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Visão geral do desenvolvimento biofarmacêutico de proteinas recombinantes usando o leite de animais transgênicos

Overview of Recombinant Biopharmaceutical Development Using the Milk of Transgenic Livestock

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Abstract

Human plasma for obtaining biotherapeutics has shown considerable limitation in terms of biosafety and scalability. Engineered animal cells in bioreactors provide an alternative source of these complex proteins. However, manufacturing animal cell bioreactor technology can just produce about grams recombinant protein per liter. Besides, animal cell bioreactor has shown a rate limitation of biosynthesis of the post translational modifications (PTMs) in human proteins necessary for physiologic function. PTMs can be provided in nature by the capacity of mammary epithelia of livestock. Transgenic livestock offer a continuous productivity of grams recombinant protein per liter in the milk. Here, we discuss how recombinant proteins made in the milk of transgenic livestock represent a prodigious, self-replicating bioreactor technology which can bring promising new biotherapeutic treatment paradigms world-wide.

Keywords*: biopharmaceuticals, post-translational modification; transgenes; livestock; milk*

Resumo

O plasma humano para a obtenção de bioterapêuticos tem mostrado limitaçoes consideráveis em termos de biossegurança e escalabilidade. Células animais cultivadas em biorreatores fornecem uma fonte alternativa para essas proteínas complexas. No entanto, a tecnologia de biorreator de célula animal pode produzir apenas cerca de gramas de proteína recombinante por litro. Além disso, o biorreator de células animais tem mostrado limitacoes na escalabilidade de biossíntese das modificações pós-translacionais (PTMs) nas proteínas humanas necessárias para a função fisiológica. As PTMs podem acontecer naturalmente no epitélio mamário do gado. O gado transgênico oferece uma produtividade contínua de gramas de proteína recombinante por litro no leite. Aqui, discutimos como as proteínas recombinantes feitas no leite de gado transgênico representam uma tecnologia de biorreator auto-replicante prodigiosa que pode trazer novos paradigmas de tratamento bioterapêutico promissores em todo o mundo.

Introduction

There is a limited amount of pathogen safe human plasma from which to derive biotherapeutics (T. Burnouf, 2016; Van Cott et al., 2004). To address that dilemma, genetically engineered animal cells cultured in bioreactors currently serve as a recombinant alternative to providing a source of these complicated medicines (Echelard et al., 2006). While being tissue specific, animal cells are used because they can biosynthesize many of the posttranslational modifications (PTMs) in human proteins necessary for physiologic function (Kaufman et al., 1989; Kaufman et al., 1986). Within the rate limitations of their biosynthesis, these necessary PTMs can be provided by the natural capacity of mammary epithelia of livestock (Archibald et al., 1990; Subramanian et al., 1996; Van Cott et al., 1999) and some of these limitations can be mitigated by transgenesis. In addition, the high secretory cell density of the mammary gland enables a continuous productivity of grams recombinant protein per liter per hour in milk over the course of months. By comparison, today's manufacturing scale animal cell bioreactor technology can only produce about grams recombinant protein per liter per week of the complex human proteins (Elhadj, 1998; Van Cott et al., 2004).

For any biotechnology, process economics and design studies show that the minimum manufacturing cost is reached at the production capacity of about 100 to 1000 kg of recombinant protein per year (Elhadj, 1998). This is largely due to the percentage of manpower costs that are minimized at

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this extraordinary manufacturing capacity. In the case of transgenic dairy livestock, this scale is reached while using only hundreds of livestock. The combination of the construction cost and fixed scale that culture facilities operate is an inherent shortcoming of this animal cell biotechnology. Further complicating this investment formula is that cell culture manufacturing facilities typically take 8 years to both construct and validate for licensed product manufacturing. In contrast, dairy herds can rapidly, flexibly and on a "per head livestock basis" be validated for manufacturing at any production scale. The "shifting needs of increasing scale to meet clinical demand" can be easily fulfilled using herd sizes and milking facilities considered moderate or small by dairy industry metrics (Echelard et al., 2006).

Biotherapies for inhaled (McElvaney et al., 1991), buccal, oral administration (Hemker et al., 1980) and for oral immune biotherapeutic tolerization (Alpan et al., 2001) are typically needed in large, daily amounts of grams per dose (de Serres & Blanco, 2014; Kasper, 1999). These types of therapies become more feasible when using transgenic dairy biomanufacturing. Importantly, the above non-intravenous routes of administration would decrease healthcare complexity and therefore would dramatically improve biotherapeutic treatment availability worldwide (Alpan et al., 2001). Thus, recombinant proteins made in the milk of transgenic livestock represent a prodigious, self-replicating bioreactor technology which can bring promising new biotherapeutic treatment paradigms world-wide (Echelard et al., 2006; Van Cott et al., 2004).

