM, and consequently, triggers mobilization of HSC^[67]. The third (C3a) and fifth protein components of complement (C5) have also an important role in the mobilization of HSC^[88]. The first cells migrating from the BM are neutrophils^[88]. C5b-C9 complex (or membrane attack complex) induces release of S1P, from red blood cells and platelets^[89]. Thus, inflammatory process and innate immunity will induce the migration of HSC from BM into PB^[76].

In another cascade involved in the mobilization of HSC, the fibrinolytic cascade, plasminogen binds to the BM extracellular matrix (ECM). Secondly, various proteins components of the ECM, including fibrin, laminin, are damaged by plasminogen after it converted into plasmin. Other proteases, such as metalloproteinases MMP-3, MMP-9, MMP-12 and MMP-13, are also activated by plasminogen in order to reduce the levels of other components of the ECM, such as collagen^[90].

Heme oxygenase 1 (HO-1) is also involved in the mobilization process of the HSC^[91]. Among other roles, HO-1 mitigates the inflammation linked to the complement by increasing the expression of complement inhibitors CD55 and CD59 on endothelial cells^[92]. HO-1 participates also in the regulation of the expression of SDF-1^[93], a major factor in the retention of HSC in BM niches^[74-77]. It has been shown recently that HO-1 influences actually negatively the adhesion and migration of neutrophils during acute inflammation^[94]. It has been suggested that negative regulators of the mobilization of HSC from BM exist. But few results have been reported so far. Serine protease inhibitors (serpins)^[95] and tissue inhibitors of metalloproteinases (TIMPs)^[96] seem to have such inhibitory effects on mobilization of HSC. HO-1 seem to influence negatively the migration of HSC^[74].

Concerning homing of progenitor cells to organs,

and not PB, other factors can be involved. For example, resveratrol enhances migration of mesenchymal stem cells to injured liver^[97].

After mobilization from the BM, the second step would be the differentiation into CNS cells of different lineages. However, mechanisms of differentiation are not totally clear yet^[98]. Neural cell adhesion molecules (*i.e.*, N-CAM), proteins regulating cell cycle transit^[99] and transcription factors^[100] have been implicated in this process, as well as molecules of the ECM^[68,69]. Ectopic expression of a specific set of transcription factors (c-Myc, Sox2, Oct4 and Klf4) can reprogram mouse embryonic and adult fibroblasts into embryonic stem-like cells^[101]. These cells were called induced pluripotent stem (iPS) cells. It has been reported that stem cells can be reprogrammed into iPS cells more effectively than into mature cells. Other types of cells can demonstrate multipotency. Among these cells, multilineage-differentiating stress-enduring (MUSE) cells^[102] and mesenchymal stem cells (MSC) have been described^[103,104]. MUSE cells are adult stem cells. They are characterized by differentiation into different lineage (mesodermal-, ectodermal- and endodermal) cells from a single cell. MUSE cells are tolerant to stress. They express markers of pluripotency, and are able of self renewal^[102]. They can be obtained from fibroblasts. Properties of MUSE cells resemble those of iPS cells, but they are devoid of tumorigenicity^[102]. MSC are a population of multipotent, self-renewing cells, mostly located in a bony niche, that regulate skeletal tissue and repair^[105].

High levels of sonic hedgehog and induction of Wnt signaling induce derivation of floor plate from pluripotent stem cells, increasing the quantity and quality of dopaminergic (DA) neurons consequently produced^[106,107]. These results led to the development of protocols for differentiation of mouse iPSCs into DA neurons^[108]. These newly generated cells expressed markers of DA neurons, such as the enzyme tyrosine hydroxylase, as well as the transcription factors Nurr1, and Pitx3. Once transplanted in a rat model of Parkinson's disease, these DA cells could repair lesions seen in the animal model^[108].

Transdifferentiation of fibroblasts to neurons has been reported. The introduction of three specific factors of neurodevelopment (Brn2, Ascl1 and Myth1l, or BAM) in mouse fibroblasts, directly generated neuronal cells, called induced neurons, or iNs^[109]. Similar approach was used with the same results by using human fibroblasts^[110]. Mouse and human iNs were characterized by immunopositivity for neuronal markers such as Tuj1, Map2, Tau and synapsin. Moreover, the combination of the iN factors with Lmx1a and FoxA2^[111] or a combination of Lmx1a and Nurr1^[112] in fibroblasts can directly generate cells with DA neuronal characteristics, named iDA neurons. The combination of the three neurodevelopmental factors (Brn2, Ascl1 and Myth1l, or BAM) with other factors implicated in the embryonic development of DA neurons enhances the transdifferentiation towards cells presenting characteristics of DA neurons^[113-118].

It is also possible to induce the transformation of

acinar exocrine cells from pancreas of cadavers into pancreatic β -cells. Cells generated that way can produce insulin, and are glucose-regulated. Once transplanted into immunocompromised diabetic mice, these cells can normalize glycemia^[119].

In our experiments, we examined the brain and other organs^[120] to assess the transgene expression outside of the BM in animals whose BM was injected with SV40-derived vector. We found that CNS cells of different lineages expressed the transgene several months after intramarrow administration of the vector, suggesting that transgene-positive cells were likely to have migrated from the BM. About 5% of DG cells were transgene-positive. BM progenitor cells were also migrating towards other organs (*i.e.*, spleen, lungs, liver). In the lungs, FLAG-expressing cells were mainly seen in the alveoli and were coexpressing markers of progenitor cells (*i.e.*, TTF-1) and of macrophages. Our studies do not determine the nature of the BM progenitors migrating to the CNS. These cells may be of hematopoietic, stromal or other origin. The hypothesis that BM cells can transdifferentiate into CNS cells of different lineages has been the topics of several conflicting reports. However, various animal models, different experimental paradigms and diverse methods have been used and could explain the discrepancies observed in these reports. In most of the studies against the hypothesis of BM to CNS transdifferentiation, the markers used are protein products of transgenes^[121-123]. Transgene delivered by numerous vectors, integrating or not, may disappear with time, particularly if they are of protein origin. By contrast, rSV40 vectors lead to long-term transgene expression^[54,124]. In other studies, DNA markers have been used to assess the engraftment of stem cells and the differentiation of donor cells in to host cells^[125,126]. DNA probes, such as the ones in FISH assays, were used in most studies sustaining the hypothesis of BM to CNS transdifferentiation. One of the important factors explaining the conflicting data appears to be the time between the transplantation of progenitor cells in the brain or in the periphery and their differentiation. Human cells transplanted into the mouse lateral ventricle differentiate into neurons in the OB only after several months^[61]. The number of transgene-positive neurons enumerated in the brain after injection of GFP-expressing HSC increases with age^[62,63]. The brief time intervals between the injection of the animals with progenitor cells and the harvest of the tissues might explain some negative results^[121]. However, these negative results might be due to the full maturity of the neurons, to the molecular marker used, and to the type of neurons as well. Our work suggest that BM-derived cells found in the DG are mature neurons, because expressing NeuN. They also have the shape of mature neurons in the considered area. However, we do not know at this point what is their physiological function and what type of synapses are established.

The settings of the experimental system might also explain the results observed. For example, some experiments include treating HSC *in vitro* with growth

factors that might modify their properties before their administration. Such treatment could potentially influence the homing of transplanted cells towards the brain or their differentiation into cells of CNS lineages. These experimental settings do not reproduce physiological conditions. By avoiding some of these treatments, we were able to transduce the BM directly without perturbing the different BM populations of cells. Thus, we can suggest that BM resident cells can transdifferentiate into cells of CNS lineages. However, additional studies would be useful to explore the functions of these cells.

Bone marrow progenitor cells express different markers of neuronal differentiation in the brain

We reported above that permanent BM-directed gene transfer using recombinant SV40-derived vectors led to expression of the transgene in mature neurons, and thus without CNS lesion indicating that BM progenitor cells can differentiate into cells of different CNS lineages. Most of transgene-expressing cells also expressed NeuN, a marker of mature neurons. However, it remained to be determined by what mechanism the cells from the BM come to be neurons. Although the observed gradual increase in transgene-expressing neurons over 16 mo suggested that the pathway involved differentiation of BM-resident cells into neurons, we could not rule out cell fusion as the principal route. Therefore, we tested here whether BM-derived progenitor cells migrating in the CNS could express markers of neuronal precursors or immature neurons. We injected SV40-derived vectors, carrying marker epitopes (FLAG or AU1), into the femoral cavities of rats or rabbits. Control animals received a control vector, SV (BUGT), injected into the BM as well. AU1- or FLAG-positive cells were seen in the DG 16 mo after injection of respectively SV (RevM10.AU1) or SV (Nef-FLAG) in the BM. In addition to cells expressing markers of mature neurons, transgene-positive cells were also positive for nestin and doublecortin, molecules expressed by developing neuronal cells. These cells were actively proliferating, as shown by short term BrdU incorporation studies (Figures 4B, 4C and 5). These results confirm that BM progenitors migrate to the CNS where they become neurons, by differentiating into neuron precursors and subsequently developing into mature neurons. Similar results were seen in the rat (Figures 4B, 4C and 5) and the rabbit (Figure 6).

This progression recapitulates the sequential stages of neurogenesis. Transgene-expressing cells positive for nestin and DCX were mainly localized at the border between SGZ and GCL, and less frequently in the GCL. This location is the one usually reported for immature neurons in the hippocampus. Cells in the SGZ have been shown to migrate into the GCL^[30].

In assessing the studies that report support for, or evidence against, BM cell transdifferentiation into other lineages^[121,122], the time between the administration of stem cells and harvest of the tissue is a critical factor^[61-63,66]. Thus, a brief time interval between injecting animals and collecting tissue may not identify markers

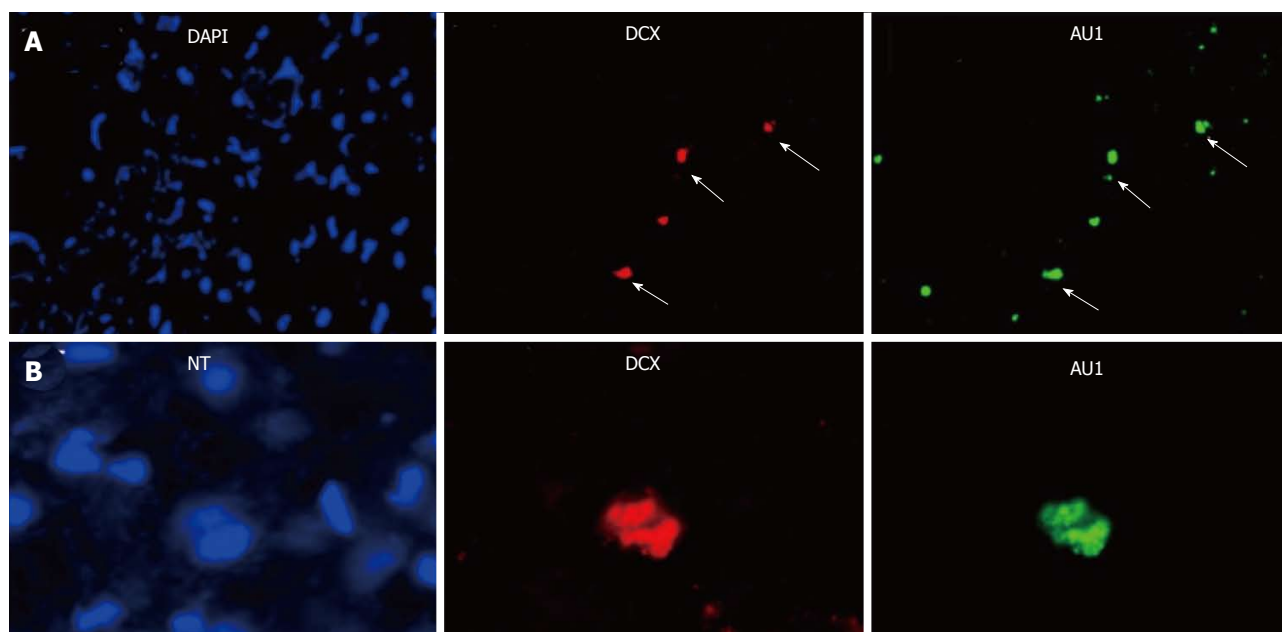


Figure 5 Transgene (AU1) expression in the hippocampus of rats whose bone marrow has been injected with SV (RevM10.AU1). A: Almost all DCX-positive cells were expressing AU1 (arrows); B: DCX+/AU1+ cells were of neuronal lineage, because they were stained by NT (arrows). No AU1 expression was seen after bone marrow injection of SV (BUGT), a control vector. DCX: Doublecortin; NT: Neurotrace.

belonging to the BM cells in the target organ studied. We have found that increases in neuronal and microglial cell populations expressing transgenes delivered to BM were gradual and protracted. No transgene-expressing neurons were seen 1 mo following BM injection, a few at 4 mo and many were at 16 mo^[51]. BM cells can undergo spontaneous fusion with other cell types, a process that is probably unlikely. Higher levels of transdifferentiation have been reported in some studies^[127]. The results observed in physiological conditions, as here, should also be compared to the ones seen during brain damage^[128-132] in order to determine the putative factors influencing the migration and differentiation of BM-derived cells in adult CNS.

FACTORS INFLUENCING NEUROGENESIS IN ADULT BRAIN AND ROLE OF SEIZURES

Factors influencing neurogenesis

Various physiological and pathological factors may increase neurogenesis in different areas of the brain. Neurogenesis is found to be increased in acute neurodegenerative disease^[28,133]. Hippocampal neurogenesis is enhanced during Alzheimer's disease^[134]. Brain injury such as stroke also leads to an increase in neurogenesis in humans^[135]. Neurogenesis may be increased following damage to the adult brain^[57,59]. Different classes of molecules have affected the rate of neurogenesis, including growth factor, hormones^[136] and neurotransmitters. Testosterone can increase generation of new neurons in the DG of adult male rodents^[136]. Blockade of neuroinflammation can restore adult neurogenesis in the hippocampus^[137].

Neuronal loss, inflammation, regeneration and gliosis following kainic acid-induced seizures

It has been reported that seizures can induce neuronal regeneration in the DG, but the extent of the process is hampered by the inflammation following the seizure. We first identified neuronal regeneration post kainic acid (KA)-induced seizure. For this purpose, we injected intraperitoneally 10 mg/kg KA in rats. KA causes tonic-clonic seizures. After the animals recovered from these seizures, they were rested for 7 d and their brains were examined for neuron loss in the HC, using either NeuN or NT as neuronal markers. We observed that neuron loss was most prominent in the CA1 and CA2/CA3 regions of the hippocampus, but loss of neurons was statistically significant, compared to control rats receiving vehicle only, in the hilus, SGZ/GCL and in CA1, CA2 and CA3.

