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[10.30-10.50]
From antibodies to Aptamers
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Monoclonal antibodies were developed three decades ago. Today they are used in a wide range of applications, including the targeted therapy of human neoplasia. There are now eight monoclonal antibody based therapeutics approved for the treatment of cancer, and, monoclonals represent one of the fastest growing categories of new drug approvals in oncology. A new class of molecules, which like monoclonal antibodies, display both specificity and high affinity for their target, is now emerging. These drugs are Aptamers, agents that, despite their functional parallels with antibodies, are nucleic acid molecules rather than glycoproteins. These aptamers have a number of properties that make them attractive as a new class of therapeutic molecules.

Aptamers were first discovered by virologists studying HIV and adenovirus. They found several small, structured RNAs that bound to viral proteins and either modulated their activity, which was vital to replication, or inhibited the function of proteins involved in cellular antiviral responses. Over the past decade, large libraries of RNA and DNA aptamers have been generated, using the systematic evolution of ligands by exponential enrichment (SELEX) process, developed by Tuerk & Gold. The targets for these aptamers vary widely, and include dyes, viral proteins, NF-kappa B, Tenascin-C, by probing antigens presented by a monolayer of glioblastoma derived cells, hepatitis C virus proteases, TNFalpha, protein kinase C, thrombin, factor VIIa, VEGF, PDGF-Beta, PSMA and nucleolin. Two aptamers are in clinical trials, and one, an anti-VEGF aptamer, is approved for the treatment of macular degeneration.

One of the ongoing clinical trials, utilises a G-rich DNA aptamer, that binds specifically to nucleolin, a protein found in the nucleus and cytoplasm of normal cells, but also expressed on the cell membrane of both tumour cells and tumour vascular, endothelial cells. This DNA aptamer, previously known as AGRO-100, and now AS1411, entered clinical trials at the Brown Cancer Centre, Louisville, Kentucky, and in a phase I clinical trial has been shown to induce both stable disease and partial responses in patients with a variety of tumour types. The properties, *in-vitro*, *in-vivo* and clinical, of this aptamer to nucleolin will be discussed.

[10.50-11.10]
Expression of a humanized antibody repertoire in transgenic rabbits
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Introduction: Polyclonal antibodies are effective treatments for many viral and bacterial infectious diseases. In addition, polyclonal rabbit and horse antibodies are being used for the depletion of lymphocytes in immunosuppressed transplant patients. However, the use of animal derived antibodies in humans is limited by their immunogenicity. While the immunogenicity of monoclonal antibodies can be reduced by various humanization strategies, non-immunogenic human polyclonal antibodies (hPABs) can only be prepared from human plasma. The supply of hPABs is limited because human blood donors cannot be hyperimmunized and anti-bacterial or anti-viral titers in hPABs are low (about 2 to 8). As a consequence, effective therapy requires the administration of large quantities of hPABs. Genetically engineered animals could provide a source of hPABs, especially specific hPAbs resulting from hyperimmunization with human pathogens, human cytokines or tumor antigens. Mice with transgenic human immunoglobulin loci have been generated and are being used for the generation of therapeutic human monoclonal antibodies. However, the expression of human immunoglobulin loci in transgenic mice is ineffi-

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cient, and generation of a human antibody repertoire in transgenic mice required inactivation of endogenous immunoglobulin loci. Similarly, expression of human immunoglobulins in transgenic cows carrying a human minichromosome was in the nanogram range.

For the effective expression of a human antibody repertoire in a farm animal it is important to consider species-specific aspects of B-cell development and antibody diversification. Generally, all vertebrates start the creation of the primary antibody repertoire by recombining V, D, and J gene segments. In mice and humans this step results in considerable diversity as hundreds of VDJ genes are randomly recombined and genes are imprecisely joined together. In most other vertebrates, including rabbits, chickens, cows and sheep, this first step of VDJ recombination does not lead to significant diversity because only a limited number of V genes are employed. To enhance diversity of the primary repertoire, these animals use a second step to modify antigen-binding regions through templated (gene conversion, Figure 1) and/or non-templated (hypermutation) mutational processes. Gene conversion creates broad diversity by modifying all three antigen-binding sites of the VDJ region. Consistent with the hypothesis that gene conversion requires high homology of V elements, rabbit and chicken Vs belong to single families.

Another important difference to consider is in immunophysiology. In mice and humans, the fetal liver, omentum and bone marrow serve as primary sites for B cell development and the process of immunoglobulin gene rearrangement appears to occur thoughout life. In rabbits diversification by gene conversion occurs in the appendix and other gut-associated lymphoid tissue, in chickens in the bursa of Fabricius, in cows in spleen. Rearrangement of Ig genes stops in the chicken at hatching and diminishes in rabbits, cows and sheep after birth .

