Genomic and EST Sequencing of the Ferret (Mustela putorius furo)

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Abstract:
The six areas of biomedical research that most commonly use ferrets (cystic fibrosis, influenza and other respiratory diseases, lung cancer, reproductive and fetal biology, neuroscience, and specific forms of cancer) are collectively responsible for over 12 billion dollars of funding from the National Institutes of Health alone for 2006. This total represents about 44% of the NIH annual grant monies. Additionally, ferrets have the potential of becoming an important system for drug and vaccine development, including validation and toxicity studies. The lack of genomic sequence hampers the interpretation of the results from existing research programs as well as the development of novel programs that rely on this kind of information. Combined sequencing of ferret genomic DNA and ESTs will allow for the development of additional resources for genomic and proteomic research and provide the genetic information necessary to improve the interpretation of results and quality of reagents for future research efforts within this model. There is strong support from the research community for this proposal, as evidenced by the accompanying 53 letters of endorsement from leading investigators in both academic and industrial institutions. These emphasize that ongoing research with ferret biomedical models is significantly limited by the lack of sequence data and derived molecular tools. *In particular, ferret sequence data is urgently needed for models of infectious respiratory diseases with pandemic potential, e.g. avian influenza and SARS corona virus. National interest in these models is evidenced by recent Congressional funding for pandemic flu preparedness (June 2006) and the Biodefense Advanced Research and Development Authority (Dec. 2006).*

I. Introduction

Ferrets have a long history as animal model subjects. In 1933, influenza-induced rhinitis was first observed in ferrets. Because this model mimics the shedding of viruses from respiratory tissue in a fashion similar to humans, their importance as an animal model was immediately apparent[1]. Ferrets belong to the Order Carnivora, which includes dogs, wolves, cats, bears, weasels and otters. At the family node, ferrets diverge from the Canidae to form the Family Mustelidae. Most closely related to the European polecat, the domestic ferret (*Mustela putorius furo*) deviated from the polecat at least 2,500 years ago. Sharing the Genus Mustela with ferrets are the ermine (*Mustela erminea*), mink (*Mustela vison*) and several weasel species. Of the two ferret species, the sequencing project would exclusively encompass the domestic ferret; the black-footed ferret (*Mustela nigripei*) is endangered.

To date, the genomes of many species have been completed sufficiently to allow for gene searching and regional homology mapping. This tool is available through the Genome Bioinformatics Site at UCSC and includes the mammalian genomes of human, chimpanzee, rhesus, dog, cow, mouse, and rat. Of these available genomes, the ferret genome would likely share the highest homology with that of the dog or cat. While these genomes can be used to complement the ferret sequence, they can

not serve as a substitute for the genome. Despite the phylogenetic relationship between ferrets and dogs, the dog sequence alone is not sufficient to further the development of the ferret animal model.

II. Specific Biological Rationale

1) Improving human health and informing human biology

Ferrets are unique in that, at the physiological level, they exhibit many similarities to humans. Presently, it is difficult to investigate human-ferret similarities at a molecular level using high-throughput techniques, mainly due to the lack of ferret-specific materials. Access to genomic sequence information will allow researchers to more accurately mimic human disease using the ferret model and investigate cellular and molecular pathways that are not accessible in humans. It can be stated with certainty that the community working with ferrets is prepared and eager to take full advantage of the genomic sequence when it becomes available (please refer to the enclosed letters of support).

Understanding the mechanisms of disease *in vivo* is the most difficult aspect of biomedical research, yet it is also essential. While examining cell cultures or working with *in vitro* systems are an excellent start to any project, researchers can not truly understand the pathogen, mutation, or condition until the living host is treated as a complete system. It is not possible to list in this document all of the scientific research that is dependent on the ferret model. A few examples must suffice to demonstrate the array of projects that have come to rely on this animal model. *One of the largest benefits of this sequencing effort will be the development of ferret-specific materials and assays to better utilize them as a model for human diseases.*

