The Large-Scale Production of Recombinant Human Serum Albumin in the Milk of Transgenic Cattle: Strategy & Implications

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Abstract: The annual demand for therapeutic human serum albumin (HSA) is estimated to be more than 500 metric tons worldwide. As a major protein in the human body, HSA plays a vital role in many physiological processes, including the maintenance of oncotic pressure and the transportation of various biomolecules and pharmaceuticals. Currently, all HSA used for clinical blood expansion purposes is isolated from pooled human blood or plasma, an unpredictably fluctuating supply that can at times fall to dangerously low levels. Furthermore, this supply is derived from thousands of different donors and can potentially result in the spread of pathogenic contaminants to recipients. The use of transgenic animals, such as cattle, as living bioreactors, provides a potential solution to this problem by enabling the large-scale production of recombinant HSA (rHSA) in a cost-effective manner. Cattle are capable of producing large amounts of milk that can potentially yield abundant quantities of a desired recombinant protein. The production of rHSA in the milk of cattle would provide an economical resource that circumvents the current dependence on blood-derived sources. One challenge to this system, however, is the presence of endogenous bovine serum albumin (BSA) in the milk. BSA is a highly conserved ortholog of HSA that necessitates a tedious and prohibitively expensive purification process, which has up to now hindered the efficient purification of rHSA from bovine milk. Here we overview our approach to humanize the endogenous BSA gene, replacing it with an rHSA minigene construct, which should allow for normal expression of rHSA in the liver, as well as exogenous expression of rHSA in the milk. The future generation of such cattle supports the potential for a safer and more reliable source of therapeutic rHSA.

Keywords: Biopharming, Transgenic cattle, Milk, Recombinant human serum albumin, TALENs, Homologous recombination, Somatic cell nuclear transfer

1. Introduction

1.1 Physical Properties and Clinical Importance of Serum Albumin

Human serum albumin (HSA) is the most abundant protein in the body, accounting for over 50% (35-50 g/L) of the total protein in blood plasma [1, 2]. It is a negatively-charged, hydrophilic, and non-glycosylated protein with three binding domains (I, II, III) and a molecular weight of approximately 66.5 kDa [1-3]. Synthesized in the liver, serum albumin functions primarily as a physiological stabilizer of oncotic pressure and as a major transport protein for various endogenous and exogenous ligands [1, 2, 4]. HSA is used extensively in the clinic as a critical component in blood volume resuscitation [2, 5-7] and for the treatment of hypoalbuminemia [1, 7], a multifactorial condition that has been shown to positively correlate with an increased risk of morbidity and mortality among hospitalized patients [2, 8]. Additionally, due to its nonimmunogenic properties and relatively long half-life, HSA is utilized as a drug delivery vehicle or excipient to help stabilize and improve the overall pharmacokinetics of an active drug molecule [2, 9]. HSA is used in various other applications, including its use as a cell culture supplement in various clinical and non-clinical procedures, such as in vitro fertilization (IVF) and recombinant protein production [1, 2, 10].

1.2 Historical Perspective of Serum Albumin
The greatest incentive for the large-scale production of HSA as a pure protein occurred during WWII, when a critical need for a more stable and less antigenic substitute for whole blood plasma became evident on the battlefield [2, 5, 11]. Initially, due to its availability in large quantities, serum albumin was purified from bovine plasma using the cold ethanol (EtOH) method, developed by E.J. Cohn and colleagues, a technique that is still widely in use [2, 11]. Unfortunately, intravenously administered bovine serum albumin (BSA) resulted in sickness and death among some of the volunteer subjects [2, 11]. Attempts to further purify surrounding proteins from the albumin samples by crystallization were ineffective and resulted in termination of the bovine albumin program in 1943 [2, 11]. Once it was recognized that the adverse reactions were due to interspecies differences and not impurities, the emphasis of the plasma substitute program quickly shifted toward the purification of HSA from donated blood, provided by the American Red Cross [2, 11]. For more than 50 years, HSA has been a standard in hospitals worldwide and will continue to be clinically important until an alternative can thoroughly prove its superiority in quality, availability, and efficacy [2, 7].