Generation of Rabbits with a human antibody repertoire. For the production of therapeutic human polyclonal antibodies we selected the rabbit for the following reasons: (i) short generation time (5-7 months), (ii) fast growth, (iii) production of high affinity, high specificity antibodies, (iv) established GMP production of therapeutic antibodies, and (v) excellent safety record (over 100,000 human transplant recipients have been treated with rabbit IgG).

Based upon the experience with transgenic mice and the further complication of the absence of compatible pathways, we speculated that the simple introduction of a human immunoglobulin gene into a

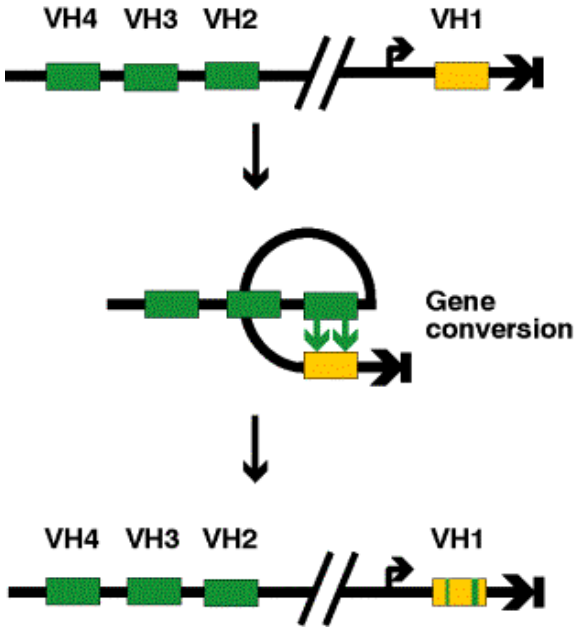

Fig. 1. Schematic representation of gene conversion. Homology between V genes facilitates conversion which starts 5‘ and ends where homology drops below threshold. DNA sequences from upstream V gene elements are introduced into the rearranged V gene. Most changes are in the CDR regions of the rearranged V gene. Gene conversion is a “CDR grafting” process.

gene-converting animal may result in impaired antibody production and diversification. To address this technical challenge, THP developed a novel approach that selectively humanizes only the immunoglobulin-coding elements of the Ig translocus while leaving the endogenous regulatory and antibody-production machinery intact (Figure 2). This human-animal translocus is a substrate for enzymes involved in DNA repair by gene conversion to support production and diversification of high titer, high affinity antibodies in gene converting animals.

For the isolation of rabbit immunoglobulin genes, BAC libraries were generated and high density filter arrays were screened with probes specific for rabbit light chain variable, κ1 joining region, and κ constant region, and probes specific for rabbit heavy chain variable, joining, μ constant, γ constant, α constant, and 3´ enhancer region. A total of 23 clones for the light chain and 56 clones for the heavy chain were isolated. Two light chain and four heavy chain BAC clones were completely sequenced (about 800 kb). Maps of the clones are shown in Figures 3 and 4, respectively.

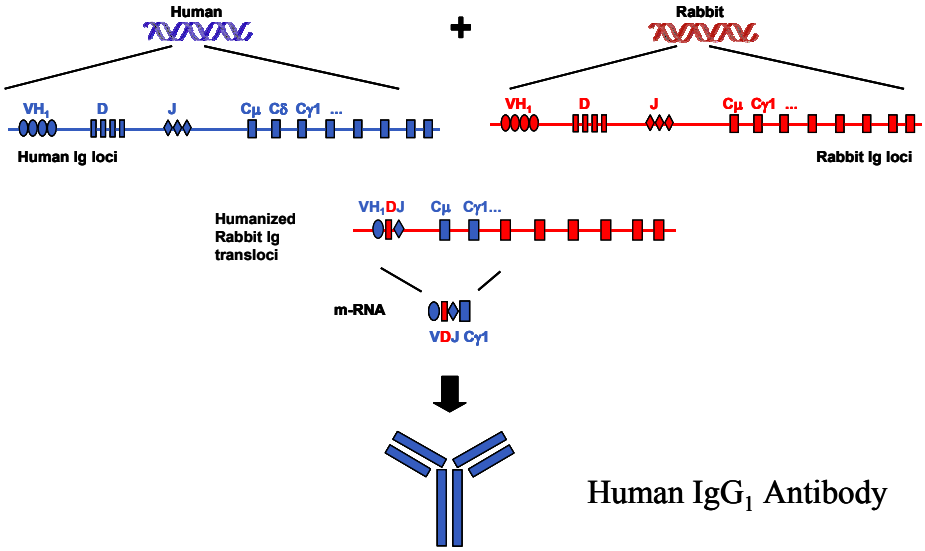

Fig. 2. Schematic representation of THP's approach to express human antibodies in animals. Coding regions in the rabbit immunoglobulin locus were replaced with corresponding human gene elements.