Influenza: Current perspectives on the biological course of influenza infection and the development of host immunity to subsequent infections would not have been identified without the use of the ferret model[2]. Infection by influenza virus in ferrets closely resembles the symptoms, viral transmission, pathogenicity and immunity development in humans[2,3]. Influenza Types A and B naturally infect ferrets, providing an opportunity for controlled and physiologically-relevant studies of immunity[4]. Additionally, human influenza viruses bind to sialic acids on the respiratory epithelial cell surfaces at α-2,6-glycosidic-linkages specifically[5], and this binding is believed to be a major factor in determining the host range specificity of influenza viral strains. Ferrets are also an animal model used in highly pathogenic H5N1 avian influenza research, including the identification of determinants for efficient human-to-human transmission, although the mechanisms responsible for this transmission remain poorly understood[6,7,8]. Recent developments concerning avian H5N1 strains of influenza highlight the importance of the ferret model for this kind of research, and influenza vaccine studies are already being performed using the ferret[9]. *The national urgency surrounding pandemic influenza preparedness is evident from the recent acts of Congress that address concerns regarding natural spread of Avian influenza(HR 5409 "Emergency Flu Response Act" 2004) as well as the potential use of influenza as an agent of bioterrorism(HR 2863 "Public Readiness and Emergency Preparedness Act" 2005)*.

SARS-CoV: The causative agent of Severe Acute Respiratory Syndrome, this coronavirus possesses a high rate of mutation and recombination that allows it to evade host immunity defenses successfully. Studies show that ferrets are susceptible to experimental infection with SARS and that the virus transmits to non-inoculated animals in shared housing. Additionally, the infected ferrets demonstrate symptoms similar to infected humans[10]. SARS vaccine studies are already underway in the ferret model[11] in hopes of preventing a potential epidemic, and the ferret model provides the only opportunity to study genetic mechanisms regulating transmission between susceptible hosts.

Cystic Fibrosis: Due to physiological differences, mice are unable to generate the spontaneous lung disease typical of cystic fibrosis as found in humans. However, ferrets possess the lung biology

required of a suitable animal model with which to study this disease. Ferrets can be selected for targeted gene mutations and somatic cell nuclear cloning, which is a common method used to study cystic fibrosis [12].

Reproductive Biology: The seasonal reproductive cycle of male[13] and female[14] ferrets is easily monitored and has provided a model system in which to study regulatory features including day length[15] and pineal hormones[16]. Ferrets have also been used to study neuroendocrine mechanisms controlling brain[17] and behavioral[18-20] sexual differentiation, pubertal development[21,22], mating-induced ovulation[23,24], steroidal feedback control of pituitary hormone secretion[25], and fertility. The menstrual cycle of ferrets is easily monitored, making them an ideal for studying the physiological and environmental factors that influence reproductive activity, fertility[26], ovulation and puberty. Embryonic transfer has already been accomplished in the ferret model[27], making these animals ideal for generating inbred colonies for research purposes. Additionally, fetal studies, such as those on fetal alcohol syndrome, are pursued using the ferret model[28,29].

Cancers: As a model system, ferrets are used extensively to study all cancers, including various kinds of carcinomas[30-32] and lymphomas[33]. In particular, the lung anatomy of the ferret offers striking similarities to human lung, providing a system to examine carotenoid absorption, metabolism, tissue distribution and biological functions as they relate to lung carcinomas[34]. These ferret lung tumors offer a unique and ideal system for studying the molecular mechanisms of carcinogenesis in lung cancer due to smoke exposure.

Neuroscience: Ferrets are born in an altricial state, and thus are valuable models for fetal brain development in humans. Studies in newborn and early postnatal ferrets have provided important information on very early events in development of the thalamus[35,36] and cerebral cortex[37,38]. In particular, studies on the visual system of ferrets have been instrumental in shaping key concepts of the interplay between intrinsic and extrinsic factors in the development of brain pathways and circuits. These studies include anatomical and molecular analyses of visual projections [39-41], physiological descriptions of visual cortical responses[42,43], misrouting of visual projections associated with albinism[44,45], and analyses of "rewired" projections in which visual projections are diverted to the auditory pathway[46,47]. Together with mice, ferrets have become the model system of choice for studies of mammalian visual system development [48,49].

Physiology: Ferrets have been used extensively to study physiological development and cellular physiology, particularly within the respiratory system[50]. An example of a physiological assay includes using autoradiography to assess epithelial cell proliferation[51], measure β-carotene metabolism, and examine skeletal structure and bone composition [52].