1.3 Limitations Surrounding the Current Source of HSA

Since the 1940s, all clinically applicable HSA used for blood-expansion purposes has been derived by EtOH fractionation of human blood that has been pooled from a multitude of donors [2, 4, 5, 11]. Despite preventive measures and extensive screening procedures, blood-derived products continue to carry an associated risk of potential contamination with human viral pathogens and prions [1, 2, 5, 12, 13]. Between the 1970s-1990s, thousands of patients contracted HIV or hepatitis after receiving treatment with contaminated blood-derived factor VIII or fibrinogen [2, 12, 14]. During the same time, hundreds of women that were undergoing IVF with culture media containing contaminated donor serum were infected with hepatitis [2, 10]. In addition, the secondary transmission of the virulent prion disorder, variant Creutzfeldt-Jakob disease (CJD), through blood and blood-derived products, such as factor VIII concentrates, have been reported in several cases worldwide [2, 13]. Consequently, many blood factors, including virtually all factor VIII, are now produced by recombinant methods, which are highly preferred over blood-derived sources [1, 2, 5, 6, 12].

Furthermore, the recall of blood-derived products due to the confirmed or suspected presence of pathogens, as well as a general lack of available donors, has caused a perpetual fluctuation in the supply of HSA [1, 2, 5, 6]. In fact, recent shortages of therapeutic HSA have caused major issues in certain parts of the world such as China, Japan, and India, where hospitals nationwide have received half as much HSA as is needed and must consequently prioritize among their patients [1, 2]. Commercially, the unit price of therapeutic HSA is relatively low ($3.00-$5.00 per gram), however, issues with maintaining its supply continue to cause a steady rise in prices [1, 2 5]. In certain cities, this has led to the development of underground economies where fraudulent and potentially dangerous “albumin” has been found to be illegally circulating the market [2, 6]. To remedy these issues, it is anticipated that the recombinant production of biopharmaceuticals will provide the market with safer, more reliable replacements of HSA and other blood-derived proteins, ultimately replacing current human-derived sources and thereby avoiding any potential for human pathogenic contamination [1, 2, 6, 12].

1.4 Recombinant Protein Expression

To avoid the potential spread of blood pathogens, there exists a continuous effort to shift towards non-human derived sources of blood products, such as HSA. The use of transgenic expression systems as living bioreactors has promoted a constructive method for the production of biotherapeutics [2, 12]. Today, recombinant proteins are produced in a variety of transgenic systems including bacteria, yeast, plants, insect cells, mammalian cells, and domesticated animals [2, 15].

Bacterial expression systems are capable of mass producing large amounts of simple proteins in a rapid and inexpensive manner [2, 15]. In addition, bacteria are easy to culture and are highly amenable to genetic modifications [1, 2, 15]. Despite these advantages, bacteria are not capable of providing the proper post-translational modifications that are often necessary for complex eukaryotic protein bioactivity [2, 15-17]. Bacterial hosts were also the earliest platform utilized for the production of recombinant HSA (rHSA). However, these attempts yielded improperly folded or processed products, resulting in insoluble, aggregated forms of non-functional rHSA protein [1, 2].
Proteins that are not efficiently produced in bacteria are often produced in yeast fermentation systems, which are also able of rapidly synthesizing high levels of protein [2, 4, 15]. As eukaryotes, yeast are able to perform some of the necessary folding and protein modifications [2, 15, 18], however, in many instances, fungal systems have proved unable to provide the appropriate mammalian-specific modifications, leading to tedious and expensive purification and processing [2, 16, 17, 19]. Limitations in the ability of eukaryotic systems to replicate human patterns of protein processing can yield immunogenic or non-functional recombinant products [2, 16]. Furthermore, proteolytic degradation of rHSA secreted into the culture broth by endogenous yeast proteases persists as an issue in these systems [1, 2]. Strategies to reduce degradation by inactivation of putative protease genes can be time-consuming and the sufficient production of rHSA protein on an industrial scale continues to be challenging [1, 2]. These limitations as well as the high cost associated with the construction and maintenance of a fermentation facility decreases the appeal of yeast as a recombinant protein expression system [2, 15].

