Proposal for Complete Sequencing of the Genome of a Marsupial: The Gray, Short-tailed Opossum, *Monodelphis domestica*

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I. Introduction and Overall Rationale

Metatherian ("marsupial") mammals are phylogenetically distinct from current mammalian biomedical models, all of which are eutherian ("placental") species. However, marsupials and eutherians are more closely related to one another than to any other vertebrate model species (i.e., birds, amphibians, fishes). Fossil evidence establishes a minimum date of 125 million years (MY) for the separation of eutherian and metatherian mammals (JI *et al.* 2002), while analyses of nuclear gene sequences suggest that metatherian / eutherian divergence may be as old as 173-190 MY (KUMAR and HEDGES 1998; WOODBURNE *et al.* 2003). To place this in context, the evolutionary gulf between mammals and the next most closely related group of non-mammalian research models, i.e., birds (chicken), is approximately 300 –350 MY. Thus, the marsupial – eutherian relationship represents a unique midpoint in age relative to existing mammalian and non-mammalian vertebrate models. As a legacy of their common ancestry, marsupials and eutherians share basic genetic mechanisms and molecular processes that represent fundamental (ancient) mammalian characteristics. Nevertheless, since their divergence, eutherian and marsupial mammals have evolved many distinctive morphologic, physiologic, and genetic variations on these elemental mammalian designs. These phylogenetically restricted differences can be used as comparative tools for examining the underlying molecular and genetic processes that are common to all mammalian species, and thereby help to reveal how variations in these mechanisms lead to differences in gene regulation, expression, and function. As the closest sister group to eutherian mammals, marsupials are also the most appropriate "outgroup" for assessing the relative antiquity or novelty of the molecular and genetic changes that have occurred among the many eutherian species (including ourselves) presently used in biomedical and evolutionary research.

A. Marsupials in the mammalian scheme. Modern mammals (Class Mammalia) form three distinct groups. The subclass Prototheria (monotremes) comprises the three egg-laying mammals: platypus and two species of echidnas. The infraclasses Metatheria (~270 species) and Eutheria (~4,600 species) together form the subclass Theria. Molecular and physical data suggest that the prototherian lineage diverged from the therian line some 10-50 MY before the metatherian / eutherian divergence (PHILLIPS and PENNY 2003; WOODBURNE *et al.* 2003), although the exact timing of this event remains unclear. In any case, during their long, independent evolutionary histories, these three mammalian lineages evolved many distinctive anatomical, physiologic, and genetic features. Of these, none so clearly distinguish them as do their reproductive characteristics. Prototherians lay eggs. Marsupials and eutherians bear live young, but exhibit characteristic differences in the timing and pattern of early development. Relative to eutherians, marsupial fetuses are born at an extremely early stage of development after a brief gestation (~2 weeks in most species). Marsupials complete the majority of their "fetal" development subsequent to birth, attached to a teat, and often (but not always) within a protective pouch. Thus, whereas eutherian development is gestationally intensive and occurs internally, primarily via the placental attachment, marsupial development is lactationally intensive and occurs largely external to the mother.

Marsupial genomes are roughly the same size as those of eutherian mammals but far more conservative with regard to chromosomal compartmentalization (discussed by SAMOLLOW and GRAVES 1998; GRAVES and WESTERMAN 2002). Diploid chromosome numbers range from 10 - 32, but in more than 90% of species examined, $2n = 14 – 22$. On average, then, marsupials package the same amount to DNA into far fewer, larger chromosomes than do their eutherian counterparts. Judged from G-banding and chromosome painting studies, the chromosomes of most marsupial are extremely similar, manifesting only minor deviations that are easily explained by a few simple internal rearrangements and/or a small number of centromeric fissions and fusions. This strong karyotypic conservation implies a parallel conservation in gene arrangements and syntenic relationships among species, both within and among marsupial families.

B. Marsupials in biomedically oriented research. Inspection of the PubMed literature database revealed 1,402 publications pertaining to the use of marsupials in experimental, biomedically oriented

research during the 5-year period ending May 31, 2003. A broader research strategy (same time frame) using the NERAC information service returned more than 3000 “hits” including abstracts and non-experimental research topics. Examples of research topics included: fetal development; nervous system development and repair; neurophysiology; immunobiology; reproductive biology, gametogenesis, and fertilization; sexual differentiation; endocrinology and physiology of lactation; cellular physiology; hormone-receptor interactions; membrane transport and signal transduction; digestive physiology; renal physiology; neuromuscular physiology and experimental pharmacology; toxicology; parasitic and infectious disease research; anatomy and biomechanics; behavioral development; and a host of genetic topics (e.g.: gene and genome evolution; gene structure, regulation, and expression; DNA repair; cancer genetics; RNA editing; sex determination; gene mapping; molecular evolution and phylogeny). Many, though not all such studies take advantage of the immature state of the newborn marsupial which, together with the extended lactational developmental period, facilitates many kinds of fetal manipulations that are impractical or impossible with eutherian species.