Economics of Transgenic Dairy Biopharmaceutical Production Versus Animal Cell Culture

We discuss below transgenic livestock examples that produce biologically functional recombinant proteins made in milk at >100 fold higher production rates (Figure 1A, 1B) than reported for analogous animal cell culture bioreactors (Subramanian et al., 1996; Van Cott et al., 2004). As a result, 300-400 transgenic dairy cows made by artificial insemination of a transgenic lineage would be expected to produce >1 metric ton of recombinant human fibrinogen annually for use in fibrin surgical tissue sealants (Calcaterra et al., 2013). For treatment of hemophilia B (Figure 1B), only hundreds of milking pigs would serve prophylactic therapy to meet worldwide clinical demand per annum (Zhao et al., 2015).

A transgenic biopharmaceutical dairy livestock facility is about 10-fold more affordable to construct than today's modern animal cell culture bioreactor facility (Echelard et al., 2006; Elhadj, 1998). In that context, an approximate savings of >$USD 4.5 B in upfront capital investment and 2 times faster construction of a USFDA approved manufacturing factory would result. Animal cell bioreactor arrays represent 90% of facility costs where purification facility costs are less than 10% of the capital investment of animal cell culture bioreactor installations. In contrast, purification facilities are the largest percentage of capital investment cost for transgenic livestock biopharmaceutical manufacturing. The USFDA/ EMEA approval of Atryn (recombinant human anti-thrombin III made in the milk of transgenic goats) has provided a "proof of practice template" for using the milk of transgenic livestock as a cost-effective vehicle for biopharmaceutical manufacturing (Echelard et al., 2006).

Making Genetically Engineered Livestock for Biotherapeutic Production in Milk

The genetic engineering of livestock of the mid-1980s showed that recombinant DNA microinjected into the pronucleus of mouse, rabbit and livestock zygotes could result in stable chromosomal integration and Mendelian germline transmission of the "transgene" (Hammer et al., 1985). For example, transgenic sheep lineages were made by micro-injection of human Factor IX (hFIX) cDNA into a pronuclear phase zygote (Schnieke et al., 1997). However, this transgenesis occurred via a random natural gene repair process and at a frequency of about 4 % in live-born animals. Thereafter, a new branch of embryonic cloning technique called Somatic Cell Nuclear Transfer (SCNT) that made "Dolly the sheep" then made "Polly the transgenic sheep" (Colman, 1999). Polly was made using an ovine β-lactoglobulin (BLG) promoter and a human Factor IX (FIX) cDNA transgene that was transfected into a primary ovine somatic cell lineage (Schnieke et al., 1997). This transgenic cell lineage was identified by using co-integration of a cell selection pressure transgene (ie. neomycin resistance). Nuclear transfer from this transgenic cell line into an enucleated wild type zygote resulted in 100% transgenesis in viable, born lambs.

Importantly, this cloning based transgenesis then made the installation of a multi-transgene array an efficient method for enhancing synthesis, PTM, stability and functionality of the targeted complex recombinant proteins made in milk. For example, multigene transgenesis in cloned pigs was used to

improve both the post-translational modification and activity of milk expressed rhFIX (Zhao et al., 2015). Multigene cloned transgenic cows containing about 500 kbp of three full length human fibrinogen genes have stably made functional human fibrinogen in the milk (Calcaterra et al., 2013). Both founder and subsequent offspring bovine lineages showed stable germline transmission of about eight copies each of the three-hF1 transgenes. While pronuclear injection generally produces healthy animals, cloning methods make special care to select somatic tissue cell lines that generate healthy animals (Forsberg et al., 2002). For the transgenic livestock discussed below, the presence of and mammary specific protein expression of the transgenes presented no ramifications of adverse health, development or impaired reproductive health.

Purification of Recombinant Biotherapeutics from Milk

The milk from genetically engineered livestock herds to manufacture biopharmaceuticals must necessarily occur from herds having specific pathogen free (SPF) pedigrees. The same modern purification technology used for deriving biotherapeutics from human plasma (Thierry Burnouf, 2016; Limentani et al., 1987; Tharakan et al., 1990; Velander et al., 1990) are very effective in purifying recombinant proteins from milk (Drews et al., 1995; Paleyanda et al., 1997; Pipe et al., 2011; Velander et al., 1992; Zhao et al., 2015). This technology serves to not only purify the target biopharmaceutical away from other milk proteins, but also to remove potential contamination of known and unknown pathogens to acceptable levels of very low risk. Purification and pathogen inactivation technology has been greatly improved since the HIV, HBV, and HCV pathogen contamination crises of human plasma derived products during the 1980s (Thierry Burnouf, 2016; Limentani et al., 1987; Velander et al., 1990). It includes the use of immunoaffinity purification and the incorporation of pathogen-inactivation steps such as wet and dry heat pasteurization, solvent detergent treatment, and nanofiltration. The combination of these steps is highly effectively in removing the activity of known classes of nucleic acid-based pathogens to undetectable levels. Specific pathogen testing and routine quarantine of plasma donations has greatly increased plasma-derived product safety, but this necessarily limits the amount of biotherapeutics that can be made from this precious resource and which cannot meet clinical demand worldwide (T. Burnouf, 2016; Van Cott et al., 2004).