Transgenic insect cells, avian eggs, and plants, in particular transgenic tobacco and rice, are other systems capable of expressing heterologous proteins [2, 6, 15, 20, 21]. These expression systems are attractive alternatives in terms of low production cost and safety, but are limited by incorrect post-translational modifications, difficult and expensive purification techniques, and relatively low levels of protein expression [2, 15, 20]. Additionally, codon-usage bias is of concern, especially in the context of recombinant protein expression using non-mammalian hosts such as bacteria, yeast, and plants [2, 20, 22, 23]. For example, certain studies have revealed differences in amino acid sequence between rHSA produced in yeast and HSA isolated from human serum that could potentially cause problems with antigenicity [2, 24]. Studies have demonstrated that rare codon usage and differences in the levels of corresponding transfer ribonucleic acids (tRNAs) in different species can affect the translational efficiency and stability of a desired recombinant protein [2, 20, 22, 23, 25]. These interspecies variances necessitate arduous optimizations that are required to overcome translational inefficiencies associated with species-specific codon bias [2].

Mammalian culture systems are capable of overcoming many of these limitations and are currently the preferred method for the production of more complex recombinant proteins that require post-translational processing [2, 20]. At present, the majority of biopharmaceuticals are produced using mammalian cells, in particular immortalized Chinese Hamster Ovary (CHO) cells, due to their high production capabilities [2, 17, 26]. A vast number of market-approved biotherapeutics are produced in CHO cells, including different blood clotting factors such as factor VIII and factor IX and various monoclonal antibodies such as Humira and Herceptin [2, 26]. Although, acceptable yields of many biotherapeutics can be achieved in this way, the cost-effective production of rHSA is still unattainable since most systems cannot compete with the high annual demand of 500 metric tons worldwide [1, 2, 15, 18]. Furthermore, these systems are often unable to compensate for the high expenses associated with the establishment, scale-up, and maintenance of cell-based culture facilities [2, 12, 16, 18].

The use of domesticated animals such as rabbits, goats, pigs, and cattle as transgenic bioreactors is actively being developed, particularly with the production of biopharmaceuticals in the milk [2, 5, 15, 16, 19, 27]. In 2009, Atryn, a recombinant form of human anti-thrombin produced by Genzyme Transgenics Corporation (GTC) in the milk of goats became the first therapeutic protein produced by “pharming” means to obtain U.S. Food and Drug Administration (FDA) approval for clinical use in humans [2, 27]. Although other biological fluids, such as blood, urine, and semen, from animals can be targeted as the source of recombinant proteins, milk has remained the most attractive due to its non-invasive availability, abundance, and relative ease of purification [2, 15, 16]. According to published data, the mammary gland is fully capable of properly post-translationally modifying recombinantly expressed human proteins, such as HSA and lactoferrin [2, 5, 19]. Furthermore, venture and annual maintenance costs associated with the establishment of a transgenic livestock farm are several orders of magnitude lower than that for a standard cell culture facility [2, 12, 15, 16]. The expression of heterologous proteins in the milk of genetically modified (GM) dairy animals provides a reliable and economically feasible approach to producing large-scale bio-therapeutic proteins. For these reasons, the use of transgenic livestock as recombinant expression systems has developed into a promising method of production for human biotherapeutics [2].

1.5 Recombinant Protein Production in Milk
The selection of a particular species for the mammary-specific production of a heterologous protein is determined by several parameters (Table I) including: the required quantity of a particular recombinant protein, the milk production capacity of the animal per natural lactation, gestation time, reproductive capacity, and associated costs [2, 28]. Milk-specific promoters and associated regulatory sequences are commonly used to direct the expression of a heterologous protein into the mammary gland, with protein expression levels being dependent on the type of sequences utilized, the type of protein to be expressed, and the integration site of the transgene [2, 18]. Due to the greater lactational capacity of larger animals, traditional dairy species such as goats and cattle are often the primary choice for the large-scale expression of recombinant proteins [2, 16, 28]. Although goats are capable of producing suitable quantities of heterologous protein, the use of the cattle mammary gland is especially appealing because of its annual capacity to produce substantial volumes of milk, with the potential to yield significant amounts of recombinant protein [2].