At the same time, we demonstrated an inflammatory infiltration in the HC by enumerating macrophage/microglial cells using antibodies against Iba-1 (quiescent and activated cells) and CD68 (activated cells), and astrocytes using antibody against GFAP. Numbers of GFAP- and Iba-1-positive cells were increased in the HCs of KA recipients, compared to controls, indicating that brain injury related to KA administration led to infiltration of the affected area by macrophages and microglia, and to an astrocytic response. Neuroinflammation was associated with an increased expression of cytokines and chemokines, particularly regulated on activation, normal T cell expressed and secreted (RANTES) and macrophage inflammatory protein 1 α (MIP-1 α), as well as C-C chemokine receptor type 5 (CCR5) itself. RANTES and MIP-1 α are both ligands of CCR5 receptors. KA-induced increases in production of CCR5 ligands and ICAM-1

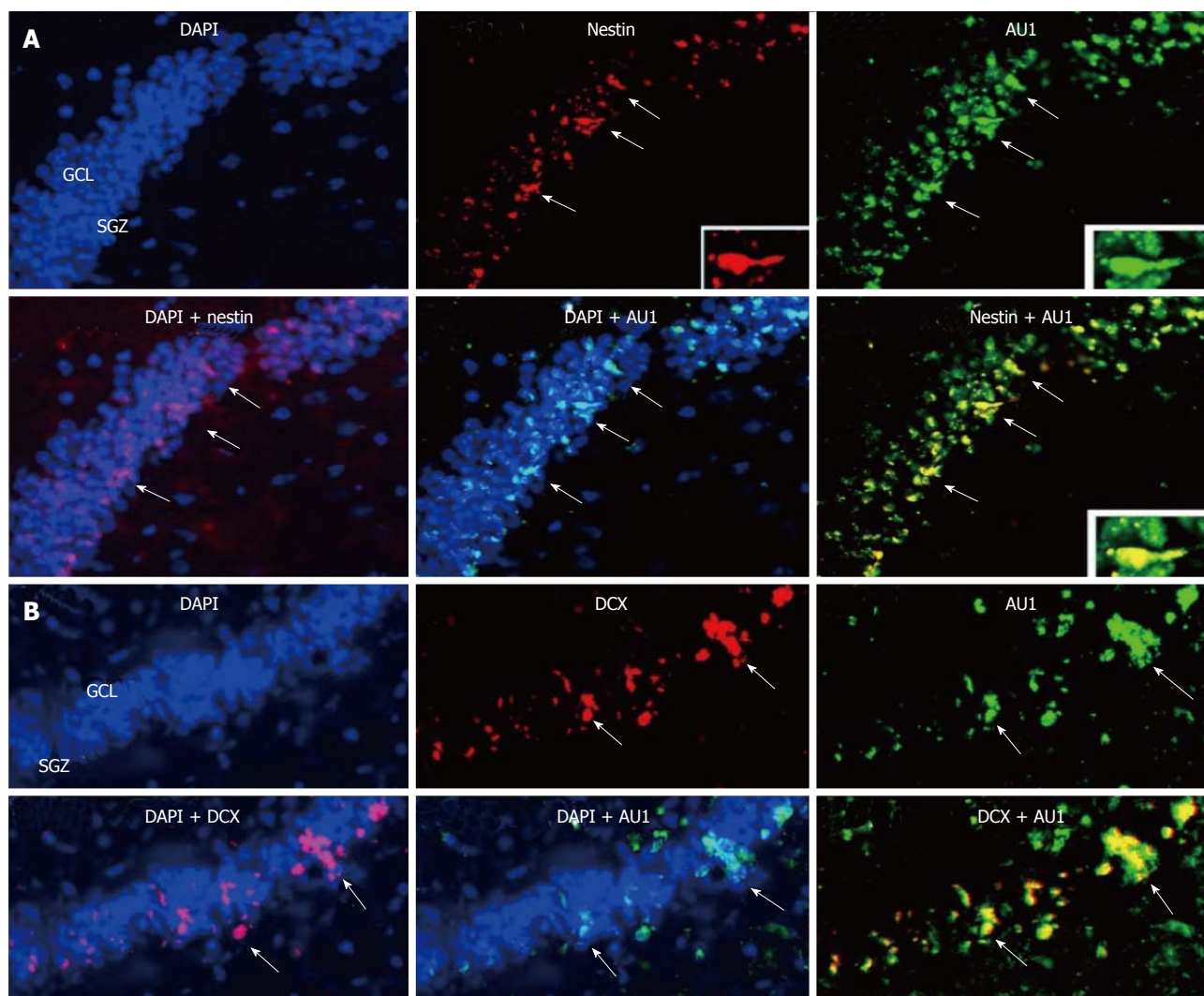


Figure 6 Migration of bone marrow derived cells to the rabbit hippocampus. Sixteen months after injection of SV (RevM10.AU1) into the rabbit bone marrow (BM), transgene-positive cells were seen in the dentate gyrus. Numerous AU1-positive cells colocalized with NeuN, a marker of postmitotic neurons (not shown). A: Nestin+/AU1+ cells were seen mainly at the border SGZ/GCL, and more rarely in the GCL (arrows); B: DCX+ positive cells expressing AU1 were detected at the border SGZ/GCL and more rarely in the GCL (arrows). SGZ: Subgranular zone; GCL: Granular cell layer; DCX: Doublecortin.

within blood vessels suggests that CCR5⁺ cells may be increased in the hippocampi of KA-treated rats, compared to control animals. We therefore enumerated CCR5⁺ cells in several areas of the HCs of rats treated with KA or saline. No CCR5⁺ cells were detected in control rat HCs. In contrast, CCR5⁺ cells were significantly more abundant throughout the hippocampi in KA recipients. In HCs of rats injected with KA, CCR5 was expressed mainly by lymphocytes, monocytes/macrophages, microglial cells, to a lesser extent by neurons, and rarely with astrocytes. This expression was seen not only in inflammatory cells in brain parenchyma, but in vessel walls as well.

KA-induced injury also elicits a regenerative response: Increased new neurons are formed following the insult. We assessed the level of cell proliferation and the populations of cells involved by labeling with BrdU. BrdU-expressing cells were seen in the GCL and SGZ of HCs from rats injected with KA. Very rare cells positive for BrdU were seen in HCs of controls. In KA recipients, 80.2% of these cells were neurons. In addition, proliferation of both

immature neurons and neural stem cells was involved, as evidenced by BrdU positivity in cells that also expressed doublecortin and nestin, respectively (Figure 7).

CCR5 regulates migration of bone marrow progenitors to the DG and limits neuroregeneration in response to injury

We reported above that KA elicits injury, inflammation and neuron regeneration in the HC. In the next step, we showed that decreasing CCR5 on bone marrow-derived cells reduces the number of KA-induced seizures, and related injury and inflammation. We used for that a bifunctional vector composed of a vector targeting CCR5 (RNAiR5) and a vector carrying a protein tag, used to evaluate transgene expression. The effect of *RNAiR5* gene (targeting CCR5) delivery to the BM on the migration of BM progenitors to the DG and their differentiation into neurons was assessed, with and without KA treatment.

Intraperitoneal injection of 10 mg/kg KA causes grade 5 tonic-clonic seizures (range of seizure duration,

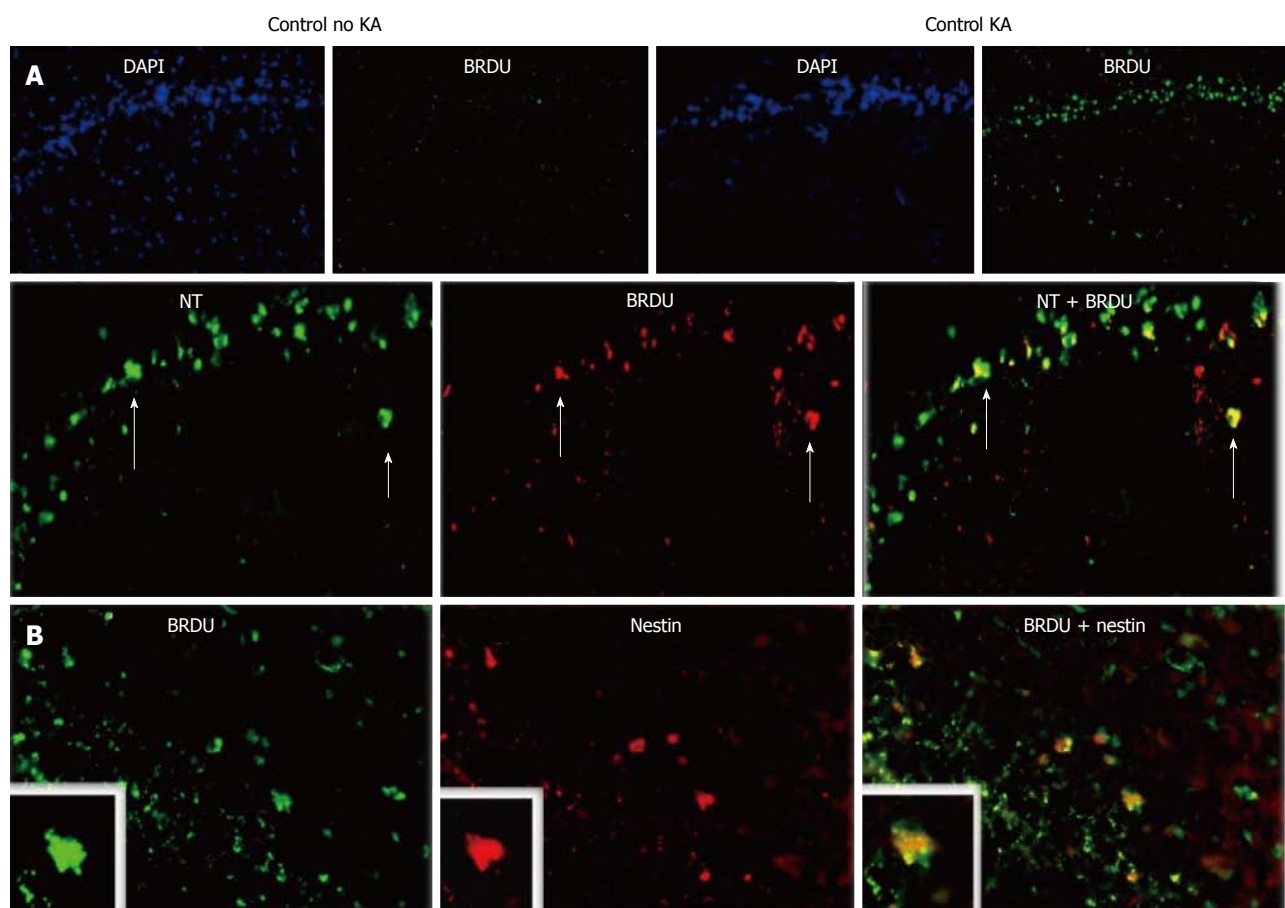


Figure 7 Kainic acid-induced regeneration in the hippocampus. Rats were injected with kainic acid (KA) intraperitoneally (*i.p.*), 10 mg/kg, and their hippocampi (HCs) analyzed by immunomicroscopy 7 d thereafter. A: Neurogenesis following KA treatment. Rats given KA or saline were injected with BrdU. Then, at 7 d post-KA, their DGs were immunostained for BrdU to visualize proliferating cells. DAPI counterstain is shown to facilitate interpretation. Arrows show neurons positive for BrdU. Double staining with Neurotrace (NT) for neurons is shown below; B: Double staining for nestin, a marker of proliferating and migrating neural cells (modified from Ref. [132]).

30–80 s) in normal rats beginning an average of 32.5 ± 4.7 min post-injection (range, 20–45 min). The average number of such seizures within a 4 h period after KA administration was 23.6 ± 3.4 (range, 21–28). Rats were given SV (RNAiR5-RevM10.AU1), the monofunctional vectors SV (RevM10.AU1) or SV (RNAiR5) intrafemorally. SV (BUGT) was used as control vector. Animals were challenged 4 mo later by *i.p.* injection of KA. Rats given RNAiR5-containing vectors were significantly protected from KA-induced seizure activity compared to rats receiving either SV (BUGT) or SV (RevM10.AU1), by all criteria applied: Time of seizure onset, number and severity of seizures and recovery time. The two RNAiR5-carrying vectors were comparable in protecting from KA-induced seizure activity, and were highly significantly different from the two control vectors [SV (RevM10.AU1), SV (BUGT)].

Four months after injection of the SV40-derived vector that does not target CCR5, and without KA administration, very few BM progenitors were seen in the DG. This is consistent with the observations reported about concerning the migration of BM-derived cells to the CNS. By contrast, in rats receiving a vector carrying the RNAi

that targets CCR5, there was a significant increase in the numbers of bone marrow-derived cells expressing neuronal markers. Thus, decreasing CCR5 led to increased bone marrow cell migration to the brain and increased DG neurons derived from those cells. However, in the absence of KA treatment, the number of cells originating from the BM was low. After KA treatment, and notwithstanding the ability of RNAiR5 to mitigate KA-induced injury, numbers of DG cells derived from the bone marrow was far greater in recipients of the vector targeting CCR5 than in recipients of an unrelated vector.

We examined, further, the influence of bone marrow-directed gene delivery of RNAiR5 on DG neuroregeneration. Proliferating cells were visualized using BrdU, and immature neurons were identified using doublecortin and nestin as markers. Again, despite the mitigating effect of bone marrow-directed RNAiR5 on DG injury and neuroinflammation, proliferation of neuronal precursors in response to KA treatment was approximately 3-fold that seen in recipients of the control vector only. Moreover, $72.2\% \pm 7.4\%$ of BrdU-positive cells were also transgene-positive, and $79.8\% \pm 6.9\%$ of transgene-positive cells were positive for BrdU. Most of these cells were of neuronal

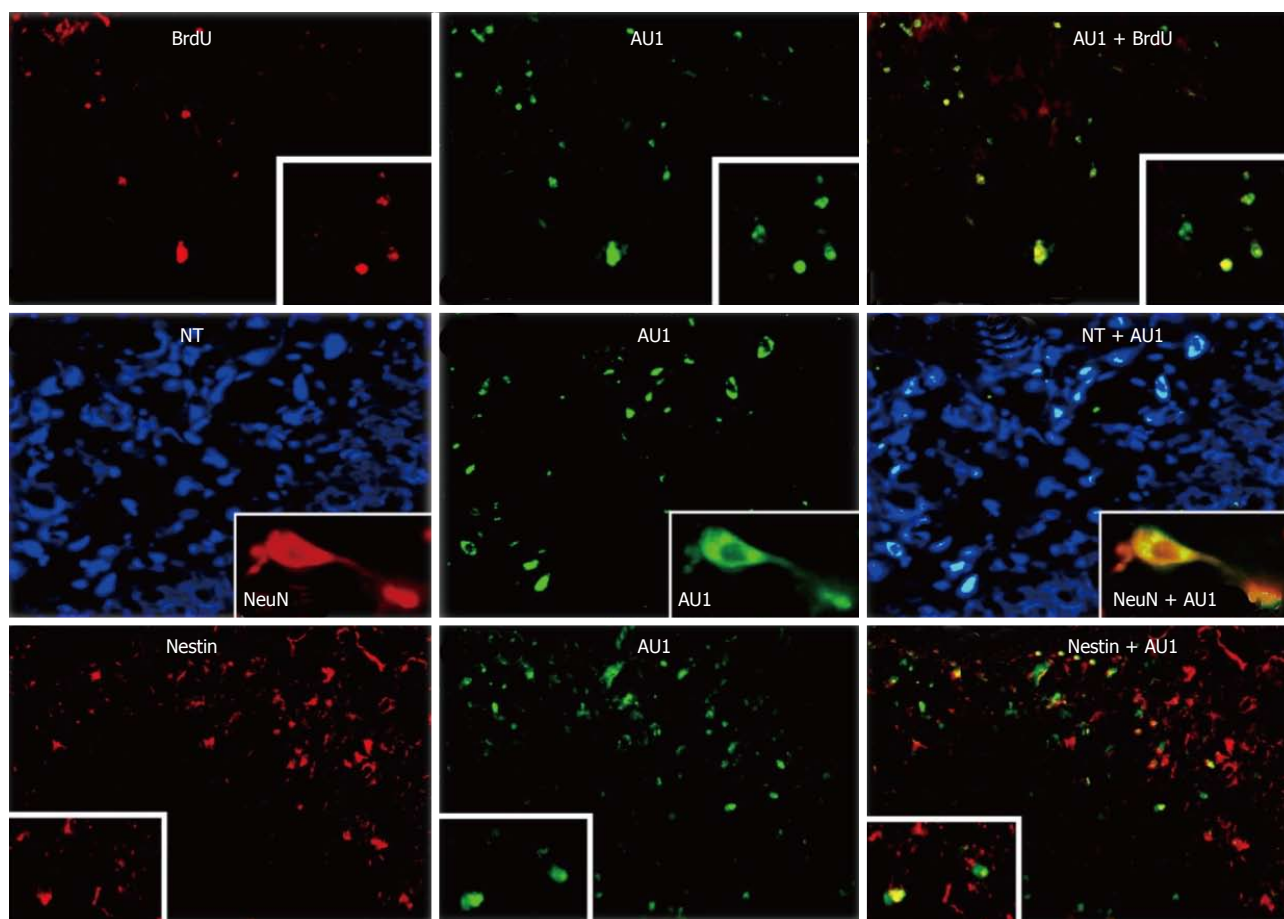


Figure 8 *RNAiR5* gene delivery to the bone marrow increases numbers of bone marrow-derived cells in the hippocampus and neuroproliferative activity in the hippocampus, in the resting state and in response to kainic acid treatment. Bone marrow-derived cells in the hippocampus (HC) were all neurons, and most proliferating cells in the HC were bone marrow-derived. Double immunostaining for AU1 plus BrdU (upper row) and nestin (lower row), and neuron identification using Neurotrace (NT) and NeuN (middle row) was performed. Shown are HC sections from kainic acid-treated rats, transduced with SV (RNAiR5-RevM10.AU1). Most of the AU1+ cells were also positive for BrdU (79.8%, \pm 6.9%) as a marker of cell proliferation, and most BrdU+ cells were also AU1+ (72.2%, \pm 7.4%). Numerous AU1+ cells were also positive for nestin, a marker of proliferating and migrating neurons, although there were as well many nestin-positive cells that did not express AU1. The equivalence of NT identification of neurons and NeuN immunostaining of neurons is illustrated in the insets in the middle row. Data are representative of 3 independent experiments (modified from Ref. [132]).

lineage, as demonstrated by immunopositivity for nestin and/or doublecortin or NeuN (Figure 8).