A typical biotherapeutic purification process consists of a filtration and or centrifugation step and three chromatography steps and is schematically represented in Figure 1A and 1B. Plasma and cell culture manufacturing make very low concentrations of complex biopharmaceuticals like Factor IX and Factor VIII (Elhadj, 1998). This makes robust purification processing more difficult (Figure 1A) in order to satisfy overall purity and the economy of product yield. The much higher levels of product protein typically made by transgenic dairy livestock in milk (Figure 1B) enable engineering flexibility in achieving the overall balance of yield with necessary purity needed by each process step. The chromatographic steps typically consist of a sequence: a low-resolution ion exchange or hydrophobic interaction chromatography product capture step, a high-resolution immunoaffinity or affinity ligand chromatography step and a high-resolution ion exchange chromatographic “polishing step”. The above cited pathogen inactivation steps are inserted at key points into the purification process to assure robust pathogen inactivation processing.

The transgenic livestock and mammalian PTMs needed for making active complex recombinant biotherapeutics

The production of a diversity of biotherapeutics in transgenic milk that were first derived from human plasma and then from recombinantly engineered animal cells in culture has been well explored. The emphasis has been on identification of the animal species best capable of making the PTMs necessary for biotherapeutic activity and thereafter the volumetric biotherapeutic production capacity. For example, pigs have been identified as a unique species for efficiently making vitamin K-dependent (VKD) proteins such as coagulation factors and a mature glycosylation pattern similar to human glycoproteins (Van Cott et al., 2004; Velander et al., 1992). In that context, pigs were observed to efficiently make the VKD coagulation cascade family glycoproteins of recombinant human protein C (rhPC)(Velander et al., 1992), recombinant human Factor IX (rhFIX)(Zhao et al., 2015), human Factor VIII (rhFVIII)(Paleyanda et al., 1997). Transgenic goats were shown to make functional recombinant human anti-thrombin III (rhATIII) (Edmunds et al., 1998; Wright et al., 1991) and recombinant human alpha-1 Antitrypsin (rhA1AT) in large amounts (Carver et al., 1993). Transgenic cows were shown to make large amounts of recombinant human fibrinogen applicable to topical surgical sealants (rhF1) (Table

1A). In each case, the requirement of necessary PTMs, biological activity and volumetric capacity needed to justify making the biotherapeutic in the milk of that livestock were respectively satisfied.

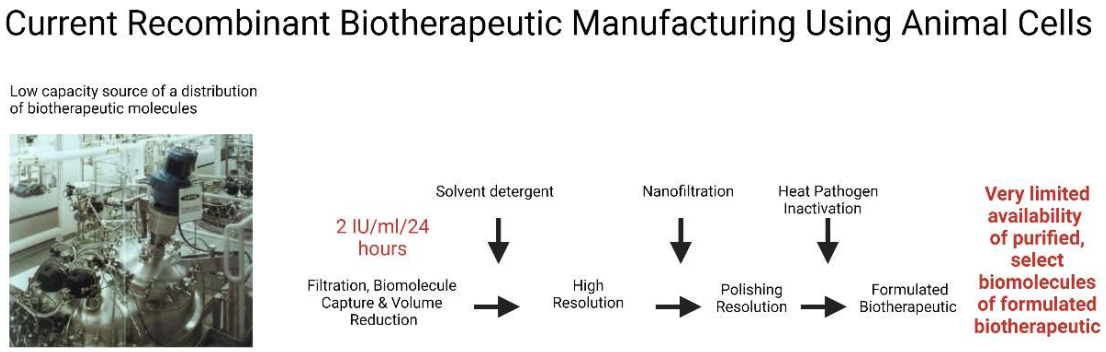

Figure 1A: Schematic representation of current Recombinant biotherapeutic manufacturing process using animal cells. Levels of biotherapeutic proteins typically made by bioreactors and plasma represent a limited source of biotherapeutic molecules. After capture, filtration and reduction, different filtration, centrifugation and heating steps are made for purification and pathogen inactivation. Biomolecules undergo different resolution steps from high to polish until the final purified biotherapeutic product, resulting in a limited level of biomolecules.

