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Novel human renal proximal tubular cell line for the production of complex proteins

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ABSTRACT

Human host cell lines for the production of biopharmaceutical proteins are of interest due to differences in the glycosylation patterns of human and animal cell lines. Specifically, sialylation, which has a major impact on half-life and immunogenicity of recombinant biopharmaceuticals, differs markedly. Here, we established and characterized an immortalized well documented and serum-free host cell line, RS, from primary human renal proximal tubular epithelial cells (RPTEC). In order to test its capacity to produce complex glycosylated proteins, stable recombinant human erythropoietin (rhEpo) producing clones were generated. The clone with highest productivity, RS-1C9 was further characterized and showed stable productivity. Biological activity was observed in in vitro assays and 28% of rhEpo glyco-isoforms produced by RS-1C9 were in range and distribution of the biological reference standard (BRP) isoform, as compared to 11.5% of a CHO based rhEpo. Additionally, cellular α-2,6 sialylation, Galactose-alpha-1,3-galactose (alpha-Gal) and N-glycolylneuraminic acid (NeuGc) patterns compare favourably to CHO cells. While productivity of RS still needs optimization, its amenability to upscaling in bioreactors, its production of glyco-isoforms that will increase yields after down-stream processing of about 2.5 fold, presence of sialylation and lack of Neu5Gc recommend RS as alternative human host cell line for production of biopharmaceuticals.

1. Introduction

Today the recombinant production processes of complex proteins mainly rely on Chinese Hamster Ovary (CHO) cell lines (Wurm, 2004). To be fully active when applied, such complex proteins must meet characteristics like complete and correct amino acid backbone, proper folding and a 'human-like' glycosylation pattern (Kwaks and Otte, 2006). Although one of the benefits of mammalian cell lines is that they have the ability to add glycan-structures to a protein, the repertoire of glycosidases and glycosyltransferases is species specific and even cell type specific (reviewed in (Berger et al., 2012)). The resulting incomplete and non-authentic glycosylation patterns can subsequently result in reduced stability and half-life of the glycoprotein or it might trigger an immune response in patients (Cai et al., 2012 and reviewed in Berger et al., 2012). Moreover, activity as well as signal transduction and cell adhesion of complex proteins (reviewed in Berger et al., 2012) depend on the glycosylation pattern of the protein. Especially, the last step of glycosylation, the terminal linkage of sialic acids on glycostructures has major effects on the in vivo half-life (Ashwell and Harford, 1982; Fukusa et al., 1989). Although CHO cells efficiently add carbohydrate chains to the proteins backbone (Sasaki et al., 1987), differences were found in the glycan structures, which correlate to the proteins' biological activity (Takeuchi et al., 1989). One difference in terms of sialylation is that proteins produced by CHO cells, only contain α-2,3 linkages between galactose and the terminal sialic acid residue of N-linked oligosaccharides due to their lack of α-2,6 sialyltransferase. In order to bypass this limitation, BHK and CHO cells were engineered to express the human α-2,6 sialyltransferase (Grabenhhorst et al., 1997; Schlenke et al., 1997; Zhang et al., 1998). In addition, CHO cells in general have a reduced potential of sialylation compared to humans (Brooks, 2004). In order to improve this phenomenon as well as productivity, the 30Kc19 gene of the silkworm (Bombyx mori) has been introduced into recombinant human erythropoietin (rhEpo) producing CHO cells (Park et al., 2012; Wang et al., 2011). Furthermore, most non human

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mammalians produce Galactose-alpha-1,3-galactose (alpha-Gal) and N-glycolylneuraminic acid (NeuGc) (Padler-Karavani and Varki, 2011). These differences in glycosylation can lead to immunogenicity and increased clearance of the recombinant product (Ghaderi et al., 2010; Padler-Karavani and Varki, 2011).

On the contrary, recombinant proteins produced in human cells are expected to perfectly match essential characteristics of the corresponding endogenous proteins like folding and post-translational modifications (Swiech et al., 2012). Thus, in vitro cultivated, highly differentiated normal human cells with secretory capacity represent optimal expression systems for complex proteins produced by the same cell type in vivo. However, the use of such cells for biotechnological applications is limited by the fact that these cells can only be propagated for a limited number of population doublings (PD) before entering the phase of replicative senescence (Hayflick and Moorhead, 1961). Therefore, the cellular life span of the cells has to be extended as well as the maintenance of differentiated features, which are essential for proper glycosylation. Erythropoietin (Epo), a major humoral regulator of erythropoiesis (Krantz and Jacobson, 1970 and reviewed in Jelkmann, 1992) represents one of the leading biopharmaceutical protein products with applications in the treatment of hypoxia and anaemia and was the first biopharmaceutical to reach blockbuster status (Wurm, 2004). Epos 165 amino acid backbone is linked to 3 N- and one O-glycan chain that make up about 40% of the proteins mass (Jelkmann, 1992). Additionally, sialic acid residues terminate glycan chains and thereby prolong the circulation time of the glycoprotein. Desialylated Epo is recognized by galactose binding receptors of the liver, namely asialoglycoprotein receptors, and rapidly cleared from the blood stream (Ashwell and Harford, 1982; Fukusa et al., 1989). In vivo, Epo is produced and secreted in the kidney by renal proximal tubular epithelial cells (RPTEC) (Shanks et al., 1996). Since different glycosylation patterns are due to specialized cells, human kidney cell line endowed with characteristics of the normal counterpart in vivo might be an ideal host system to produce this complex protein with high quality.

