Human leukemia inhibitory factor produced by the ExpressTec method from rice (*Oryza sativa* L.) is active in human neural stem cells and mouse induced pluripotent stem cells

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Stem cell-based therapy has the potential to treat an array of human diseases. However, to study the therapeutic potential and safety of these cells, a scalable cell culture medium is needed that is free of human or bovine-derived serum proteins. Thus, cost-effective recombinant serum proteins and cytokines are needed to produce such mediums. One such cytokine, leukemia inhibitory factor (LIF), has been shown to be a critical paracrine factor that maintains stem cell pluripotency in murine embryonic stem cells and human naïve stem cells while simultaneously inhibiting differentiation. We recently produced recombinant human LIF (rhLIF) in a rice-based protein expression system known as ExpressTec.[12] We described expression of rice-derived rhLIF and demonstrated its biological equivalency to *E. coli*-derived rhLIF in traditional and embryonic mouse stem cell systems. Here we describe the expression yield of rice-derived rhLIF and the scale up production capacity. We provide further evidence of the efficacy of rice-derived rhLIF in additional stem cell systems including human neural stem cells and mouse induced pluripotent stem (iPS) cells. The expression level, biological activity, and potential for production at commercial scale of rice-derived rhLIF provides a proof-of-principal for ExpressTec-derived proteins to produce regulatory-friendly, high performance, and dependable stem cell media.

Introduction

The vast therapeutic potential of stem cells has come into the forefront of regenerative and cell-based medicine. Administration of various human pluripotent or more differentiated multipotent stem cell types has shown therapeutic benefit in a variety of animal models of debilitating diseases in the past decade.[1,2] The promising results attained from these in vivo studies has fueled enthusiasm for stem cell therapy and thus widened the scope of application in human disease.[3] As the rate of translation of these therapeutic cells from the laboratory to the clinic is expected to increase in the coming years, major scientific and regulatory challenges exist and must be addressed in order to both facilitate the "bench to bedside" process of this nascent technology as well as enhance safety of the final cell product.[4]

One such challenge is the expansion of a homogeneous, self-renewing population of stem cells in a regulatory-friendly, economically feasible, and scalable cell culture medium.[5,6] Protein supplementation, such as with serum albumin and transferrin required to maintain adequate cell proliferation and high viability in vitro, has traditionally been met using bovine-derived serum or isolated proteins. However, the use in clinical settings necessitates minimization of these reagents for safety and regulatory reasons. Proteins isolated from human serum represent a useful strategy to replace

bovine-derived components. Nevertheless, the risk of transmitting new or emerging blood-borne pathogens to the recipient patients still exists.[7] Further, unpredictable integrity of starting material as well as minute differences in purification protocols can produce serum protein preparations of varying qualities, thus bringing the requirement of reproducibly high cell performance into question.

Use of recombinant versions of vital serum proteins minimizes the risk of adventitious agent contamination. However, it proves costly due to the combination of relatively high protein concentrations required in medium formulations,[8] the cost of the recombinant protein,[9] and the volumes of growth medium needed to produce a clinically relevant doses of stem cells per patient.[10] Thus, current mainstream platforms for the production of recombinant proteins, although suitable for producing regulatory-friendly human proteins with consistent performance, lack the economical sustainability for therapeutic stem cell expansion.

ExpressTec, a proprietary rice- (*Oryza sativa* L.)-based expression system developed by Ventria Bioscience Inc, has the ability to produce large complex glycoproteins that are completely free of human or animal pathogens or contaminating byproducts such as endotoxin. Due to the unlimited production scale, high recombinant protein expression yield, and low operational costs, ExpressTec has been shown to produce some of the most cost-effective recombinant proteins on the market.[11]