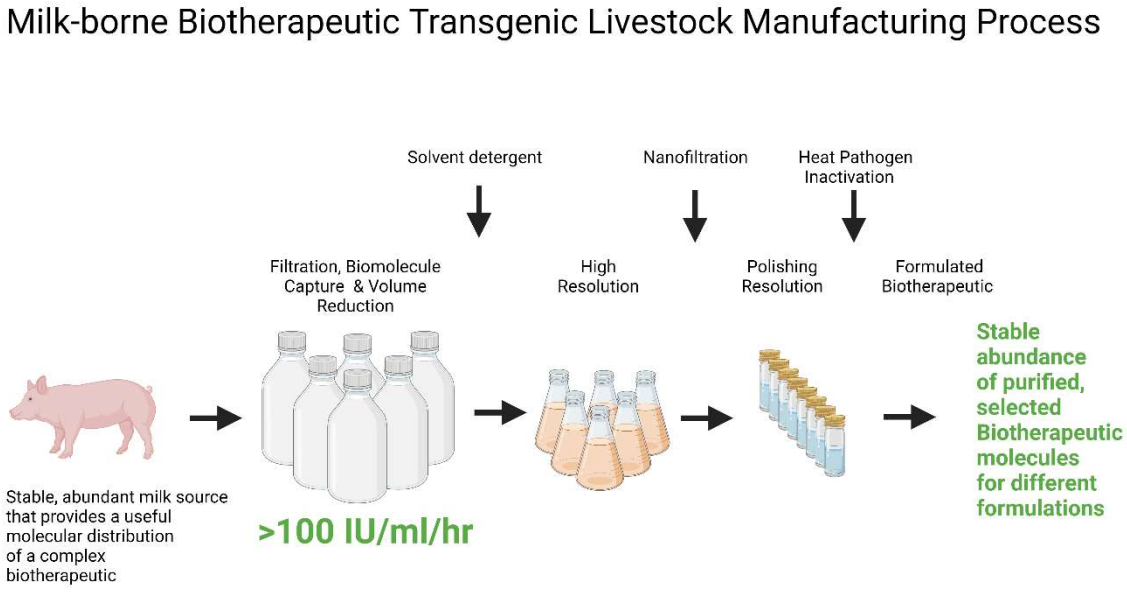

Figure 1B: Schematic representation of Milk-borne Biotherapeutic Manufacturing process using transgenic Livestock. Levels of biotherapeutic proteins typically obtained from mammary glands represent stable and abundant milk sources. After capture and volume reduction, different filtration, centrifugation and heating steps are made for purification and pathogen inactivation. Biomolecules undergo different resolution steps from high to polish until the final purified biotherapeutic product, resulting in an abundant level of biomolecules.

The above protein examples have complex physiologic activities such as stopping hemorrhage or mitigating vascular inflammatory pathogenesis caused by infection or traumatic injury. The diversity of PTMs needed to impart these biotherapeutic functions are specific to mammalian biochemistry and are also tissue specific within any animal. Table 1B presents some of these needed PTMs useful to a discussion of

the above proteins made in the milk to livestock. In summary, it is noted that significant, species-specific differences in PTM capabilities of the pig versus ruminant mammary gland have been observed.

Recombinant human A1AT

Plasma alpha-1 antitrypsin (A1AT) is a Mr= 52 kDa, single-chain glycoprotein encoded by the SERPINA1 gene and consisting of 418 amino acid residues and is synthesized in the liver. Normal plasma levels are similar to plasma immunoglobulin levels of about 1.5-3.5 g/l and during acute phase inflammation may rapidly rise 4-fold. A1AT is a member of the serpin superfamily of protease inhibitors and plays a central role as an anti-inflammatory by inhibiting neutrophil elastase and other activated serine proteases associated with infection. A1AT's role in regulating inflammation has been observed in the pathologies of cystic fibrosis (McElvaney et al., 1991), chronic obstructive pulmonary disease, emphysema, chronic liver disease, and acute respiratory distress syndrome arising from viral pulmonary infection. Due to its normally very high plasma concentration, A1AT augmentation therapy for A1AT deficiency and other anti-inflammatory therapies typically requires multiple series of gram doses. This underscores the need for developing rhA1AT sources that can meet the large clinical demand that plasma-derived A1AT biotherapy cannot satisfy (Brantly et al., 1988; Santangelo et al., 2017; Stoller et al., 1993).

The expression of rhA1AT at very high levels of up to 70 g/l in the milk of transgenic sheep was achieved after an extensive studies of transgene promoter and gene elements in transgenic mice. These studies of mammary specific expression elucidated a combination of an optimized 5'-downstream untranslated regions of the sheep BLG gene and several intron regions of the hA1AT gene that were combined into A1AT transgene platform. Transgenic sheep having this "mini-gene" construction gave expression levels of rhA1AT that represented about 50% of total whey protein where cloned Transgenic sheep showed expression levels of 33 g/l (Carver et al., 1993) (Table 1A). The inhibitory activity of the rhA1AT to neutrophil elastase was similar to that of plasma-derived but possessed a ruminant whey protein glycopattern. Future clinical evaluation will depend on further advancement of a purification process for making inhaled rhA1AT products for treatment of inherited deficiency, acute respiratory syndromes and topical wound therapies.