Bacterial infection: Ferrets are also used for public health studies involving bacterial infections including pneumococcal transmission[53], and interest has been shown in using them for vaccine studies. In fact, there is no other pneumococcal animal transmission model available. While these models are not as well developed as those for influenza, given the public health importance of Streptococcus pneumoniae (community acquired pneumonia, otitis media, sepsis, meningitis, etc.) the potential of the ferret in studying these diseases should not be overlooked.

Generation of ferret sequence data will lead to new and more sophisticated research projects in both existing ferret models of human disease and basic research in fundamental aspects of mammalian and human biology. Realizing the full potential of ferret biomedical models requires access to molecular tools, which are virtually non-existent at present. Such tools include expression arrays, proteins, antibodies and immunological reagents. A concerted sequencing endeavor is the only means to begin to build these tools in an expeditious manner.

2) Provide new surrogate systems for human experimentation such as new disease models

As described above, ferrets already provide an experimental animal model and surrogate system for a wide range of research centered on human biology. Ferret sequence information will encourage and facilitate new experimental strategies for existing models and possibly lead to the development of new models for disease.

Two examples of this are non-human primates (*Macaca sp.*) and the mouse (*Mus musculus*). Non-human primates are not an ideal animal system due to challenges of infection, availability, size, ethical guidelines, lengthy gestation periods and risk to the handlers. Ethical considerations aside, non-human primates have a prohibitive cost for purchase and husbandry, making them the most expensive animal model available. In contrast to macaques, for models of infectious respiratory diseases such as influenza, the anatomy and cellular distribution of the ferret respiratory system better reflects the course of disease seen in humans. When studying pneumonia in macaques, for example, H5N1 virus attaches to both type I and type II pneumocytes, while H5N1 attaches to only type II pneumocytes in human and ferret[54], leading to differences in pathology, disease progression, and immunohistochemistry profiles.

A wealth of reagents and immunological data are available to researchers using the mouse model, and many of these were derived directly from sequence data. However the physiological relevance of the results needs to be considered, a limitation arising from the pitfalls of using a manipulated animal system (genetically altered or inbred) as well as mouse-adapted virus models. While the cost of acquiring and maintaining mouse colonies has considerable economic merit, husbandry costs associated with ferrets are similarly far less expensive than the cost of non-human primates but provide better physiological relevance than murine models (Table 1). Ferrets are largely available and the demand for this animal model continues to grow, particularly with increased research emphasis in influenza, SARS, and biodefense.

Table 1. Research husbandry comparing domestic ferret to other research models.

Model Species	Average Weight (kg)	Space Req./ Animal (in^3)	Purchase Cost ($)	Per Diem Cost ($)	Total Cost ($) for 1 Animal for 1 Year
Rhesus Macaque (*M. mulatta*)	6.5	1548[b]	5,340-7,700[c]	12.91[c]	10,052-12,412
Pigtail Macaque (*M. nemestrina*)	9.0	1548[b]	3,690-6,655[c]	12.91[c]	8,402-11,367
Ferret (*M. putorius furo*)	2.0	504[b]	98-168[a]	4.54[a]	1,755-1,825
Mouse (*M. musculus*)	.025	50[b]	41-366[a]	0.81[a]	336-661

[a]Values from the University of Washington's Department of Comparative Medicine, 2006
[b]Values from the University of Washington's IACUC Guides to Animal Use, 2006
[c]Values from the Washington National Primate Research Center, 2006

As ferret research expands and becomes more established, so too will the pedigree of available animals. Radiation hybrid maps of the ferret genome, complimented with chromosome painting, are powerful tools for comparative genomics. Despite their breadth of biomedical research involvement, ferret genomic and EST sequence data are essentially non-existent. A banded karyotype was published in 1983 [55], but chromosomes are numbered differently according to the consulted source. Projecting chromosomal homology from related species with extensive genomic resources for the ferret promises to generate a tremendous amount of genetic understanding. Recent research shows too that the genome of the ferret is highly conserved and has not evolved too greatly from that of the ancestral

carnivore karyotype [56]. Sequence from the ferret genome will be necessary to generate low or high density genetic maps for the ferret using either single nucleotide polymorphisms or simple tandem repeats. The presence of these genetic maps will enable investigators to undertake linkage mapping studies necessary for identifying genes responsible for specific genetic diseases.