We recently used this expression system to produce recombinant human leukemia inhibitory factor (LIF), an IL-6 cytokine family factor required in a variety of human and mouse stem cell systems.[12] LIF signaling, via gp130/STAT3-dependent signaling, enhances stem cell state regulator transcription and simultaneously inhibits cell differentiation.[13,14] Removal or decreased LIF concentrations can induce rapid cessation of growth and subsequent differentiation in LIF-dependent stem cells.[15] LIF reactivity across species has been well-characterized, as human LIF will bind the mouse LIF receptor.[16] Thus,

by producing the human isoform, we would be able to utilize this rice-derived human recombinant LIF (rhLIF) in the highly LIF-sensitive mouse embryonic stem (ESC) cell and induced pluripotent stem (iPS) cell systems,[17,18] LIF-dependent naïve human stem cells,[19,20] and other human stem cell systems that are responsive to LIF supplementation.[21-24]

Rice-derived rhLIF was highly active as determined by a modified M1 cell differentiation assay and was sufficient in retaining pluripotency of C57BL/6 N Lex3.13 mouse ESCs through multiple subcultures.[12] Here, we provide further evidence of the utility of our rice-derived rhLIF and provide insight into the expression level and the subsequent scalability of the ExpressTec system. Further, we expand the application of the rice-derived rhLIF to mouse iPS cells as well as human neural stem cells. High expression of biologically active recombinant proteins combined with the low cost of operation and virtually limitless scale has the potential to enable high quality animal component-free stem cell medium components with reliable and reproducible performance. Thus, inclusion of these ExpressTec-produced proteins can improve the affordability of new stem cell therapies and facilitate the translation of this promising technology to clinical practice.

High Level Expression of hLIF in Rice Grain

Recently, we reported the successful expression of human LIF protein in rice using a seed endosperm cell-specific promoter and a synthetic gene sequence encoding the hLIF protein with codons biased toward the rice proteome.[12] We showed that this rice-derived rhLIF was extensively glycosylated as evidenced by a smear region ranging from 19–50 kDa via western blot of protein extracts. The purified rice-derived rhLIF showed apparent molecular weights from 20–30 kDa after a purification process comprised of ammonium sulfate precipitation and concanavalin A chromatography.[12] The heterogeneity of rice-derived rhLIF was likely due

to glycosylation, since the amino acid sequence of hLIF has five potential N-glycosylation sites, and higher plants have the similar post-translational modification systems as mammalian cells.[12,25]

In order to determine the expression level of rhLIF protein in rice seeds, we first examined the extractability of rhLIF under different pH conditions (**Fig. 1**). Leveraging the high isoelectric point (pI) of human LIF, we extracted protein from rice seeds expressing rhLIF with low pH buffers (0.1 M NaOAc buffer at pH 4 and 5) as well as PBS buffer, pH 7.4. One hundred mg each of milled rice seed flour was extracted in 1 ml of different extraction buffers for 30 min at room temperature, followed by clarification. The total amount of soluble proteins (TSP) extracted in different buffers was determined using Coomassie (Bradford) Protein Assay kit (Thermo Scientific). Under each extraction condition, the transgenic rice seeds showed a smear region between 20 and 37 kDa, which is absent in non-transgenic rice seeds (**Fig. 1A**). This smear region of proteins was confirmed to be recombinant hLIF by western blot probed with an anti-hLIF antibody (**Fig. 1B**). These extractions also demonstrated that the higher pH of the extraction buffer extracted greater levels of rice host cell proteins. For example, the PBS buffer, pH 7.4 and 0.1 M NaOAc buffer, pH 4.0 extracted 546 μg, and 215 μg TSPs per 0.1 g of seed flour, respectively (data not shown). The different solubility and extractability of rhLIF and rice host cell proteins under acetic condition make rhLIF clearly visible, despite reduced total protein. In contrast, in PBS buffer at pH 7.4, many endogenous rice proteins are soluble and extractable, masking visible rhLIF. The large difference of TSPs from different extraction conditions can significantly impact the estimation of rhLIF expression in reference to TSPs.