Recombinant Antithrombin III

Antithrombin III (ATIII) is a Mr = 58 kDa glycoprotein encoded by the SerpinC1 gene consisting of 464 amino acid residues that is synthesized by hepatocytes and occurs in human plasma at about 120-150 ug/ml. ATIII is a member of the serpin superfamily that inhibits thrombin and other activated serine proteases of the coagulation system (Hsu & Moosavi, 2021). The antithrombin deficiency is the cause of the high incidence of thrombophilia (Hsu & Moosavi, 2021; Maclean & Tait, 2007) associated with life-threatening thrombosis (Hsu & Moosavi, 2021; Maclean & Tait, 2007).

The production of recombinant human ATIII in the milk of cloned goats was demonstrated at 5.8 g/l (Baguisi; Behboodi; Melican; Pollock et al., 1999) (Table 1A). The physiologic binding of ATIII by heparin receptors on the vascular endothelium makes extravascular reservoirs of ATIII needed for the control of thrombin activity at wound sites. The rhATIII from transgenic goat milk had similar in vitro thrombin inhibitory activity to human plasma-derived ATIII and four-fold more affinity for heparin than plasma-derived ATIII. The rATIII possessed a ruminant whey protein glycopattern that included high mannose N-linked carbohydrate having no sialylation (Edmunds; Van Patten; Pollock; Hanson et al., 1998). Circulation clearance behavior is more rapid for non-sialylated glycostructures. Goat derived rhATIII was approved by the USFDA under the brand name ATRYN^{TM}. ATryn is indicated for the prevention of peri-operative and peri-partum thromboembolic events in hereditary antithrombin deficient patients.

Recombinant human Protein C

Human protein C (hPC) is a Mr = 62 kDa heterodimeric, trace plasma Vitamin K dependent (VKD) glycoprotein family member (Esmon, 1989; Griffin et al., 1981). It is a structurally and

Table 1A: History and Lead Candidates for Biopharmaceutical Development Using the Milk of Livestock

	Species	**Transgene/Promoter**	**Protein**				**Authors, Year**
			Name	tissue	presentation	max expression	
Livestock	Goat	β-casein–hAT	ATIII	mammary gland	milk	5.8 g/l	(Baguisi et al., 1999)
Mouse		WAP-hPC	hPC	mammary gland	milk	0.7 g/l	(Drohan et al., 2005)
		WAP-hPC	hPC	mammary gland	milk	1.8 g/l	(Drews et al., 1995)
Livestock	Pig	WAP-rhPC	hPC	mammary gland	milk	0.4 g/l	(Velander et al., 1992)
	Pig	WAP-PC1	hPC	mammary gland	milk	1.2 g/l	(Van Cott et al., 2001)
Mouse		WAP/rFVIII- rVWF-AAT	hFVIII	mammary gland	milk	0.2 g/l	(Pipe et al., 2011)
Livestock	Pig	WAP/rFVIII	hFVIII	mammary gland	milk	0.003 g/l	(Paleyanda et al., 1997)
Mouse		BLG-FIXDA3'	hFIX	mammary gland	milk	0.12 g/l	(Yull et al., 1995)
Livestock	Pig	WAP-FIX	hFIX	mammary gland	milk	2.3g/l	(Zhao et al., 2015)
Mouse		BLG-AATB	hA1AT	mammary gland	milk	21 g/l	(Carver et al., 1993)
Livestock	Sheep	BLG-AATB	hA1AT	mammary gland	milk	33 g/l	(Carver et al., 1993)
Mouse		BLG-hF1 α-β-y	hF1 (α-β-y)	mammary gland	milk	2.0 g/l	(Prunkard et al., 1996)
		oBLG-hF1 α-β-y	hF1 (α-β-y)	mammary gland	milk	2.0 g/l	(Butler et al., 1997)
Livestock	Cow	aS-1-casein hF1	hF1 (α-β-y)	mammary gland	milk	4.0 g/l	(Calcaterra et al., 2010)
	Ewe	oBLG- hF1 α-β-y	hF1	mammary gland	milk	5.0 g/l	(Butler et al., 1997)

History and Lead Candidates for Biopharmaceutical Development Using the Milk of Livestock. Transgene and promoter are expressed in mammary glands of mice and livestock. Maximum quantification of the recombinant protein present in milk of transgenic animals.

WAP: Whey Acidic Protein, WAP-hPC: Whey Acidic Protein and human Protein C, hAT: human antithrombin, ATIII: antithrombin III, hPC:human protein C, rhPC: recombinant Protein C, PC1: protein C, rFVIII- rVWF-AAT: recombinant Factor VIII and recombinant Von Willebrand Factor and recombinant Alpha 1 Antitrypsin, BLG-FIXDA3': Beta-Lactoglobulin and factor IX, WAP-FIX: Whey Acidic Protein and Factor IX, BLG-AATB: Beta-Lactoglobulin and human alpha 1 antitrypsin, BLG-hF1 α-β-y: Beta-Lactoglobulin and Fibrinogen alpha, beta and gamma chains, aS-1-casein hF1: alpha s1-Casein and human Fibrinogen, oBLG- hF1 α-β-y: Beta-Lactoglobulin and Fibrinogen alpha, beta and gamma chains.