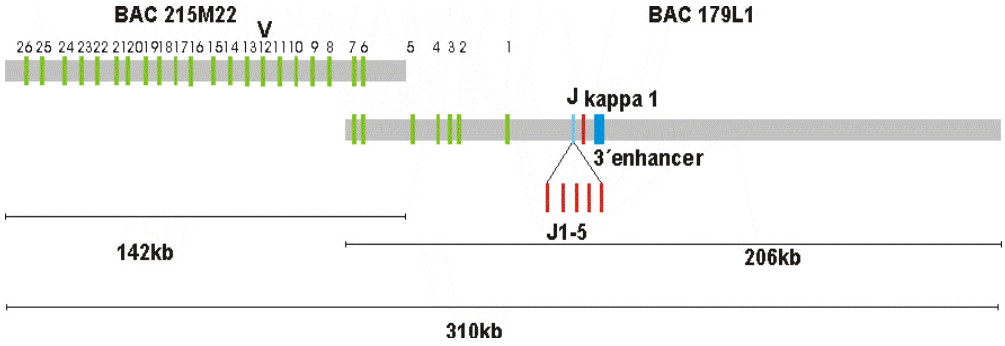

Fig. 3. *Rabbit light chain*; overlapping BAC clones 215M22 and 179L1 containing rabbit Ck, 3' enhancer, rabbit J segments, and 26 Vk segments.

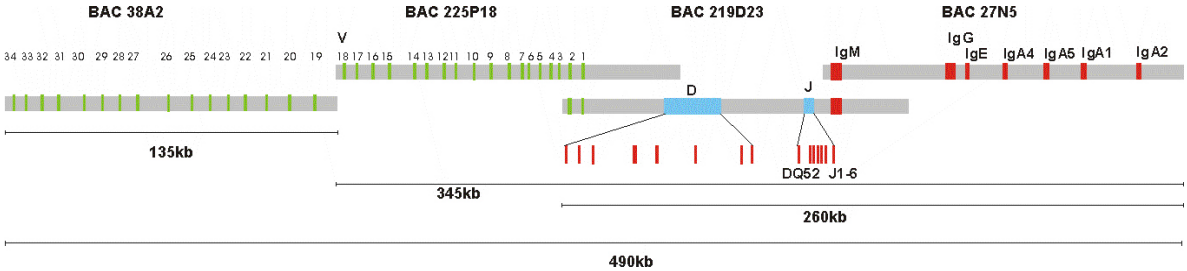

Fig. 4. *Rabbit heavy chain;* four BAC clones—38A2, 225P18, 219D23, and 27N5—containing several rabbit Cα, Cγ and Cμ segments, rabbit Jh and Dh segments, and 34 rabbit Vh segments. BAC clones 225P18, 219D23, and 27N5 are overlapping.

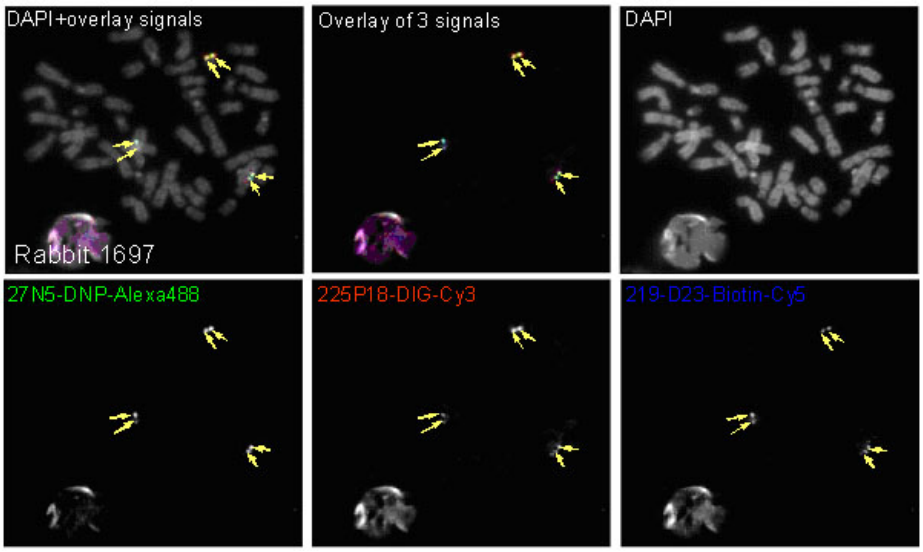

Fig. 5. *FISH analysis of heavy chain founder animal.* Chromosomes were hybridized with three differently labeled probes (27N5, 225P18 and 219D23). These probes are overlapping BACs of the humanized heavy chain transgene. With each probe 6 signals can be seen: four (4) for the duplicated endogenous loci and two (2) for the duplicated transgenic locus.