In order to proof this hypothesis we isolated human RPTECs from kidney tissue biopsies. The cells were maintained in vitro under culture conditions lacking animal derived products under full documentation and immortalization was initiated using SV40 early region. After characterization of the cellular phenotype, we used the cell line for production of recombinant human Epo in small and large scale and the produced protein was characterized in terms of its glycosylation pattern. Thereby, we have shown that immortalized human RPTECs have a high potential as novel host for the production of complex biopharmaceuticals.

2. Material and methods

2.1. Cell lines

The local ethic commission approved the study and the patients gave their informed consent (Biobank Graz). Thus, the study was performed in accordance with the Declaration of Helsinki.

nRPTEC: Normal human renal proximal tubular epithelial cells (nRPTEC) were isolated as described in detail previously (Wieser et al., 2008) and cultivated in ProxUp-Pri medium (Evercyte GmbH).

RS cells: nRPTEC were transfected with a plasmid encoding SV40 early region (Voglauer et al., 2005) using Lipofectamine 2000 (Invitrogen) according to the manufacturers instructions and positively transfected cells were selected by outgrowth of SV40 early region overexpressing cell clones. These cells were cultivated as mass culture designated RS cells and grown in OptiPro serum-free medium (OptiPro SFM, Gibco) supplemented with 4 millimolar (mM) L-glutamine (Sigma Aldrich) in culture flasks (Greiner Bio One) pre-coated with human collagen (Sigma Aldrich) for at least 30 min prior to passaging the cells.

2.2. Plasmids and transfection

The plasmid pDEPT carrying the early region of SV40 (large T/small t), including the SV40 promoter/enhancer sequences, is described in detail by (Banerji et al., 1983). The plasmid pEPO was constructed using the pCI-neo (Promega) backbone habouring a CMV enhancer/promoter and SV40 late poly(A). The human codon-optimized human erythropoietin sequence was integrated using the cloning sites MluI and XbaI. Codon optimization and vector cloning was performed by Geneart.

All transfections were done using Lipofectamine2000 (Life Technologies) according to the manufacturers' protocols.

2.3. Culture conditions

2.3.1. Cultivation in roux flasks

All cell lines used within this study were cultivated at 37 °C in a humidified atmosphere containing 7% CO_2. Confluent monolayers were passaged with a split ratio of 1:2–1:4 depending on the cell line. Therefore, the cells were detached using 0.1% trypsin-0.02% EDTA (Sigma), which was inactivated by addition of soybean trypsin inhibitor (1 mg/ml, Sigma), followed by a centrifugation step (5 min, 170 g) and transfer of the resuspended cells to new culture vessels.

2.3.2. Cultivation in spinner flasks

Spinner flask cultivation was performed as described previously (Fliedl and Kaisermayer, 2011). Briefly, prior to cultivation glass vessel (Techne, Abington, England) were siliconised using Sigmacote (Sigma Aldrich) to prevent adhesion of microcarrier to the glass surface. Microcarriers (Cytotex 3; Sigma Aldrich) were washed in calcium and magnesium free-phosphate buffered saline (PBS) (PAA, Pasching, Austria) and autoclaved.

The spinner flasks were prepared 24 h before inoculation with 50% of medium and the appropriate amount of carriers to reach a concentration of 3 g/l in the final working volume of 60 ml. Flasks were incubated at 7% CO_2 for pH adjustment.

After detachment of cells, spinner flasks were inoculated with 2×10^5 cells/ml. Cultivation was performed at 37 °C and 50 rotations per minute. Samples were taken daily to determine cell concentration, cell morphology and metabolite concentrations. Cultivation medium was replaced as necessary to maintain a residual glucose concentration higher than 1 g/l.

2.4. Cell counting and calculation of population doublings (PD)

For T-flask cultures cell number and viability were determined using a ViCell (Beckman Coulter). To establish a growth curve, cells were passaged twice a week and population doublings (PD) were calculated using the following equation: $PD = 3.32 (\log X_2 - \log X_1) + B$. ($X_1$ is the cell number at the beginning of the incubation time, X_2 is the cell number at the end of the incubation time, B PD at the beginning of the incubation time).

In microcarrier cultures 1 ml of suspension was used to determine the total cell concentration. The carriers were left to settle, the supernatant was removed and the carriers were resuspended in 1 ml of 0.1% crystal violet in 0.1 M citric acid. After a minimum incubation period of 1.5 h the released nuclei were counted in a haemocytometer.

2.5. Microscopy and photography

Cell morphology was documented by photographs taken at 100-fold magnification using an Olympus microscope (IMT-2) and an Olympus digital camera (XC 50). Cells on microcarriers were stained using haematoxylin solution (haematoxylin 0.9 g/l, sodium