ADDENDUM

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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tem 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.\textsuperscript{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,\textsuperscript{4} the cost of the recombinant protein,\textsuperscript{9} and the volumes of growth medium needed to produce a clinically relevant doses of stem cells per patient.\textsuperscript{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- (\textit{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.\textsuperscript{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.\textsuperscript{12} LIF signaling, via gp130/STAT3-dependent signaling, enhances stem cell state regulator transcription and simultaneously inhibits cell differentiation.\textsuperscript{13,14} Removal or decreased LIF concentrations can induce rapid cessation of growth and subsequent differentiation in LIF-dependent stem cells.\textsuperscript{15} LIF reactivity across species has been well-characterized, as human LIF will bind the mouse LIF receptor.\textsuperscript{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) and induced pluripotent stem (iPS) cell systems,\textsuperscript{17,18} LIF-dependent naïve human stem cells,\textsuperscript{19,20} and other human stem cell systems that are responsive to LIF supplementation.\textsuperscript{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 NCrl mouse ESCs through multiple subcultures.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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
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.\(^20\) 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.
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 iPSC (Fig. 3A). These results indicate that mouse iPSC 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 = 0.00006) 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 inherently 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 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 kilogram 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, albumin, and lactoferrin 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

No potential conflict of interest was disclosed.

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