In addition, the ferret model has the potential for generating inbred lines and knockout models. Compared to the macaque species, the ferret has a much higher rate of fecundity. Macaques give birth to one infant generally once a year under proper conditions and have a gestation period averaging 165 days. The ferret can reproduce up to three times annually with an average of 15 kits produced per year. This lends itself to creating different lines of ferrets that may have defined phenotypic characteristics or the development of knock-out animals. In fact, several laboratories are in the process of developing ferret colonies for their research, and knockouts have already been generated in this model (unpublished data). Obviously, these resources can not be developed at the same rate in ferrets as in the rodent animal models, but resources could be developed substantially faster than in macaques. Additionally, macaques are also almost exclusively out-bred, but the reproductive system of the ferret can also be used to generate in-bred lines of animals. Ferrets are also used effectively in embryo transfer[57] and cloning[58] procedures, allowing further possibility in the production of manipulated animals as a model.

As was seen in the case of the macaque genome, provision of the requisite tools and data are likely to lead to an increase in interest in ferret genetics (private communication from George Weinstock). Similar to the situation of the macaque in HIV research, the ferret's unique position in terms of research on potentially epidemic diseases such as SARS and influenza underscore the importance of generating genomic and gene-specific sequence information to aid in the development of species-specific research resources. With the urgency of these diseases on the rise, it is preferable to have a lower coverage genome produced in a shorter time frame rather than a whole genome that would take considerably longer to generate.

Access to genomic and EST sequence data will allow us to understand how the ferret model relates to human disease and will allow us to utilize this model in a more cost and resource effective matter than other mammalian models for these human diseases. The sequencing proposed would identify targets that can be used to develop lines of animals that have a phenotype specific to the disease for which they will be used as a model.

3) Expand our evolutionary understanding of both human and non-human organisms

The phylogenetic position of the ferret makes it a prime genetic target for the study of evolution because ferrets are positioned between humans / non-human primates and the rodent species along the evolutionary continuum. Fortunately, both primates and rodents have completed genomic data, allowing for detailed comparisons across mammalian genomes. Ferrets, as the ideal intermediary, would only compliment this comparison and provide answers to evolutionary questions. Ferrets are thought to have highly conserved coding regions relative to the ancestral carnivore. For this reason, both the dog and cat genomes can be used to leverage the ferret genome. By examining deviation in the 18s genetic code of animals in the Class Mammalia, investigators can assess species divergence on an evolutionary time course.

As discussed earlier, comparison of genomic sequences between human, chimp, rhesus, dog, cat, ferret and the rodents will generate many opportunities for the analysis of individual genes, gene families, families of repetitive elements, isoforms, splice variants and other components of the genome. With the addition of the ferret genome, the picture of genome evolution will become more focused. *Generating the genomic sequence will also benefit research in comparative evolution, ultimately facilitating our understanding of ferrets as a model for human disease*

III. Strategic Issues

1) Demand for new sequence data and rationale for ferret sequence completion

Access to extensive genomic sequence from ferret will significantly benefit on-going research. In particular, the ferret's unique position as a model for influenza and SARS and the potential for epidemics of these diseases in the near term adds to the urgency of this research. Sequence from humans, primates or rodents will not provide any additional information for members of the Order Carnivora but the ferret sequence could directly inform the genomes of its closest phylogenetic neighbors. The ferret shares its Family (Mustelidae) with several species that are also used for biomedical research, such as martens, weasels and otters. *Ferret-specific reagents developed from the ferret genome and EST data would lend themselves for use in these animal models.*

2) Suitability of organism for experimentation

Ferrets are highly amenable to experimentation and investigation. This species is available in large numbers from a variety of institutions and breeding operations. While routine production of transgenic ferrets is not yet possible, development of transgenic technology using ferrets will be stimulated by genomic sequence availability. Stem cell research will also exploit the strengths of ferret models over the coming years, making them even more valuable as a model system.

