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ГЕННО-КЛЕТОЧНАЯ ТЕРАПИЯ ПЕЧЕНОЧНОЙ НЕДОСТАТОЧНОСТИ. ЭКСПЕРИМЕНТАЛЬНАЯ ЧАСТЬ 1

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Описан метод получения гепатоцитоподобных клеток из мультипотентных мезенхимальных стромальных клеток жировой ткани путем трансфекции последних вектором, несущим ген, кодирующий гепатоцитарный фактор роста.

Полученные гепатоцитоподобные клетки имеют морфологические и биохимические показатели, тождественные показателям гепатоцитов. Работа является первой частью исследований возможности генно-клеточной терапии тяжелых поражений печени.

INDUCED PLURIPOTENT STEM CELLS FROM ADULT HUMAN ADIPOSE STEM CELLS FREE OF VECTOR AND TRANSGENE SEQUENCES

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We show here a novel strategy to transfect human MMSC cells by using of the new type of episomal self-destructing vector system to produce induced pluripotent stem cells. The number iPS colonies and hence the number of reprogramming events was significantly higher for our new method (221.88+/-38.28 colonies, 2.24+/-0.77%.) when compared with the standard protocol (118.20 +/- 38.28 colonies, 1.14 +/- 0.77% p=0.97). MMSC iPS cells were demonstrated to express markers of pluripotency and could differentiate to the three germ layers, both in vitro and in vivo, to cells representative of the three germ lineages. Our findings confirm that MMSC are an ideal candidate as a readily accessible somatic cell type for high efficiency establishment of iPS cell lines, especially when using new "push pull" protocol.

Since the first publications, reporting the reprogramming of somatic cells to induced pluripotent stem cells (iPS cells) by the ectopic expression of the transcription factors, high expectations regarding their potential use for regenerative medicine have emerged. However, there are two major problems impeding the use of these cells in medicine. First of all, human iPS cell derivation required vectors that integrate into the genome, which can create mutations and limit the utility of the cells in both clinical and research applications. In addition, the effectiveness of the known methods of transfection are less than 0.5%.

Well-known attempts to use a nonintegrating episomal vector further reducing the efficiency of transfection, but remain the only alternative to vectors that integrate into the genome.

So further development of this direction requires the improvement of vectors, transfection methods and search for more suitable cells for reprogramming in the IPS/

To solve these problems we developed an approach assumes the derivation of human iPS cells from adipose stem cells which presumably have a greater potency for the reprogramming. After self-destruction of the new type of episomal vector, iPS cells are completely free of vector and transgene sequences and are similar to human embryonic stem (ES) cells in proliferative and developmental potential. These results removes one obstacle to the clinical application of human iPS cells. Furthermore, the transfection of multipotent mesenchymal stromal cells using a new developed "push-pull" method allowed to raise the efficiency of transfection up to 2%.

Materials and methods

Cell culture. Human MMS cells and iPS cells were maintained in DMEM/F12 culture medium supplemented with 20% knockout serum replacer, 0.1 mM non-essential amino acids (all from Sigma, USA), 1 mM L-glutamine, 0.1 mM ß-mercaptoethanol and 100 ng/ml zebrafish basic fibroblast growth factor (zbFGF). The feeder-free culture on matrigel (BD Biosciences, MA) with conditioned medium was carried out as previously described [11] except with 100 ng/ml zbFGF.

Construction of expression vectors. The cDNAs for the open reading frames (ORFs) of human *OCT4*, *SOX2*, *NANOG*, *LIN28*, *c-Myc*, and *KLF4* genes were obtained by direct PCR of human ES cell cDNA. The cDNA for the SV40 large T (*SV40 LT*) antigen ORF was obtained from plasmid pBABE-puro SV40 LT (plasmid 13970, Addgene, Cambridge, MA), and the cDNA for the *hTERT* gene ORF was cloned from plasmid pBABE-hygro-hTERT (plasmid 1773, Addgene Cambridge, MA). The internal ribosome entry site 1 (IRES1) was derived from plasmid pIRESpuro3 (Clontech, Mountain View, CA), while IRES2 was obtained from plasmid pIRES2EGFP (Clontech).

The foot-and-mouth disease virus-derived F2A peptide coding sequence (5′ ggttccggaaaaattgtcgctcctgtcaaacaaactcttaactttgatttactcaaactggctggggatgtagaaagcaatccaggtcca 3′) was chemically synthesized and placed in frame with the upstream *OCT4* ORF (no stop codon) and the downstream *SOX2* ORF via PCR. Transgenes were cloned into a 1 modified lentiviral vector *(S5)* or the oriP/EBNA1-based pCEP4 episomal vector (Invitrogen) for reprogramming

Reprogramming human somatic cells. For reprogramming with oriP/EBNA1-based episomal vectors, episomal plasmids were cotransfected into cells in serum-free DMEM.

After incubation for 5 h at 37 ° C in a CO2 incubator, transfection mixture was removed and replaced with normal medium. After 18 hours of incubation, the transfection procedure was repeated. Total this cycle (push-pull), repeated three times, thus improving the efficiency of transfection (Fig 1).