We reported that bone marrow-directed gene delivery of RNAi targeting CCR5 using a recombinant SV40-derived vector results in high levels of gene modification of bone marrow-derived cells, particularly Sca-1+ cells. As a consequence of the effectiveness of this approach to downregulating CCR5, our data suggest that the cell membrane chemokine receptor, CCR5, influences greatly the regulation of the traffic of BM progenitors towards the CNS, both in the basal state and in response to injury. Furthermore, reduction in CCR5 expression in circulating cells provides profound neuroprotection from, in this case, excitotoxic neuronal injury. CCR5 and its ligands enhance CNS inflammation and seizure activity, and may result in increased CNS injury as a result.

Inflammatory cell infiltration of the CNS entails adhesion of lymphocytes and monocytes in the blood to cerebrovascular endothelium, mediated by endothelial cell production of chemokines, clustering of integrins and migration of peripheral blood mononuclear cells

(PBMC) through the vascular endothelium into the brain. CCR5 and its ligands, RANTES and MIP-1 α , are known to be involved in this process^[138-144]. Thus, such transmigration is stimulated by RANTES but decreased by anti-CCR5 antibodies^[142,143]. Our data are consistent with these findings. Expression of MIP-1 α and RANTES on brain microvessels and endothelial cells is greatly increased after KA administration, and we showed that reducing cell membrane CCR5 probably decreases PBMC adhesion to CCR5 ligands^[145]. Lower levels of CCR5 on PBMC membranes following BM-directed gene delivery also decreases production of those chemokines by brain vascular endothelium. Migration of CCR5⁺ microglia and monocyte-derived macrophages is stimulated by CCR5 ligands and these cells, in turn, stimulate both endothelial activation and production of proinflammatory cytokines^[141]. Our results show also that the initial production of CCR5 ligands after KA administration is unaltered in rats injected with both control and rSV40s vectors targeting CCR5, but this changed over time between the recipients of control

vector and rSV40 vectors reducing CCR5. Thus, CCR5 is involved in a multiplicative effect of chemotaxis, stimulation of chemotaxis and then more chemotaxis. Altering this cycle by reductions in PBMC CCR5 may thus have neuroprotective and antiinflammatory effects that are disproportionate to the magnitude of the decrease in CCR5.

These results showing the interaction between CCR5 on PBMC and its ligands at the vascular level emphasize the role of vascular inflammation in KA-induced seizures. It has been reported that vascular inflammation and leukocyte-endothelial adhesion can participate in the development of seizures. $\alpha 4$ integrin and VCAM-1 antibodies can mitigate leukocyte-vascular interaction and prevent pilocarpine-induced seizures^[146]. It has been recently suggested that blood-brain barrier (BBB) breakdown can induce epileptiform activity^[147,148]. BBB disruption has been described after KA administration^[149-151]. During KA-induced seizures, disruption of the BBB is characterized by disappearance of tight junction ZO-1 and occludin, recruitment of neutrophils^[149], increase in the production of tissue plasminogen activator and NO^[150]. Activation of astrocytes, for example by glutamate agonists, can influence vessel permeability^[149]. Another chemokine, MCP-1 (CCL2), can induce BBB opening and KA-induced upregulation of MCP-1 mediates recruitment of macrophages and granulocytes^[151]. Increase of MCP-1 in blood vessels of HCs after seizures might lead to modifications of permeability of the BBB^[152]. Enhanced permeability of BBB can increase the access of KA to the parenchyma^[146]. In the pilocarpine model, it has been suggested that the effect of the drug can be to allow focal BBB leakage, which then synergizes with the CNS effects of pilocarpine to induce seizures. Leukocyte adhesion blockade prevented BBB opening in the pilocarpine model^[146]. In the present results, much less leakage of vascular contents from blood vessels was seen in HCs of rats given rSV40s vectors targeting CCR5, suggesting that the experimental reduction of the interaction between CCR5 on PBMC and CCR5 ligands on vessels limited BBB leakage.

BBB leakage is partly mediated by leukocytes through different mechanisms: Generation of oxygen free radicals, enhanced production of cytokines and chemokines, vascular alterations, release of cytotoxic enzymes. Adhesion of leukocytes to endothelium produces changes in small GTPases involved in cytoskeletal organization, and in calcium signaling, as well as in activation of kinases^[146,153]. Our demonstration that inhibition of interactions between CCR5 on PBMC and CCR5 ligands in vessels prevents BBB disruption after KA is consistent with prior observations about the effects of leukocyte adhesion on vascular permeability^[146]. Thus, our results show that CCR5 influences the synergistic interactions between leukocyte adhesion, endothelial activation, BBB leakage and seizure activity.

CCL3 (MIP-1 α)^[154,155] and CCL5 (RANTES)^[156,157] can be expressed by brain endothelial cells. Different mechanisms can be responsible for the induction of

CCL5 and CCL3 in the endothelium. Several studies have demonstrated the involvement of TNF- α and IL1- β in seizure activity^[129,158-160]. TNF- α and IL1- β can be rapidly produced by microglial cells^[161]. TNF- α and IL1- β stimulate expression of CCL5 in endothelial cells^[156,157,162]. TNF- α can also activate NF kappaB, the role of which has been underscored in seizure activity, including KA-induced neurotoxicity^[163]. TNF- α -induced CCL5 transcription involves cis-regulatory promoter elements, *i.e.*, NF- κ B, C/EBP β , NF-IL-6, NF-AT and the cAMP response element, CRE^[164]. TNF- α can induce NF- κ B, but apparently not AP-1, activity in endothelial cells^[165]. Other mechanisms upregulating CCL5 in endothelial cells involve HIF-1 α , JNK-2 and AP-1 (JunD). Lysophosphatidylcholine can induce rapid expression of CCL5 in endothelial cells by activating of multiple kinases^[166]. Increase of PLA2 activity has been reported in different models of epilepsy^[167] including kainic acid treatment^[168-170].

The type of benefit from decreasing CCR5 described in the present study has been seen in studies in which TAK-779, an inhibitor of CCR5, mitigates CNS damage due to ischemia^[171]. Targeting MIP-1 α may also protect from experimental autoimmune encephalomyelitis^[172] and other types of CNS injury^[173-176]. Another important consequence of BM-delivered, RNAi-mediated, decrease in CCR5 expression in blood cells is increased migration of BM progenitors to the DG, where they become neurons. These new neurons are generated in absence of lesions, but also can assist in repairing insults to the CNS^[177-179]. BM progenitors can migrate to the brain and become neurons by transdifferentiation or other processes^[44-48]. Inflammation may limit the ability of the brain to repair itself by neurogenesis, as the generation and survival of new neurons is inversely proportional to the magnitude of the inflammation^[128,180], and is facilitated by administration of inflammation inhibitors such as indomethacin^[137].

Our results underscore the relationship between ongoing neurogenesis and BM-derived cells. In those studies, we found almost no such cells in the DG at 4 mo after gene delivery to the BM, but numerous cells 15 mo after intramarrow inoculation of the vector. Our current data show that decreasing CCR5 expression on bone marrow-derived cells both increased the basal level of neurogenesis from BM-derived progenitors and greatly amplified the regenerative response to excitotoxic injury. This observation is even more striking in light of the fact that recipients of the vectors targeting CCR5 had much less severe neuron loss than did controls. Furthermore, the level of neuron proliferation, as measured by BrdU incorporation, was also several-fold higher in recipients of vectors that contained RNAiR5 than in controls. It is likely, therefore, that targeting CCR5 by gene transfer or pharmacologic means may promote ongoing neurogenesis and neuroregenerative responses to injury. It might be due to an inhibition of inflammation, known for limiting neurogenesis^[128,137,180]. However, a direct role of CCR5 in regulating neuron regeneration cannot be excluded. It is not known whether the cell population(s) in the bone

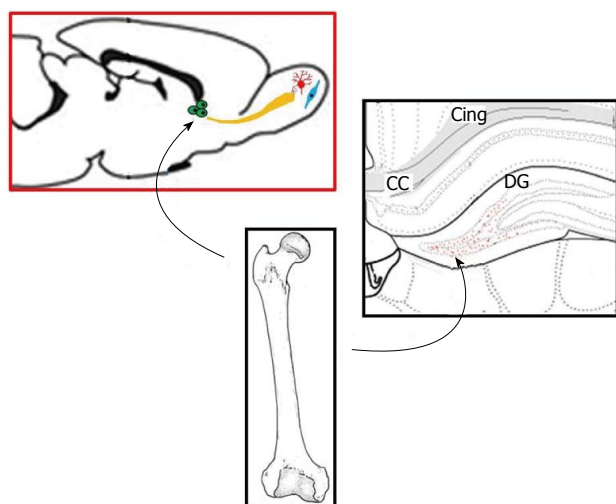


Figure 9 Figure suggesting the participation of bone marrow progenitor cells to neurogenesis in adults. DG: Dentate gyrus.

marrow that provide these neuron precursors are of hematopoietic or other origin.

Thus, the consequences of acquired deficiency in CCR5 highlights the role of CCR5 in neuroinflammation, excitotoxic injury, chemotaxis and astrocyte proliferation, and decreases in CCR5 may provide pronounced neuroprotection from such injury. In the present study, cells in the treated animals expressed less CCR5 (molecule per cell) than those from control animals. Even if the cells have not become negative for CCR5, the decreased expression of this receptor did affect their function. The distinction between a complete shutdown of CCR5 and reduced expression due to the RNAi is important, because if marginal reductions in a target can result in a disproportionate loss of function (due for example to reduced total avidity at the cell surface) there is greater hope for the use of such vectors in other *in vivo* applications.

In conclusion, our data demonstrate the centrality of CCR5 and its ligands in mediating injury-induced inflammation, and suggest that decreasing levels of CCR5 may have as its consequences neuroprotection and enhanced neuroregeneration. We confirm here that BM progenitor cells participate in neurogenesis in the adult brain, and migrate towards the DG and SVZ (Figure 9). SV40-based gene delivery of RNAi targeting CCR5 to the BM results in downregulating CCR5 in circulating cells. Consequently, the inhibition of interactions between CCR5 on peripheral blood mononuclear cells and CCR5 ligands in vessels prevents BBB disruption after KA treatment. The decrease of leukocyte-vascular interaction affects vascular permeability, thus, infiltration of parenchyma by inflammatory cells, and reduces neuroinflammation. Subsequently, our results imply that CCR5 influences the interactions between leukocyte adhesion, endothelial activation, BBB leakage and seizure activity. However, given the redundancy of cytokines and chemokines, CCR5 might be just one of the components implicated in the interaction between leukocytes and vessels, and

other chemokines, or other molecules, might be involved as well. For example, receptors for IL-1 and TNF- α are upregulated rapidly during seizures^[160]. The magnitude of seizure activity impacts on the inflammatory responses that follow seizures^[181-183]. Microglial activation, and production of IL-1 β , IL6, TNF- α and free radical species, directly affect the process of post-seizure neurogenesis^[181-184] and the survival of the neurons that are produced as a result^[159,182,185,186].

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Mucosal-associated invariant T cells from induced pluripotent stem cells: A novel approach for modeling human diseases

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Abstract

Mice have frequently been used to model human diseases involving immune dysregulation such as autoimmune and inflammatory diseases. These models help elucidate

the mechanisms underlying the disease and in the development of novel therapies. However, if mice are deficient in certain cells and/or effectors associated with human diseases, how can their functions be investigated in this species? Mucosal-associated invariant T (MAIT) cells, a novel innate-like T cell family member, are a good example. MAIT cells are abundant in humans but scarce in laboratory mice. MAIT cells harbor an invariant T cell receptor and recognize nonpeptidic antigens vitamin B2 metabolites from bacteria and yeasts. Recent studies have shown that MAIT cells play a pivotal role in human diseases such as bacterial infections and autoimmune and inflammatory diseases. MAIT cells possess granulysin, a human-specific effector molecule, but granulysin and its homologue are absent in mice. Furthermore, MAIT cells show poor proliferation *in vitro*. To overcome these problems and further our knowledge of MAIT cells, we have established a method to expand MAIT cells via induced pluripotent stem cells (iPSCs). In this review, we describe recent advances in the field of MAIT cell research and our approach for human disease modeling with iPSC-derived MAIT cells.

Key words: Mucosal-associated invariant T cells; Induced pluripotent stem cells; Differentiation; Adoptive transfer; Inflammatory diseases; Autoimmune diseases; Disease modeling; Infectious diseases; Immunocompromised mouse

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Core tip: Mucosal-associated invariant T (MAIT) cells, a novel innate-like T cell subset abundant in humans, play a pivotal role in immune-dysregulated diseases. However, MAIT cells are quite rare in laboratory mice and show poor proliferation *in vitro*. This makes it difficult to delineate their physiological functions in health and disease. Therefore, we developed a method to generate

human MAIT cells from induced pluripotent stem cells [redifferentiation of MAIT (reMAIT) cells]. Given that reMAIT cells harbor characteristics quasi-identical to those found in MAIT cells from human peripheral blood, they will be useful to model human diseases in animals.

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INTRODUCTION

T cells are distinguished from other lymphocytes, such as B cells and natural killer cells, by the expression of T cell receptors (TCRs) on the cell surface. T cells have been well-characterized as central players in adaptive immunity, so-called conventional T cells. The TCRs in conventional T cells consist of a heterodimer of α -chain and β -chain and are highly diverse owing to gene rearrangement together with insertion and/or deletion of nucleotides at the junctions between the gene segments, enabling them to recognize a wide variety of peptide antigens presented on major histocompatibility complex (MHC) molecules, which are also highly polymorphic^[1]. In recent years, however, non-conventional type T cells termed "innate-like" T cells have received keen attention in immune homeostasis and diseases^[2]. In contrast to conventional T cells, innate-like T cells express a limited set (semi-invariant) of TCRs and recognize nonpeptidic antigens presented on evolutionarily conserved non-classical MHC molecules^[3,4]. Innate-like T cells develop in the thymus, similar to conventional T cells^[5]. There is a time lag between the initial antigen exposure and execution of the maximum effector function in conventional T cell responses. Given that conventional T cells transit from naïve to effector/memory stage through the recognition of peptidic antigens, these T cells are ready to be activated and to expand upon receiving secondary stimuli to exert effector functions. In marked contrast, innate-like T cells have already acquired such immune competence when leave the thymus. This may be relevant to the fact that innate-like T cells, but not conventional T cells, express the transcription factor promyelocytic leukemia zinc finger (PLZF), which directs effector differentiation of these cells during thymic development^[5-7]. Thus far, it has been appreciated that the *raison-d'être* of innate-like T cells consists in filling a gap between innate and adaptive immunity^[8].

Mucosal-associated invariant T (MAIT) cells and natural killer T (NKT) cells are representatives of innate-like T cells expressing semi-invariant $\alpha\beta$ TCR in mammals^[2]. Because the discovery of NKT cell ligands has preceded that of MAIT cells, most of our knowledge on diseases has been made with NKT cells abundant in laboratory mice (but quite few in humans). NKT cells play a pivotal role in the suppression

of tumor growth and/or metastasis, and in ameliorating or aggravating autoimmune diseases^[9,10]. NKT cells produce a plethora of cytokines, including Th1-, Th2- and Th17-cytokines, upon stimulation, and MAIT cells also have a similar potential^[11,12]. Although they are different in many aspects such as antigens, restriction molecules for development and/or differentiation, and abundance, they are common in that they play a critical role in infectious diseases and autoimmune and inflammatory diseases. Regardless of their importance, it was not until recently that some information on MAIT cells has become available. In the last couple of years, there has been exciting progress regarding the functions of MAIT cells in the immunology field and in clinical settings. There are, however, some difficulties in studying MAIT cells, in that the frequency of MAIT cells is much lower in laboratory mice than in humans, and that MAIT cells show extremely poor proliferation *in vitro* with any T cell stimulants tested to date. Here, we provide an overview of recent advances in the study on MAIT cells and introduce our approach with induced pluripotent stem cell (iPSC) technology to overcome the experimental difficulties in MAIT cell study.