Table 1B: Post-translational modifications

Protein	Total Amino acids	Mr weight	Post-translational modifications					References
			Serine phosphorylation	Tyrosine sulfation	N and O-linked glycosylation	disulfide bridging	γ-carboxylation	
ATIII	464	52.6 kDa	by FAM20C		Asn128, Asn167, Asn187, and Asn224			(Zahn-Zabal et al., 2020)
rhPC	461	52.1 kDa			Thr19, Asn139, Asn290, Asn355, and Asn371	light chain and a heavy chain disulfide-linked	9 gla domain	(Zahn-Zabal et al., 2020)
hFVIII	2351	~260-280 kDa		Tyr-1699 for binding vWF	Asn60, Asn258, Asn601, Asn776, Asn803, Asn847, Asn919, Asn962, Asn982, Asn1020, Asn1024, Asn1074, Asn1085, Asn1204, Asn1274, Asn1278, Asn1301, Asn1319, Asn1431, Asn1461, Asn1829, and Asn2137			(Zahn-Zabal et al., 2020)
hFIX	461	51.8 kDa			Thr85, Ser99, Ser107, Asn203, Thr205, Asn213, Thr215, and Thr225	light chain and a heavy chain disulfide-linked	12 gla domain	(Zahn-Zabal et al., 2020)
hA1AT	418	46.7 kDa			Asn70, Asn107, Thr109, Asn271, and Thr273			
hF1		~340 kDa				Heterohexameric linked		(Farrell et al., 1991; Meh et al., 2001; Zahn-Zabal et al., 2020)
Aα chain	866	66.5 kDa				2Aα-2 Bβ-2 γ’		
Bβ chain	491	52 k Da				2Aα-2 Bβ-2 γ		
γ’ chain		47 kDa		Tyr -422, Tyr -418				
γ chain	453	46.5 kDa		Only in γ’				
rVWF	2813	320 kDa monomer >1M kDa multimer			Asn99, Asn156, Asn211, Asn368, Asn666, Asn857, Asn1147, Asn1231, Thr1248, Thr1255, Thr1256, Ser1263, Thr1468, Thr1477, Ser1486, Thr1487, Asn1515, Asn1574, Thr1679, Asn2223, Asn2290, Thr2298, Asn2357, Asn2400, Asn2546, Asn2585, and Asn2790	All cysteine residues are involved in intrachain or multimer interchain disulfide bonds		(Zahn-Zabal et al., 2020)

Post-translational modifications of recombinant proteins expressed in milk of transgenic mice and livestock. Amino acid and relative molecular weights are shown. Post-translational modifications are described for each recombinant protein: Serine phosphorylation, Tyrosine sulfation, N and O-linked glycosylation, disulfide bridging and gamma-carboxylation.
“Mr”: Relative Molecular Weight by SDS-PAGE, ATIII: antithrombin III, rhPC: recombinant human Protein C, hFVIII: human Factor VIII, hFIX: human Factor IX, hA1AT: human Alpha 1 antitrypsin, rVWF: recombinant Von Willebrand Factor, FAM20C:Family with sequence similarity 20, member C, , hF1 γ’ chain: human fibrinogen gamma prime chain, hF1 γ chain: human fibrinogen gamma chain, Tyr: Tyrosine, Asn: Asparagine, Thr: Threonine, Ser: Serine, Gla: Vitamin K-dependent carboxylation/gamma-carboxyglutamic.

functionally complex vitamin K dependent protein family member having nine gamma-carboxy glutamic acid (gla) residues in the first 35 amino terminal amino acids. Relative to other VKD procoagulant proteins, hPC is very slowly synthesized by hepatocytes to occur at only 4 ug/ml in human plasma. Its unique VKD PTM dependency is illustrated by the impact of the VK competitive inhibitor warfarin. Warfarin inactivates the γ-carboxylation PTM that is essential for procoagulant cascade protein activity as well as hPC functionality and its activation to aPC. Paradoxically, a 24-hour period is needed once warfarin anticoagulant therapy is withdrawn before plasma levels of biologically active hPC are recovered. The procoagulant VKD protein activities come back much faster than does hPC anticoagulant activity to cause a procoagulant activity imbalance with prothrombotic risk. This imbalanced period having dysfunctional hPC can result in profound vascular thrombosis.