The visible rhLIF proteins shown on a Coomassie blue-stained SDS-PAGE gel indicated that the expression level of rhLIF was relatively high (**Fig. 1A**). To provide further insight, we determined the expression level of rhLIF in rice grain by immuno-dot-blot (**Fig. 2**). Equal volumes
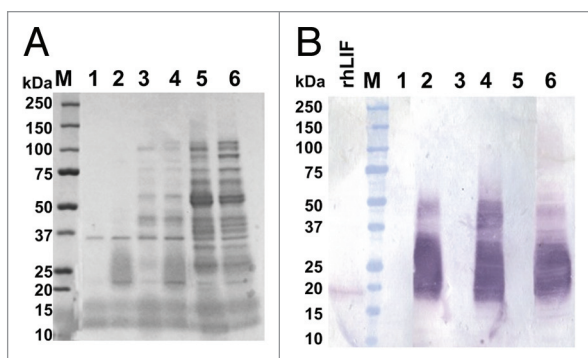
Figure 1. SDS-PAGE and western blot analyses of rice crude protein extracts in different buffers. (**A**) Coomassie blue-stained SDS-PAGE gel. Total soluble proteins were extracted from rice flour of transgenic lines expressing rhLIF and non-transgenic line Bengal with different buffers at a 1:10 ratio (g/ml) of buffer to rice flour. Total soluble proteins without any protein concentration, enrichment or purification were resolved on 4–20% Tris-glycine SDS-PAGE gels (Life Technologies). M, Molecular weight standard; Lanes 1, 3, and 5 are non-transgenic Bengal seed proteins extracted with 0.1 M NaOAc buffer pH 4, 5, and PBS buffer, pH 7.4, respectively; Lanes 2, 4, and 6 are transgenic rice seed proteins containing rhLIF extracted with 0.1 M NaOAc buffer pH 4, 5, and PBS buffer, pH 7.4, respectively. (**B**) western blot immuno-detection of anti-hLIF antibody. M, Molecular weight standard; rhLIF, 50 ng of purified *E. coli*-derived rhLIF protein (Millipore); Lanes 1, 3, and 5 are non-transgenic rice seed proteins extracted with 0.1 M NaOAc at pH 4.0, 0.1M NaOAc at pH 5.0, and PBS, pH 7.4, respectively; Lanes 2, 4, and 6 are transgenic rice seed proteins containing rhLIF extracted with 0.1 M NaOAc buffer pH 4, 0.1 M NaOAc at pH 5.0, and PBS buffer, pH 7.4, respectively.
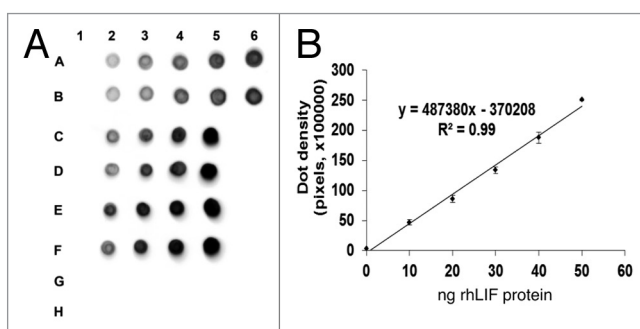
Figure 2. Expression level determination of rhLIF in rice seeds by immune dot-blots. (**A**) Immuno-dot-blot of crude protein extracts of transgenic rice seeds expressing rhLIF and purified standard rhLIF protein. Dots A1-A6 and B1-B6, duplicate of purified rhLIF protein standards at 0, 10, 20, 30, 40, and 50 ng, respectively; dots C1-C5 and D1-D5, duplicate of 0, 1/20 diluted, 1/10 diluted, 1/5 diluted, and non-diluted crude protein extracts in PBS buffer, pH 7.4 of transgenic rice seeds, respectively; dots E1–E5 and F1–F5, duplicate of 0, 1/20 diluted, 1/10 diluted, 1/5 diluted, and non-diluted crude protein extracts in 0.1M NaOAc buffer, pH 4.0 of transgenic rice seeds, respectively; dots G1–G5, 0, 1/20 diluted, 1/10 diluted, 1/5 diluted, and non-diluted crude protein extracts in PBS buffer, pH 7.4 of non-transgenic rice seeds, respectively; dots H1–H5, 0, 1/20 diluted, 1/10 diluted, 1/5 diluted, and non-diluted crude protein extracts in 0.1 M NaOAc buffer, pH 4.0 of non-transgenic rice seeds, respectively. (**B**) Standard curve of quantity of purified rhLIF and immune dot densitometry. A standard curve of rhLIF derived from *E. coli* (dots A1-A6 and B1-B6) was used to calculate rhLIF expression in rice grain.