C. *Monodelphis domestica* as a target for genome sequencing. Because all marsupials are equally phylogenetically distant from all eutherians, the “evolutionary position” argument in favor of a marsupial genome sequencing initiative applies equally to virtually any marsupial species. However, the choice of a species to target for genome sequencing must also take into account practical aspects of research utility: i.e., availability of animal resources to researchers, amenability of the species to experimental applications and manipulations, existing genetic resources, and breadth and depth of the research community that currently uses or would potentially utilize the resource. On these grounds one marsupial species, the gray, short-tailed opossum (*Monodelphis domestica*), stands out from all others used in research anywhere in the world.

Monodelphis domestica (a.k.a. the laboratory opossum) is a small South American marsupial that is widely used as a model organism for comparative research on a broad range of topics that are relevant to human development, physiology, and disease susceptibility (reviewed by VANDEBERG and ROBINSON 1997; VANDEBERG 1999). It is small (100-175 grams), grows rapidly (mature at 5-6 months), is highly prolific (average litter size ~8; up to 3 litters per year), breeds year-round (no seasonality), and has simple husbandry requirements (rodent cages and commercial feed). *M. domestica* have been raised as pedigreed laboratory animals in the U.S. and elsewhere for nearly 25 years. Initiated from a small founding stock in 1979, the research colony at the Southwest Foundation for Biomedical Research (SFBR) in San Antonio, TX has been repeatedly infused with new genetic material from wild caught populations and exhibits high genetic diversity (VANDEBERG and ROBINSON 1997; P. Samollow unpublished). More than 70,000 animals, including members of outbred and partially inbred strains, have been produced from the SFBR colony alone, and the distribution of experimental animals and breeding stock has resulted in the establishment of many research colonies worldwide.

Because of its favorable physical and reproductive characteristics *M. domestica* has become the predominant laboratory-bred research marsupial in the world today (SAMOLLOW and GRAVES 1998; current database search results). Recent examples of its use in basic biologic and biomedical research include: gene and genome evolution; photo-biology and DNA repair; molecular characterization of ultraviolet radiation-induced neoplasias; structure and evolution of mammalian antigen receptors; genomic imprinting; developmental neurobiology (neuromotor, neuro-sensory); neurophysiology; normal and regenerative development of the central nervous system and peripheral nervous structures; craniofacial and dental ontogeny and evolution; skeletal and skeleto-muscular development; reproductive endocrinology, behavior, and anatomy; lipoprotein metabolism and response to dietary fat and cholesterol; and more (references available from P. Samollow).

This is not to say that *M. domestica* is the only candidate for a genome sequencing project; the tammar wallaby (*Macropus eugenii*) is also a possibility. It is the primary colony-bred marsupial research model in Australia and will undoubtedly continue to be the focus of major research programs in the future. Unfortunately, physical and reproductive limitations related to its size (4.5-8.5 kg), low fecundity (litter size =1), and long generation time (females and males mature in 12 and 18 months,

respectively), coupled with the need to maintain it in outdoor "animal parks", have impeded its full development as a conventional laboratory animal. Nevertheless, it is important to realize that the evolutionary distance between *M. domestica* and *M. eugenii* is approximately 60 million years, placing these among the most distantly related of marsupial species (KIRSCH *et al.* 1997; SPRINGER 1997; GRAVES and WESTERMAN 2002). This degree of divergence is equivalent to that between humans and mice, suggesting that comparisons of genomic structures and patterns of gene regulation and expression between these two model species would be useful and informative.

Considering this huge evolutionary distance, and the substantial research communities utilizing these two species, we believe that genome sequencing projects for both *M. domestica* and *M. eugenii* would be highly desirable, and we urge the NHGRI to consider this possibility. We realize, however, that a single organization is unlikely to be prepared to support two marsupial genome projects simultaneously. Given *M. domestica*'s status as a genuine laboratory species, its use as a model organism for comparative research on topics that are relevant to human development, physiology, and disease susceptibility, and its broad availability from research colonies in North and South America, Europe, and Australia, we firmly believe that it is the more logical choice for genome sequencing at this time. Availability of the genome sequence of *M. domestica* will impact more laboratories, more investigators, and a broader spectrum of research worldwide than that of any other marsupial species (please see comments in the accompanying support letters in the Appendix).

II. Biological Rationales for the Utility of Marsupial (*M. domestica*) Genome Sequence Data

A. / B. Improving human health / Informing human biology. Marsupials are used in a wide range of comparative research applications that are relevant to human development, physiology, and disease susceptibility. In this section we summarize a few examples of current research foci that hold outstanding promise for contributing to our understanding of basic mammalian biology and, by extension, the processes that impinge upon health-related physiologic characteristics, disease susceptibilities, and developmental anomalies in humans.