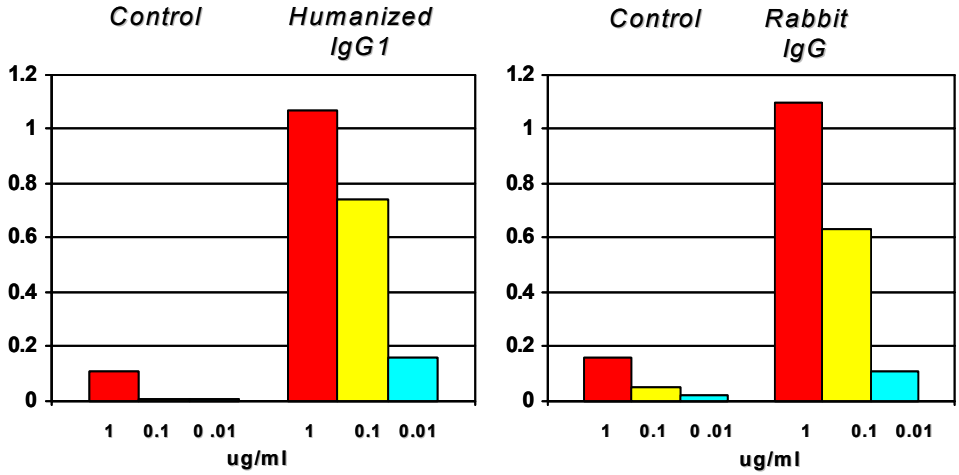

Fig. 6. *Antigen-specific antibody detection by ELISA*. ELISA plates were coated with KLH, blocked and incubated with normal human or rabbit IgG as control and humanized IgG1 or rabbit IgG purified from serum of immunized transgenic rabbits. Bound antibody was detected with a biotinylated secondary antibody specific for rabbit and human IgG, respectively. HRP was detected with TMB and the optical density (OD) was measured at 450nm.

Large DNA fragments can be manipulated by homologous recombination in *E. coli* DY380 containing genes encoding the recombination enzymes Recαγ under the control of a temperature inducible promoter. THP used this ET cloning procedure to humanize rabbit light and heavy chain immunoglobulin genes. Rabbit Cκ1, Cμ, Cγ, and J were replaced with corresponding human counterparts. The light chain V-region was built with human Vk1 family members, the heavy chain V-region with human VH3 gene elements. The final constructs contain rabbit control elements (i.e., promoters, enhancers, etc.) and human gene segments encoding human immunoglobulin constant regions, joining and

variable elements. These transgenic constructs support fully human antibody production in transgenic animals.

Transgenic founder animals were generated through injection of DNA constructs into pronuclei of fertilized oocytes (about 3000/construct) and subsequent embryo transfer into foster mothers. About 10 founder lines were generated with each construct. In addition to the successful introduction of a functional transgene, genetic engineering of animals for the production of human antibodies requires inactivation of endogenous antibody expression. For the rabbit, natural mutants impaired in heavy chain expression (Alicia rabbits) and light chain expression (Basilea rabbits) have been identified. The Alicia strain has a 10 kb deletion and lacks the VH1 and VH2 gene segment, and therefore has highly impaired Ig heavy chain expression. Rabbits heterozygous for the Alicia allele express solely the wild type allele. The second strain of rabbits, Basilea, do not express the dominant κ1 light chain because of a κ1 mRNA splice site mutation. Homozygous Ali/Bas animals can be used as natural knockouts for the efficient expression of humanized immunoglobulin transgenes. Live founders were crossed with homozygous Ali/Bas rabbits. Based on the level of expression of humanized immunoglobulin in F1 and F2 animals several transgenic lines were expanded.
Integration of transgenes into the rabbit genome was visualized by FISH using the corresponding BACs as probes (Figure 5). All probes hybridize with endogenous immunoglobulin loci as well as transgenic loci. Therefore, hybridization of metaphase chromosomes from wildtype rabbits results in four signals (two alleles, two chromosomes each). A single copy of the HC or LC transgene results in two additional signals (one copy, two chromosomes).