The potent anticoagulation activity of activated hPC (aPC) and potential for causing bleeding makes hPC an inherently safer biotherapeutic for anticoagulation than aPC. hPC has two essential regulatory roles that are both centered about its occupation of extravascular reservoirs occurring on the surface of vascular endothelial cells. The activation of hPC to aPC by thrombin is staged at the vascular endothelial protein C receptor (EPCR). EPCR juxtaposed to the thrombomodulin cell membrane protein receptor that thrombin occupies. A properly formed gla domain is required for its vascular endothelial protein C receptor (EPCR) reservoir occupation. This reservoir is needed to maintain hPC:aPC cytoprotective, anti-apoptotic activity in response to profound endothelial cell stress caused by sustained exposure to thrombin. The endothelial cell stress caused by thrombin can lead to cell death that launches profound disseminated intravascular coagulation (DIC). DIC onset is in part prevented by the maintenance of the hPC:aPC:EPCR reservoir at the vascular endothelial surface along with the actions of A1AT anti-inflammatory activity. Further complicating cell stress induced death is the exponential activation of the coagulation cascade requiring an additional role of a sustained hPC:aPC:EPCR reservoir. This is a source of anticoagulation activity needed after vascular injury induced coagulation by tenase and prothrombinase. Thrombin feedback from that coagulation event generates aPC needed to inactivate both “tenase” and “prothrombinase” complexes at the injured vascular surface. In the case of life-threatening hemorrhage, aPC mediated inactivation of both of these complexes shuts down the formation of any continued surge of thrombin to prevent thrombosis from an uncontrolled generation of fibrin.

The biotherapeutic needs for hPC are diverse and include the treatment of bacterial, viral and trauma induced sepsis and DIC. The amounts of hPC biotherapy are clinically difficult to assess and the amounts that can be derived from plasma are limited since it is a trace plasma protein. Furthermore, the need to prophylactically maintain systemic endothelial cell reservoirs to prevent DIC onset would likely require gram hPC doses. Congenital hPC deficiency has been effectively treated using plasma human Protein C replacement in gram doses (Dinarvand, Moser, 2019; Gupta & Patibandla, 2021; Marlar & Mastovich, 1990). Mutations in the PROC gene have been associated with thrombotic events like neonatal purpura fulminans, and recurrent venous thrombosis (Egami et al., 2021; Gupta & Patibandla, 2021).

Biologically active rhPC has been produced in the mammary glands of swine ((Lee et al., 1995; Van Cott et al., 2001; Velander et al., 1992) and mice (Drews et al., 1995; Drohan et al., 2005) (Table 1A). Biologically active recombinant human Protein C (rhPC) has been made in the milk of Swine at 0.2 to 0.4 g/l by using the hPC cDNA and the mouse whey acidic protein promoter (Velander et al., 1992). The immunopurified rhPC had anticoagulant activity equivalent to the physiological protein C derived from human plasma. Importantly, the expression of both biologically active hPC and FIX demonstrate a unique capacity of the pig mammary gland to γ-carboxylate at high levels.

Multigene Transgenesis: Recombinant human Coagulation Factor IX and Engineering of PTM

Factor IX (FIX) is a Mr = 56 kDa VKD-glycoprotein zymogen of the serine protease Factor IXa (Bajaj et al., 1992). Like hPC, it is a trace plasma protein made by hepatocytes occurring in plasma at only 5 ug/ml. The FIX gene lies on the X chromosome and mutations results in the bleeding diathesis observed in hemophilia B. Congenital FIX deficient patients have been effectively treated by using plasma-derived and recombinant human Factor IX replacement as well as gene therapy (Orlova et al., 2012). However, the manufacture of rhFIX made by the culture of Chinese Hamster Cells is characterized by very low levels of active protein due to the difficulty in VKD biosynthesis of γ-carboxylation. Thus, the cell culture manufacturing capacity of rhFIX has limits use worldwide.

Recombinant human Factor IX (rhFIX) has been produced in the mammary glands of transgenic sheep and pigs. In comparison to animal cell culture, these transgenic livestock showed high-level production of rhFIX from 0.12 g/l in sheep milk (Yull et al., 1995) and 2.3 g/l in pig milk (Yull et al., 1995; Zhao et al., 2015) (Table 1A). However, only the pig milk was demonstrated to have significant rhFIX coagulation activity.

The rhFIX in milk of swine was produced about 3,000-fold higher levels than provided by industrial bioreactors at about 1 to 2 g/l in milk of pigs (Zhao et al., 2015). In contrast to sheep expression, about 100-200 ug/ml of functional rhFIX was selectively purified by a sequence of immunoaffinity and γ-carboxylation selective chromatography. While this purified rhFIX had excellent γ-carboxylation, some was inactive due to the lack of the removal of its propeptide (pro-rhFIX). An engineered co-expression of a soluble furin species increased the purified rhFIX specific activity by 25% to a native value of about 200 u/mg. In general, the furin was expressed in milk at a ratio of about 1 furin:100 rhFIX which resulted in the complete removal of the propeptide from pro-rhFIX. This represents the first known engineering of furin-based proteolysis in the mammary gland of a livestock..