UVR-induced neoplasia: *M. domestica* is the only laboratory animal known in which ultraviolet radiation (UVR) is a complete carcinogen (ROBINSON *et al.* 1994; 1998b). This characteristic has led to *M. domestica*'s development as a unique model for UVR-induced melanoma (humans also develop melanoma in response to UVR alone) and to the establishment of cell lines that are useful for studies of metastatic progression, immunologic maturation, and the testing of anti-neoplastic therapies (e.g., ROBINSON *et al.* 1998a; CHAN *et al.* 2001; 2002; WANG *et al.* 2001; WANG and VANDEBERG 2003). Low-level UVR exposure also leads to the initiation and progression of aggressive corneal sarcoma and the development of hyperkeratotic (pre-neoplastic) skin lesions (VANDEBERG *et al.* 1994a; 1994b; KUSEWITT *et al.* 1997). Susceptibilities to both of these diseases are variable and strongly heritable, indicating major genetic influences in the development of these conditions. Studies of mutation profiles in *M. domestica* homologs of human *TP53*, *CDKN2A*, *NRAS*, *HRAS*, and *KRAS* in melanoma (CHAN *et al.* 2002) and corneal sarcoma (KUSEWITT *et al.* 1999; 2000) cells have revealed similarities to and differences from those in human neoplasias. Deeper insight concerning the molecular basis of carcinogenesis and differential susceptibility to these tumors will require scrutiny of a much broader range of genes involved in cell cycle regulation, DNA repair, cell mobility and adhesion, immune surveillance, apoptosis pathways, transcription regulation, and other molecular phenomena involved in cell proliferation, tumor promotion and growth, and metastatic spread. Such studies would benefit greatly from the availability of sequence data from numerous regions of the *M. domestica* genome; i.e., full genome data.

Immunogenetics: The molecular components of the eutherian and metatherian immune systems are fundamentally similar, yet the immune responses of *M. domestica* and other marsupials exhibit notable differences from those typically seen in eutherian species (reviewed by STONE *et al.* 1996). For example, while *M. domestica* exhibits a primary humoral response that is similar to that in eutherians, the secondary response differs, being much weaker and less persistent. Similarly, allogeneic skin transplants are rejected rapidly by naïve hosts but, in contrast to the eutherian model, second set grafts

are not rejected more quickly (STONE *et al.* 1997). Other differences include the observation that *M. domestica* do not exhibit a mixed lymphocyte culture response (STONE *et al.* 1998), nor do their T-cells show a typical T-cell helper effect during secondary response. These functional distinctions imply that *M. domestica* possesses differences in the genetic architecture of the major histocompatability (MHC) Class I and Class II loci, or in the properties of T-cells and their receptors (STONE *et al.* 1998; 1999), that could provide a distinct alternative to the standard eutherian paradigm for hypothesis-driven experimentation on genetic mechanisms in mammalian immune function.

Characterizations of *M. domestica* immunoglobulin heavy and light chains (MILLER and BELOV 2000) have demonstrated that the isotypes IgG and IgE, previously known only from eutherian mammals, appeared prior to metatherian / eutherian divergence. In addition, the opossum T-cell receptor (TCR) alpha and beta chains (BAKER *et al.* 2001) and several MHC class I and class II loci (O'HUIGIN *et al.* 1998; MISKA and MILLER 1999; STONE *et al.* 1999; R. Miller unpublished) have been identified, and *RAG1* and *TdT*, genes which are important in Ig and TCR gene rearrangement, have been characterized (MILLER and ROSENBERG 1997; GUTH *et al.* 1998). Nonetheless, investigations of the immune system of any marsupial species are hampered by the shortage of reagents to identify markers of specific cell lineages, and to detect specific immune mediators such as cytokines. For example, the homologues of important cell markers such as CD4 and CD8 and cytokines such as Interleukin-2 have yet to be cloned from any marsupial. Such reagent development would be dramatically enhanced by the availability of the *M. domestica* genome sequence and would expand opportunities to analyze the large number of rapidly evolving gene families present in the mammalian immune system. Contrasts of such immune system components between marsupials and eutherian mammals will help illuminate the contributions of gene family diversity, gene structural differences, and levels of genetic variation to differences in immune system function.