Transfected MMSC (~$1.0*10^5$ cells per transfection) were directly plated to $3*10^{-cm}$ MEF-seeded dishes in culture medium. Culture medium was exchanged every other day. On day 4 posttransfection, the culture medium was replaced with human ES cell culture medium. Human ES cell culture medium conditioned with MEFs (CM) was used to sustain the reprogramming culture at 8 to 10 days after plating when the MEFs began to deteriorate. Colonies with morphology similar to iPS colonies were readily visible on day 18 posttransfection . To examine the presence or absence of human iPS colonies, one of the three 10^{-cm} dishes of reprogramming culture was stained with alkaline phosphatase (Millipore, Billerica, MA) between day 18 and 20 posttransfection. Due to the presence of many non-iPS colonies with morphology similar to human iPS colonies, the remaining two 10^{-cm} dishes of reprogramming culture were passaged between day 25 and 30 posttransfection to fresh 10^{-cm} MEF dishes at ratio of 1:3 with collagenase (1 mg/ml, Sigma) treatment. Human iPS colonies with morphology highly similar to human ES cells could be readily discernible from non-iPS colonies upon passaging, and were individually picked for expansion and analysis.

PCR analysis of episomal vectors. Purification of episomal DNA from human iPS cells and MMSC was carried out with QIAprep Spin Miniprep Kit (Qiagen) and Proteinase K (Sigma, MA) digestion. Genomic DNA was isolated using the traditional phenol/chloroform extraction method. Due to the nature of purification methods, the purified genomic DNA was likely contaminated with residual amount of episomal DNA from the same cells, and likewise, the purified episomal DNA was contaminated with small amount of genomic DNA. All the PCR reactions were carried out with pfx DNA polymerase. For each PCR reaction, ~0.1 µg genomic DNA or episomal DNA from $1 * 10^4$ cells equivalent was added as template. Genomic DNA and episomal DNA from nontransfected cells were used as negative controls

Teratoma formation. To examine the developmental potential of human iPS cells generated with episomal vectors *in vivo*, iPS cells grown on MEFs were collected by collagenase treatment, and injected into hind limb muscles of 6-week-old immunocompromised SCID-beige mice (approximately one 10-cm dish with 50 to 80% confluency per mouse) (Charles River Laboratory, Wilmington, MA). After five to ten weeks, teratomas were dissected and fixed in 4% paraformaldehyde. Samples were embedded in paraffin and processed with hematoxylin and eosin staining.

Experimental procedures

Reprogramming efficiencies with current nonintegrating methods are several orders of magnitude lower (0.001%) than those achieved with integrating vectors (0.1%–1%), most likely because factor expression is not maintained for a sufficient length of time to allow complete epigenetic remodeling. Derived from the Epstein-Barr virus, oriP/EBNA1 vectors are well suited for introducing reprogramming factors into human somatic cells, as these plasmids can be transfected without the need for viral packaging and can be subsequently removed from cells by culturing in the absence of drug selection. The stable extrachromosomal replication of oriP/EBNA1 vectors in mammalian cells requires only a cis-acting oriP element [3] and a trans-acting EBNA1 gene [10]. The oriP/EBNA1 vectors replicate only once per cell cycle, and with drug selection can be established as stable episomes in about 1% of the initial transfected cells [6, 7]. If drug selection is subsequently removed, the episomes are lost at ~5% per cell generation owing to defects in plasmid synthesis and partitioning; thus, cells devoid of plasmids can be easily isolated. OCT4, SOX2, NANOG, and LIN28 are sufficient to reprogram human embryonic, neonatal, and adult fibroblasts to iPS cells [2, 4, 9], but the reprogramming efficiency is low (<0.01% for newborn foreskin fibroblasts) [4]. Such low efficiency makes it difficult to reprogram with oriP/EBNA1-based vectors because the stable transfection efficiency is almost two orders of magnitude less than that of our lentiviral vectors.

We found that the internal ribosome entry site 2 (IRES2) supported higher reprogramming efficiency., IRES2 was chosen to coexpress reprogramming factors because linkers have less effect on reprogramming efficiency when used to coexpress *NANOG* and *LIN28*. Using IRES2-mediated expression of OCT4, SOX2, NANOG, and LIN28, we improved the reprogramming efficiency for adult human adipose stem by about 10-fold (~0.1%) over what we had previously reported. The addition of c-Myc and KLF4 further improved the reprogramming efficiency to more than 1%, the highest efficiencies we have achieved for these cells.

Thus, we cloned all six reprogramming factors (*OCT4*, *SOX2*, *NANOG*, *LIN28*, c-*Myc*, and *KLF4*) into an oriP/EBNA1 vector using IRES2 for coexpression.

With oriP/EBNA1-based episomal vectors, exogenous DNA is not integrated into the human iPS cell genome, and owing to the gradual loss of cellular episomal vectors in the absence of drug selection, vector- and transgene-free human iPS cells can be isolated through subcloning without further genetic manipulation. Similar to mouse studies based on nonintegrating reprogramming methods [9], the current reprogramming efficiency of MMSC with oriP/EBNA1 vectors is low (about three to six colonies per 10^6 input cells). These frequencies are, however, sufficient to recover iPS cells from a reasonable number of starting cells, and MMSC are easy to obtain and culture.