from serial dilutions of 1, 1/5, 1/10, and 1/20 of protein extracts extracted with PBS buffer, pH 7.4 or 0.1 M NaOAc buffer, pH 4.0 were spotted onto nitrocellulose membranes along with known amounts of purified *E. coli*-derived rhLIF (Millipore). The membrane was blocked and probed for rhLIF as described previously.[12]

Densitometry of 1/10 and 1/20 diluted protein extracts were in line with the range of standard proteins (**Fig. 2A**), and the dot intensity of these samples was compared against the standard curve produced from the purified rhLIF (**Fig. 2B**) to determine rhLIF expression level. It was calculated that the PBS buffer, pH 7.4 and 0.1 M

NaOAc buffer, pH 4.0 extracted 103 μg and 66 μg of rhLIF from 100 mg of rice seed flour, respectively (**Fig. 2**). Thus, the amount of rhLIF proteins extracted in PBS buffer, pH 7.4 was 56% more than that extracted in 0.1 M NaOAc, pH 4.0, estimating expression level as a percent of TSP of 18.8% and 30.6%, respectively. These results indicated the measurement of recombinant protein expression level in reference to total soluble proteins is biased toward the extraction buffers. Therefore, we choose to determine the rhLIF expression level based on the biomass, which is more consistent than the TSP-based approach.[26] With the estimated amount of rhLIF in PBS buffer extraction, we calculated the expression level of rhLIF protein in rice seed to be 0.103 ± 0.009% of dry seed weight or about 1.03 g of rhLIF in 1 kg of dry rice seeds. This expression level of rhLIF is 10-fold higher than the suggested critical limit of plant-derived recombinant protein expression level for commercial viability (0.01% mass weight[27]).

Potent in Vitro Activity of rhLIF on Mouse and Human Stem Cell Systems

Our previous study demonstrated that the biological activity of the rhLIF protein was equivalent in a modified M1 cell proliferation inhibition assay as well as in the C57BL/6 N Lex3.13 mouse embryonic stem cell system.12 To provide further evidence of application of the rhLIF in stem cell systems, we tested the ability of rice-derived rhLIF to maintain pluripotency in mouse iPS cells. Like mouse ESCs, iPS cells require the presence of LIF to maintain the stem cell state in culture. Mouse embryonic fibroblast-derived iPS cells were cultured either in the presence of rice-derived rhLIF, *E. coli*-derived rhLIF, or without LIF for multiple passages (**Fig. 3**). We examined the retention of pluripotency by determining mRNA expression levels of the transcription factors, *Pou5f1* (Oct4), *Nanog*, and *Zfp42* (Rex1) on passage 5. Mouse iPS cells cultured in the presence of either LIF proteins showed significantly higher expression of the transcription

factors than the cells grown without LIF (**Fig. 3A**). Consistent with mouse ESCs,[12] the rice-derived rhLIF was statistically indistinguishable from the *E. coli*-derived rhLIF in the expression of mRNAs encoding the stem cell state regulators in mouse iPS cells (**Fig. 3A**). These results indicate that mouse iPS cells cultured in rice-derived rhLIF maintain pluripotency to the same extent as currently marketed *E. coli*-derived rhLIF.

Similarly, human neural stem cells (NSCs) have been shown to be responsive to LIF supplementation in that LIF supplementation induced neural progenitor cell proliferation and survival.[23] We therefore exposed H9 embryonic stem cell (ESC)-derived neural stem cells to 10 ng/mL LIF for 96 h in order to compare rhLIF to human LIF produced in *E. coli*. When cultured in DMEM/F12 supplemented with 2 mM Glutamax, 1% N-2, 10 ng/mL bFGF and EGF, H9-derived NSCs exhibited a doubling time of 58.97 ± 4.43 h (**Fig. 3B**). However, addition of human LIF, produced either in *E. coli* or rice, exhibited a significant 41% ($P = 0.0006$) and 43% ($P = 0.00006$) reduction in doubling time to 35.63 and 34.75 h, respectively.