PHENOTYPIC FEATURES OF MAIT CELLS

MAIT cells are probably one of the most abundant T cell subsets in humans^[13]. However, until quite recently, MAIT cells had been hidden behind conventional T cells because they are indistinguishable from other T cell populations by standard T cell phenotyping using cell surface markers such as CD3, CD4 and CD8. MAIT cells are distinguished from conventional T cells and other T cell subsets such as NKT cells and $\gamma\delta$ T cells by the expression of an invariant TCR α chain, V α 7.2-J α 33 in humans and V α 19-J α 33 in mice, paired with a limited repertoire of TCR β chains; V β 13 and V β 2 are preferentially used in humans and homologous V β 8 and V β 6 in mice (Figure 1)^[13,14]. Together with invariant TCR α V α 7.2, human MAIT cells express a C-type lectin CD161 and interleukin (IL)-18 receptor α chain (IL-18R α) as specific markers^[15,16]. Primarily, MAIT cells are defined as CD3⁺, V α 7.2⁺, CD161⁺ and IL-18R α ⁺. MAIT cells can further be classified into CD8⁺ (most abundant), CD4⁻CD8⁻ [double negative (DN)] and CD4⁺ phenotypes (very few) in healthy human subjects^[13,17]. In addition, MAIT cells display CD45RA⁻, CD45RO⁺, CD95^{high}, and CD62L^{low} as their effector/memory T cell phenotype, and α 4 β 7 integrin⁺, CCR9^{int}, CCR7⁻, CCR5^{high}, CXCR6^{high}, and CCR6^{high}, suggesting MAIT cells home to the intestines and liver^[11,18,19]. High expression levels of CD161 in MAIT cells are accompanied by ROR γ t, IL-23R and IL-21R, markers associated with Th17/Tc17 type T cells^[11,19,20]. Furthermore, MAIT cells possess PLZF, indicating the capacity to promptly produce cytokines upon stimulation without priming^[7,17] and CD26⁺, a serine exodipeptidase, which processes chemokines in the extracellular matrix^[20,21]. Accordingly, MAIT cells have the potential to release a variety of cytokines under various conditions: Interferon (IFN)- γ , tumor necrosis factor (TNF)- α , IL-2, IL-4, IL-10, IL-17, IL-22, granzymes, and

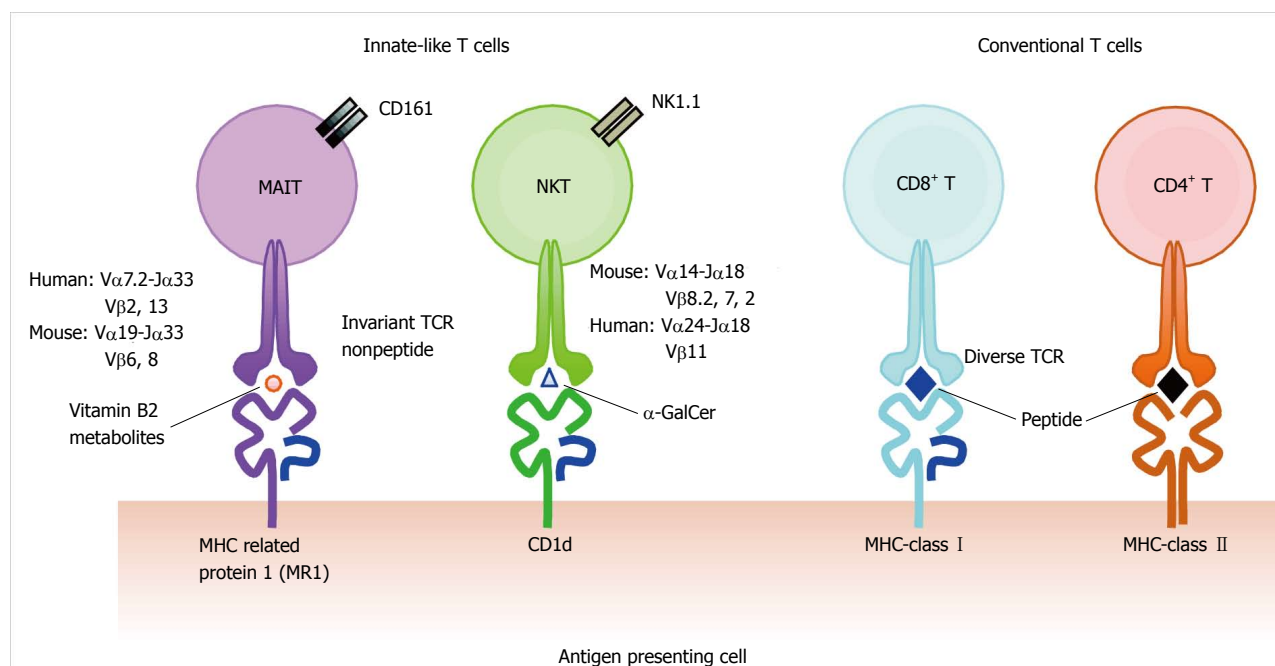


Figure 1 Comparison of the T cell receptors and the antigen presenting molecules among $\alpha\beta$ T cell subsets. Invariant T cell subsets consist of mucosal-associated invariant T (MAIT) cells and natural killer T (NKT) cells expressing invariant TCRs. MAIT cells and NKT cells recognize vitamin B2 metabolites on MR1, and α -galactosylceramide (α -GalCer) on CD1d, respectively. In contrast, conventional CD8⁺ and CD4⁺ T cells possess divergent TCRs and recognize a variety of peptides on major histocompatibility complex-class I and class II, respectively. TCRs: T cell receptors; MHC: Major histocompatibility complex.

others, which anticipates the multifaceted roles in health and diseases^[11,12,22].

MAIT CELLS AND MR1

The TCR of MAIT cells recognizes derivatives of vitamin B2 presented on the monomorphic MHC class-related molecule 1, MR1^[18,23] (Figure 1). MR1 mRNA is expressed ubiquitously in all types of cells, whereas the MR1 protein are not always on the cell surface but mainly in the endoplasmic reticulum^[24,25]. Although vitamin B2 derivatives are exogenous ligands from the biosynthetic pathway that some bacteria and yeasts possess, they are indispensable for the development of MAIT cells, because MAIT cells are absent in germ-free mice^[18]. TCRs for MAIT cells and MR1 are highly conserved during evolution, which suggests the functional and physiological importance of MAIT cells and MR1 in animals^[26]. Indeed, mouse and human MR1 molecules crossover part of the antigen presentation and activation in MAIT cells^[26].

MAIT cell development is dependent on MR1. Lymphoid progenitors derived from CD34⁺ hematopoietic stem cells in the bone marrow migrate to the thymus, wherein they undergo random rearrangement at the TCR loci. MAIT cell progenitors harboring the TCR V α 7.2-J α 33 are selected from CD4/CD8 double positive thymocytes that express MR1 loaded with unknown endogenous ligands^[18,27]. MAIT cells then egress from the thymus as naïve cells and further differentiate into effector/memory cells by recognizing commensal microflora-derived vitamin B2 metabolites bound to MR1 at mucosal sites^[18,19].

MAIT CELLS IN HEALTH AND DISEASES

MAIT cells consist of 1%-10% of T cells in the peripheral blood and of T cells in the intestinal lamina propria and 20%-50% in T cells of the liver, but they are at least 10 times less abundant in laboratory mice^[11,28]. MAIT cells are already present in the tissues of second trimester fetuses. Fetal MAIT cells exhibit a naïve phenotype but have potential functions in the activation and secretion of cytokines upon antigen stimulation^[29]. Although MAIT cells still showing a naïve phenotype and are low in frequency at birth, most of them have acquired a memory phenotype by 3 mo of age, and their frequency increases with age and reaches adult levels within 8-10 years after birth^[11]. This corresponds to the expansion and maturation of MAIT cells by commensal microflora colonizing after birth. The highest number of MAIT cells in PBMC is observed in adults aged 30-50 years, notably in females of reproductive age^[30]. MAIT cells, especially CD8⁺ MAIT cells as the most abundant subset, decrease drastically with age, implying an association with waning immunity in the elderly^[22,30].

The diseases in which a potential implication of MAIT cells has been reported are summarized in Table 1. A well-defined function of MAIT cells in disease settings is the control of infections with bacteria and/or yeasts. MAIT cells are activated by bacteria-infected cells in a MR1-dependent manner, followed by release of proinflammatory cytokines and cytotoxic granules, and eventually killing the infected cells^[16,31-33]. MAIT cells also express multidrug resistance transporter (ABCB1), which implies that MAIT cells are highly resistant to xenobiotics produced by bacteria^[11].

Table 1 Clinical relevance of mucosal-associated invariant T cells

Disease categories	Diseases or status	Features relevant to the diseases	Ref.
Infectious diseases	Pneumopathy	Decrease in frequency and absolute number of MAIT cells in peripheral blood	[16]
	Tuberculosis (<i>Mycobacterium tuberculosis</i>)	Decrease in frequency and absolute number of MAIT cells in peripheral blood Enriched in the lung	[16,37,92]
	HIV/AIDS (opportunistic infection)	Decrease in frequency of MAIT cells in peripheral blood, guts, and lymph nodes Failure of recovery of blood MAIT cells with successful cART Long-term cART restore colonic but not blood MAIT cell levels MAIT cells are depleted but retain functionality	[38-43,93,94]
	Sepsis (severe bacterial infection)	Decrease in frequency and absolute number of MAIT cells in peripheral blood of patients	[95]
	<i>P. aeruginosa</i> infection with cystic fibrosis	Decrease in frequency of MAIT cells in peripheral blood of cystic fibrosis patients with <i>P. aeruginosa</i> infection	[96]
	Cholera (<i>Vibrio cholera</i> O1)	Activation of MAIT cells in the acute phase No change of blood MAIT cell frequency in adult patients, but persistently decreased in child patients	[97]
Autoimmune diseases	Multiple sclerosis	Accumulation of MAIT cells in the central nervous system lesions Decrease in frequency of MAIT cells in peripheral blood Increased CD161 ^{high} CD8 ⁺ T cells in peripheral blood Accumulation of MAIT cells in the peripheral nerves	[44-46,48] [98] [44]
	Chronic inflammatory demyelinating polyneuropathy		
	Psoriatic and rheumatoid arthritis	Enrichment of CD161 ^{high} CD8 ⁺ T cells in the joints and secretion of IL-17 from those cells	[99]
	Rheumatoid arthritis		
	Inflammatory bowel disease	Decrease in frequency and absolute number of MAIT cells (in particular, in CD8 ⁺ and DN subsets) in peripheral blood Increased MAIT cell levels in the synovial fluid Decrease in CD8 ⁺ MAIT cells in peripheral blood of CD and UC patients Accumulation of MAIT cells in the inflamed ileon of patients with CD Reduced IFN- γ production in CD patients and increased IL-17 production in CD and UC patients Fewer MAIT cells in the inflamed ileon of patients with CD and UC Increased apoptosis in MAIT cells	[53] [49] [100]
	Psoriasis	MAIT cells reside in not only the dermis of patients but also that of health donors. MAIT cells may contribute IL-17 production in the dermis of patients	[51]
	Celiac disease	Decrease in frequency of MAIT cells in peripheral blood and guts of adult and pediatric patients	[52]
	Systemic lupus erythematosus	Decrease in frequency and absolute number of MAIT cells (in particular, in CD8 ⁺ and DN subsets) in peripheral blood Reduced IFN- γ production Elevated expression of PD-1 in MAIT cells	[53]
Inflammatory diseases	Asthma	Decrease in frequency of MAIT cells in blood, sputum, and endobronchial biopsy	[101]
	Diabetes type 2/obesity	Decrease in frequency of MAIT cells in peripheral blood Circulating MAIT cells display an activate phenotype MAIT cells are more abundant in adipose tissue	[55,56]
	Acute cholecystitis	Decrease in frequency and absolute number of MAIT cells in peripheral blood	[102]
	Fibromyalgia syndrome <i>vs</i> Spondyloarthritis <i>vs</i> Rheumatoid arthritis	Defined analysis of MAIT cell phenotype among three diseases that exhibit a similar clinical manifestation Decrease in frequency of MAIT cells in three diseases Three diseases are able to distinguish by surface marker expression	[57]
Tissue transplant	Cutaneous acute graft- <i>vs</i> -host disease	Infiltration of CD8 ⁺ T cells, CD161 ⁺ , CCR6 ⁺ , ROR γ t ⁺ in the epidermis and dermis of patients with GVHD	[103]
	Hemodialyzed and kidney transplant	Decrease in frequency of MAIT cells in peripheral blood Implication for the susceptibility to infections in the patients	[104]
Tumors	Kidney and brain tumors	Presence of MAIT cells in tumors	[58]
Physiological change	Fetus	Rare and immature in the thymus, spleen, mesenteric lymph nodes Mature and enriched in the guts, liver, and lung	[29]
	Neonate/infant	Naïve phenotype at birth. Acquisition of effector/memory phenotype and increase in frequency and number with age	[11,30]
	Adult	Maximum levels in the third and fourth decenniums Higher amounts in females with reproductive age than in males	[30]
	Aging	Decrease in CD8 ⁺ MAIT cells and increase in CD4 ⁺ MAIT cells with age Th2 shift in cytokine profile in elderly	[22,30]

CD: Crohn's disease; UC: Ulcerative colitis; MAIT: Mucosal-associated invariant T; HIV: Human immunodeficiency virus; AIDS: Acquired immunodeficiency syndrome; *P. aeruginosa*: *Pseudomonas aeruginosa*.

Although MAIT cells are extremely rare in laboratory mice, *Francisella tularensis*-infected mice revealed a massive expansion of MAIT cells in infected tissues earlier than the migration of conventional CD4⁺ and CD8⁺ T cells^[34], which suggests their unique function in host defense against bacterial infection. V α 19 iTCR (invariant TCR) transgenic mice (a MAIT-cell-enriched mouse model) and MR1 knockout mice (a MAIT-cell-deficient model), MAIT cells seemed to prevent the growth of bacteria such as *Mycobacterium abscessus*, *M. bovis* (BCG), *Escherichia coli* and *Klebsiella pneumoniae*^[16,35-37]. Accordingly, patients with bacterial infections such as tuberculosis and pneumopathies showed a decrease in MAIT cells in circulating blood, which might reflect their infiltration into the diseased sites^[16,37]. In HIV-infected patients, MAIT cells were also depleted from the circulating blood irrespective of the disease stage (acute or chronic infection), and even with combinatorial anti-retroviral therapy^[38-43]. Although it is believed that CD4⁺ T cell depletion causes immunodeficiency in HIV-infected patients, innate immune cells such as MAIT cells could play a crucial role in prevention of opportunistic infections with bacteria and/or fungi, which is a manifestation of AIDS.

Multiple sclerosis (MS) is a demyelinating disease of the central nervous system caused by autoreactive T cells. Although it is suggested that myelin-specific CD4⁺ T cells might play a central role in MS pathogenesis, recent studies have indicated that MAIT cells accumulate in brain lesions concomitantly with a decrease in peripheral blood in MS patients^[44-48]. This evidence indicates that MAIT cells may play a pivotal role in MS pathology, but the underlying mechanisms are yet to be elucidated. An increase in IL-18 in the serum of MS patients could signify that MAIT cells tend to migrate into the brain^[46]. In conjunction with the high levels of IL-17 and IFN- γ secretion from MAIT cells in MS patients, one study has demonstrated that MAIT cells in MS exhibited proinflammatory profiles^[45], but another interpreted that these MAIT cells exhibited a regulatory function to suppress the pathogenic Th1 response^[48]. Accordingly, a novel animal model will be required to examine the direct contribution of MAIT cells in MS pathogenesis, as will be described later in this review.

Inflammatory bowel diseases (IBDs), such as Crohn's disease and ulcerative colitis (UC), are autoimmune diseases in which the potential contribution of MAIT cells is suggested owing to their anti-microbial activity, intestinal homing, and capacity to promptly induce both Th1- and Th17-cytokines. Similar to MS patients, a decrease in MAIT cells in the peripheral blood concomitant with an increase in MAIT cells in the injured ileal regions of IBD patients has been reported^[49]. In addition, peripheral blood MAIT cells from IBD patients showed more activated and proliferative state compared with that in healthy controls, suggesting that such alterations impinge on their functions. In fact, MAIT cells from the IBD patients produced significantly more IL-17 than from healthy donors, whereas there was no difference in IL-2 and TNF- α production^[49]. MAIT cells from UC patients produced more IL-22, a Th17-cytokine,

than controls. Upon binding to its cognate receptors on respiratory and gut epithelial cells, IL-22 evoked the expression of mucin and antimicrobial peptides, both of which play a critical role in the protection of epithelial cells from bacteria and/or fungal invasion^[50]. Expression of these proteins may in turn enhance the protection and accelerate healing of cellular damage, implying the tissue protective functions of MAIT cells^[49].

Numerous studies have reported possible implications of MAIT cells in psoriasis^[51], celiac disease^[52], systemic lupus erythematosus^[53], diabetes^[54,55] and obesity^[55,56]. In our recent study, MAIT cells were shown to be useful to distinguish diseases that manifest similar symptoms such as fibromyalgia syndrome, rheumatoid arthritis, and spondyloarthritis by measuring the expression of cell surface antigens, in particular, chemokine receptors associated with homing^[57]. MAIT cells tend to migrate toward peripheral tissues, particularly in inflammatory conditions, because they express a variety of chemokine and cytokine receptors. Most of these studies have implied that immune-mediated tissue damage is induced by the pathogenic proinflammatory features of MAIT cells. In contrast, MAIT cells may protect against the damage caused by inflammation, as described above for UC. Furthermore, a subset of MAIT cells (CD56⁻) accumulated in kidney and brain tumors and may operate in tumor immune responses^[58].