Antibody expression in transgsenic rabbits was analyzed by ELISA. In some instances, founder animals (F0) with undetectable levels of humanized immunoglobulin generated F1 offspring which expressed the transgene efficiently. These results indicate that some founder animals are somatic mosaics. This is consistent with the observation that some founders transmit their transgene to less than half of their offspring (e.g., germline mosaics). Human antibody expression increased dramatically in second and third generation animals and increased further in homozygous knock-out animals. In some lines expression of human immunoglobulin was normal (as high as rabbit immunoglobulin expression in wildtype rabbits).

Transgenic rabbits have been used for immunization studies with KLH or Botulinum Toxin A. Human and rabbit antibodies were purified from serum and analyzed by ELISA. Titration of purified fractions revealed that rabbit and human IgG have similar antigen-specific titers against botulinum toxin and KLH (Figure 6).

Antibody diversification of humanized IgG1 in transgenic rabbits was analyzed by sequencing of PCR amplified VDJ and VJ genes. For this purpose VDJ sequences were compared to genomic V gene sequences. The analysis revealed VDJ sequences containing continuous stretches of DNA which are probably derived from upstream donor V genes indicating gene conversion. Additional sequence diversification by hypermutation was also observed. Similar results were obtained with VJ genes. Taken together, sequence analysis of rearranged transgenes showed diversification by gene conversion and hypermutation. These results deomonstrate the functional expression of THP transgenes in rabbit B-cells as both gene conversion and hypermutation are antigen-driven processes.

Conclusion: A novel engineering approach for the expression of a diversified human antibody repertoire in gene-converting animals was developed. Humanized rabbit immunoglobulin loci competed efficiently with endogenous loci. In knock-out rabbits expression of humanized rabbit immunoglobulin loci appeared to be normal. Analysis of antibody genes in B-cells demonstrated that transgenic rabbits expressed a polyclonal human antibody repertoire diversified by gene conversion and hypermutation. Following immunization THP rabbits respond with the production of high-titer antigen specific human antibodies.

[11.10-11.30]

***In vitro* affinity maturation of lead candidates from the Human Combinatorial Antibody Library HuCAL GOLD®**

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In the development of antibodies for therapeutic applications very high affinity is most often a desired antibody feature. Due to its fully synthetic design HuCAL® antibody sequences are completely modular, which facilitates a ‘plug-and-play’ approach to

engineering these antibodies by CDR exchange. Antibody optimization is therefore restricted to the CDRs and thus, does not compromise the germline configuration of the frameworks.

By means of a case study the affinity maturation process by CDR exchange is illustrated. Data on final lead antibodies from several programs are shown reaching dissociation constants down to the low picomolar range.

[11.30-11.50]

Generation and characterization of a recombinant blocking anti-glycoprotein VI single chain antibody fragment

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Glycoprotein VI (GPVI) is a key receptor for platelet activation by collagen. GPVI play a critical role in haemostasis but its defect has minimal hemorrhagic consequences. It has been clearly involved in the formation of thrombi on atherosclerotic lesion that are responsible for acute ischemic events [1]. The inhibition of the collagen-GPVI interaction thus represents a new target to develop antithrombotic agents.

Antibodies are so far the more potent GPVI antagonists described and they are an interesting starting material to develop antithombotic compounds. The aim of this study was to generate and characterize a single-chain anti-GPVI antibody fragment (scFv).

In this attempt, the cDNA encoding the VH and VL domains of the murine monoclonal IgG 9O12 [2] were cloned and assembled. The scFv cDNA was cloned into a bacterial periplasmic expression vector. The functional recombinant scFv was purified by a single step affinity-chromatography using immobilized recombinant GPVI, with a yield of 200 µg scFv per liter of bacterial culture. The purity of the scFv preparations was checked by SDS-PAGE, Western-Blot and MALDI TOF mass spectrometry.

The scFv exhibits a high affinity for rGPVI as shown by Plasmon resonance surface analysis (10^{-9} M) similar to the affinity of the parental IgG and Fab fragments. The scFv also retained the capacity to bind to platelet GPVI as determined by flow cytometry on freshly isolated human platelets. Futhermore the scFv retained the inhibitory properties of the 9O12 Fab: inhibition of GPVI binding to immobilized collagen, inhibition of collagen-induced platelet aggregation and procoagulant activity.

The scFv derived from the 9O12 antibody thus presents *in vitro* the characteristics expected for an antithrombotic molecule.