Low Cost and Scale-Up Capability of Producing Rice-Derived rhLIF and other Proteins

Given the reported successes of stem cell-based therapies in animal models, it is anticipated that increasing numbers of stem cell based therapies will enter clinical testing in the near future. Thus, large quantities of recombinant proteins such as human LIF will be in demand for the expansion of these clinical-grade therapeutic cells. With our high expression level of rhLIF in rice (about 1 g per kg of rice seeds), in combination with the inherent extremely low cost of production, we are able to scale-up the production of rhLIF in a cost-sensitive manner. We have developed the genetically stable homozygous transgenic line at the R4 generation with over 400 kg of seeds. Furthermore, we have demonstrated the ease and long storage times (over two years) of rice grain
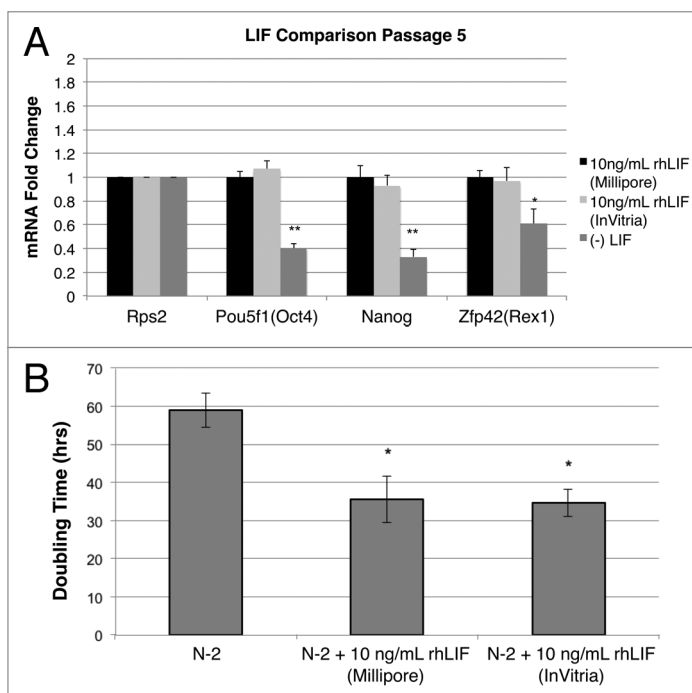

Figure 3. rhLIF efficacy in mouse iPS cells and human neural stem cells. (**A**) Expression of pluripotency regulators in mouse iPS cells. Mouse primary iPS cells-WP5 (Stemgent) were maintained on 0.1% gelatin-coated 6-well plates without feeder cells in Embryo Max DMEM (Millipore) supplemented with 15% ESC-qualified fetal bovine serum (Hyclone/Thermo), 2 mM L-glutamine (Gibco), 0.1 mM β-mercaptoethanol (Sigma), 0.1 mM MEM non-essential amino acid stock (Gibco), and 1000U mouse leukemia inhibitory factor (LIF, Millipore, ESG1106). qRT-PCR analysis of *Pou5f1* (Oct4), *Nanog*, and *Zfp42* (Rex1) on passage 5 in mouse iPS cells cultured in either *E. coli*-derived rhLIF, rice-derived rhLIF, or without LIF. Mouse iPS cells cultured in either *E. coli* or rice-derived LIF are indistinguishable in mRNA expression of pluripotency regulators (*$P < 0.05$; **$P < 0.01$). (**B**) Doubling time of human neural stem cells cultured in rhLIF. H9-embryonic-derived neural stem cells were maintained in DMEM/F12, 2 mM Glutamax, 25 ng/mL bFGF and 1% N-2 (Life Technologies) on poly-L-ornithine/laminin-coated (Sigma Aldrich) plates. Cells were plated at a starting density of $0.1–0.2 \times 10^5$ viable cells/cm^2 in either N-2 growth medium or N-2 growth medium supplemented with 10 ng/mL *E. coli* or rice-derived rhLIF for 96 h in 48-well plates coated with the same attachment matrix. Cells were harvested by exposure to TrypLE with 1 mM EDTA in DPBS and cells were counted. Doubling time of cells was calculated using the formula LN(2)/μ where μ is equal to LN (final cell density/initial cell density)/1/time (in hrs). Addition of either *E. coli* or rice-derived rhLIF induced a significant increase in viable cell density after 96 h in H9-derived NSCs ($P = 0.0005$ and 0.00006, respectively). Data presented is cumulative from 3 experiments, 8 replicates per experiment. Error bars represent standard deviation between assays.