Genomic imprinting: Aberrant expression of imprinted genes can result in developmental failures, neurodevelopmental and neurobehavioral disorders, and neoplastic disease (MURPHY and JIRTLE 2003). For example, abnormalities in imprinted inheritance occur in genetic diseases such as Prader-Willi syndrome, Angelman syndrome, and Beckwith-Weidemann syndrome, as well as in multiple types of pediatric and adult cancers. Imprinting abnormalities are also found in certain endocrine disorders, including type 1 diabetes, transient neonatal diabetes, and pseudohypoparathyroidism. Imprinted genes have been identified in eutherian and metatherian mammals (including *M. domestica*: O'NEILL *et al.* 2000) but not in prototherian mammals, nor in birds or other non-mammalian vertebrates (KILLIAN *et al.* 2000; 2001). This suggests that genomic imprinting arose in the common ancestor of metatherian and eutherian mammals after divergence from the prototherian lineage. Marsupials are thus the only non-eutherian species available for comparative studies of phylogenetic variants of the imprinting phenomenon. Beyond such intra-therian comparisons, comparisons of the genomic sequences of imprinted domains of therian mammals with orthologous, non-imprinted regions of prototherians, offers a potentially powerful bioinformatics approach for identifying structural features that led to the "imprintability" of genes and their *cis*-acting regulatory elements during therian evolution (MURPHY and JIRTLE 2003). The ability to use this phylogenetic approach to investigate imprinting and its regulation will be greatly facilitated by the genomic sequence of *M. domestica*. Comparative genomic studies of these kinds will not only further our understanding of the molecular evolution of imprinting, but will also help characterize the role of imprinting dysregulation in human disease development and assist in the discovery of novel therapeutic targets.

Central nervous system regeneration: Several research groups have documented anatomical and biochemical features of neuronal growth and axon targeting during normal and experimentally perturbed development of *M. domestica* newborns. An exciting result of these studies was the discovery that newborn opossums possess the ability to heal complete transections of the spinal cord (reviewed by NICHOLLS and SAUNDERS 1996; NICHOLLS *et al.* 1999). More recent work has revealed that regenerating axons not only target appropriate motoneuronal connections after crossing the surgical lesion (LEPRE *et al.* 1998), but that the regeneration process is complete, resulting in virtually normal locomotory (walking, climbing, swimming) behavior in weanlings and adults (SAUNDERS *et al.*

1998). Most exciting from a therapeutic viewpoint is that the developmental time "window" for regeneration competence can be extended significantly by blocking the actions of certain myelin-associated proteins (VARGA *et al.* 1995; NICHOLLS *et al.* 1999). Ongoing studies (letter from J.G. Nicholls; Appendix) concerning changes in gene expression and regulation during critical periods when neuronal growth and/or regrowth can or cannot occur, may furnish insights that lead to the manipulation of genes or their products to promote neural regeneration in spinal cord or peripheral nerve injury patients.

X-chromosome inactivation: The pattern of X-chromosome inactivation (XCI) and the regulation of X-linked gene expression in marsupials differ in important ways from those in eutherian mammals, most obviously by being non-random and incomplete (COOPER *et al.* 1990; 1993). These phylogenetic distinctions can be exploited to better understand the fundamental processes of X-chromosome imprinting and gene silencing. For example, non-random paternal X-inactivation also characterizes some early differentiating extra-embryonic tissues in eutherian embryos, and it has been suggested that the deterministic, paternal X-inactivation system of marsupials may represent a fundamental inactivation system upon which the random inactivation pattern of eutherian somatic cells has been elaborated (MIGEON *et al.* 1989). Rapid advances in understanding the regulation and molecular function of *XIST*, a *cis*-acting gene that initiates the XCI process in eutherian females (BROCKDORFF and DUTHIE 1998; MIGEON 1998; BROCKDORFF 2002), suggest testable hypotheses concerning the existence and function of a marsupial *XIST* homolog (if any) in the X-inactivation process. Similarly, marsupials offer the only models for examining the normal reactivation of "inactivated" genes during pre- and postpartum development (SAMOLLOW *et al.* 1995).

Progress in these and other promising research areas has been hindered by a dearth of modern genetic and genomic marsupial resources. Indeed, the production of such resources is cited in many of the accompanying support letters (Appendix) as the most important objective for advancing specific research applications of marsupial models. Steps toward mitigation of this resource shortage for *M. domestica* are evident in the recent production of a first generation linkage map for *M. domestica* and a high quality *M. domestica* BAC library (see section III.2). Nevertheless, the potential of *M. domestica* and other marsupial species for comparative genomic applications, and the implications of this research for better understanding of basic mammalian biology and specific human characteristics, cannot be realized without access to full genomic sequence data.

C. / D. Informing the human sequence / Providing connections between the sequences of humans and other species. Differences in the regulation of gene expression and mechanisms of gene interaction that underlie distinctions in biochemical, physiologic, reproductive, and developmental characteristics of marsupial and eutherian species have evolved primarily as modifications of ancient genetic mechanisms that were present in the common therian (mammals exclusive of prototherians) ancestor. Past comparisons of eutherian and metatherian genome features using "pre-genomic era" gene mapping approaches, have already demonstrated the power of the comparative approach to provide insights and suggest novel hypotheses regarding such questions as the evolution of sex chromosomes and the genetic bases of sex-determining mechanisms; the evolution of X-linked and autosomal gene regulation and expression patterns; the evolution of gene structure; and the multilevel nature of X-chromosome inactivation (extensive references for each of these topics can be found in SAMOLLOW and GRAVES 1998).