<table>
<thead>
<tr>
<th>Host Animal</th>
<th>Annual Milk Production (liters)</th>
<th>Annual Recombinant Protein Production (kg per lactating animal)</th>
<th>Average Gestational Period (days)</th>
<th>Average Number of Offspring</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mice</td>
<td>0.001</td>
<td>0.000201</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>Rabbits</td>
<td>4</td>
<td>0.004</td>
<td>31</td>
<td>8</td>
</tr>
<tr>
<td>Pigs</td>
<td>1.20</td>
<td>0.120</td>
<td>114</td>
<td>9</td>
</tr>
<tr>
<td>Sheep</td>
<td>200</td>
<td>0.100</td>
<td>147</td>
<td>2</td>
</tr>
<tr>
<td>Goats</td>
<td>800</td>
<td>1</td>
<td>150</td>
<td>2</td>
</tr>
<tr>
<td>Cattle</td>
<td>10,000</td>
<td>10</td>
<td>285</td>
<td>1</td>
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In comparison to blood products derived from thousands of human donors, it is generally accepted that recombinant milk-derived products provide a safer source of biopharmaceuticals. Prion disorders are not considered to be a risk in transgenic dairy animal production systems since prions do not naturally occur in the mammary gland [2, 5] and have only been documented to be transmitted through the milk of sheep that are severely infected with mastitis [2, 29]. According to The World Health Organization (WHO), tests conducted on mice using milk from BSE-infected cattle have not been shown to exhibit transmissible properties [2]. In 2007, Hematech reported successful production of healthy cattle lacking expression of the prion gene, an exciting development in the field of agriculture and biotechnology [2, 30]. These prion-free cattle could potentially contribute advantageously to the phenotype of transgenic livestock used for biopharming purposes [2]. Furthermore, the FDA regulations have prohibited the inclusion of mammalian protein in feed intended for ruminants since 1997, and in 2008, this ban was expanded to prohibit high risk tissue materials in all animal feed [2]. Finally, transgenic cattle would be maintained under highly-controlled, closed conditions where their health would be carefully monitored [2]. Taken together, the currently enforced regulations and the application of risk-minimization precautions should further diminish any concerns regarding transmission of bovine spongiform encephalopathy (BSE) [2].

### 1.6 Production of rHSA in Bovine Milk

Previous efforts undertaken to efficiently purify rHSA from the milk of transgenic dairy cattle [2, 5] have been hindered by the presence of significant amounts of BSA, which naturally enters the milk as a serum transudate [2, 31-33]. Due to its relatively small size and high concentration in the blood, albumin is able to passively infiltrate the paracellular spaces of the mammary alveolar epithelium from surrounding capillaries and is present in the milk at a concentration of 0.2-0.4 g/L [2, 32, 34]. At the amino acid level, HSA and BSA are 77% identical and 87% homologous (Table II), and thus highly conserved in terms of sequence, structure, and function [2, 3]. Exploiting slight differences in amino acid composition, the isolation of rHSA from endogenous BSA is mainly achieved using affinity chromatography techniques, such as antibody- or synthetic chemical-coupled columns that specifically bind to HSA [2, 35]. The cost, long-term quality, and scalability associated with these types of columns are critical parameters to consider [1, 2, 35]. Since rHSA intended for clinical use is required in large dosages that typically exceed 10 g/vial, it is challenging to meet expected standards of purity on an
industrial scale while remaining cost-effective [1, 2]. Affinity tags fused to HSA can be used to facilitate purification of the protein, however, the removal of fused tags requires expensive proteases that must cleave the tag with 100% efficiency to avoid any potential issues with altered biological activity and adverse immunological reactions [2, 36]. While this technique is extremely useful for small-scale research applications, it is impractical for the development of recombinant proteins on an industrial scale.

<table>
<thead>
<tr>
<th>Amino Acid Residue Distribution</th>
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<tr>
<td><strong>Residue</strong></td>
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<tr>
<td><strong>HSA</strong></td>
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<td><strong>BSA</strong></td>
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It is anticipated that the elimination of endogenous BSA in the starting material should facilitate the simple and inexpensive purification of rHSA from bovine milk. In the absence of endogenous BSA, conventional dairy-handling procedures can be utilized to efficiently isolate rHSA from surrounding components that are naturally present in bovine milk. These techniques include the removal of fat molecules by centrifugation and standard skimming methods, the precipitation of casein proteins by rennin (chymosin) treatment, and finally the use of size-exclusion filtration to remove other whey proteins as well as any somatic cells, bacteria, and viral particles that may be present [2, 18, 19, 28, 33, 35].