As detailed above, there is no doubt about the importance of understanding the functions of MAIT cells in health and disease. However, the following questions still remain to be answered: What are the underlying mechanisms in immune regulation, particularly in innate immunity? And what are the molecules that control the functions of MAIT cells other than vitamin B2 metabolites? Although laboratory mice are useful to model human diseases, the study of MAIT cells is quite limited owing to their paucity in mice^[18]. Furthermore, MAIT cells hardly propagate *in vitro*^[11]. A recent paper, however, has showed the potential for MAIT cells to proliferate in response to *E. coli* and to anti-CD3/CD28/CD2 antibodies^[33]. The expansion most likely depends on a precise balance between proliferation and activation-induced cell death, because MAIT cells are highly sensitive to activation-induced cell death^[33,38,59]. To overcome these difficulties, we attempted to produce human MAIT cells through iPSC technology.

GENERATION OF MAIT CELLS USING MAIT CELL-DERIVED iPSCS

iPSCs may be established from a variety of somatic cells^[60-62] and be differentiated into T cells, as can embryonic stem cells (ESCs)^[63-65]. Nonetheless, it is near-impossible to obtain a monoclonal T cell with an antigenic specificity. This is primarily due to the fact that iPSCs and ESCs carry the germline configuration of *TCR α* and *TCR β* , which are subject to random gene rearrangement during T cell differentiation, resulting in the generation of polyclonal T cells (Figure 2)^[65]. Although iPSCs have been

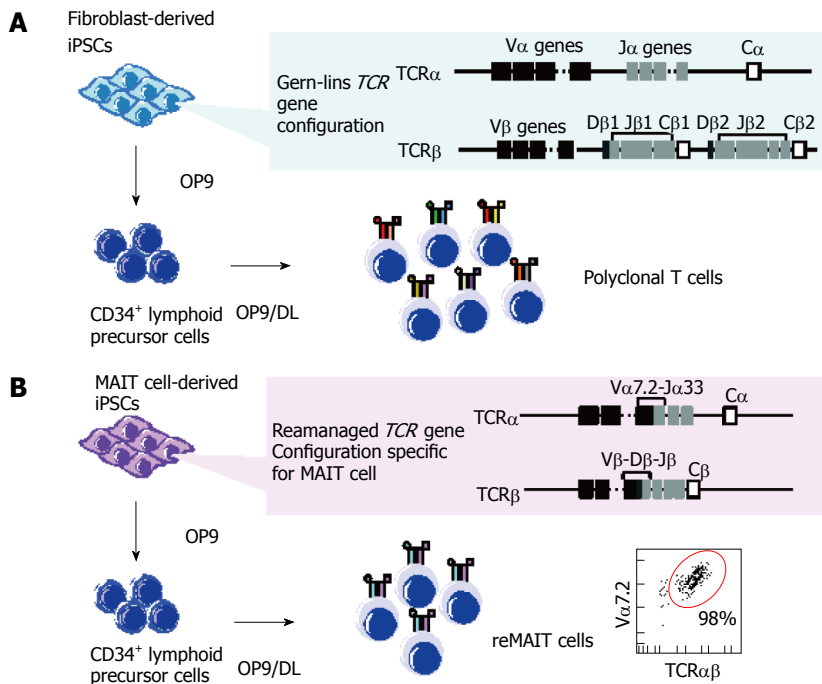


Figure 2 Scheme for T cell differentiation from induced pluripotent stem cells. Induced pluripotent stem cells (iPSCs) derived from normal somatic cells such as fibroblasts possess the germline configuration at T cell receptor (TCR) loci, whereas those from T cells harbor rearranged configurations (A); Upon differentiation in the T-cell-permissive conditions, the resulting T cells possess diverse sets of TCR repertoires; polyclonal T cells. In contrast, mucosal-associated invariant T (MAIT) cells-derived iPSCs exclusively confer MAIT cells in the same differentiation conditions. Note that MAIT cell-derived iPSCs possess a rearranged Vα7.2-Jα33 specific for MAIT cells in the genome (B).

established with terminally differentiated T cells in PBMC, the authors did not address whether or not differentiation of these iPSCs into T cells culminated in regeneration of an antigen-specific T cell clone^[66-68]. Recently, however, iPSCs have been established from tumor antigen-specific or HIV-specific CD8⁺ T cells with intention to rejuvenate T cells harboring the original epitopes, although the efficiency of such redifferentiation into the original clone remains unclear^[69,70]. Well before these reports, we have shown that the progeny of a cloned mouse from NKT cells possessed an in-frame rearranged *TCRα* (Vα14-Jα18) specific for NKT cells in the genome, and an increased number of NKT cells^[71]. This indicated that in-frame rearranged *TCRα* (Vα14-Jα18) had a strong impact on the destiny of T cells in the thymus. Such a notion has been explored further *in vitro*. ESCs prepared through nuclear transfer with hepatic NKT cells (ntESCs), harboring in-frame rearranged *TCRα* (Vα14-Jα18), gave rise to T lymphocytes exclusively comprising NKT cells (> 94%) when ntESCs were subjected to the OP9/OP9-DL system, which is well-known to promote T cell lineage differentiation from pluripotent stem cells^[64,65,72,73]. We have exploited a corollary that iPSCs derived from MAIT cells would efficiently redifferentiate into MAIT cells under the same conditions, because MAIT cells are innate-like T cells, and these iPSCs possess a rearranged *TCRα* (Vα7.2-Jα33), specific for MAIT cells^[21]. This turned out to be the case.

MAIT cells purified from umbilical cord blood (CB-MAIT) as TCR Vα7.2⁺ cells were reprogrammed with Sendai virus (SeV) vector harboring four reprogramming factors (*Oct4*, *Sox2*, *Klf4* and *c-Myc*) (MAIT-iPSCs) without any proliferative stimulation as used in reprogramming of antigen-specific CD8⁺ T cells^[69,70]. SeV is superior to other viruses, such as lentivirus, in that SeV

does not integrate into the host genome, thus leaving the genomic DNA free from interruptions^[21,67,69,74]. As expected, MAIT-iPSCs successfully redifferentiated into MAIT cell-like cells expressing Vα7.2, CD3, CD161, and IL-18Rα (reMAIT cells) with high efficiency (> 98%) in T-cell-permissive conditions (Figure 2)^[21]. reMAIT cells generally display a naïve phenotype, but express a high level of CCR6 (a receptor directing mucosal tissue homing and IL-17 expression), recapitulating that CB-MAIT cells that are still in an immature stage prior to exposure to commensal flora^[16,17,21,75,76]. Furthermore, reMAIT cells produce an array of cytokines, chemokines, and cytotoxic granules, such as granulysin, perforin and granzyme A, in an MR1-dependent manner. reMAIT cells also protect mice from Mycobacterial infection upon adoptive transfer, holding a promise to realize cell therapy with these cells^[21]. Taken together, reMAIT cells should function as innate-like T cells, although they are still immature^[16,17,21].

FUTURE PERSPECTIVES – DISEASE MODELING USING MAIT CELLS DIFFERENTIATED FROM iPSCS

reMAIT cells generated from iPSCs will be useful not only for deciphering their immunological functions *in vivo* but also for creating novel disease models in animals. Two types of genetically engineered mice, MR1-knockout mice and TCR transgenic mice, have been widely used to delineate the roles of MAIT cells *in vivo* (Tables 2 and 3). Originally, MR1-knockout mice (MR1^{-/-}) were generated to assess the roles of MR1 in the selection and expansion of MAIT cells *in vivo*^[18]. MR1-knockout mice possessed severely decreased TCR Vα19-Jα33

Table 2 Mice used in study for mucosal-associated invariant T cells

	Genotype	Characteristics	Ref.
Knockout mice	MR1 ^{-/-}	Impaired development of MAIT cells	[79]
Transgenic mice	Vα19 iTCR Tg	Enriched MAIT cells	[17,78-80]
	Vβ6 Vβ8 Tg	Increase of MAIT cells	[17]

MAIT: Mucosal-associated invariant T; iTCR: Invariant T cell receptor.

Table 3 Mucosal-associated invariant T cells in the diseases

Category	Mouse strains	Disease model	Phenotype	Ref.
Bacterial infection	MR1 ^{-/-}	<i>Escherichia coli</i>	Increase in the bacterial burden	[16]
	Vα19 iTCR Tg	<i>Micobacterium abscessus</i>	Repression of the bacterial burden	
	Vβ6 Vβ8 Tg			
	MR1 ^{-/-}	<i>Klebsiella pneumoniae</i>	Increased susceptibility to <i>K. pneumoniae</i> infection	[36]
Autoimmune diseases	MR1 ^{-/-}	<i>Mycobacterium bovis</i> BCG	Enhanced bacterial growth at the early stage of infection	[35]
		<i>Francisella tularensis</i>	Delayed adaptive immune reaction	[34]
	Vα19 iTCR Tg	Experimental autoimmune encephalomyelitis (model of MS)	Suppressed disease induction and progression	[78]
	MR1 ^{-/-}	Collagen-induced arthritis (model of rheumatoid arthritis)	Improved CIA score	[86]
	Adoptive transfer Jα33 ⁺ MAIT cells into BALB/c B10.R.III	TNBS induced colitis	Improved disease index	[105]
		Spondyloarthropathy by IL-23	Enthesitis induced by IL-22 produced from IL-23R ⁺ RORγt ⁺ CD4 ⁺ CD8 ⁻ T cells (MAIT cells?) in the entheses	[91]
Others	Vα19 iTCR Tg NOD	Non-obese diabetes	Delayed disease onset	[106]
	Vα19 iTCR Tg	Delayed-type hypersensitivity to sheep erythrocytes (type IV allergy)	Suppression of the disease	[106]

MAIT: Mucosal-associated invariant T; IL: Interleukin; CIA: Collagen-induced arthritis; iTCR: Invariant T cell receptor.

expression compared with their littermate controls^[18]. Thus far, MR1-knockout mice have been used as a model devoid of MAIT cells. MR1-knockout mice may have shed light on the roles of MAIT cells *in vivo*. However, the findings from the mice were too complicated to interpret, maybe because in part of the insufficient number of MAIT cells in the control, and the lack of an appropriate reagent to detect mouse MAIT cells. An MR1 tetramer that has been created recently is useful to detect MAIT cells in mice and humans^[77], but tetramer-positive cells may not always be functional cells. Three groups have independently reported Vα19 iTCR transgenic mice as a MAIT cell-enriched model^[17,78-80]. Two reports indicated that MAIT cells in Vα19 iTCR transgenic mice harbored an effector/memory phenotype; CD44^{high}CD69⁺CD25⁺ICOS⁺ and NK1.1^[78-80]. With the ligand-loaded MR1 tetramer, it was found that approximately 40%-50% of MAIT cells were CD4⁺, and the rest being comprised of DN cells and fewer CD8⁺ MAIT cells^[77], whereas in humans few CD4⁺ MAIT cells are present. Such a difference in CD4 or CD8 usage between mouse and human may reflect their physiological roles. In contrast, Martin *et al.*^[17] showed that MAIT cells from their Vα19iTCR transgenic mice were DN and CD8⁺ with few CD4⁺. Furthermore, NK1.1, CD25, CD69, and ICOS were not present in MAIT cells. Such inconsistency demonstrated that MAIT cells

are different in nature from those in transgenic mice. It is plausible that such an alteration stems from the differences in transgenes or commensal flora utilized. Should it be the case, the transgenic mouse may not be adequate to delineate the functions of MAIT cells^[17]. Therefore, it is indispensable to create a novel animal model to address the physiological roles of MAIT cells in health and diseases, and harnessing the results of animals for clinical applications.

In this context, use of humanized mice can be envisaged, because the human cells in question can be engrafted and their functions and development may be examined *in vivo*^[81]. To study the physiological roles *in vivo*, reMAIT cells were adoptively transferred to NOD/SCID or NOG (NOD/Shi-scid IL2Rγ^{null}) mice, both of which are devoid of mature B, T cells, and the later deficient in NK cells, functional macrophages, and dendritic cells^[21]. reMAIT cells migrated and engrafted in tissues such as the intestines, bone marrow, liver, and spleen, which probably mirrors the distribution of MAIT cells in humans^[18,71]. In addition, reMAIT cells dramatically changed the phenotype from naïve to mature concomitant with the expression of the chemokine receptors required for the tissue-specific homing. Moreover, reMAIT cells appeared to proliferate in mice, whereas they did not *in vitro*. These results indicated that reMAIT cells from iPSCs responded to external

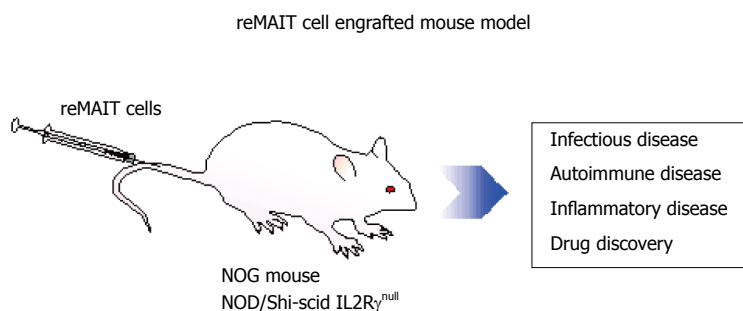


Figure 3 Utility of mucosal-associated invariant T cells from induced pluripotent stem cells (redifferentiation of mucosal-associated invariant T cells) for modeling human diseases. Severely immunocompromised mice received MAIT cells from induced pluripotent stem cells. reMAIT cells are useful for deciphering the physiological functions of MAIT cells in health and disease. MAIT: Mucosal-associated invariant T; reMAIT: Redifferentiation of MAIT.

cues, migrated to different tissues, and proliferated in mice. Such interactions most likely occur *via* chemokine receptors on reMAIT cells and *via* mouse MR1 bound with ligands from commensal flora or with an endogenous one. The data suggest that the function of reMAIT cells could be assessed *in vivo*, which opens up new horizons for modeling human diseases in mice.

Accordingly, the protective mechanisms of MAIT cells against bacterial infection have been examined using reMAIT cells^[71]. Upon adoptive transfer, reMAIT cells protected mice from *M. abscessus*, as demonstrated by a decrease in bacterial burden. Such a protective activity mirrors that observed with MAIT cells from PBMCs^[16]. Granulysin has been identified as an effector molecule in the control of mycobacterial infection. Granulysin is present together with granzymes and perforin in the cytolytic granules of cytotoxic cells such as CD8⁺ T and NK cells as well as MAIT cells^[32,82]. Granulysin plays a crucial role not only in the destruction of infected cells but also in killing pathogens^[83,84]. Given that mice are devoid of granulysin and its homologue^[85], mice harboring reMAIT cells could serve as a novel model to decipher the roles of human-specific factors.

There is accumulating evidence that MAIT cells play a pivotal role in inflammatory and autoimmune diseases. Nonetheless, delineating how MAIT cells are implicated in these diseases has to await the advent of appropriate animal models. Experimental autoimmune encephalomyelitis (EAE) and collagen-induced arthritis (CIA) are animal models for MS and RA, respectively. By using V α 19 iTCR transgenic mice and/or MR1-knockout mice, the implication of MAIT cells in autoimmune diseases has been investigated^[78,86]. In V α 19 iTCR mice, the severity of EAE was ameliorated in both induction and progression of demyelination compared with control littermates^[78,86]. In marked contrast, the severity of CIA was improved in MR1-knockout mice, whereas adoptive transfer of MAIT cells from V α 19 iTCR transgenic mice resulted in aggravation of the disease^[78,86]. EAE and CIA are intended to induce autoreactive T cells, especially focused on Th17 or Th1 responses, through hyperimmunization of putative target antigens (myelin basic proteins or type II collagen) with Freund's adjuvant. Induced T cells could migrate to target tissues and secrete proinflammatory or anti-inflammatory cytokines, which may further worsen tissue damage or help resolve the damage. It has been believed that such mechanisms recapitulate the etiology and pathology

of human diseases. Nonetheless, it is not appropriate to use such mice for disease modeling because MAIT cells do not react with peptide antigens, although they may respond to the components of adjuvant such as those from *M. tuberculosis*. Furthermore, the paucity of murine MAIT cells is another issue. Even though V α 19 iTCR transgenic mice can be used in a disease model, the nature of transgenic MAIT cells may be different from that present in the control. Given that MAIT cells are competent to produce a plethora of cytokines, a nature prerequisite for immunoregulatory functions, the above disease models may not be suitable for deciphering the etiology and pathology, in that such a crucial feature of MAIT cells is largely overlooked or distorted.