Acquisition and distribution of *M. domestica* genome sequence data will propel comparative genetic analysis far beyond the limitations of crude mapping-based studies by providing greatly improved power for inferring the origins of mammalian-specific genetic and genomic structures and molecular process (genetic and epigenetic functions). Genome sequence information will help to determine which novel eutherian gene functions are paralleled by novel changes in gene sequence and structure, and which gene structures and functions have been co-conserved over a much longer time period – i.e., since before the metatherian / eutherian divergence. *M. domestica* genome sequence will also enhance the utility of non-mammalian vertebrate (e.g., chicken, *Fugu*, zebrafish) genomes and those of the several eutherian species already sequenced or in production by providing a "benchmark"

that will help clarify which and what kind of sequence and structural changes occurred before and subsequent to the divergence of mammalian ancestors from other vertebrate progenitors.

E. Expanding our understanding of basic biological processes relevant to human health. Genes of the MHC and related loci play pivotal roles in molecular mechanisms at work in a broad range of processes that influence disease susceptibility and general health. Much of what is known about the relationships among these various mechanisms and MHC gene function has been inferred from the experimental manipulation of eutherian animal models. Some of the functional differences in metatherian and eutherian immune responses (section II.A / II.B) could result from differences in the genetic architecture (type, number, and/or genetic variation) of MHC genes and gene families. Thus, information about the organization and function of marsupial MHC genes can be expected to contribute significantly to our understanding of fundamental immune mechanisms that are common to all mammalian (and perhaps other vertebrate) species.

The nature of marsupial reproduction provides unique opportunities to investigate, postnatally, early developmental events in the immune system that occur *in utero* in eutherian species. In *M. domestica* (at least) lymphoid development occurs entirely postnatally. At birth the thymus is undifferentiated epithelial tissue, and *RAG* gene expression, which is necessary for rearrangement of the Ig and TCR genes, is not detectable until day 7 post-birth (R. Miller unpublished). *M. domestica* also provides a model to study unique maternal-fetal interactions that may occur prenatally in marsupials and during maternal transmission of immunity (and possibly disease) to offspring during the extended lacational period. Furthermore, the importance of the innate immune system in early marsupial development is unknown but is certain to yield insights by contrast to those of birds and eutherian mammals. Finally, the lack of extensive placental development in most marsupials (the bandicoots, family Peramelidae, are exceptional in having significant placentation) suggests strategies whereby maternal-fetal interactions and correlations between gestational development and MHC gene expression (e.g., maternal tolerance to fetal antigens) could be profitably investigated.

M. domestica genome sequence data also have important implications for investigating mechanisms of infectious disease and the evolution of host-parasite interactions. For example, *M. domestica* is a member of the Didelphidae, the most species rich of all marsupial families (~63 species). Within the Didelphidae are several opossum species, including *M. domestica,* that are important hosts and reservoirs for the parasite *Trypanosoma cruzi*, the causative agent of Chagas' disease. According to the CDC an estimated 15-18 million people are infected with *T. cruzi*, and 50,000 die annually. There is evidence that the relationship between *T. cruzi* and marsupials may be as old as 65 million years, while the parasite has been infecting eutherian hosts for less than 5 million years (SCHOFIELD 2000). This is consistent with the observation that *T. cruzi* is less pathogenic to opossums than to humans. Such a relationship provides an important model in which to study host-pathogen interactions and the evolution of pathogenicity.

Elucidation of the evolutionary history and molecular mechanisms of genomic imprinting will help define the pathways for intervention in a growing category of epigenetic disease caused by the aberrant expression of imprintable genes. Bioinformatic studies have revealed a paucity of Alu and MIR SINEs (GREALLY 2002) and CR1 LINEs (J. Greally unpublished) in imprinted regions of the human genome. While Alu SINEs entered the genome since our divergence from marsupials, both MIR SINEs (JURKA *et al.* 1995) and CR1 LINEs (HAAS *et al.* 2001) are common to all mammals and possibly other vertebrates indicating that regions currently undergoing genomic imprinting were established prior to the mammalian radiation. The availability of whole genome sequence data from *M. domestica* would enable testing of the hypothesis that genomic imprinting is an ancient gene regulatory process. Moreover, limited studies of imprinting in marsupials suggest that some but not all loci that are imprinted in eutherians undergo imprinting in marsupials (KILLIAN *et al.* 2000). The identification of additional *M. domestica* orthologs of eutherian imprinted genes would allow the application of allelic discrimination techniques to test their imprinting in tissues from these animals, and to correlate imprinting (or its absence) with sequence content at these loci. This approach would yield insights into

the evolution and mechanism of imprinting that would be difficult to achieve by other approaches.