To avoid this issue, we have developed a "push-pull" method, consists of several cycles of transfection with the accumulation of transfected cells.

We isolated MMSC from healthy volunteers as described previously [1]. While transduction efficiencies were not significantly different among the different MMS cell lines (approximately 71.0+/-5.4%), the number iPS colonies and hence the number of reprogramming events was significantly higher for our new method (221.88+/-38.28 colonies, 2.24+/-0.77%. Fig 1) when compared with the standard protocol (118.20 +/- 38.28 colonies, 1.14 +/- 0.77% p=0.97). MMSC iPS cells were demonstrated to express markers of pluripotency and could differentiate to the three germ layers, both in vitro and in vivo, to cells representative of the three germ lineages. Our findings confirm that MMSC are an ideal candidate as a readily accessible somatic cell type for high efficiency establishment of iPS cell lines, especially when using new "push pull" protocol.

Thus, we got a good material for the development of tissue engineering designs and use in the clinic.

This work was performed under MT Agreement from 03.15.2010

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ИНДУЦИРОВАННЫЕ ПЛЮРИПОТЕНТНЫЕ КЛЕТКИ, ПОЛУЧЕННЫЕ ИЗ СТВОЛОВЫХ КЛЕТОК ЖИРОВОЙ ТКАНИ, СВОБОДНЫЕ ОТ СЛЕДОВ ВЕКТОРА И ТРАНСГЕННЫХ ПОСЛЕДОВАТЕЛЬНОСТЕЙ

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Представлена новая стратегия получения индуцированных плюрипотентных стволовых клеток (иПК) путем трансфекции мультипотентных мезенхимальных стромальных клеток человека (ММСК) с использованием эписомного саморазрушающегося вектора. Число колоний иПК и, следовательно, число событий перепрограммирования была значительно выше для нового метода (221,88+/-38,28 колоний, 2,24+/-0,77%.) по сравнению со стандартным протоколом (118,20+/-38,28 колоний, 1,14+/-0,77% p=0,97). Было продемонстрировано что полученные иПК репрезентируют маркеры плюрипотентности и могут дифференцироваться в три зародышевых листка как in vitro так и in vivo и свободны от вектора или трансгенных последовательностей. Полученные данные подтверждают, что ММСК являются идеальным кандидатом для создания иКП с высокой степенью эффективности.

ТОПИЧЕСКИЕ СТЕРОИДЫ И АНТИГИСТАМИННЫЕ ПРЕПАРАТЫ В ЛЕЧЕНИИ АЛЛЕРГИЧЕСКОГО РИНИТА ПРИ БЕРЕМЕННОСТИ

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Беременность - это особое состояние организма женщины, при котором многие физиологические процессы изменяют свое течение. Сдвиги в метаболизме, эндокринной, нервной и иммунной системе направлены на создание гармоничных взаимоотношений между организмом матери и организмом развивающегося плода. Факторы внешней среды, развитие патологических процессов, а также лекарственные вещества, попадающие в организм, могут оказывать токсическое действие на развивающийся плод.

Ежегодный рост заболеваемости аллергическим ринитом (АР) приводит к тому, что все больше и больше беременных пациенток обращаются за помощью к специалистам-оториноларингологам. Несмотря на возрастающее количество пациентов, до сих пор не существует стандартизированного документа, регламентирующего лечение аллергического ринита у беременных, и врач амбулаторного звена встает перед сложным выбором: что назначить такой пациентке для купирования симптомов АР и при этом не оказать нежелательные влияния на плод? Симптомы АР безусловно пагубно сказываются на развитии беременности. Так, затруднение носового дыхания может приводить к снижению газообмена плода, обострению хронических гнойных заболеваний ЛОР органов ассоциированных с АР у матери, это в свою очередь приводит к необходимости лечения с применением антибактериальной терапии, пагубно сказывающийся на развивающегося в утробе ребенка. Поэтому глубоко ошибочным оказывается мнение пациентов и даже некоторых врачей о том, что при беременности следует отказаться от лечения АР и прекратить прием лекарственных препаратов вследствие их возможных побочных эффектов.

Исследования эффективности терапии АР у беременных довольно редки из-за особой этической стороны данного вопроса, а в доступной литературе вопрос лечения АР у данной категории больных освещен весьма скудно.

Поэтому нам показалось актуальным в этом обзоре рассмотреть некоторые аспекты терапии АР у беременных.

Согласно клиническим рекомендациям по диагностике и лечению аллергического ринита (СПб 2004) в настоящее время существует три основных нехирургических метода лечения АР: элиминация аллергенов, медикаментозная терапия и специфическая иммунотерапия (СИТ).

Элиминация аллергенов является довольно сложной задачей. Во-первых, далеко не всегда удается выявить причинно значимый аллерген. Во-вторых, даже такое радикальное мероприятие как, например, удаление животного при аллергии к эпидермису и тщательная уборка в помещении в некоторых случаях являются недостаточными для