Exploring a disease model with reMAIT cells could further our knowledge of the etiology and pathology of MS. It has been reported that inflammatory demyelinating lesions are infiltrated by IL-17-expressing T cells in the mouse brain when they received cerebrospinal fluid from a progressive MS patients^[87]. A longitudinal study in MS patients indicated massive expansion of MAIT cells or MAIT cell-like cells, harboring canonical or atypical TCR V α and β chains but do not react with bacterial antigens, could play an important role in the onset and the formation of early active MS lesions^[47]. The above data implied the presence of yet-to-be-identified ligands responsible for the negative effects of MAIT cells in disease. Use of reMAIT cells could make it possible to examine whether or not sole ligands for MR1 or any epigenetic modifications of MAIT cells are responsible for disease. In either case, mice with reMAIT cells are useful to identify such ligands and to create a novel autoimmune disease model.

Should MAIT cells play a pivotal role in autoimmune diseases, it is tempting to anticipate that granulysin *per se* or in combination with granzymes and perforin exerts a cytolytic activity against the target tissue. In line with this hypothesis, granulysin may play crucial roles in transplant rejection and epidermal necrosis in toxic epidermal necrolysis and Stevens-Johnson syndrome^[85,88,89]. Furthermore, combined with the ectopic expression of human cytokines and/or chemokines, mice with reMAIT cells could be further fine-tuned to mimic human diseases by controlling tissue migration^[90,91]. Provided such an exquisite model is available, we can go to the next step of drug discovery and/or screening. Compounds that interfere either with the development of MAIT cells or the function of MAIT cells can be screened

in such a mouse model (Figure 3).

CONCLUSION

Recent studies have shed light on the unique properties of MAIT cells and on their possible involvement in a variety of human diseases, although MAIT cells have been overlooked behind conventional T cells and other innate immune cells for a long time. The paucity of MAIT cells in laboratory mice and their extremely poor proliferative capacity are the biggest obstacles to fully understand the function of MAIT cells in health and diseases. Reprogramming and redifferentiation of MAIT cells from iPSCs have overcome these difficulties. Furthermore, mice with reMAIT cells will pave the way for unveiling the mechanisms underlying the diseases and open up new horizons in medical research.

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Retinoblastoma tumor suppressor functions shared by stem cell and cancer cell strategies

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Abstract

Carcinogenic transformation of somatic cells resembles nuclear reprogramming toward the generation of pluripotent stem cells. These events share eternal escape from cellular senescence, continuous self-renewal in limited but certain population of cells, and refractoriness to terminal differentiation while maintaining the potential to differentiate into cells of one or multiple lineages. As represented by several oncogenes those appeared to be first keys to pluripotency, carcinogenesis and nuclear reprogramming seem to share a number of core mechanisms. The retinoblastoma tumor suppressor product retinoblastoma (RB) seems to be critically involved in both events in highly complicated manners. However, disentangling such complicated interactions has enabled us to better understand how stem cell strategies are shared by cancer cells. This review covers recent findings on RB functions related to stem cells and stem cell-like behaviors of cancer cells.

Key words: Stem cells; Cancer; Retinoblastoma; Cancer stem cells

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Core tip: Carcinogenic transformation of somatic cells resembles nuclear reprogramming toward the generation of pluripotent stem cells. The retinoblastoma tumor suppressor product retinoblastoma (RB) seems to be critically involved in both events in highly complicated manners. This review covers recent findings on RB functions related to stem cells and stem cell-like behaviors of cancer cells.

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INTRODUCTION

The cell-of-origin in various types of cancers has been one of the most important areas of research in modern cancer biology, because deep understanding of this can help design of future cancer therapies^[1-4]. Many studies have indicated that distinct cells-of-origin give rise to distinct features of cancers, and can often predict the prognosis of patients^[5,6]. However, the debate on cell-of-origin of each cancer is often controversial, because cancer phenotypes do not stereotypically reflect phenotypes of their true cells-of-origin. This is at least partially due to the high developmental plasticity that is acquired after tumor initiation and during tumor progression.

The cancer stem cell hypothesis proposes a model in which like in normal tissues, cancer cells obey the hierarchy of development where stem cell-like cancer cells are placed at the top. This hypothesis does not always explain the cell-of-origin of a specific cancer; however, it has led us to the idea that cancer cells prefer to employ stem cell strategies in order to maintain tumor-initiating clones^[7,8]. This theory has been reinforced by many findings, including the switchable cell fates of cancer cells, the inseparable relationship between pluripotency and teratogenicity, the requirement for oncogenic elements for the generation of induced pluripotent stem (iPS) cells, and the oncogenic activities of many embryonic genes^[9,10].

Sophisticated gene-engineered and tumor-grafted mouse models have been used to trace the cells-of-origin for specific cancers, for instance in case of prostate adenocarcinomas, to basal or luminal cells^[2]. During these investigations, the existence of unexpectedly high levels of developmental plasticity became apparent, when comparing the cell-of-origin to its resultant tumor. In addition, researchers found switchable cell fates in cultured cancer cell lines that were induced by artificially altering the status of genes, such as tumor suppressor genes.

Although the significance of epigenetic alterations is still unclear, carcinogenesis results from the step-wise accumulation of readable lesions in the genome^[11]. Given the gain of developmental plasticity, even if it is transient, is essential for carcinogenesis, then the next question should be as follows: Which genes are mechanistically involved in this gain of plasticity?

This question is not satisfactorily answered yet, but a part of answer may be informed by the switchable cell fate of cancer cells. The retinoblastoma tumor suppressor gene (RB) is closely implicated in the change in developmental phenotypes of many types of cancers, including lung cancer, breast cancer, prostate cancer, osteosarcoma, and soft tissue sarcoma. This phenomenon has been attributed to the physical or genetic interaction between the RB gene product (RB) and tissue-specific transcription factors^[12]. However, emerging evidence indicates that inactivation of RB in particular genetic backgrounds or in certain contexts can lead cells to an undifferentiated state that resembles

that of most immature cells, such as embryonic stem cells^[13-15].

We also know that the targeted inactivation of RB, in combination with p53, provides strong experimental tools to determine the cell-of-origin of various types of cancers^[16-18]. Indeed, these two tumor suppressor pathways are the most commonly inactivated in human cancers, and simultaneous inactivation is sufficient to induce cancers from various types of somatic cells^[19]. Therefore, one of the optimal ways to understand RB function in the context of full carcinogenesis would be to determine RB functions in a p53-deficient genetic background.

This review briefly summarizes the well-established functions of RB in mammalian cells, presents cross-species evidence for the possible link between RB function and the control of stem cell activities, and describes findings that may explain the molecular mechanisms underlying this link. The RB locus was identified more than a quarter century ago; however, researchers are still providing new wineskins to new wines.

CELL CYCLE-DEPENDENT AND INDEPENDENT FUNCTIONS OF RB

Cell cycle control by RB

The RB gene was first identified as a tumor suppressor in the childhood malignancies retinoblastoma and osteosarcoma^[20]. Somatic RB loss typically causes unilateral retinoblastoma with no obvious risk for other types of malignancies. However, germline RB mutation often results in bilateral retinoblastoma, and carriers are at very high risk of various types of cancer over their lifetimes^[21]. Therefore, researchers proposed that RB might be involved in the core mechanisms of tumorigenesis. Indeed, unveiling the functions of RB in controlling cell cycle progression provided a big breakthrough to the field of cancer research^[22].

A primary RB function in cell cycle control is exerted at the G₁/S transition. RB undergoes dephosphorylation at the end of the M phase with the aid of protein phosphatases (PPs) and resumes its phosphorylated state during the G₁ phase by the action of cyclin D/cyclin-dependent kinase (CDK) 4 or 6 complexes^[23]. Most of cellular mitogenic signals converge on the transcriptional upregulation of D-type cyclins. This could be one reason that cells in the G₁ phase are most vulnerable to extracellular growth stimuli^[23,24].

Phosphorylation of RB alters its three dimensional (3D) structure. This results primarily in the loss of binding affinity to E2F family transcription factors^[25,26]. Among nine identified E2F family members (E2F1, 2, 3A, 3B, 4-8), RB was shown to bind to at least E2F1, 2, and 3A. Each of these three family members is able to positively transactivate genes, including cyclin E^[27]. Upregulation of cyclin E in cooperation with CDK2 further promotes RB phosphorylation. This enables cells to cross the boundary between G₁ and S. Further, with the aid of cyclin A, RB attains the maximal level of phosphorylation before cells enter the M phase^[23]. In addition, when bound to

hypophosphorylated RB, E2Fs form a transcriptional repressor complex that recruits histone deacetylase (HDAC) to epigenetically silence gene transcription^[28]. Therefore, the phosphorylation status of RB dramatically changes the expression of E2F-targeted genes. The function of RB in restricting the G₁/S transition is also mediated by its binding to SKP2, which destabilizes p27^{KIP1} by enhancing the ubiquitin-proteasome system when freed from phosphorylated RB^[29,30]. This represents one of E2F-independent functions of RB in the control of cell cycle progression.

RB plays pivotal roles also in M phase, which is most typically represented by the impact of RB inactivation on the chromosomal instability (CIN). E2Fs target a number of M phase genes including MAD2 which functions by inhibiting the anaphase promoting complex/cyclosome (APC/C)-cell division cycle 20 (CDC20) complex. This complex regulates spindle assembly^[31]. RB also controls the M phase by directly binding to cohesin and condensin II, two critical regulators of centromeric functions^[32].

"How many total RB functions are cell cycle-dependent?" is an intriguing question. RB mutants found in partially penetrant retinoblastomas (low grade retinoblastomas with limited genetic inheritance) or retinomas that failed to inhibit the cell cycle but retained the ability to promote terminal differentiation suggested that RB functions in cell cycle control and differentiation might be distinct^[33]. In addition, phenotypic analyses of *Rb*-deficient mice simultaneously lacking an *E2F* family member allowed at least partial discrimination of the E2F-dependent function from the E2F-independent function^[34]. However, since E2Fs target both cell cycle-related and cell cycle-unrelated genes, discrimination of cell cycle-dependent functions from cell cycle-independent functions of RB based on the E2F-dependency is difficult.

Artificial and acute alteration of RB status in a wild type genetic background often greatly affects the cell cycle. For instance, it induces cell cycle exit (quiescence or cellular senescence), or inversely cell cycle re-entry^[35,36]. This change in the cell cycle control is highly drastic, thus can mask cell cycle-independent phenotypes associated with altered RB activity. However, based on the experience of analyzing *Rb*-deficient mice, our group discovered several genetic backgrounds that allow mice or cells to exhibit cell cycle-independent phenotypes following RB inactivation^[14,15,37,38]. The cell cycle-independent phenotypes include gain of undifferentiated phenotypes and altered chemo-resistance^[15]. Therefore, we thought that control of the undifferentiated state of cells might represent at least a part of the cell cycle-independent functions of RB.

Cell cycle-independent functions of RB

Whole genome sequencing studies have revealed that the *RB* loci undergoes the fourth most frequent loss-of-heterozygosity (LOH) found in whole human tumors, following *CDK42A*, *PTEN*, and *SMAD4*. Thus, *RB* mutations definitely can be "driver mutations"^[39]. However, the type

of tumors in which *RB* mutations occur at their initiation is highly limited, including only retinoblastomas, small cell lung cancers (SCLC), osteosarcomas, and familial melanoma. In other types, in the vast majority of tumors, RB functions are largely maintained during initiation but these functions typically collapse while tumors undergo malignant progression^[40].

The question of why RB mutation is rare in the majority of cancers has yielded many interesting answers. Lack of RB in many cell types has already been linked to apoptosis through E2Fs, ARF, and p53^[41]. In addition, RB residing in mitochondria directly interacts with Bax, and thus regulates apoptosis in a completely E2F-independent and cell cycle-independent manner^[42,43]. These pathways represent a disadvantage in carcinogenesis upon RB inactivation when it occurs at early steps. The 3T3 cells lacking *Rb* are less susceptible to Ras-transformation, indicating that preservation of RB functions in the interaction of mitogenic signals and the cell cycle might be important for tumor initiation^[44].

Our group demonstrated that *Rb*-heterozygous mice generate adenomas or low-grade adenocarcinomas derived from calcitonin-producing cells (C cells) of neuro endocrine origin that exhibit whole evidence of DNA damage response and cellular senescence. However, the genetic background of a homozygous lack of *N-ras* allowed these *Rb*-deficient C cell tumors to progress to highly invasive and metastatic adenocarcinomas^[14,37]. RB appeared to regulate isoprenylation of the N-Ras protein. Isoprenylation (farnesyl moiety-transfer and geranylgeranyl moiety-transfer) is the chemical reaction that is essential for the initial maturation of this protein. RB loss causes intermediate level upregulation of N-Ras under particular culture conditions, which induces a DNA damage response and subsequently cellular senescence, thus antagonizing full carcinogenesis in a manner similar to oncogene-induced senescence (OIS). The mechanism whereby RB controls N-Ras isoprenylation involves E2F-dependent regulation of sterol regulatory element binding protein (SREBP) transcription factors and direct drive by E2Fs in some of mevalonate (MVA) pathway genes^[14]. This study indicated a case that RB influences intra-cellular signaling primarily by controlling metabolic pathways. Many reports, including ours, have directly implicated RB in the control of cellular metabolism^[36,45,46]. This may indicate that RB is simultaneously involved in the control of the cell cycle and metabolic regulation. This may further explain why cell cycle progression and cellular metabolism are tightly coupled. Cells never diminish their volume after rounds of cell division. This is because cells very strictly double biomass up to the time of mitosis, just as they strictly conserve genome size. From this point of view, cellular metabolism for biomass synthesis would not be passively controlled by the demands of cell cycle progression; rather, they are both actively and presumably simultaneously regulated by a common mechanism.

The outcomes of RB inactivation during malignant progression entail not only facilitated G₁/S transition, but

also many events critical for malignant cell behaviors. These include increased cell motility, angiogenesis, inflammatory response, metabolic rewiring, gain of undifferentiated developmental features, lineage change and altered drug resistance^[36,45]. Since acquisition of these abilities is not always associated with cell cycle progression, many of these, except G₁/S control, may more or less represent cell cycle-independent functions of RB. Particularly, gain of undifferentiated developmental features following RB inactivation and lineage changes presumably related to the developmental plasticity of tumors are central interests of this review article.

We need to be very careful in defining what is cell cycle-independent function of RB and what is not. E2F targets contain not only cell cycle-related genes. In addition, many LxCxE proteins that bind to RB are chromatin modifiers; hence, their roles in control of the cell cycle and differentiation are barely discernible. We later discuss the experimental system by which we addressed cell cycle-independent functions of RB in the context of regulation of undifferentiated cell states. In the next paragraph, we introduce accumulating biological evidence implicating RB in multiple stem cell systems. Later, we discuss possible cell cycle-dependent and independent mechanisms underlying RB functions in stem cells.

CROSS-SPECIES EVIDENCE THAT LINK RB TO STEM CELLS

Figure 1 shows a phylogenetic tree of genes in the RB family (Figure 1). RB gene orthologues do not exist in the genome of many of unicellular organisms, including yeasts, but appear in almost all multicellular organisms^[47]. In addition, the component including RB alone and/or E2F transcription factors and DP protein(s) are well conserved from plants to animals^[48]. This indicates that RB might be involved not only in cell-autonomous proliferation control but also in some machineries unique to multicellular organisms. Stem cell conservation attained by asymmetric cell division is apparently unique to multicellular organisms. Therefore, we present cross-species evidence that may link RB to stem cells.

Mammalian cells

Analysis of RB functions in embryonic development and embryonic stem cells provided a substantial amount of information regarding its potential roles in stem cells. The role of RB in various adult tissue stem cells was once thoroughly summarized by Sage^[19]. Our current review focuses on the cells-of-origins in which RB inactivation likely gives the first cue for clonal expansion of stem or progenitor cells during carcinogenesis.

Retinoblastoma develops from the retina composed of multiple lineages of cells. The cell-of-origin of retinoblastoma is quite controversial because of the complexity of retinal development and tumor phenotype. More in concrete, findings that contribute to the controversy include the

reported appearance of differentiated and undifferentiated developmental markers of mixed lineages within the same tumor derived from retinoblastoma patients and Rb-deficient mice^[49]. Human retinoblastomas show typically differentiated features^[50], and post-mitotic retinal cells can be the cell-of-origin in mouse models^[51]. These findings suggest that promoting dedifferentiation and increasing flexibility of fate determination are initially attained by RB inactivation in retinal cells. In other words, RB inactivation in retinal somatic cells endows them with the ability to differentiate to multiple types of cells. The dispute over cell-of-origin might be resolved, since cone precursors have been shown to be specifically vulnerable to Rb-deficiency-induced clonal expansion^[52]. However, given that the cell-of-origin has been truly traced to one lineage in retinoblastoma development, the findings on mixed and normally inconsistent developmental features co-existing in retinoblastoma tumor cells imply that RB-deficiency not only initiates tumors of a particular cell-of-origin, but also increases plasticity in lineage specification and probably induces dedifferentiation so that tumor cells employ multiple stem or progenitor cell strategies to fit to the retinal microenvironment.