expressing recombinant proteins.[28] These storage conditions eliminate the need for immediate downstream processing and guarantee continuous supply to the market.

Similar to the high level expression of rhLIF in rice grains, Ventria Bioscience has expressed other recombinant proteins including human lysozyme, lactoferrin, serum albumin, and transferrin at the expression level from 0.1–1.5% of dry seed weight or 1–15 g of recombinant protein per kg of dry seeds.[26,28-30] The high level expression of these recombinant proteins are achieved through the exploitation of

advanced genetic strategies, including the use of a strong rice seed endosperm-specific promoter, transcriptional factors and enhancers, codon optimization of heterologous genes, and compartmentalizing recombinant proteins into specialized endosperm protein bodies through intracellular trafficking and/or targeting.

Conclusions

Translation of stem cell therapy to the clinic will necessitate the generation

of scalable, high performance, and economically feasible animal component-free media. Recombinant proteins used to generate these products must be readily available in kilogram quantities, highly active, pure, and affordable. Using the rhLIF protein as a proof-of-principle, we provide justification for inclusion of ExpressTec-derived recombinant proteins in stem cell culture medium. Our studies described here indicate that the rhLIF expression in rice seed is very high. Analysis of the rice-derived rhLIF demonstrates an expression level of 0.1% of dry seed weight. Because of the scaling potential of ExpressTec, we have the capability to produce kilograms of rice-derived rhLIF at a fraction of the cost of *E. coli*-driven production. Further, we demonstrate equivalency in biological activity in a variety of stem cell systems of both human and mouse origin in comparison to the *E. coli*-derived rhLIF. Thus ExpressTec recombinant human LIF can be produced more cheaply at scale than *E. coli*-derived LIF, is animal

product-free, and is endotoxin-free. These advantages make this product compelling for cost-effective studies of stem cells in support of cell-based therapies.

In addition to encouraging the results obtained from rhLIF, we have also demonstrated biological equivalency of ExpressTec-derived human transferrin,[9] albumin, and lactoferrin[31] to the native versions. These components can be combined into optimized mixtures to produce animal component-free serum replacements that have the ability to expand stem cells in the undifferentiated state with the high fidelity expected of clinical cell preparations. The cost effective and large scale source of ExpressTec-derived recombinant proteins enables the feasibility to provide volumes of defined media that will be required by cell therapies. Thus inclusion of ExpressTec-derived proteins in stem cell mediums can enhance the safety, affordability, and reliability of novel stem cell therapies and potentially facilitate the translation of these new therapies to

first line treatments for many devastating diseases.

Disclosure of Potential Conflicts of Interest

Acknowledgments

References

1. Xu XL, Yi F, Pan HZ, Duan SL, Ding ZC, Yuan GH, Qu J, Zhang HC, Liu GH. Progress and prospects in stem cell therapy. Acta Pharmacol Sin 2013; 34:741-6; PMID:23736002; http://dx.doi.org/10.1038/aps.2013.77

2. Inoue H, Nagata N, Kurokawa H, Yamanaka S. iPS cells: a game changer for future medicine. EMBO J 2014; 33:409-17; PMID:24500035; http://dx.doi.org/10.1002/embj.201387098

3. Harding J, Mirochnitchenko O. Preclinical studies for induced pluripotent stem cell-based therapeutics. J Biol Chem 2014; 289:4585-93; PMID:24362021; http://dx.doi.org/10.1074/jbc.R113.463737