1.7 Transgenic Animal Technologies

For over two decades, the generation of transgenic animals has been accomplished using pronuclear injection or the microinjection of foreign DNA directly into the pronuclei of oocytes [2, 16, 28, 37]. Germline transmission of transgenes by pronuclear injection was applied to create the first transgenic livestock in 1985 [2, 38, 39]. This method has been widely used since then despite significant limitations, including random integration into the host genome, significant loss in the rate of blastocyst development, and the frequent incidence of mosaicism, where only a certain percentage of the animal’s cells will have incorporated the transgene [2, 12, 16, 39]. Furthermore, this technique is very inefficient with integration of DNA into the host genome approaching 1% on average in large domestic animals [2, 12, 16, 28].

More recently, the application of GM somatic cells as nuclear donors in somatic cell nuclear transfer (SCNT) has provided a wide range of possibilities for the introduction of transgenes into the germline, including precise gene-targeting and a substantial improvement in the production of live transgenic animals [2, 16, 28]. The donor somatic nucleus, introduced into an enucleated oocyte, is subsequently reprogrammed by the recipient oocyte’s cytoplasm, resulting in a reconstructed embryo with totipotent potential or the ability to give rise to all cells of an organism, including embryonic and extraembryonic tissues [2, 40]. Dolly the sheep was the first mammal to be cloned from a somatic cell nucleus in 1997 at the Roslin Institute and several other species have since followed [2, 40-42]. Cloning of cattle by SCNT has demonstrated great potential for a variety of agricultural and biomedical applications, including the production of therapeutic proteins in transgenic cattle [2, 40]. While successful and widely used, the full-term developmental potential of SCNT-derived offspring using adult cells remains low, with an overall efficiency rate of about 1-5% in most mammalian species [2, 39, 40, 43]. Furthermore, SCNT is frequently associated with pre-, peri-, and postnatal loss and a high incidence of developmental abnormalities [2, 39, 40, 43, 44]. This low cloning efficiency is generally attributed to an incomplete erasure of the highly differentiated and globally methylated somatic cell epigenome, which hinders proper reprogramming of the donor cell nucleus by the oocyte to that of a totipotent ‘embryonic’ state [2, 43, 44]. The use of dedifferentiated nuclear donors, such as stem cells or induced pluripotent stem (iPS) cells, is hypothesized to increase the efficiency and overall success rates of SCNT by providing an epigenetic landscape that is more readily reprogrammed in the nuclear transfer process [2, 41, 43]. Unfortunately, the derivation of embryonic stem (ES) cells and true iPS,
that can maintain pluripotency without the continuous expression of exogenous factors, has not been successful in livestock species [2, 12, 39, 41, 45].

Until recently, unlike homologous recombination (HR) or homology-directed repair (HDR) in ES cells, HR in primary somatic cells is extremely inefficient, occurring at very low frequencies of less than 0.0001% [2, 41, 46]. Site-specific nucleases, such as transcription activator-like effector nucleases (TALENs), allow for precisely targeted genome modifications and can be used to significantly improve HR efficiency in a variety of species and cell types [2, 47-50]. Chimeric TALEN proteins function as heterodimers that consist of a site-specific DNA binding domain conjugated to a nuclease cleavage domain [2, 51]. Dimerization of the engineered TALEN proteins to the target site results in the activation of the nuclease domains and the subsequent introduction of a double-strand break (DSB) [2, 51-53]. Two cellular pathways exist to repair the effected DSB: non-homologous end joining (NHEJ) or HDR [2, 51-53]. In the absence of an exogenous donor template, the cell will employ NHEJ, a mutagenic repair mechanism which can result in the introduction of small insertions and deletions (indels) at the target site [2, 51-53]. In many instances, this can result in a frameshift mutation leading to disrupted expression of a targeted gene [2, 51, 53]. Alternatively, in the presence of a double-stranded DNA (dsDNA) template containing regions of homology to the target site, the cell will preferentially perform HDR of the introduced DSB, resulting in targeted modification of the genome with [2, 51, 53].

TALENs and other genome-editing proteins provide a powerful and rapidly advancing platform for genome engineering and are already demonstrating their potential to completely revolutionize the fields of molecular biology and biotechnology. In conjunction with biopharming, site-specific nuclease technology enables the efficient creation of sophisticated genetic modifications with significant biomedical and agricultural implications.

