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Review

Generation of patient-specific pluripotent stem cells and directed differentiation of embryonic stem cells for regenerative medicine

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Abstract

Embryonic stem(ES) cells are pluripotent cells that can give rise to derivatives of all three embryonic germ layers. Due to its characteristics, the patient-specific ES cells are of great potential for transplantation therapies. Several strategies can reprogramme somatic cells back to pluripotent stem cells: nuclear transfer, fusion with ES cells, treatment with cell extract and induction by specific factors. Considering the future clinical use, the differentiation from ES to neurons, cardiomyocytes and many other types of cells currently provide basic cognition and experience to regenerative medicine. This article will review two courses, the reprogramming of differentiated cells and the differentiation of ES cells to specific cell types.

Key words: stem cell; reprogramming; differentiation; regenerative medicine

INTRODUCTION

Stem cells are of great capability in regenerative medicine due to their characteristics of long-term proliferation and pluripotency. They are promising donor sources for cell transplantation therapies in diseases such as diabetes, Alzheimer's disease, Parkinson's disease, heart failure and so on. But the transplantation immunorejection holds this therapy back. Fortunately some strategies have already been figured out to get round this tough problem(Fig1). In summary, these strategies intended to solve this problem by reprogramming the patient's mature cell back into the pluripotent state. That is to generate patient-specific pluripotent stem cells by means of nuclear transfer, cellular fusion, the use of cell extracts and induction by defined factors.

As for the clinical use of ES cells, basic methodologies for large-scale cultures as well as numerous differentiation protocols have been developed that permit the generation of many tissue and cell types **in vitro** (Fig 2).

REPROGRAMMING OF DIFFERENTIATED CELLS

Reprogramming by nuclear transfer

It was once considered that the mammalian somatic cell did not have the potential to reverse to embryonic state until birth of Dolly[1]. So far a dozen kinds of animals were born from somatic cell nuclear transfer[2-15], even including those from terminal differentiated cells[16-18], so nuclear transfer was proved to be competent in reprogramming. Although the birth rate in most mammalian species studied is only 1%-5%, the establishment rate of embryonic stem cells(ESCs) from blastocysts by nuclear transfer(ntESCs) is much higher than that from animal cloning[19]. It is probably the inappropriate reprogramming in placenta that contributes to the abnormalities of cloned animals such as large offspring

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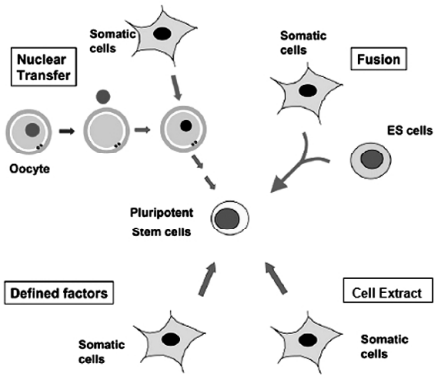

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Fig 1 Currently Available Methods to Generate Pluripotent Stem Cells from Adult Somatic

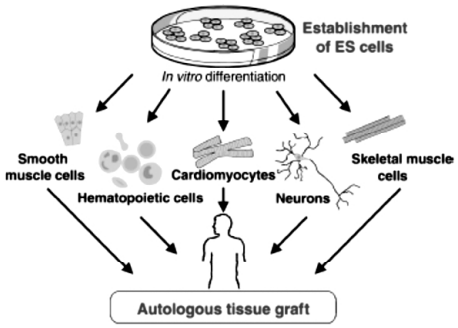

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Fig 2 ES cells differentiate to various cell types to generate autologous tissue graft for transplantation

syndrome, enlarged placental and perinatal death[20, 21]. What's more, biallelic expressions of X-linked genes in the placentas of dead clones were described[22]. Because the ntESCs are established from the inner cell mass (ICM) of the reconstructed blastocyst, it seems that the aberrant epigenetic modifications in trophectoderm will not affect ntES cell lines. Detailed molecular characterization and developmental potency of mouse ntESCs were examined. Wakayama *et al*[23] reported that the ntESC lines were identical to those derived from fertilized blastocysts in terms of pluripotency markers expression, DNA methylation regions and global gene expression profiles. Similarly, Brambrink *et al*[24] also found that mouse ntESCs derived from cloned and fertilized blastocysts were transcriptionally and functionally indistinguishable. In addition, ntESCs were able to differentiate *in vivo* into all functional embryonic tissue types and can give rise to pups via injection into blastocyst or the tetraploid placenta complementation method[25].