There are several other types of cancers in which RB loss occurs prevalently at initiation: Osteosarcoma and SCLC. In these malignancies, discussions of the cell-of-origin and the role of RB in tumor initiation are inseparable similarly as in case of retinoblastoma. Mesenchymal stem cells or osteo-progenitors can be the cell-of-origin of osteosarcoma. The direct interaction between RB and osteoblast transcription factor Runx2 might at least partially explain the ability of RB to suppress tumor initiation in a lineage-specific manner. Rb deficiency in these cells often cooperates with p53 loss-of-function to generate osteosarcomas^[53]. p53-deficiency is in some case sufficient to induce osteosarcoma, and p53-deficient osteosarcomas can be converted to brown fat tumors (hibernomas) with subsequent RB inactivation^[12]. This has been attributed to increased expression of PPAR γ which is governed by E2Fs. However, this fate change might also be mechanistically supported by function of RB to influence on cell fate decision that may occur prior to the decision of commitment to terminal differentiation. Hence, in osteosarcoma, like in retinoblastoma, RB might function in mesenchymal stem cells or osteo-progenitors to contribute to suppress the plasticity in lineage specification.

There are also debates on the cell-of-origin of SCLC. Again, simultaneous inactivation of RB and p53 is sufficient to induce SCLC in mouse lungs^[3]. A study of cell type-specific deletion of RB and p53 indicated that neuroendocrine cells more often gave rise to SCLC than alveolar type II cells^[54,55]. From this and other evidence, neuroendocrine cells are believed to be the predominant cell-of-origin of SCLC. The differential roles of RB and p53 in the formation of SCLC will be discussed later (see below).

The role of RB in controlling pluripotency was first addressed by testing whether tumor suppressor depletion facilitates iPS induction^[56]. This study identified p53 but not RB to be an influential molecule in iPS induction.

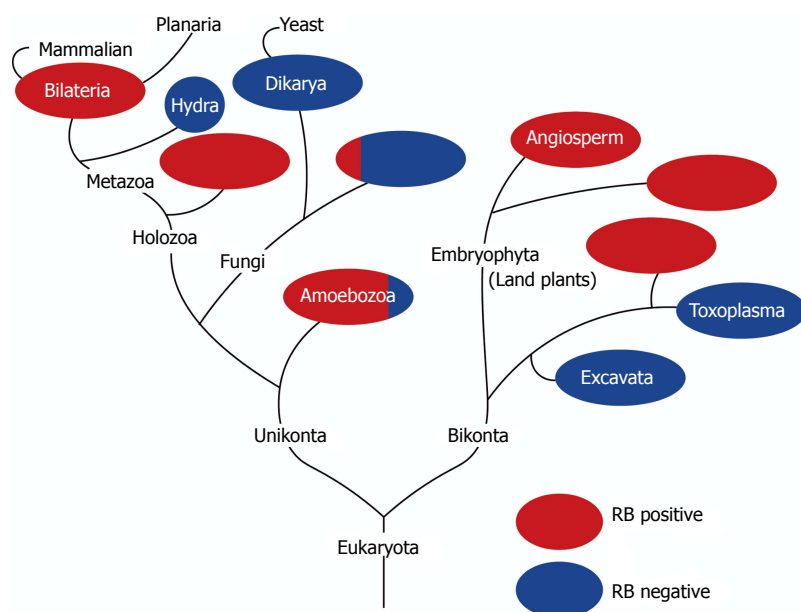


Figure 1 Evolution of the retinoblastoma gene in eukaryotic super-groups. Modified from Desvoyes *et al.*^[67]. RB: Retinoblastoma.

However, two later studies indicated that RB suppresses iPS induction from fibroblasts. A screen for short hairpin RNAs (shRNAs) that enhance iPS induction efficiency identified RB^[57]. Another study indicated that RB cleavage by caspase 3/8 is critical for iPS induction^[58]. More recently, RB was directly implicated in the transcriptional control of *Oct4* and *Sox2*^[59]. These findings indicate that RB inactivation leads to a state favorable for iPS induction by facilitating the induction of embryonic genes that induce a pluripotent state. This function of pRB may partly explain how stem cell functions can be shared by cancer cells. We indeed often observed increased expression of *Oct4* and *Sox2* in spheres induced by RB inactivation^[59]. The effect of RB inactivation on the cell cycle control is also contributable to iPS cell induction. We will discuss later many of the signals required for iPS induction are possibly controlled by pRB (see below).

MEFs lacking all members of the RB family can form embryoid body-like 3D structures in suspended culture conditions that express higher levels of embryonic genes and can form teratoma-like tumors when inoculated into immune-deficient mice^[13]. Since mostly tumor-derived spheres express higher levels of embryonic genes than when cultured under 2D conditions, this observation might result from carcinogenic change in MEFs.

Kareta *et al.*^[59] (our study) demonstrated that RB inactivation in *p53*^{-/-} MEFs give rise to sphere formation without carcinogenic conversion. Sphere formation in the absence of serum and in the presence of limited growth factors (bFGF and EGF) is thought to represent increased self-renewal/symmetric cell division. Preceding this observation, we analyzed C cell adenocarcinomas developed in *Rb*-heterozygous mice that simultaneously lacked *p53*, *Ink4a*, *Arf* or *Cdkn1a* (*p21*). *Rb*-heterozygous mice typically develop low-grade C cell adenocarcinomas. Simultaneous lack of an additional gene allowed *Rb*-deficient C cells to develop full brown tumors at seemingly similar levels. However, as compared to other

genotypes of C cell tumors that were all calcitonin-positive, thyroid tumors that developed in *Rb*^{+/-} *p53*^{-/-} mice showed virtually no expression of calcitonin. However, earlier neuroendocrine lineage markers, including synaptophysin, were expressed. These findings indicated that simultaneous inactivation of *Rb* and *p53* induced a highly undifferentiated status in neuroendocrine cells that were originally destined to develop into C cells. We then analyzed *Rb*^{-/-}; *p53*^{-/-} MEFs in comparison with *Rb*^{+/-}; *p53*^{-/-} MEFs. They showed insignificant differences in cell cycle progression; however, *Rb*^{-/-}; *p53*^{-/-} MEFs showed significantly higher self-renewal activity and increased expression of embryonic genes. This may represent a cell cycle-independent function of RB in controlling stem cell-like features. Lastly, we screened an FDA-approved drug library and found that some drugs reported to be effective as cancer stem cell therapies were also effective in cells lacking *Rb* and *p53*^[15].

Tumor cell fate

Here, we focus on four tumor types (SCLC, breast cancer, prostate cancer, and soft tissue sarcoma) in which RB inactivation during tumor progression presumably contributes to the increased developmental plasticity of tumor cells.

p53 is the primary gene mutated in SCLC (75%-90%)^[60]. *p53* mutations are found also in normal bronchioles of patients, suggesting that this mutation most likely occurs at tumor initiation^[61]. The second most frequent mutation occurs at *RB* loci. Recent comprehensive genomic profiling of SCLC revealed that bi-allelic losses at *p53* and *RB* loci were 100% and 93%, respectively^[62]. This signifies that the simultaneous inactivation in *p53* and *RB* occurs in almost 90% of all cases. This study showed a previously unexpected high frequency of *RB* inactivation in SCLC. In terms of determining *RB* function in SCLC development, other gene mutations relevant to *RB* loss in *RB*-intact SCLC (7% of *p53*-mutated cases) should be noted. Semenova *et al.*^[3] discussed possible *RB* functions in *p53*-mutated SCLC cells.

They pointed out that pluripotency genes such as *OCT4* and *SOX2* are frequently amplified in SCLC^[63] and that enhancers of zeta 2 (*EZH2*), which is implicated in the neural stem cell maintenance, are very often upregulated in SCLC following RB inactivation^[64,65]. Finally, they concluded that "RB loss (in SCLC) is associated with an increase in cell plasticity"^[3]. Although more evidence is needed to finally accept this notion, it is definitely an attractive hypothesis.

Recent publication from Engelman group demonstrated that gain of resistance to tyrosine kinase inhibitors in non-small-cell lung cancers (NSCLCs) harboring epidermal growth factor receptor (EGFR) mutation is associated with fundamental histological transformation from NSCLC to SCLC at a certain frequency (5%-15%). Surprisingly, in such subset of resistant cancers, RB was lost at 100% frequency^[66]. This report indicates that the developmental plasticity enhanced by RB inactivation is coupled to gain of drug resistance. The mechanism of drug resistance associated with NSCLC-to-SCLC conversion is currently unknown, but it would be definitely attractive to investigate RB functions in this context.

Breast cancers are frequently characterized by RB pathway inactivation, and low RB expression is a hallmark of basal-like breast cancers^[67-69]. RB inactivation in luminal type breast cancer induces tamoxifen resistance^[70], possibly owing to a gain-of-undifferentiated status following RB inactivation. Simultaneous inactivation of p53 and RB is prevalent in basal-like cancers^[71]. Basal-like and luminal type breast cancers have been recently suggested to stem from common luminal progenitor cells^[72]. Because p53 mutations are common in most breast cancer types^[73], RB status might be one of the determinants of basal-like or luminal type cancers. If so, RB could be implicated in determining the fate of breast cancers.

RB was also implicated in epithelial-to-mesenchymal transition (EMT) in breast cancer cells. Taya group demonstrated that RB depletion in a luminal type MCF-7 breast cancer cells induced EMT and overexpression of RB inhibited the EMT in MCF10A non-tumorigenic breast mammary epithelial cells^[74]. They also demonstrated that RB controls transcription of *SLUG* and *ZEB-1* in cooperation with the transcription factor activator protein 2 α .

RB is deeply implicated in prostate cancer development, especially during its progression. Although RB inactivation is observed in only 5% of primary prostate cancers, its rate rises to 40% in metastatic tumors. Furthermore, the RB signaling pathway is altered in 34% and 74% of primary tumors and metastatic loci, respectively^[75]. One study demonstrated a high rate of RB loci deletion and DNA methylation in the RB promoter in metastatic castration-resistant prostate cancers (CRPC)^[76]. RB depletion in hormone-dependent human prostate cancer cells induces androgen-independent cell growth through upregulation of androgen receptor (AR) in an E2F1-dependent manner^[77]. RB and p130 are involved in the regulation of *EZH2* transcription in prostate cancer cells, whose upregulation is often observed during prostate cancer progression^[78]. Loss of RB in prostate cancer

cells increases the expression of nucleolar and spindle-associated protein 1 (*NUSAP1*), which is associated with a poor prognosis in prostate cancer^[79]. Recently, we observed that RB depletion in androgen-dependent prostate cancer cells induces several lipid metabolism-related genes and some typical malignant features, including tumor spheroid formation. These observations indicate that inactivation of RB strongly promotes prostate cancer progression.

Simultaneous mutations in RB and p53 are frequently found in human soft tissue sarcomas^[80]. Conditional inactivation of both tumor suppressors by subcutaneous injection of AdCMVC into *p53^{fllox/fllox}; Rb^{fllox/fllox}* mice induced undifferentiated high-grade pleomorphic type sarcomas from locally resident cells. Inactivation of p53 but not RB is sufficient to induce well-differentiated sarcomas, such as rhabdomyosarcoma and leiomyosarcoma, but typically not sufficient to induce undifferentiated types of tumors^[81]. These findings indicate that RB inactivation does not directly contribute to the initiation of sarcoma development, but rather does contribute to converting well-differentiated types of tumors to undifferentiated types in a p53-deficient background. We recapitulated this finding in an *in vitro* culture system. We first developed poorly spherogenic p53-null soft tissue sarcoma cell lines from soft tissue sarcomas that subcutaneously developed in approximately 10% of *p53^{-/-}* mice with a C57BL/6 background. Additional depletion of Rb successfully induced less differentiated highly spherogenic and less differentiated sarcomas from "poorly spherogenic" p53-null soft tissue sarcoma cell lines. These findings indicate a possibility that in soft tissue sarcomas, RB directs both the self-renewal and plasticity of developmental features.

Planarians

Relying on a large population of pluripotent adult stem cells, planarians exhibit extraordinary high regenerative capacities. Zhu and Pearson presented a comprehensive study on the RB system in these organisms^[82]. Planarians possess unexpectedly few RB system components: A single Rb family member, single E2F (*E2F4-1*), and single DP. They are primarily expressed in planarian stem cells, and knockdown of any of these components significantly phenocopied the stem cell loss induced by irradiation or RNAi against stem cell-specific genes. The RB system was found to be indispensable for planarian stem cell self-renewal and survival; however, it was dispensable for late differentiation. Interestingly, planarians have 20 homologs to cyclin genes and none of them is homologous to cyclin E. An HDAC1 and a cyclin D homolog are expressed specifically in planarian stem cells, and knockdown of either of them induced deficiencies in stem cell functions. The simplicity of the RB system and ease of visualizing stem cell behaviors in planarians make this a valuable system for the RB research field, especially with regard to stem cell functions. Additionally, the molecular mechanism whereby the RB system contributes to the extraordinarily high regenerative capacity of this creature is great of interest.

Plant cells

RB family proteins and their binding partners had existed before multicellular organisms appeared on the earth, and are shared by plants and animals^[82]. *Arabidopsis thaliana* has one ortholog of an RB family protein, which was named retinoblastoma-related protein (RBR)^[83]. Inactivation of RBR led to the expansion of root stem cells without affecting the ability of progenitor cells (descendants) to self-renew and differentiate^[48]. The mechanism whereby RBR controls maintenance of root stem cells seems to involve two transcription factors shortroot (SHR) and scarecrow (SCR). SCR interacts with RBR through an LxCxE motif. Thus, surprisingly, the role of LxCxE motifs is well conserved between animals and plants. More surprisingly, the RBR status affects the function of the SHR/SCR complex to spatiotemporally control the expression of a plant homolog of D-type cyclin (CYC D6;1). CYC D6;1, in cooperation with its corresponding cyclin-dependent kinase (CDK), promotes phosphorylation of RBR^[83]. These findings indicate that in plants, the RB ortholog exerts the function of controlling stem cells through the regulation of well-conserved cell cycle machinery. Further investigation in this field might unveil many unexpected aspects of RB functions in the control of stem cells.

MECHANISTIC ASPECT OF RB FUNCTIONS IN CONTROLLING STEM CELL ACTIVITIES IN MAMMALIAN CELLS

Cell cycle

Analysis of the cell cycle status in embryonic stem (ES) cells provided valuable information on how its alteration might contribute to the acquisition of increased self-renewal and pluripotency. Conklin and Sage^[84] provided a concise perspective on the possible roles of RB in maintaining the ES cell functions. ES cells have a rapid cell cycle. Because of the prolonged or continuous expression of cyclin E and A family members and lower or no expression of many CDK inhibitors, human and mouse ES cells maintain high levels of activity in multiple CDKs. pRB is consequently hyperphosphorylated for longer periods in ES cells than in normally cycling cells. No obvious cell cycle phenotype in mouse ES cells following inactivation of all RB family members might be consistent with this view^[85,86].

An extraordinarily shorter G₁ phase as compared to the relatively prolonged S phase in ES cells would be beneficial to lessen the susceptibility to differentiate upon receiving stimuli to lender cells to do so. As G₁ as well is the period that is most vulnerable to mitogenic signals that directs cells to decide to proliferate, arrest or senesce, this phase could be the most critical one in the decision to differentiate upon various stimuli. Keeping the G₁ phase shorter could be the primary role of RB hyperphosphorylation in ES cells. Consistent with this view, overexpression of the constitutively active

(non-phosphorylatable) form of RB (RB^{7LP}) in human ES cells induced cell cycle arrest, followed by spontaneous differentiation and p53-dependent cell death^[87].

The difference in undifferentiated behaviors between *Rb*^{+/+}; *p53*^{-/-} and *Rb*^{-/-}; *p53*^{-/-} MEFs predicted that there could be cell cycle-independent functions of RB in controlling stemness. *p53*^{-/-} MEFs exhibited similar proliferation phenotypes regardless of Rb genotype; however, *Rb*^{-/-}; *p53*^{-/-} showed increased sphere forming activity relative to *Rb*^{+/+}; *p53*^{-/-} MEFs^[15]. The same phenomenon was observed in mouse soft tissue sarcoma cells and mouse mammary gland epithelial cells. Given that RB-deficiency has no or very little impact on cell proliferation in a *p53*-null genetic background, the mechanism whereby RB-deficiency effects stem cell-like features of multiple types of cells can be separated from that governs cell proliferation.