2. Transgenic Strategy for rHSA Cattle

As an initial step toward the future large-scale production of rHSA in cattle, we review here the transgenic strategy used to modify the bovine genome, with the aim of humanizing the endogenous BSA gene by a TALEN-mediated targeted integration. Our approach to disrupt and replace BSA gene expression with that of rHSA required the design and development of a specific targeting construct, which will ultimately allow for the directed expression of rHSA in both the liver and the mammary gland. Following this strategy, the elimination of BSA from bovine milk can aid to simplify and economize the downstream purification of rHSA for therapeutic applications.

(1) The gene-targeting construct, termed pHSA-neo, was designed to target the first exon of the BSA gene as an 11.5-kb unit, resulting in the simultaneous disruption of endogenous BSA expression (Figure 1 A/B/D) [31]. The pHSA-neo construct (Figure 1 A), which contains a direct repeat of two HSA minigenes, is flanked by regions of bovine homology specific to the BSA locus on chromosome 6, each approximately 1 kb in length [31]. The first rHSA coding sequence is a promoterless cDNA that will, following proper HR, be driven by the endogenous BSA promoter and will direct rHSA transgene expression to the liver and ultimately into the blood [31]. The second downstream rHSA cDNA is under the control of a milk-specific, α-lactalbumin promoter, and will direct rHSA expression to the mammary gland [31]. A previously characterized region of the bovine α-lactalbumin promoter, comparable to that cloned into pHSA-neo, was chosen for this system due to its ability to efficiently direct the expression of randomly integrated transgenes into the milk of mice [2, 54]. The targeting vector has been designed in this way to maintain potential BSA regulatory sequences, located in the upstream promoter region and/or downstream intronic regions of the endogenous gene, which will remain to optimally regulate expression of the rHSA transgene [31].

(2) The pHSA-neo construct was targeted to the BSA locus in primary male and female bovine fibroblasts by TALEN-stimulated homology-directed repair (HDR) [31]. The TALENs (Cellectis) were designed to target the BSA locus immediately downstream the endogenous ATG initiation codon (Figure 1 C) [31]. Their targeting efficiency was evaluated in bovine fibroblasts and determined to be approximately 8%, by sequencing of the amplified TALEN target site for the presence of any introduced indels [31]. Based on an average transfection efficiency of 70.4% for a similarly sized eGFP-expressing plasmid, we estimated the actual indel frequency to be approximately 11.8% [31]. Bovine fibroblasts, cotransfected using nanoparticles (jetPRIME) with the pHSA-neo targeting construct and the TALENs, were incubated at 30°C for 72 hours,
a technique which has been shown to significantly increase the targeting efficiency of site-specific nucleases [31, 55]. About 13 days post-transfection, neomycin-resistant clones were isolated by ring-cloning for further expansion and analysis [31]. Genomic DNA extracted from the clones was subjected to PCR and sequencing analysis to confirm the precise integration of the pHSA-neo construct [31]. Using TALEN technology to significantly stimulate HR in bovine fibroblasts, we were able to achieve monoallelic integration of the 11.5 kb construct at the BSA locus with an average frequency of 11% [31].

Figure 1. Humanizing the BSA locus using TALEN-stimulated HR [2, 31]. A) Structure of the 11.5 kb pHSA-neo targeting construct containing two HSA minigenes and 1 kb targeting arms. The bovine α-lactalbumin promoter is 2 kb in size. Diagram is not drawn to scale. In addition, the donor construct contains a centrally located floxed neomycin-resistance cassette for positive clone selection. B) Structure of the BSA gene on chromosome 6. Targeting arms are homologous to a region immediately upstream of the endogenous 5′ translation initiation codon and intron 2 C) Binding of the left and right TALENs to the target site within the first exon of the BSA gene. To stimulate HR, double strand breaks are expected to be produced by the FokI nuclease domains in the 15-bp spacer region. D) Positive recombination results in the deletion of an 835-bp region of DNA, removing bovine exons 1, intron 1, and exon 2 and thereby disrupting the endogenous BSA gene and replacing the 5′ coding region of the BSA gene with two copies of an HSA minigene. HSA1 will be under the control of the endogenous BSA promoter and will direct rHSA expression to the liver and ultimately the blood and HSA2 will be specifically expressed in the mammary gland under the bovine α-lactalbumin promoter.