Accordingly it is proposed that the patient-specific embryonic stem cells can be generated from blastocysts cloned from the host's own cell nuclei, with the process termed "therapeutic cloning". This strategy holds great promise for the treatment of many human diseases[19]. One considerable issue for generating human ntESCs is the availability of human oocytes. Recently Dieter Egli *et al*[26] were able to reprogramme somatic cells using mouse zygotes temporarily arrested in mitosis, and produced embryonic stem cell lines from embryonic and somatic donor cells. Currently this method has only been demonstrated with mouse zygotes. However, it does raise the possibility that discarded human IVF zygotes and perhaps human embryonic blastomeres could potentially be used as recipients for human ntES cell derivation instead of oocytes with strict limitations.

Reprogramming by cell fusion

Miller and Ruddle demonstrated in 1976 that the fusion of pluripotent embryonic carcinoma cells(EC) with thymocytes resulted in the formation of pluripotent hybrids[27]. Tada *et al*[28] hybridized adult thymocytes with embryonic stem(ES) cells, resulting in teratomas formation. A recent report using hESCs also demonstrated that hESCs can reprogram the transcriptional state of somatic nuclei[29]. One technical barrier to this method is the removal of ES genome from the hybridized cells. Despite Tada and colleagues developed a system to remove selected chromosomes from hybrid cells[30]. It is still uncertain about the reprogramming state and immune compatibility if all of the ES chromosomes were removed.

Reprogramming by cell extracts

Cell-free system provides an alternative way to reprogramming. Taranger *et al*[31] reported that when permeabilized 293T cells were exposed to the extracts derived from undifferentiated embryonal carcinoma (EC) cells, they observed the formation of colonies with phenotypical organization of ESCs, as well as upregulation of pluripotency genes and downregulation of somatic genes. Lately they demonstrated by exposing 293T cells to EC cell extracts, reprogramming of DNA methylation and histone modifications occurred in the regulatory regions of Oct4 and Nanog[32]. It would be interesting to see whether similar reprogramming can be achieved with primary cells of adult individuals.

Another phenomenon of reprogramming was observed with *Xenopus* egg extracts. Human cells[33] or porcine fibroblast cells[34] treated with extracts from *Xenopus* laevis eggs began to express OCT4. But no stable reprogramming was seen in reversibly permeabilized somatic cells that were subsequently passaged in culture[33].

Reprogramming by Defined Factors

Successful reprogramming of somatic cells by nuclear transfer or fusion with ES cells indicates that unfertilized eggs and ES cells contain factors that can induce pluripotency. It is hypothesized that these factors play important roles in the maintenance of ES cell identity also play pivotal roles in the induction of pluripotency in somatic cells. Based on this hypothesis, 24 different candidate factors were examined for their ability to induce pluripotency[35]. The result demonstrated that retrovirus-mediated introduction of four transcription factors, Oct-3/4, Sox2, c-Myc, and KLF4, induced mouse embryonic or adult fibroblasts to reprogramme back to induced pluripotent stem(iPS) cells, which are similar to ES cells in morphology, proliferation, and teratoma formation. However, they are different with regards to gene expression and DNA methylation patterns, and fail to produce adult chimaeras. With a better pluripotent marker, Nanog or Oct4, a series of researches got significant improvement[36-38]. Three groups generated iPS cells competent for adult and germline chimeras, which proved the pluripotency of the iPS cell lines. Moreover, they detected the gene expression profiles and epigenetics state. Wernig *et al*[38] reported that global transcriptional profiles, chromatin configuration and demethylation tolerance of iPS cells were similar to that of ES cells. Maherali *et al*. showed reactivation of a somatically silenced X chromosome[36]. While at the same time, Okita *et al*[39] obseved that approximately 20% of the offspring developed tumours attributable to reactivation of the c-myc transgene[37]. Then the authors suggested screening for other factors to replace the four genes in order to apply iPS cells to regenerative medicine. In addition, considering that the transgenic method may hinder the human therapeutic use of iPS cells, a recent paper demonstrated that reprogrammed pluripotent cells can be isolated based upon morphological criteria.

Remarkable progress has achieved using specific factors in human induced pluripotent stem cells[40, 41], which is a significant turning point in nuclear reprogramming research with broad implications for generating patient-specific pluripotent stem cells for research and therapeutic applications[42].