3) Project Overview

2X-genome sequencing scheme: The genome of the ferret is estimated to be similar in size to that of macaques and humans (approximately 3000 Mb), but currently no extensive genetic or physical maps exist. The strategy to be employed would be to generate 2X whole-genome shotgun coverage. Shotgun reads will be generated from libraries with end pairing to be maintained at high (>90%) fidelity within the assembly. Subsequent ordering and orientation of the resulting contigs can be achieved through the use of either a combination of a BAC contig map and BAC end sequencing, use of bidirectional EST reads, and/or alignment to a complete genome of a related carnivore such as that of the dog. While a BAC library has already been generated for the ferret through Children's Hospital Oakland Research Institute (library CHORI-237), there is currently no plan in place for fingerprinting, generation of a minimum tiling path, or BAC end sequencing. However, now that genomes have been completed for a number of other species, these completed sequences could be used to generate a comparative map of the resulting ferret contigs using homology.

In addition, we propose that support be allocated for finishing of specific regions of scientific interest within the genome. Ideally, recommendations for these regions would be taken from members of the scientific community who use ferrets in their research programs. These recommendations could be prioritized by an oversight group formed from the community and sequenced accordingly.

In general, all of the methodology proposed here has been successfully implemented in the sequencing of other genomes. The entire project would require approximately 6 million successful reads which could be performed at a high-throughput genome sequencing center in less than 6 months.

EST Sequencing: In order to construct community resources such as a custom, ferret-specific oligonucleotide microarray and to aid in the resolution of highly repetitive regions of the genome, EST cloning and sequencing will need to be performed. The construction of positionally cloned, normalized cDNA libraries using ferret total RNA is the best method available to generate ESTs. Plasmids containing inserts >1kb will be plated, picked, replicated and bi-directionally sequenced. This data can be used not only to aid in the ordering and orientation of the contigs resulting from the genome project, but also to generate probe sequences from the 3'UTR reads[59] for a ferret-specific microarray. We suggest completing a

total of 100,000 reads (50,000 per direction) with sequence quality of 90% 100 PHRED 20 or better.

4) Other partial support

At the present time, there is no additional support for a ferret sequencing project. That said, a large number of government institutes are currently supporting ongoing research in ferret animal models for influenza and SARS (NIAID and NCID), cystic fibrosis (NHLBI), behavioral and developmental biology (NIDOCD, NEI, NIMH, NIDCR, NICHHD, NIDDK, and NIAAA), cancer (NCI), visual system development and function (NEI), and epilepsy (NINDS). Government preparedness efforts against pandemic influenza also have an urgent interest in the ferret sequence as a means to improve this crucial disease model. The ability of NHGRI to leverage support requires a strong rating as to the project's importance. It will be more difficult to obtain this support if the project is perceived as anything other than high priority. In addition, the ferret sequence data will enable these agencies to pursue further development of reagents (e.g. proteins and antibodies) and analytical resources (e.g. microarrays).

IV. Final Comments

We propose the ferret as a priority organism because of the large existing base of research already underway, particularly in such as influenza, SARS and biodefense. The resulting data would provide immediate benefit to investigators currently using the ferret model in their research as well as new opportunities for model development. The genomic data would also make the ferret model amenable to studies involving genetic mapping of disease, potentially replacing more expensive, less readily available models such as macaques. With vaccine studies already underway for H5N1 influenza viruses[60,61] and SARS coronavirus[62] in the ferret model, the genome information would also provide inroads to pharmacogenomic studies and a wider application of genetic studies in general within this model. Higher quality, more complete sequencing of the genome could be undertaken at a later date if sufficient interest in the completed genome should arise.

Genomic sequence of the ferret will undoubtedly shed light on many evolutionary details of the phylogenic tree and aid in the interpretation of the meaning of human comparisons to mammals. Other organisms may be more desirable from an evolutionist standpoint; however, we believe that the immediate benefit for human biomedical models that will be gained from the ferret sequence project outweigh any reservations based on evolutionary considerations.

The strength of the ferret genome project is its direct relevance to the goals of "development of innovative and improved methods of diagnosis, treatment or prevention" of human disease. It offers novel opportunities to increase our understanding of human biology, because it is closer to humans than the mouse or rat, and it is more useful as a model system than non-human primates. The evidence indicates that the ferret research community would benefit significantly by applying genetic and genomic methods in their studies, despite the minimal state of the resources. Likewise a concerted sequencing effort for the ferret will bootstrap the development of many other molecular tools. *The ferret sequencing project should be viewed as an investment in this research.* It will certainly lead to further and more rapid development of genetic methods, applications in academic and industrial research and a greater understanding of human disease.

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