(3) Bovine fibroblasts from the targeted male cell line, heterozygous for pHSA-neo at the BSA locus, were used as nuclear donors in SCNT to generate transgenic bovine blastocysts [31]. The modified fibroblasts were G1-synchronized by cell culture confluency to coincide with the cell cycle status of the recipient oocyte. Bovine oocytes, aspirated slaughterhouse-sourced ovaries, were incubated in cytochalasin B and Hoechst stain following the removal of surrounding cumulus cells. Enucleation was confirmed by the presence of the UV illuminated metaphase II (MII) plate and first polar body within the micropipette (Figure 2). The donor fibroblasts were transferred to the perivitelline space of the enucleated oocyte and fibroblast-oocyte couplets were reconstructed by electrofusion. Fused embryos were chemically activated with ionomycin.
and 6-dimethylaminopurine (DMAP). Activated embryos were cultured for 8 days in modified synthetic oviduct fluid in an atmosphere of 38.5 °C, 5% CO2, 5% O2, and 90% N2 for blastocyst development [31]. The resulting blastocysts were lysed for DNA extraction and subjected to PCR analysis to confirm the presence of the rHSA modification [31]. The blastocyst development rate using targeted cells was observed to be approximately 10%, with 50% of the analyzed blastocysts exhibiting the desired rHSA modification [31].

Figure 2. Overview of SCNT using bovine oocytes [2]. a) Oocyte with surrounding layer of cumulus cells, known as the cumulus-oocyte complex (COC). b) Enucleation of an oocyte. The Hoechst-stained MII plate and first polar body are illuminated by UV in the enucleation pipette. c) Developing blastocysts incubated in an atmosphere of 38.5 °C, 5% CO2, 5% O2, and 90% N2 following effective fusion with donor nuclei.

3. Conclusion

Biopharming technology is expected to eventually decrease our dependence on current blood-derived sources of HSA, which continuously fluctuate in supply and also carry an associated risk of potential pathogenic contamination. Fulfilling the annual global demand for clinical HSA requires a readily scalable system capable of producing high levels of rHSA that can be easily and inexpensively purified. The expression of recombinant proteins has been investigated in many systems, including the bovine mammary gland which can serve as a high-capacity bioreactor for the largescale production of rHSA [1, 2, 5]. A challenge posed by this system, however, is the presence of significant amounts of BSA in the milk as a result of normal leak-through from the bloodstream [32]. Consequently, the high-levels of structural similarity between BSA and HSA render rHSA difficult and expensive to purify from bovine milk [31]. To address this issue, we developed a transgenic strategy aimed at replacing endogenous BSA with rHSA in the blood, while simultaneously providing a sequence for milk-specific rHSA expression [31]. GM cows that are bred to homozygosity for the transgene locus will produce milk that contains rHSA as a result of normal serum transudation and by mammary synthesis, as a function of our integrated α-lactalbumin promoter [31].

Healthy transgenic rHSA mice, exclusively expressing HSA in the place of endogenous mouse serum albumin (MSA), have been developed by New Century Pharmaceuticals to aid in the evaluation of therapeutic compounds intended for clinical use in humans [2, 31, 56]. The even tighter homology that exists between HSA and BSA, as opposed to HSA and MSA, reinforces the belief that HSA will be capable of supporting life in transgenic cattle [2, 31].

Following standard cattle husbandry techniques, the transgenic, SCNT-derived blastocysts can be transferred to a hormonally synchronized female for a 9 month gestation period [2]. Homozygous rHSA cows, generated through the crossbreeding of heterozygous male and female founders are anticipated to produce an annual yield of 10-20 kilograms of rHSA per cow pre-purification, following the onset of natural lactation [2]. In theory, a farm containing 1,000 GM cows, each expressing about 1-2 g/L rHSA and up to 10,000 liters of milk, could potentially provide 10-20 metric tons of the worldwide demand for HSA every year [2]. The existence of 25-50 of these transgenic farms could potentially fulfill the annual global requirement of 500 metric tons [2].

Ultimately, the future establishment of such GM cattle provides the potential for a reliable, inexpensive, and quality-controlled source of rHSA, overcoming current blood-derived concerns and stabilizing the ever-increasing global demand for therapeutic HSA.

References