MULTILINEAGE DIFFERENTIATION FROM ESCs

Differentiation of ESCs to neural cells

Differentiation of ESC to neuronal and glial cells is specifically important for cell therapies of neurodegenerative disorders. The differentiation of mES cells into neuronal cells was first reported in 1995[43-45]. Later alternative protocols were established. Okabe *et al*[46] conducted sequential culture of EBs in serum followed by serum-free medium. Tropepe *et al*[47] cultured ES at low density in serum-free medium in the presence of LIF, and generated a population that has been called primitive neural stem cells. Similarly Ying *et al*[48] demonstrated that up to 60% of the cells in monolayer cultures formed neuroectoderm in serum-free cultures using ES cells with GFP targeted to Sox1. Others differentiated ES cells directly on stromal cells in the absence of serum[49-50]. In addition, Wichterle *et al*[51] successfully generated cells that displayed many of the characteristics of motor neurons using the coculture approach together with the appropriate signaling molecules and selection steps. In a word each of the three major neural cell types of the central nervous system-neurons, astrocytes and oligodendrocytes, can be generated, and relatively pure populations of each can be isolated when cultured under appropriate conditions[46,50].

Several animal model researches provide indications for hES replacement therapy. Brustle *et al*[52] transplanted ES-derived neural cells into rats and demonstrated the incorporation of donor derived neurons, astrocytes, and oligodendrocytes into the brains of the recipient animals. Later the same group showed that ES derived oligodendrocytes could form myelin sheaths when transplanted into myelin-deficient rat model of multiple sclerosis[53]. Using nonhuman primates model, neurons generated from monkey ESCs were transplanted into 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated (MPTP-treated) monkeys. Behavioral studies and functional imaging revealed that the transplanted cells functioned as dopaminergic neurons and attenuated MPTP-induced neurological symptoms[54].

For the ultimate target of cell therapy in human neurodegenerative disease, three CNS cell types have been generated from hES cells using several different protocols[55,56], and the differentiation of hESC into highly enriched cultures of neuronal progenitors was achieved using a two-step protocol[57]. Other researchers have differentiated hESC lines H1 and H9 into neuroepithelial cells using a chemically defined adherent colony culture system[58]. Recently Zhu *et al*[59] showed the feasibility of labeling neural stem cells from humans, and this may facilitate further research and application. However it remains to be seen whether transplanted hES-derived neural cells can persist and function over long periods.

Differentiation of ESCs to keratinocytes

ES cells can give rise to epithelial cells that express markers of keratinocytes[60]. **In vitro** differentiation and enrichment of keratinocytes of murine ES cells were acquired by seeding on extracellular matrix(ECM) in the presence of Bone Morphogenic Protein-4(BMP-4) or ascorbate[61]. Moreover hESCs injected into scid mice

could generate keratinocytes and be analyzed by the successive appearance of markers[62]. A recent report showed immortalized keratinocytes by transduction with the E6E7 genes of HPV16[63].

Differentiation of ESCs to cardiomyocytes

As early as 1996 Klug *et al*[64] first demonstrated that murine ES cell-derived cardiomyocytes selected for α-cardiac myosin heavy chain(MHC) expression could incorporate and survive in the hearts of dystrophic mice. Nowadays more researches focus on the hES induction and clinical application. Cardiac muscle has been derived from either spontaneously differentiating hES cells or from co-culture systems. Kehat and colleagues[65] isolated beating cardiomyocyte foci from spontaneously differentiating human embryoid bodies. They showed that the cells had properties of fetal or neonatal cardiocytes due to the observation of the subcellular distribution of gap junctions, myofibrillar organization and electrical activity. ESC-derived cardiomyocytes can be enriched by selection markers including alpha-myosin heavy chain, cardiac troponins, atrial natriuretic factor as well as transcription factors typical of cardiomyocytes, Nkx2.5, GATA4 and MEF2[65-70]. In addition coculture methodologies have also been used to produce differentiated cardiomyocytes from hESCs[71,72].

The hESC-derived cardiomyocytes are capable of integrating apparently normally when transplanted into rodent and porcine heart muscle, forming gap junction connections between hESC myocytes or the recipient mouse adult cardiomyocytes[73-75]. Analysis of the animals indicated improved cardiac function in those transplanted with the ES cell-derived cells compared to controls that received cell-free media.

Although these researches advance the prospect of hESCs being used in the clinical treatment of cardiac infarcts, it is still unclear to what extent the improvement is due to the myocyte function of the cells rather than to indirect effects such as induced vascular development at the site of injection. Thus, basic human and nonhuman embryonic stem cell research should continue to aim at clinical replacement without ethical issues and cellular rejection.