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[11.50-12.10]

Customized discovery of rare and unique therapeutic antibodies

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With access to seven of the leading commercially available Ab phage display libraries as well as a host of optimization technologies, XOMA is uniquely positioned to discover rare and unique human therapeutic mAbs that meet or exceed design goals. Since each library has distinctive characteristics and non overlapping repertoires, XOMA maximizes success of each program through customized parallel use of multiple libraries and multiple strategies. For non-human mAbs, XOMA applies our clinically validated Human Engineering™ technology to rapidly convert leads to product candidates. Multiple examples illustrating the success of this customized approach will be described.

[12.10-12.30]

Developing novel therapeutic human antibody leads using phage display technology

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Phage libraries displaying antibody fragments are the fastest routes to obtaining human antibodies. Dyax's proprietary antibody phage and phagemid libraries are combining a unique combination of human VH and VL sequences from non-immunized human donors and synthetic diversity in key antigen contact sites. Controlled phage display selection methods on purified targets and cells are carried out using automation technology and high throughput

screening is implemented to identify antibody leads which display a variety of desired properties such as high affinity, cell binding, and functional activity.

[12.30-12.50]

Antibody variant screening and candidate selection performed on the path to clinical manufacturing using GPEx™ cell line engineering technology

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Introduction: The speed at which an antibody product progresses into clinical trials is of vital importance for both small biotechnology as well as the biopharma groups of large pharmaceutical companies. For mammalian cell lines, two major impacts on the project timeline are the ability to quickly identify a product candidate and subsequently engineer a stable, high expressing cell line for that product. The advent of various computer based antibody design methodologies and antibody discovery technologies for development of therapeutics has resulted in large numbers of antibody variants that must be screened in order to identify the best clinical candidate.

For these variant candidates, proper screening requires milligram levels of antibody production in a mammalian cell for testing of the candidate material. This testing is initially performed *in vitro*, but in many instances animal studies are required for identification of the best potential clinical candidate. Animal studies can require multi-gram levels of the antibody variants. Production and screening of these variants and selection of a final clinical candidate is a time consuming process in most research groups, and is generally in conflict with what are now common aggressive timelines for moving products forward into the clinic. Typically, the initial variants are produced from a transient expression system, and then after clinical candidate selection the cell line engineering process is restarted, a stable cell line is produced, and the final clinical candidate cell line is master cell banked for manufacturing. The GPEx™ (Gene Product Expression) technology allows antibody variants to be screened as part of the manufacturing cell line generation process. Milligram to multi-gram levels of antibody are produced from an initial stable pool of clonal cell lines for variant characterization and selection. Final clonal cell line selection from this pooled line either proceeds in parallel with variant *in vitro*/*in vivo* screening or after clinical candidate selection has been completed. This method of variant selection eliminates the need for a total re-start in cell line development after the clinical candidate has been identified.

The GPEx™ technology utilizes retrovectors to stably insert single copies of genes into dividing mammalian cells [1]. Retrovectors deliver genes coded as RNA that, upon entry into the cell, are reverse-transcribed to DNA and integrated stably into the host cell genome. Two enzymes, reverse transcriptase and integrase, provided in the vector particle, allow conversion to DNA and gene insertion. These integrated genetic inserts are maintained through subsequent cell divisions as if they were endogenous cellular genes [2].

Characteristics of GPEx™ that Permit Cell Line Development Flexibility

- The gene inserts target "active" regions of the cell genome allowing for high levels of antibody production from the cell pools and lines [3-5].
- Antibiotic selection and gene amplification are not required, resulting in shorter clonal cell line development timelines.
- The high transduction efficiency, coupled with no antibiotic selection requirement, allows easy titration of heavy (HC) and light chains (LC) genes to the correct gene ratio to yield maximum antibody production and cell line stability. Gene ratio titrating can be accomplished through specific screening during clonal selection or an individual transduction of a specific chain, if required.
- Stable cell pools producing milligram to gram quantities of variant antibodies prior to selection of a high-expressing clone allows for screening of variants and optimal selection of final clinical candidates.
- The technology inserts each copy of the transgene at a different genomic location producing very stable gene insertions and stable expression from both pooled and clonal selected cell lines.