Examination of genomic sequence characteristics that make certain loci on the X chromosome more or less susceptible to XCI in eutherian mammals indicates that chromosomal position relative to the centromere and to the X-inactivation center are important determinants of susceptibility to XCI, while sequence content in the flanking regions (particularly microsatellites and LTR retroelements) are also predictive of inactivation status (J. Greally unpublished). L1 LINEs have been proposed as strong candidates for propagating XCI in eutherians (LYON 1998) based on their accumulation on the X chromosome (BOYLE *et al.* 1990; BAILEY *et al.* 2000). A systematic analysis of X-linked genes in *M. domestica* would determine whether escape from XCI occurs in marsupials, and define the role (if any) of L1 LINES or other sequence characteristics in the marsupial paternal XCI process. Such comparisons would allow us to contrast the sequence features that characterize the X chromosome as a whole in marsupials and eutherians, and thereby shed light on the molecular underpinnings of the different outcomes of XCI in these two groups. As genes that fail to inactivate in humans are likely to be responsible for Turner syndrome and possibly some sex-specific phenotypic differences (BROWN and GREALLY 2003), such insights have practical clinical applications.

M. domestica may also provide a novel model system for examining the physical and molecular processes underlying meiotic recombination. Linkage data from all eight autosome pairs indicate that meiotic recombination in *M. domestica* is strongly male biased (SAMOLLOW *et al.* submitted), a pattern that is contrary to that in eutherian mammals and other vertebrates. Specifically, male *M. domestica* exhibit twice as much recombination as females. Qualitatively similar but less extreme findings in other marsupials (BENNETT *et al.* 1986; ZENGER *et al.* 2002) suggest that reduced female recombination might be a common metatherian attribute. Genome-wide examination of the physical basis of these dramatic sex-specific recombination differences in *M. domestica* could lead to better understanding of the sequence characteristics and molecular events that differentially promote or inhibit chiasma formation and distribution between the sexes, and help to clarify the evolutionary forces that determine recombination rates within and among species.

F. Providing additional surrogate systems for human experimentation. As mentioned (II.A / II.B), *M. domestica* holds promise as a model for understanding the basic biology of several pathologic conditions that afflict humans. It is an obvious model for studying the differential expression of genes during spinal cord regeneration, and as the only laboratory species in which malignant melanoma can be induced by UVR alone, *M. domestica* holds potential as a model for studies of metastatic progression and immunologic surveillance, and for the testing of anti-neoplastic therapies and preventative treatments such as sunscreens or other pharmaceuticals. In addition, results from dietary challenge studies (e.g., RAINWATER *et al.* 2001) indicate that diet-induced variation in certain plasma lipoprotein phenotypes, which correspond to important risk factors for atherosclerotic disease in humans, are strongly influenced by genetic differences among individual *M. domestica*. A major portion of the variation in human plasma cholesterol levels is responsive to diet, but dietary response genes have been difficult to detect in humans or in animal models. The results from *M. domestica* are consistent with the hypothesis that at least three unlinked genes have major effects on the lipoprotein phenotypes that are similar to those in humans, and suggest that *M. domestica* may provide a unique animal model for studying the genetic control of diet-induced changes in lipoprotein metabolism.

G. Facilitating the ability to do experiments, e.g. "direct" genetics or positional mapping, in additional organisms. An NIH-funded project is underway to complete a 2.5-5.0 cM (centiMorgan) linkage map of the *M. domestica* genome (see section III.2 for details) that can be used for the genetic mapping of physiologically and developmentally important QTLs. As QTLs for health-related traits are detected and mapped, whole genome sequence data will become critically important not only for positional cloning and identification of these QTLs within the *M. domestica* genome, but also for facilitating extrapolation of the positions of these QTLs to specific genomic regions in other species including humans. These complementary approaches comprise a strong research strategy for the identification of marsupial homologs of hitherto unidentified human genes with potential implications for

human health, and ultimately for the localization of these genes within the human genome. Although positional cloning and interspecific extrapolation are possible without whole-genome sequence data, the availability of such data will greatly accelerate the pace both of these important activities.

H. Expanding our understanding of evolutionary processes in general, and of human evolution in particular. The phylogenetic position of marsupials comprises the fundamental rational for proposing a marsupial genome sequencing effort. By providing an evolutionarily closer alternative to non-mammalian vertebrate model species (e.g., chicken, zebrafish, *Fugu*, frog), the *M. domestica* genome sequence will enable the examination of differences in the structures of genes, diversity of gene families, and the accumulation and distribution of repetitive elements among model genomes at several levels of evolutionary divergence, and to relate these differences to characteristics of gene expression, function, and epigenetic regulation. In many cases, the genomes of eutherian mammals may be too similar to one another to provide sufficient structural modifications and evolutionary novelties to yield meaningful insights into the contribution of structural changes to certain functional differences among species. Similarly, the evolutionary distance between eutherian and avian genomes will likely be too great to inform us about many such relationships as well. Comparative genome analysis, as powerful as it is, can be limited by a lack of taxonomic sampling; thus strategic selection of genomes can improve the accuracy and resolution of comparative studies (POLLOCK *et al.* 2000). The clear intermediacy of marsupial genome data will provide a much closer, yet not too close, comparator to the genomes of eutherian mammals, and an alternate reference for comparisons between mammalian and avian (and more distant vertebrate) genomes.

