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White Paper

Authentic Recombinant Activin A and BMP-4 Production Using Proteintech's Proprietary Human Cell Line Expression Technology

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Summary

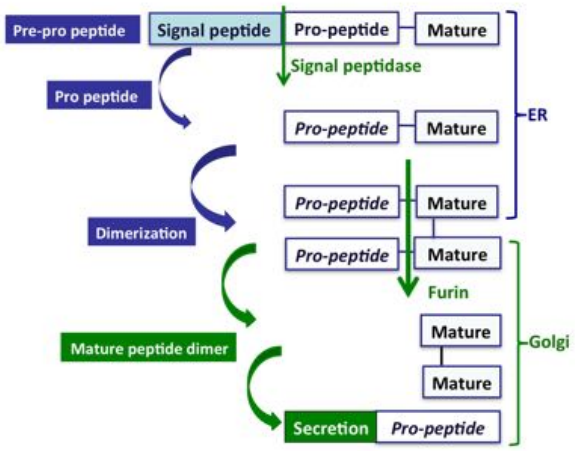
Human TGF-β superfamily cytokine expression and secretion involves multi-step posttranslational processing *in vivo*. The *in vitro* production these proteins including Activin A and BMP-4 in non-human cell expression systems lacking this processing machinery (*E. coli*, SF9, CHO) often results in a mixture of precursor and mature proteins misformed disulfide bonds, and missing or incorrect glycosylation. To meet the increasing demands for recombinant human Activin A and BMP-4 for stem cell and cell therapy research, Proteintech has utilized their proprietary human cell (HEK293) expression system for production of HumanKine® Activin A and BMP-4 as the correct, mature, fully processed, active protein with authentic folding, glycosylation, subunit assembly, and disulfide linkages.

Introduction

The transforming growth factor- beta (TGF- β) superfamily of cytokines are a large group of structurally related signaling proteins that includes the activin/inhibin family, bone morphogenetic proteins (BMPs), growth differentiation factors (GDFs), the TGF- β subfamily, and the glial cell line- derived neurotrophic factor (GDNF) family. These proteins are involved in the regulation of essential cellular and developmental processes such as growth, development, tissue homeostasis and regulation of the immune system.

The use of human recombinant TGF- β family proteins have become widespread in stem cell research, cell therapy research and clinical applications due to their important role in development and differentiation. However, the production of recombinant TGF- β proteins in sufficient quantities can be challenging, since ligand members of the TGF- β superfamily all undergo multi- step processing during maturation and secretion **(Figure 1).**

Figure 1. Proteolytic Processing of TGF beta Superfamily Ligands



These proteins are all synthesized as large precursors (preproproteins) with N-terminal peptides that are proteolytically cleaved in two steps to yield glycosylated carboxy-terminal mature protein homodimers. Each subunit contains 4 to 7 cysteine residues forming multiple intrasubunit disulfide bonds, and one cysteine forming a disulfide bond with the other subunit to stabilize the dimer. Since these ligands are only active as homo-or heterodimers, the correct folding, glycosylation and subunit association is critical to their biological activity.

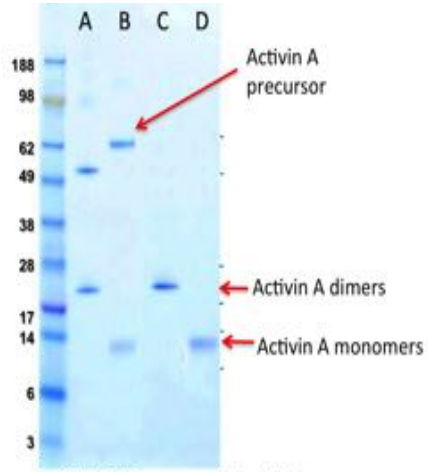
Currently, TGF-β family proteins are predominantly produced in non- human cells (*E. coli*, SF9, CHO) often resulting in a mixture of precursor and mature proteins, misformed disulfide bonds, and missing or incorrect glycosylation. In addition, a number of important cytokines are not commercially available at all due to expression systems with inadequate proteolytic processing, protein folding or other post-translational modifications such as correct glycosylation that do not occur in the non- human cell expression systems (Aono, Hazama et al.).