CHO Cell Line Development: GPEx™ cell lines expressing antibodies are produced as shown in Figures 1. For generation of antibody producing cell lines, an initial transduction of CHO cells is performed using a retrovector containing the LC gene. The LC expressing pool of cells is then transduced with a retrovector containing the HC gene. Upon completion of both LC and HC transductions, the

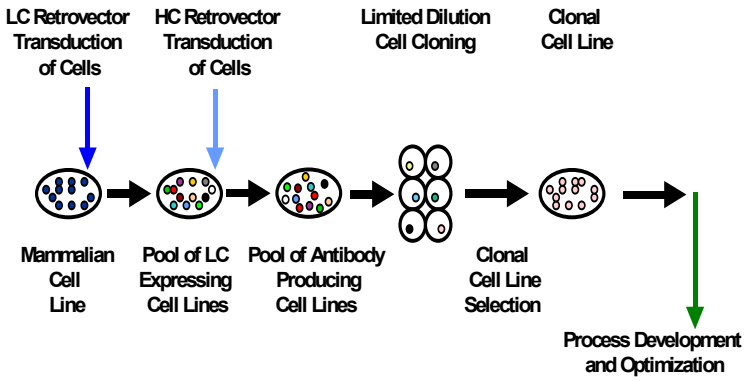

Fig. 1. Antibody cell line development method.

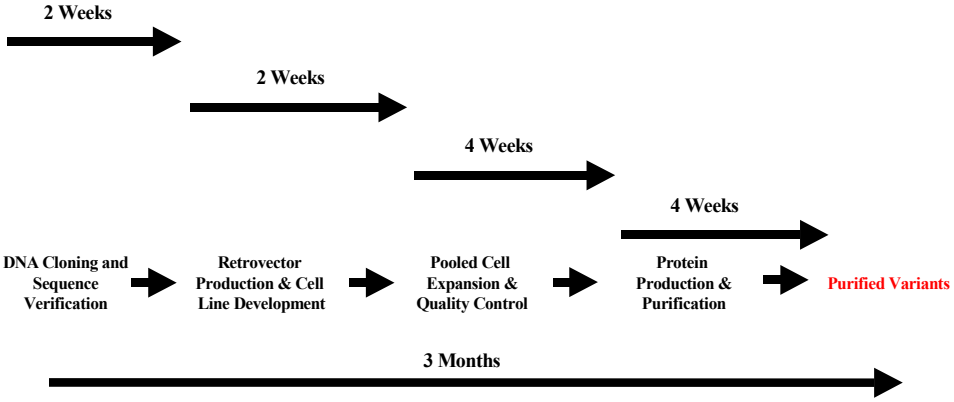

Fig. 2. Process timeline for variant production from a pooled cell line.

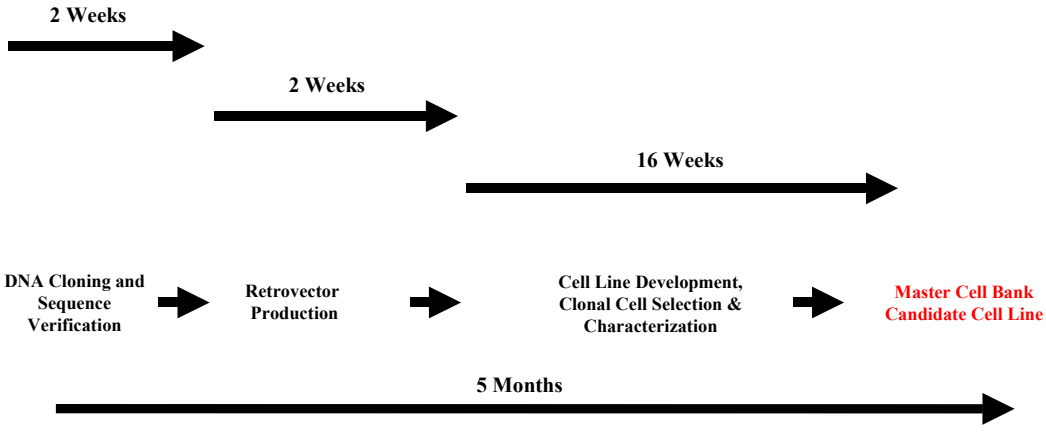

Fig. 3. Process timeline from cDNA to start of master cell banking using the GPEx™ cell line development method.

Table 1
Clonal cell line production during bioreactor manufacturing

Antibody Product	Specific Productivity (P/C/D)	Final Titer (g/L)	Maximum Cell Density ($x10^6$ Cells/ml)
Antibody 1	35	1.0	3.1
Antibody 2	19	0.8	4.4
Antibody 3	35	1.2	3.3
Antibody 4	20	0.7	3.0
Antibody 5	35	1.4	3.5
Antibody 6	31	0.7	2.8
Antibody 7	23	1.1	3.5
Antibody 8	35	1.1	2.3
Antibody 9	27	1.2	4.5

resulting pool of cells produces functional antibody. These stable pools can be expanded for antibody production and analysis. Single cell clones are isolated from the pool using limited dilution cloning. Cells are only cultured in serum-free medium; however, 2% fetal bovine serum is typically used for approximately 10-14 days during the limited dilution cloning step. Cells are typically cultured at 37˚ C and 5% CO_2. Fed-batch development is completed with a single round of analysis using generic conditions and commercially available media/supplements (HyClone, Logan, UT).