4. Volz KS, Miljan E, Khoo A, Cooke JP. Development of pluripotent stem cells for vascular therapy. Vascul Pharmacol 2012; 56:288-96; PMID:22387745; http://dx.doi.org/10.1016/j.vph.2012.02.010

5. Jeon S, Choi SH, Wolosin JM, Chung SH, Joo CK. Regeneration of the corneal epithelium with conjunctival epithelial equivalents generated in serum- and feeder-cell-free media. Mol Vis 2013; 19:2542-50; PMID:24357922

6. Patrikoski M, Juntunen M, Boucher S, Campbell A, Vemuri MC, Mannerström B, Miettinen S. Development of fully defined xeno-free culture system for the preparation and propagation of cell therapy-compliant human adipose stem cells. Stem Cell Res Ther 2013; 4:27; PMID:23497764; http://dx.doi.org/10.1186/scrt175

7. Belay E. H.R., Schonberger L, *Creutzfeldt-Jakob disease surveillance and diagnosis.* Dis 2005; 41:834-6

8. Price PBG. Serum-free media for neural cell cultures, in Protocols for neural cell culture, R.A. Fedoroff S, Editor., Humana Press, Inc.: Totowa, NJ. p. 255-263.

9. Steere AN, Bobst CE, Zhang D, Pettit SC, Kaltashov IA, Huang N, Mason AB. Biochemical and structural characterization of recombinant human serum transferrin from rice (Oryza sativa L.). J Inorg Biochem 2012; 116:37-44; PMID:23010327; http://dx.doi.org/10.1016/j.jinorgbio.2012.07.005

10. Kami D, Watakabe K, Yamazaki-Inoue M, Minami K, Kitani T, Itakura Y, Toyoda M, Sakurai T, Umezawa A, Gojo S. Large-scale cell production of stem cells for clinical application using the automated cell processing machine. BMC Biotechnol 2013; 13:102; PMID:24228851; http://dx.doi.org/10.1186/1472-6750-13-102

11. Zhang D, Lee HF, Pettit SC, Zaro JL, Huang N, Shen WC. Characterization of transferrin receptor-mediated endocytosis and cellular iron delivery of recombinant human serum transferrin from rice (Oryza sativa L.). BMC Biotechnol 2012; 12:92; PMID:23194296; http://dx.doi.org/10.1186/1472-6750-12-92

12. Youngblood BA, Alfano R, Pettit SC, Zhang D, Dallmann HG, Huang N, Macdonald CC. Application of recombinant human leukemia inhibitory factor (LIF) produced in rice (Oryza sativa L.) for maintenance of mouse embryonic stem cells. J Biotechnol 2014; 172:67-72; PMID:24380819; http://dx.doi.org/10.1016/j.jbiotec.2013.12.012

13. Burdon T, Smith A, Savatier P. Signalling, cell cycle and pluripotency in embryonic stem cells. Trends Cell Biol 2002; 12:432-8; PMID:12220864; http://dx.doi.org/10.1016/S0962-8924(02)02352-8

14. Chambers I, Tomlinson SR. The transcriptional foundation of pluripotency. Development 2009; 136:2311-22; PMID:19542351; http://dx.doi.org/10.1242/dev.024398

15. Barnes J, Lim JM, Godard A, Blanchard F, Wells L, Steet R. Extensive mannose phosphorylation on leukemia inhibitory factor (LIF) controls its extracellular levels by multiple mechanisms. J Biol Chem 2011; 286:24855-64; PMID:21613225; http://dx.doi.org/10.1074/jbc.M111.221432

16. Owczarek CM, Layton MJ, Metcalf D, Lock P, Willson TA, Gough NM, Nicola NA. Inter-species chimeras of leukaemia inhibitory factor define a major human receptor-binding determinant. EMBO J 1993; 12:3487-95; PMID:8253075

17. Pan G, Thomson JA. Nanog and transcriptional networks in embryonic stem cell pluripotency. Cell Res 2007; 17:42-9; PMID:17211451; http://dx.doi.org/10.1038/sj.cr.7310125