III. Strategic Issues in Acquiring New Sequence Data

A. Size of the research community / Demand for sequence data. An idea of the breadth of research activity that would be enhanced by the availability of the *M. domestica* genome sequence can be inferred from the range of research topics that have been mentioned in foregoing sections of this document. But perhaps more informative in this regard are the views of the large and rapidly growing community of investigators who utilize marsupial models in their research programs and who deem the acquisition of marsupial (especially *M. domestica*) genome sequence data as essential to the further development and expansion of their own research capabilities. Over the past few weeks, we have received 44 letters from individual investigators and laboratory groups (total number of signatories = 50) in Australia, Canada, Finland, Germany, Italy, Sweden, the United Kingdom, and the United States expressing their enthusiasm for *M. domestica* as a model system and strongly advocating sequencing of the *M. domestica* genome (Appendix). Most of these people use *M. domestica* as a research model, but it is significant that many letters were received from investigators who presently utilize other (i.e., Australian) marsupial species, or do not use marsupials directly but wish to use marsupial genome data in their own research programs.

In addition to indicating the enthusiasm and magnitude of the community, these letters reveal the breadth of research activity that would be enhanced by the production and availability of a full *M. domestica* genome sequence. Areas of active research represented by these signatories (in parentheses) include: evolution, organization, and expression of immunoglobulin, MHC, and other immunologically related gene families (Belov, Deane, Skow); comparative and molecular genetics of genomic imprinting (Killian, R. Nicholls, M. O'Neill, Vrana); photobiology, UVR carcinogenesis, and DNA repair (Gale, Nairn, VandeBerg, Walter); regulation of X-linked gene expression during gametogenesis and early development (McCarrey); evolution and functional significance of repetitive DNA sequences (Baker & Parish, Hughes); molecular mechanisms of neural crest cell migration (Smith); comparative genetics and molecular biology of craniofacial development and dental patterning (Jernvall, Thesleff, Weiss); comparative genomics, phylogenetic reconstruction, and gene/genome evolution (Jansa, Li, Voss); chromosome / chromatin structure, genomic instability, and evolutionary rearrangement (R. O'Neill); evolution of vertebrate sex determining mechanisms, sex-specific gene expression, and sexual differentiation (Mackay, Ullmann); maternal / fetal interaction and the evolution of viviparity (Kennedy); developmental and comparative neurobiology (Cabana, Krubitzer, Sakaguchi,

Selwood), central nervous system growth and regeneration (J. Nicholls, Saunders *et al.*); evolution of olfactory receptor families (Srotmann *et al.*); evolution, structure, and function of egg, sperm, and fertilization proteins (O'Rand, Rodger); genetics of diet-induced dyslipoproteinemia (Kammerer, VandeBerg); marsupial genomics and quantitative trait mapping (Zenger).

B. Suitability of *Monodelphis domestica* for experimentation. *Monodelphis domestica* is the only fully developed laboratory marsupial in existence. As discussed above (I.1), it is small, easily maintained in conventional animal rooms, has a short generation time (6 months), and reproduces prolifically and readily throughout the year. Origins of laboratory colonies, standard methods for care and maintenance, blood collection and surgical procedures, techniques for recovery of timed embryos, and the timetable of embryonic development are well established and have been described in detail (reviewed by VANDEBERG and ROBINSON 1997; VANDEBERG 1999). These animals are very healthy in captivity, with a spontaneous morbidity and mortality profile similar to those of traditional laboratory species. What little spontaneous pathology does occur has been documented and described (HUBBARD *et al.* 1997). Colony bred *M. domestica* are parasite free and exhibit no specific infectious disease problems.

Availability of animal and DNA resources: The total number and exact locations of all *M. domestica* research colonies is not known to us, but colonies derived from the original SFBR colony have been established in at least seven countries (Australia, Brazil, Canada, Italy, Switzerland, UK, and several in the US). The SFBR colony, which remains the largest and genetically most diverse in the world, maintains a steady state of approximately 2,400 animals including outbred stocks and partially inbred strains (inbreeding coefficients 0.65-0.85) derived from founders originating from several ecologically diverse and geographically separated (regions up to 2,400 km apart) in Brazil and Bolivia (VANDEBERG and ROBINSON 1997; VANDEBERG 1999). The colony is fully pedigreed and all pedigree records are maintained in an extensive pedigree management database. DNA is readily available from frozen tissue archives or the living colony animals. This species is common, though not abundant, throughout its range (Brazil, Bolivia, Paraguay) making it possible to procure additional animals for future colony expansions. It is not endangered or threatened.