Quiescence and apoptosis

In contrast to mouse ES cells, inactivation of all RB family members in human ES cells exhibited abnormal quiescence, featured by G₂/M arrest and cell death^[87]. Cell death depended on the p53-p21 module, similar to RB overexpression. Thus, in human ES cells, both hypo- and hyper-activation of RB are counteracted by cell cycle arrest and p53 pathway activation, indicating a critical role for RB in the homeostatic control of ES cell activities. p53 is typically expressed in human ES cells at a low level^[88]. This might be beneficial for ES cells so that they are not too sensitive to alterations in RB activity status. Mouse hematopoietic stem cells lacking all RB family proteins exhibit impaired quiescence control and apoptosis in lymphoid progenitor cells^[89]. In MEFs, the absence of p53 endowed RB-deficient cells significant increase in self-renewal activity when cultured in the presence of limited growth factors^[15]. However, in a *p53*^{-/-} background, the RB status did not impact cell proliferation of MEFs under regular culture conditions. It should be noted again that in mouse ES cells, lack of RB family proteins generated no difference in the cell cycle^[85,86]. These findings indicate that RB may control stemness beyond its role in cell cycle control.

Cellular senescence

Cellular senescence is believed to be strongly inhibited in stem cells, otherwise irreversible growth arrest could lead to total elimination of a stem cell pool from tissues. RB plays pivotal roles in inducing and maintaining cellular senescence, not only by controlling required transcription of genes, but also by being involved in senescence-associated heterochromatic foci (SAHF)^[90]. Therefore, hyperactivation of RB can be harmful to stem cells, as was shown in a human ES cell study^[87].

Surprisingly, loss of RB function, as well, can induce cellular senescence. As mentioned above, thyroid tumors that developed in *Rb*-heterozygous mice were typically low-grade adenocarcinomas or adenomas of

C cell origin; however, the genetic background lacking *N-ras*^[37] or either *Ink4a*, *Arf*, *Suvh39*^[14], *ATM*^[38] or *p53*^[15] allow these tumors to develop into highly invasive and metastatic type medullary adenocarcinomas. The immunohistochemical observation of Rb-deficient C cell tumors lacking no other genes revealed whole evidence of cellular senescence, including increased expression of p16^{Ink4a} and HP-1, and positive β -galactosidase staining^[14]. Since a simultaneous lack of genes mediating DNA damage response (*ATM*) and cellular senescence (*Ink4a*) allowed malignant progression of C cell tumors, we concluded that these cellular responses prevented Rb-deficient premalignant cells from developing into malignant cells. Analysis of *Rb-N-ras* DKO mice revealed that the mechanism whereby RB loss induces a DNA damage response involved p130^[14]. Another system in which RB loss possibly induces cellular senescence under particular culture conditions is MEFs. *Rb* loss alone does not allow MEFs to escape senescence when cultured at low density. However, simultaneous loss of *N-ras* or *Ink4a* allowed MEFs to escape cellular senescence upon low cell density plating^[14]. The reason why *N-ras* loci are associated with susceptibility to senescence was explained by the E2F-dependent control of Ras isoprenylation^[14].

There are three other tumor suppressors whose loss of function can induce cellular senescence in particular contexts. These are PTEN, VHL and NF1^[90-93]. Somatic cells are protected from carcinogenesis when these tumor suppressor genes are inactivated at early steps of carcinogenesis. This could be the major reason that RB mutations are detected in only limited types of cancer at their initiation.

ES cells, iPS cells, and tissue stem cells seem to confer lower RB activity in order to accelerate self-renewal and to keep undifferentiated state. We do not know whether cellular senescence machineries are simultaneously suppressed during the period that RB function is suppressed in stem cells. Unlike *Rb*^{-/-}; *p53*^{-/-} MEFs, *Rb*^{-/-}; *Ink4a*^{-/-} MEFs did not form spheres^[15]. However, regarding iPS induction, the *Ink4a/Arf* locus appeared to be a barrier to reprogramming^[94]. Therefore, RB functions in controlling cellular senescence could be intimately involved in the regulation of stemness.

Chromosomal instability

Chromosomal instability (CIN) might be one of the events that are seemingly not shared by normal stem cells and cancer cells. In tumor cells, RB inactivation, especially when combined with p53 mutation, led cells to accumulate chromosomal aberrations^[95]. Inactivation of all RB family members in human ES cells causes CIN; however, clonal expansion of these cells was blocked by G2/M arrest and cell death^[87]. Presumably, then, normal stem cells are much less tolerant of CIN than cancer cells. RB status could influence CIN through its effects on Mad2 transcription and its direct interaction with cohesion and condensin II^[96]. The rapid cell cycle

in ES cells might increase the risk of accumulating DNA damage due to hyper-replication and nucleotide deficiency^[97]. How ES cells are protected from these risks and why cancer cells tolerate CIN needs to be clarified.

Epigenetics

Through LxCxE motifs, RB interacts with numerous chromatin modifiers, including DNMT1, SUV39H1, Suv4-20H1, BRN1, BRG1, HDAC, and KDM5A/JARID1A/RBP2^[36]. Among these, KDM5A might mediate RB function to control stemness. KDM5A demethylates tri- and dimethylated lysine 4 in histone H3. ES cells lacking KDM5A failed to maintain OCT4 and NANOG expression upon stimulation to promote differentiation^[98]. KDM5A controls RB-dependent myogenic differentiation, at least partially, through the regulation of mitochondrial function^[99]. Importantly, loss of KDM5A in Rb-heterozygous mice attenuated pituitary tumorigenesis^[98]. KDM5A was first identified in a screen to find proteins that bind to pRB mutants unable to bind to E2Fs^[100]. pRB mutants unable to bind to KDM5A failed to control differentiation. The mechanism whereby KDM5A controls mitochondrial function involves PGC-1/PPAR γ c1a^[99]. It is of great interest to determine whether other genes under the influence of RB-KDM5A axis control stemness based on the effect on their epigenetic status.

Our experience with the stem cell-like behaviors exhibited by RB-p53 double deficient cells indicated that some of stem cell-like features are not reversed by RB reconstitution. This suggests that the effect of RB deficiency on stem cell-like behaviors might depend on its role in epigenetic control. Many chromatin modifiers carrying LxCxE motifs may be involved in the epigenetic function of pRB; however, so far, only some of them have been characterized regarding their role in controlling stem cell functions. We demonstrated that Rb in mouse cells exerts its influence on the epigenetic control of *Ink4a*, *Shc*, and *FoxO6* through DNMT1; however, its significance in the control of stemness has not yet been elucidated^[38].

Tissue specific transcription factor

RB plays critical roles in the terminal differentiation of cells owing to its genetic and physical interaction with tissue-specific transcription factors, including MYOD, C/EBP, GR, GATA-1, PU-1, CBFA-1, PDX1, RUNX2, and NF-IL6^[36]. It is totally unknown whether the interaction between RB and these factors has any role in tissue stem cells. Myoblast regeneration induced by RB and ARF depletion in post-mitotic muscle cells^[101] may involve the elimination of MYOD functions because there is a physical interaction between RB and MYOD. Additionally, the physical interaction of RB and RUNX2 may explain dedifferentiation in osteoblasts that would occur upon development of osteosarcoma.

The interaction of RB with ID2, KDM5A, and EID1 may govern differentiation in a less tissue-specific manner.

ID2, when overexpressed in Nestin-expressing cells, induced precocious neural stem cell depletion^[102]. RB is to some extent dispensable in brain development during the embryonic stage since Rb-deficient embryonic brains exhibited almost normal development, except for ectopic cell cycle entry and cell death in cortical neurons^[103,104]. However, the role of RB in adult neural stem cells has not been sufficiently addressed yet.

Embryonic gene

There are reports that NANOG and SOX2 induce hyperphosphorylation of pRB through regulation of CDC25A and CDK6^[84]. OCT4, in cooperation with miR-335, induces hyperphosphorylation of RB by suppressing PP1 through NIP1 and CCNF^[105]. Following RB hyperphosphorylation, freed E2Fs transactivate OCT4-targeted genes. These findings indicate the general function of RB in orchestrating the embryonic gene network. The most surprising finding regarding the role of RB in the embryonic gene network was recently made by the Wernig and Sage laboratory^[59]. They discovered that RB is directly involved in the transcriptional control of OCT4 and SOX2. They also demonstrated that Sox2 deletion attenuated pituitary tumorigenesis in Rb-heterozygous mice. These findings clearly revealed the strong influence of RB on the embryonic gene network.

These findings also explain the upregulation of a series of embryonic genes in MEFs lacking all RB family members^[13] and also in those lacking Rb and p53^[15] when forming embryoid body or spheres under nutrition-restricted and floating conditions. However, there was no evidence of upregulation of these genes when cells were cultured under 2D culture conditions in the presence of serum. This implies that regulation of OCT4 and SOX2 by RB is influenced by the environment where cells are placed and other genes that play a critical role in allowing RB to control embryonic genes. It would be of great interest to survey such genes.

Metabolism

There are significant similarities between stem cell metabolism and cancer cell metabolic reprogramming (rewiring)^[106,107]. RB functions in various metabolic pathways have attracted attention from cancer researchers^[36,45,46,108]. Given that RB is the central molecule in cell cycle control, it is reasonable that RB also responds to demands for increasing biomass that is coupled to the cell cycle. It is well known that RB controls thymidine kinase 1 (TK1) and dihydrofolate reductase (DHFR), both of which are required for nucleotide synthesis^[109]. However, the biomass needed for doubling the cell size during the S phase contains not only nucleotides but also amino acids, lipids, and many other carbon metabolites. pRB undergoes post-translational modification by various nutrient signals involving SIRT1 and AMPK and by cyclin/CDK complexes stimulated by various mitogenic signals^[110,111]. RB regulates the transcription or the activity of wide range of enzymes, signaling molecules, and transcription factors, including OXPHOS genes, MVA pathway genes, UCP-1, SOD2, ASCT2, GLS1, PKA, AKT, mTOR, PDK4, PGC-1, ERR, FOXc2, HIF-1, BNIP3,

SREBP-1,2, PPAR and KDM5A^[12,14,112-124]. Genes encoding many of these factors are supposed to be driven by E2F transcription factors. Myc is also a well-established downstream molecule of RB, and is implicated in the transcriptional control of GLUT1, HK2, PKM2, and LDH-A^[45].

OXPHOS activity is significantly correlated to the efficacy of iPS induction^[125]. Besides its interaction with OCT4 and SOX2, RB might be implicated in nuclear reprogramming through its influence on OXPHOS.

HIF-1 α activation explains why hypoxia facilitates iPS induction and self-renewal of tumor cells^[126,127]. Additionally, this molecule was implicated in the long-term maintenance of hematopoietic stem cell (LT-HSC) populations^[128]. Thus, RB status presumably has a big impact on glycolysis and TCA activity through the functional interaction with HIF-1. We have recently identified an HIF-1-independent target of RB in the glycolytic pathway. It is of interest to address how RB and HIF-1 cooperate in the control of the glycolytic pathway.

Recent studies from two independent research groups highlighted the link between RB status and glutaminolysis in *Drosophila* and mammalian cells^[116,129]. Activation of the glutamine pathway not only fuels the TCA cycle, but also contributes to control of the cellular reactive oxygen species (ROS) level by regulating glutathione synthesis^[130]. This may give both stem cells and cancer cells an advantage in terms of the maintenance of stemness. Additionally, ASCT2 in cooperation with GLUT1 appears to regulate human hematopoietic stem cell lineage specification^[131]. Increased dependency on glutamine metabolism is frequently observed in cancers, and has been linked to the emergence of drug resistance^[132,133]. Glutamine metabolism could be an important link between stem cells and cancer cells.

While addressing the question of why Ras proteins are activated after RB loss, we discovered that RB controls a number of enzymes involved in the MVA pathway. This study has been extended to another study that addresses the role of the MVA pathway in controlling stem cell-like activity in prostate cancer cells. The mechanism that links RB to Ras include SREBP-1,2. The characterization of Rb-Srebp-1 DKO mice revealed that RB status has a big impact on the control of fatty acid quality. We also identified molecules that explain differential self-renewal activity between p53^{-/-} cells and Rb-deficient p53^{-/-} cells (see above). These include enzymes involved in the glycolytic pathway.

Inflammation

RB status has been linked to pro-inflammatory phenotypes in breast cancer cells^[68]. This study highlighted COX2 as a target of the E2F transcription factor. However, our reassessment of these data revealed that many pro-inflammatory cytokines and chemokines, including IL-6, CCL2, and CCL5, are upregulated when breast cancers express lower levels of RB. A similar observation was made in mice lacking Rb in the back skin of p21^{-/-} mice^[134]. The tumors derived from these mice exhibited high levels of pro-inflammatory cytokines and evidence of infiltration by

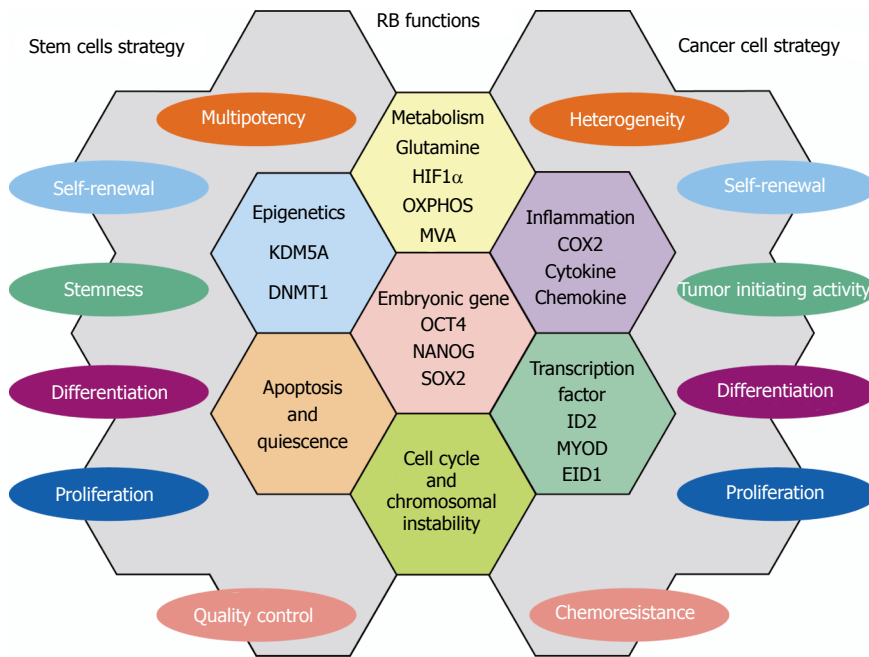


Figure 2 A schematic representation of molecular functions of retinoblastoma that may be shared by stem cells and cancer cells. RB: Retinoblastoma; MVA: Mevalonate.

immune cells. Indeed, many cytokines and chemokines, and even their receptors, are often regulated by E2F transcription factors^[135,136].

Among cytokines and chemokines, IL-6 and CCL2 are drawing attention as strong inducers of iPS cells. A study demonstrated that IL-6 is more potent than c-Myc in terms of their abilities to induce iPS cells^[137]. Another study demonstrated that CCL2 plays pivotal role in the maintenance of pluripotency in ES and iPS cells^[138]. There are many reports indicating that pro-inflammatory status is critical for cancer stem cells to evolve^[139-142]. Many of pro-inflammatory factors stimulates JAK/STAT3 pathway, thus contribute to enhance self-renewal of stem cells and possibly cancer stem cells. This could be one of core mechanisms that are shared by stem cells and cancer cells. Our recent efforts revealed that the RB status in soft tissue sarcoma, breast and prostate cancer cells significantly alter the pro-inflammatory status of these cells, and this significantly enhances self-renewal activity and chemo-resistance.

RB may control innate immunity as well. The RB-E2F1 complex appears to regulate toll-like receptor 3 (TLR3)^[143]. In *Drosophila*, RB, in cooperation with dCAP-D3, upregulates innate immunity^[144]. Several reports demonstrated that RB-deficiency in tumors attenuates the innate immune response, thereby promoting tumor development^[145]. There is also a report that showed a positive role for innate immunity in inducing iPS cells^[146].

CONCLUSION

In this manuscript, we described a part of numerous RB functions that are employed commonly as strategies to control stem cells and suppress cancer cells. These findings may help readers to understand how stem cell strategies are shared by cancer (stem) cells. Figure 2 demonstrates RB functions that may be shared by

stem cells and cancer cells. Although RB is one of tumor suppressors those have been characterized for long time and by big number of cancer researchers, our curiosity on its hidden roles in various biological events never fades away. In the near future, more number of stem cell and regenerative medicine researchers may stand up by this molecule. Although beyond the scope of this article, RB is implicated in the regeneration of many tissues/organs^[19]. Discovery of its target in the context of the undifferentiated state of cancer cells or in drug resistance may lead us to develop powerful tools in both cancer therapy and regenerative medicine.

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