18. Tang Y, Tian XC. JAK-STAT3 and somatic cell reprogramming. JAKSTAT 2013; 2:e24935; PMID:24470976; http://dx.doi.org/10.4161/jkst.24935

19. Hirai H, Firpo M, Kikyo N. Establishment of LIF-dependent human iPS cells closely related to basic FGF-dependent authentic iPS cells. PLoS One 2012; 7:e39022; PMID:22720020; http://dx.doi.org/10.1371/journal.pone.0039022

20. Hanna J, Cheng AW, Saha K, Kim J, Lengner CJ, Soldner F, Cassady JP, Muffat J, Carey BW, Jaenisch R. Human embryonic stem cells with biological and epigenetic characteristics similar to those of mouse ESCs. Proc Natl Acad Sci U S A 2010; 107:9222-7; PMID:20442331; http://dx.doi.org/10.1073/pnas.1004584107

21. Piravar Z, Jeddi-Tehrani M, Sadeghi MR, Mohazzab A, Eidi A, Akhondi MM. In vitro Culture of Human Testicular Stem Cells on Feeder-Free Condition. J Reprod Infertil 2013; 14:17-22; PMID:23926556

22. Goharbakhsh L, Mohazzab A, Salehkhou S, Heidari M, Zarnani AH, Parivar K, Akhondi MM. Isolation and culture of human spermatogonial stem cells derived from testis biopsy. Avicenna J Med Biotechnol 2013; 5:54-61; PMID:23626877

23. Majumder A, Banerjee S, Harrill JA, Machacek DW, Mohamad O, Bacanamwo M, Mundy WR, Wei L, Dhara SK, Stice SL. *Neurotrophic effects of leukemia inhibitory factor on neural cells derived from human embryonic stem cells.* Stem Cells 2012; 30:2387-99; PMID:22899336; http://dx.doi.org/10.1002/stem.1201

24. Atari M, Gil-Recio C, Fabregat M, García-Fernández D, Barajas M, Carrasco MA, Jung HS, Alfaro FH, Casals N, Prosper F, et al. Dental pulp of the third molar: a new source of pluripotent-like stem cells. J Cell Sci 2012; 125:3343-56; PMID:22467856; http://dx.doi.org/10.1242/jcs.096537

25. Gomord V, Fitchette AC, Menu-Bouaouiche L, Saint-Jore-Dupas C, Plasson C, Michaud D, Faye L. Plant-specific glycosylation patterns in the context of therapeutic protein production. Plant Biotechnol J 2010; 8:564-87; PMID:20233335; http://dx.doi.org/10.1111/j.1467-7652.2009.00497.x

26. Zhang D, Nandi S, Bryan P, Pettit S, Nguyen D, Santos MA, Huang N. Expression, purification, and characterization of recombinant human transferrin from rice (Oryza sativa L.). Protein Expr Purif 2010; 74:69-79; PMID:20447458; http://dx.doi.org/10.1016/j.pep.2010.04.019

27. Farran I, Sánchez-Serrano JJ, Medina JF, Prieto J, Mingo-Castel AM. Targeted expression of human serum albumin to potato tubers. Transgenic Res 2002; 11:337-46; PMID:12212837; http://dx.doi.org/10.1023/A:1016356510770

28. Zhang D. Plant seed-derived human transferrin: expression, characterisation and applications. OA Biotechnology 2013; 2:17; http://dx.doi.org/10.13172/2052-0069-2-2-580

29. Huang J. Expression of natural antimicrobial human lysozyme in rice grains. Mol Breed 2002; 10: 83-94; http://dx.doi.org/10.1023/A:1020355511981

30. Huang N. Y.D., *ExpressTec: high level expression of biopharmaceuticals in cereal grain.* In: Knablein J (ed) Modern Biopharmaceuticals; Design, Development and Optimization, Wiley-Vch 2005: p. 931-47.

31. Pettit S. S.M., Tanner T, Huang N, *Enhanced growth and productivity of hybridoma with recombinant human serum albumin and lactoferrin.* BioProcessing J 2009; 8:50-5