To meet the demands for recombinant human TGF- β family proteins, HumanZyme has utilized their human cell (HEK293) expression system with native human cellular manufacturing capabilities to produce TGF- β family proteins with correct proteolytic processing, folding, glycosylation, subunit assembly, and disulfide linkages. In this Whitepaper we describe the details of production of HumanKine® Activin A and BMP- 4 using our proprietary cell expression system.

HumanKine® Activin A

Activin A belongs to the TGF- β superfamily and exhibits a wide range of biological activities including regulation of cellular proliferation and differentiation, and promotion of neuronal survival. Since Activin A has also been shown to be essential to maintaining pluripotency in *in vitro* stem cell culture, its use is widespread in stem cell and cell therapy research.

Figure 2. HumanKine® Activin A vs. CHO- expressed



Purified CHO expressed Activin A from a commercial source (A) without DTT, (B) with DTT; HumanKine Activin A, (C) without DTT, (D) with DTT

Activin A, like other members of the TGF-β family, is a disulfide-linked dimeric protein produced as precursor with an amino-terminal propeptide that is enzymatically cleaved to release the carboxy- terminal bioactive ligand, and as such is difficult to produce in an *in vitro* expression system. Essentially, the following criteria need to be met (Pangas and Woodruff):

1) Correct processing of the subunit peptides from prepropeptide to mature, biologically active peptide

2) Correct assembly of monomers into active dimers

3) Expression at sufficiently high levels for purification

4) Final products must be bioactive using established assays.

In addition, the correct N- linked glycosylation of recombinant Activin A has recently been shown to be necessary for dimer assembly and secretion of the bioactive protein, so an expression system must also be capable of correct glycosylation (Antenos, Stemler et al.). The expression of Activin A in CHO cells has been reported (Pangas and Woodruff), however numerous challenges with its expression at high levels were observed including low yields, cellular toxicity, and high levels of uncleaved high molecular

weight precursor forms of Activin A (Pangas and Woodruff), (Papakonstantinou, Harris et al.). Expression of a fully active Activin A in *E. coli* is not possible, since bacterial machinery is not capable of the complex proteolytic processing or correct folding and disulfide linkages to produce active protein. Consequently, bacterial expression usually produces large quantities of the inactive mature protein into inclusion bodies, followed by solubilization and refolding processes that may or may not alter the biologic activity of the recombinant protein.

Activin A is expressed from a stable cell culture of engineered human HEK293 cells into culture media as a fully active, correctly folded homodimer of 25- 26 kD. As shown in **Figure 2**, HumanKine® Activin A analyzed on a SDS- PAGE gel shows only the correct, mature, fully processed protein, while the CHO-expressed source shows higher molecular weight precursor protein and other contaminants.

In addition, since researchers require consistency from lot to lot for long- term research studies or scale-up needs Proteintech has shown this proprietary expression system to be robust and stable. **Figure 3a and 3b** show the Lot-to-lot consistency of Activin A

Figure 3a. Lot-to-lot Consistency of HumanKine® Activin A Purity

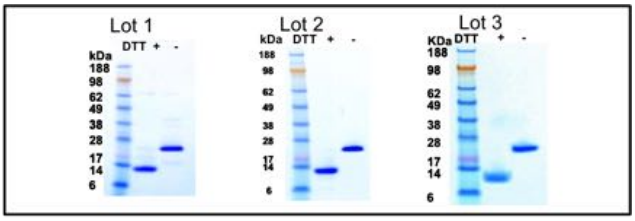
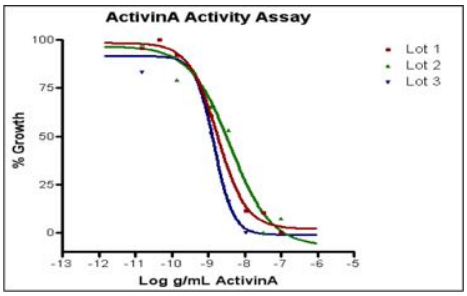


Figure 3b. Lot-to-lot Consistency of HumanKine® Activin A Purity



production activity and purity, with biological activity determined by the dose- dependent inhibition of proliferation of the MPC- 11 (mouse plasmocytoma) cell line (Phillips, Brauman et al. 1999) and purity determined by SDS PAGE gel electrophoresis.