Pooled Cell Line Production: The ability to produce substantial amounts of antibody prior to clonal selection is an advantage of this cell line development method. Stable cell pools producing 50 – 280 mg/L of over 25 different antibodies and Fc fusions in standard serum-free overgrowth cultures without feeding have been generated using GPEx™. Transfer of the pools to fed-batch conditions results in a 2 – 4 fold increase over the initial production levels. These production levels allow milligram to multigram quantities to be made from 1 - 100 liter production vessels at this early stage in the cell line development process. A timeline for production of antibody variants from pooled cell lines is shown in figure 2.

Clonal Cell Line Production and Manufacturing: After completing limited dilution cloning and initial clonal selection on the pooled cell lines, the top clones are identified and analyzed in generic fed-batch conditions. The top clone is identified from the fed-batch analysis and moved into bioreactor manufacturing using the same fed-batch conditions. Results of fed-batch bioreactor runs for nine different antibody producing cell lines are shown in Table 1. Antibody production, specific productivity (picograms/cell/day) and maximum cell density were recorded for each of the batches. The ability to manufacture clinical material without detailed cell line process development and achieve greater than 1 g/L levels of production dramatically reduces timelines for production of clinical product. More detailed process development can then be performed on the cell line while the early clinical analysis is being performed. A timeline from gene to master cell bank candidate selection is shown in Figure 3.

Summary: GPEx™ cell line engineering technology is a flexible method for cell line development capable of shortening the time required to get recombinant proteins, especially antibodies, into clinical trials. Large quantities of recombinant antibody can be produced from pooled cell lines along the path to candidate cell line selection, which in turn shortens timelines and eliminates the need for separate, large-scale transient transfection experiments. GPEx™ cell lines producing antibodies express at levels of 0.7-1.4 g/L in fed-batch bioreactor manufacturing using generic conditions. These levels are on the upper end of production needed to produce initial clinical material, and these levels can be significantly increased with more detailed cell line process development, ideally during the initial clinical trial(s).

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[12.50-13.10]
Making the VelocImmune mouse using Velocigene technology

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We have engineered a second-generation human antibody-producing mouse we call the *VelocImmune*

mouse. This mouse produces human variable domain antibodies from the endogenous mouse loci. Using Velocigene technology to manipulate bacterial artificial chromosomes, we have replaced 3 megabases of the mouse variable domain sequences from the heavy and light chain immunoglobulin loci with up to 1 megabase of the human variable domain loci. The *VelocImmune* mouse appears to have a completely normal immune system, B cell populations, antibody levels, and somatic hypermutation rates, as well as normal responses to antigens. We hypothesize that the wild type phenotype of these mice is a direct consequence of the fact that this mouse produces antibodies from the endogenous loci using the mouse constant domains. The combination of *VelocImmune* with Velocigene to produce Velocimice will allow the rapid generation of mice that are not tolerized to highly conserved antigens, thus expanding the repertoire of monoclonal antibodies. Humanization of the antigen loci further facilitates the drug discovery process by allowing the researcher to use the final human-specific antibody candidates in mouse models for efficacy and toxicology research.

[13.10-13.30]

A focused antibody library for improved hapten recognition

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In this study we have explored the correlation that exists between paratope structure and antigen preferences to create a focused scFv repertoire biased for haptens. A fluorescein-specific scFv (FITC8), which displays a characteristic hapten-binding pocket in its paratope, was used as structural backbone for library construction. A combinatorial antibody library, designated the cavity library, was created by introducing restricted variability at mainly centrally located, cavity-lining residues. These diversity-carrying residues were rationally selected based on a model structure of FITC8 and on known antibody structure-function relationships. The library was screened, using phage display, yielding diverse and highly specific binders to four different haptens. The behavior of the cavity library was compared to a conventional library, having diversity spread onto a greater area including more peripherally located residues. This resulted in the isolation of binders that, in contrast to the selected clones of the cavity library, were not able to bind the soluble hapten in absence of the carrier protein. Thus, by focusing diversity to those residues that are most likely to interact with the hapten, we have created a library with improved hapten recognition. The results further support the notion that it is possible to create antibody libraries that are biased for antigens of pre-defined size.