Linkage map: An NIH-funded research program (RR 14214: *Genomic Resource Development in the Laboratory Opossum*; P.B. Samollow, P.I.) is underway to complete a 2.5-5.0 cM (centiMorgan) linkage map of the *M. domestica* genome, comprised of both anonymous markers and functional gene loci. The current linkage map comprises approximately 90 loci distributed among 8 autosomal linkage groups and the X-chromosome. Another 130 genetic markers currently are being added to the map, and it is anticipated that approximately 350-400 markers will be mapped by the end of the grant period.

BAC library: A high quality (10X, 155 kb+ average insert size) BAC library of the *M. domestica* genome has been completed through the NIH BAC Resource Network (NBRN). The library was constructed by Dr. Chris Amemiya using a fully pedigreed male *M. domestica* from the SFBR colony. This animal was from the original founder line, Population 1, which is the most widely distributed of all *M. domestica* lines on a worldwide basis. Stored DNA from this animal is available for future studies.

Additional resources: Normal and tumor-derived cell lines, as well as small-insert genomic and cDNA libraries, are available from individual investigators and/or commercial sources.

C. The rationale for the complete sequence of the organism. Broad comparisons of coding and non-coding regions will be necessary for examining the abundances and distributions of members of gene families and repetitive elements, to examine the roles of genome-wide sequence structures as foci of recombination, to provide target material for the isolation and/or amplification of specific sequences for many different kinds of studies, and to enable whole genome analyses of *M. domestica* genome structure in comparisons to other mammalian (eutherian and prototherian) and more distant vertebrate species. Neither the targeting of specific coding regions, nor the piecemeal examination of BAC clones will have the power to reveal the whole genome changes that have accompanied the

specializations of gene structure and function that occurred during the divergent evolution of eutherian and metatherian mammals.

Whole genome sequence data will provide the basis for the construction of novel expression microarrays based on predicted gene sequences. Expression microarrays will be important for the examination of gene expression patterns associated with alternate phenotypic or disease states in the *M. domestica* model. This is a critically important capability that cannot be achieved effectively using eutherian-based arrays because of inter-infraclass sequence divergence. Microarrays based on whole genome sequence will have the advantage of including all genes in the genome. In addition, such arrays can be used in novel "fast-track" approaches to the positional cloning of QTLs that contribute to normal and abnormal physiologic and developmental variation and are detected and localized by QTL linkage mapping approaches.

D. Sequencing strategy and cost. Experience and insights gained while recently mapping and sequencing the mouse genome lead us to propose a combined strategy for the *M. domestica* genome. Genome size of this species is approximately 15-20% larger than in human and mouse (C. Amemiya, unpublished). An ~8-fold whole genome shotgun component consisting of both small and large insert clones (see Table 1) will provide the community of users with rapid access to most of the *M. domestica* genome sequence. A BAC clone-based physical map, along with paired end sequences from fosmids and the mapped BAC clones, will provide a framework by which the genome sequence can be accurately assembled, and ordered and oriented on the chromosomes. In addition, we wish to confirm the anchoring to the genome by doing FISH with fosmids from major supercontigs. We expect that this project will cost approximately $50M. As with the mouse genome sequence, we would expect that the proposed approach would result in anchored “supercontigs” (sequence contigs connected by at least two read-pair links) of greater than 10 Mb in average length (MGSC 2002).

Table 1. Proposed whole genome shotgun sequencing of the *Monodelphis domestica* genome.

Clone type	Insert size	No. of reads	Seq coverage	Phys coverage
plasmid	4 kb	33M	6.0x	18x
plasmid	10 kb	8M	1.5x	11x
fosmid	40 kb	1.6M	0.3x	9x
BAC	150 kb	0.7M	0.15x	14x
TOTALS	---	43.3M	7.95x	52x

Note: based on a genome size of 3.6Gb and an average sequence read length of 650 bp; Physical coverage calculation assumes that all clones contribute read pairs.

The resulting genome sequence will be of sufficient contiguity and quality for preliminary analyses and gene discovery by comparative methods. To improve on the utility of the sequence for these and other types of analyses, we advocate a round of computer-directed “automated finishing” in which oligonucleotides are algorithmically selected to extend sequence contigs into gap regions. BAC and fosmid clones would serve as the templates for the necessary sequencing reactions. The methods, computational tools, and laboratory pipelines for automated finishing are already in place at the Washington University Genome Sequencing Center and Whitehead Institute / MIT Center for Genome Research.

At this point, manual finishing could be employed for targeted regions (or the whole genome, if deemed necessary) to further improve contiguity and sequence accuracy. Our experience with finishing the mouse genome from a combined approach and initial finishing of the *C. briggsae* genome from a whole genome shotgun approach suggests that most of this work would involve using PCR to sequence and/or size regions that were missing, ambiguous, or repetitive in content.

5. Are there other (partial) sources of funding available or being sought for this sequencing project? None are known at this time.

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