BMP- 4

Another member of the TGF- β family, bone morphogenetic proteins (BMPs) were originally identified as regulators of cartilage and bone formation, but have since been found to also play important roles in the morphogenesis of tissues and organs during embryonic development.

BMP proteins are synthesized as inactive 30- 38 kDa preproprotides of 400-425 amino acids. Like all members of the TGF- B superfamily, a complex proteolytic path produces a mature, secreted, biologically active 100- 140 aminoid C-terminal peptide as a dimer stabilized by a disulfide bond. BMP proteins have 7 cysteine residues per subunit, with 6 forming intermolecular disulfide linkages and one forming a disulfide bond with the other subunit to stabilize the dimer. The peptide monomers form the characteristic cysteine knot structure consisting of six conserved cysteine residues with N-linked glycosylation.

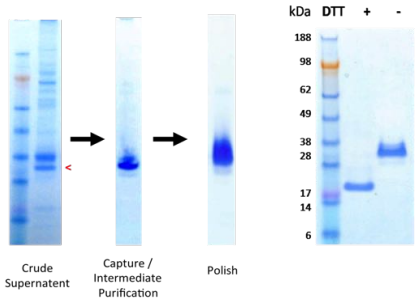


Figure 4. 2-Step Purification of HumanKine BMP-4

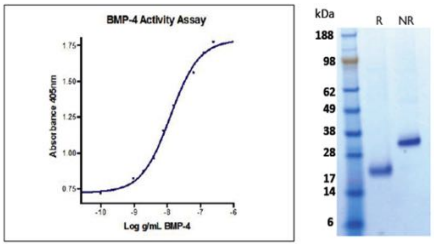


Figure5. Activity and Purity of HumanKine BMP- 4

Human recombinant BMP proteins are widely used in stem cell and cell therapy applications, and recently BMP- 2 and BMP- 7 have been approved by the FDA for certain clinical uses (Lo, Ulery et al.). Recombinant BMP- 4 is used *in vitro* in the differentiation of human embryonic stem cell cultures (hESCs), and induced pluripotent stem cells (iPSCs). Production of BMP proteins for both research and clinical use has been challenging, due to the complex proteolytic processing preceding secretionofthemature,activeprotein. BMP proteins were first expressed in CHO cells, albeit with low expression levels. Bacterial expression in *E. coli* produces much larger yields of protein, however typically without glycosylation. Bacterial expression also sequesters BMP-4 protein in inclusion bodies that need to be refolded using denaturing buffers (Bessa, Casal et al.).

Proteintech has streamlined the expression and purification of BMP- 4 from a human cell line (HEK293) to produce purified, correctly processed, folded, glycosylated, and disulfide linked protein in sufficient quantities for research use **(Figure 4)**. The active, correctly folded and disulfide linked, glycosylated monomer is expressed from the HEK293 host and is efficiently purified from the culture media in a 2- step process.

The purified protein migrates at an apparent molecular weight of 34 kDa in non-denaturing conditions, and its activity was determined by the dose- dependent induction of alkaline phosphatase production in the ATDC-5 (Mouse chondrogenic) cell line (Figure 5).

Summary

The TGF-β superfamily of proteins including Activin A and BMP-4 undergo a complex proteolytic processing and N-linked glycosylation for correct maturation and secretion of the active protein. TGF-β family recombinant proteins have been difficult to produce commercially, due to their proteolytic multi-step processing during maturation, secretion of a dimeric disulfide- linked active form, and other problematic production issues such as cell toxicity and large precursor contamination.
HumanZyme has developed a unique and robust human cell (HEK- 293) expression system for the correct and biologically active expression of TGF- β superfamily proteins, including Activin A and BMP-4.
Purification of these proteins has been simplified to isolate the correct dimer from culture media.

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