Multigene Transgenesis: Recombinant human Fibrinogen

Fibrinogen is a 340-kDa plasma glycoprotein protein composed of a symmetric, hexameric structure of pairs of three Aα, Bβ and γ polypeptide chains (Doolittle, 1984; Gorkun et al., 1997). Butler et al., expressed rhF1 In the milk of transgenic mice using cDNA sequences controlled by the WAP promoter platform (Butler et al., 1997). Prunkard et al., showed the production of functional rhF1 from the genomic sequences in the mammary gland of transgenic mice controlled by the sheep BLG promoter sequence, but some unassembled rhF1 beta chains were secreted (Prunkard et al., 1996). Later, Butler et al., reported that transgenic mice secreted fully assembled fibrinogen molecules into milk at 0.2 g/L concentration, using a murine WAP promoter directing the expression of each of the three hF1 cDNAs (Butler et al., 2004). This effort was enhanced in sheep expression by the gene rescue and stabilization of fibrinogen gene rescue by co-expression of a BLG α1-antitrypsin construct (Cottingham & Mccreath, 2003; Yull et al., 1995).

Importantly, Calcaterra and colleagues showed that 4 g/l of fully assembled rhF1 were consistently expressed across lactation of a cloned transgenic Swiss Brown cow with no detectable free fibrinogen chains (Calcaterra et al., 2013). A bovine αS1 5'-UTR promoter platform was used to stoichiometrically express each of genomic fibrinogen sequences of the three Aα, Bβ and γ hF1 genes. This was the first transgenic milk expression documenting the native translational ratio of the γ and γ' chains as appears in human plasma. This ratio has been shown to a pleotropic physiologic role. While the rhF1 possessed a ruminant whey protein glycosylation pattern, it's use was intended for topical hemostasis in the treatment of trauma and not infusion therapy to restore circulating plasma fibrinogen levels. The purified rhF1 from the lineage of two generations of these transgenic cows was fully functional in preclinical studies of topical fibrin tissue sealant treatment in grade V+ liver trauma pig wound models.

Multigene Transgenesis: High levels of Recombinant human Coagulation Factor VIII expression by and Gene Rescue, Engineered Protein Stability, Co-expression of VWF

Coagulation Factor VIII (FVIII) is a Mr = 280 kDa glycoprotein with a complex PTM proteolytic processing (Hoyer, 1994; Kaufman et al., 1989). The FVIII gene consists of 105 kBpr coding for 2351 amino acid residues and is synthesized by hepatocytes. FVIII is present in plasma at only 200ng/ml but is a potent cofactor for Factor IX that accelerates by >1000-fold the conversion of factor X to activated Factor Xa. This "tenase" complex is dysfunctional in severe type A hemophilia. Tenase dysfunction limits the necessary high level formation of thrombin needed for achieving hemostasis after an initial FVIIa-FXa catalyzed, low level thrombin catalyzed formation of fibrin (Mazurkiewicz-Pisarek et al., 2016). Hemophilia A occurs in 1/5000 male births (Hoyer, 1994; Kasper, 1999). In the most economically developed countries, Hemophilia has been effectively managed by using recombinant human Factor VIII prophylactic replacement and very recently gene therapy in some patients (Nathwani, 2019; Raso & Hermans, 2018). Like rhFIX, limitations in cell culture productivity ultimately restrict worldwide accessibility to rhFVIII therapy.

The difficulty in making rhFVIII in any animal cell results in multiple iterations involving the manipulation of its biosynthesis. This molecular engineering effort must address limitations at the levels of transcription, translation, post translational proteolytical processing, trans-golgi secretory efficiency and general stability of biological structure and function. Transgenic pigs making levels of 2 to 20 ug/ml of the native full length FVIII molecule showed functional instability (Paleyanda et al., 1997). Subsequently to that study, a combination of gene rescue used to address transcriptional restrictions, the expression of a re-engineered FVIII B-domain used to better enable proteolytic processing while co-expression of human von Willdebrand Factor at high levels to stabilize the final proteolytically processed FVIII. This complex rhFVIII expression effort was studied in the transgenic mice (Pipe et al., 2011). About 0.3 g/l or higher with native biological activity was stably secreted into in milk of these transgenic mice. Future work will use this multi-gene platform in transgenic pigs to address the numerous biosynthetic restrictions that making rhFVIII presents.

Summary

The use of the milk of livestock to make recombinant biotherapeutics was developed to conquer four main obstacles that largely and still prevent world-wide clinical needs from being met. The eventual conquering of the following challenges has been demonstrated from a proof-of-concept perspective:

1. Relative to human plasma, a greatly reduced pathogen risk, SPF source with a more rapid scaleup to biotherapeutic manufacturing capacity generally needed to satisfy world clinical demand.
2. An animal cell-based manufacturing that provides acceptable mammalian PTM signatures needed to impart the complex physiologic biotherapeutic functionality.
3. A scaleup to capacities that can potentially provide a worldwide clinical abundance needed both for more cost-effective IV administration and also for decreased healthcare complexity that can be achieved by inhaled and oral methods of biotherapeutic delivery as well as biotherapeutic tolerization by oral administration.

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