Differentiation of ESCs to hematopoietic lineage

Several studies have documented hematopoietic development of hES cell either through coculture with mouse bone marrow stromal cells[76] or the generation of Ebs[77, 78]. The hematopoietic potential of hES cells was shown by B-cell markers expression[79]. Endothelial differentiation has also been demonstrated in hES cell differentiation cultures[80]. ES-cell derived vascular cells are also able to organize into vessel-like structures in EBs[81], in explant cultures[82], or cultured on collagen I[83].

Differentiation of ESCs to pancreatic-islet cells

Type 1 diabetes results from the autoimmune destruction of β-cells in pancreatic islets, and can be reversed by pancreatic or islet cell transplantation. Lumelsky and colleagues[84] used five-step protocol to differentiate mouse ESC into insulin-secreting structures similar to pancreatic islets. Their experimental strategy involves the transfer of serum-induced EBs to serum-free medium followed by treatment with FGF and factors that promote maturation of endocrine cells. These differentiated cells expressed low levels of insulin, glucagon, or somatostatin. However, transplantation of these insulin-producing cells into streptozotocin(STZ)-induced diabetic mice failed to correct the diabetic phenotype. With some modifications of the five-step protocol, islet-like clusters were produced from spontaneously differentiating hESCs[85]. Recently, Shi and co-workers have developed a three-step differentiation protocol based on the combination induction by activin A, all trans-retinoic acid and other factors. Transplantation of the insulin-producing cells was sufficient to normalize blood glucose levels in diabetic mice. Nevertheless tumor formation was observed in the kidney of some of the mice transplanted with the induced cells[86]. In order to enrich for β-cells from heterogeneous serum-induced cultures, a cell trapping system was developed for the differentiation of the mouse ESC into insulin-secreting cells[87, 88].

Although great progress have been achieved in generating cells containing insulin from ESC, the insulin staining could be artifactual and reflected insulin uptake from culture media[89-91]. So these findings highlight the need to substantiate insulin production in these differentiated cells by additional assays to confirm its endogenous production[92].

Differentiation of ESCs to hepatic cells

mES-derived hepatic cells show hepatic-restricted transcripts and proteins by a multistep protocol that included the addition of specific growth factors[93]. Hepatocyte like cells generated with this protocol were subsequently shown to contain albumin protein and produce urea and can successfully integrate and function in host liver following transplantation[94-97]. These data suggest that mES cells can differentiate into all three lineages of the liver(hepatocytes, bile duct epithelial and oval cells). Differentiation and isolation of hepatic-like cells from hES cells were demonstrated by treating with sodium butyrate or using the reporter gene EGFP fused to an albumin promoter[98-99]. This suggests that with the appropriate markers, it will be possible to select cells capable of forming liver, gut, and other endodermal tissues.

Differentiation of ESCs to alveolar lung

Denham M *et al*[100] cocultured mESC with embryonic day 11.5(E11.5) lung buds and observed that mESC derivatives could incorporate into the reforming of pseudoglandular-like tubular ducts, displaying pan-keratin and surfactant protein C(Sftpc) immunoreactivity. Later they reported that hESC derivatives induced with the same inductive nich formed pan-keratin-positive epithelial tubules at high frequency and detected human-specific SFTPC immunoreactivity[101]. These findings are encouraging for further research on the use of hESCs for a wide range of respiratory-specific diseases.

Differentiation of ESCs to other cells

Besides various lineages differentiated from ESCs above, cell populations representing the osteogenic[102-104], chrondrogenic[105], and adipogenic[106] lineages have been generated from ES cells too.

CONCLUSION

This review mainly focuses on the current available methods to generate pluripotent stem cells from adult somatic cells and the differentiation of ES cells to various kinds of cell types. Because each method has advantages and disadvantages comparing with others, as well as the molecular mechanisms underlying nuclear reprogramming and pluripotency being as yet still unclear, there is strong demand for detailed and in-depth research before clinical use. On the other hand, initial functional and morphological studies of ES cell derived neurons, cardiomyocytes, insulin-producing cells and other types of cells have been performed. The knowledge and experience from animal model will positively advance the development of tissue engineering.

Besides technological barriers, there are ethical concerns at the same time relating to the use of human volunteers in early clinical stem-cell-based research, stem cell quality control, safety and efficiency *in vivo*, etc. So integrity of scientific and ethical issues will be fostered.

In general, with further improvement in ESCs-based therapeutic strategies, the cure for diabetes, Parkinson's and Alzheimers diseases seems possible, and heart failure may be reversed or prevented by regenerative medicine. Undoubtedly more research will provide clinicians with powerful tools, from bench to bedside.

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