Animal Cloning: A Risk Assessment

Center for Veterinary Medicine
U. S. Food and Drug Administration
Department of Health and Human Services
7500 Standish Place
Rockville, MD 20855

1/15/2008

Table of Contents

Appendices

Preface

Preface

The following Risk Assessment is the result of a multi-year effort by staff from the US Food and Drug Administration's (FDA's) Center for Veterinary Medicine (CVM or the Center). Since the late 1990s, CVM has been gathering data and meeting with clone producers and other stakeholders interested in cloning to discuss the safety and regulatory implications of somatic cell nuclear transfer (SCNT), the process most commonly used to generate animal clones during this time period. In the fall of 2000, CVM tasked the National Academy of Sciences (NAS) to perform an independent, scientific review of the available data on the safety of cloning, including holding a public meeting to identify science-based concerns and elicit data and information on clones and their food products from the scientific community. In July of 2001, the Center issued a CVM Update requesting that clone producers not introduce meat or milk from clones or their progeny into food or feed until the NAS report had been completed, and the agency had had a chance to complete its own review of the safety of those food products.[1]

In October of 2002, NAS issued its report "*Animal Biotechnology: Science-Based Concerns*." Following an overview of the available data on animal clones, the report indicated that the most likely mechanism for generating hazards to clones would stem from reprogramming of the donor cell genome, and that any harms that might result from that reprogramming would be observed early in a clone's development. They further noted that there were no published data comparing the composition of meat or milk from clones with that from conventionally-bred animals. Nonetheless, the report concluded that there is "no evidence that food products derived from adult somatic cell clones or their progeny pose a hazard (i.e., there is no evidence that they present a food safety concern)" (page 65).

This Risk Assessment is CVM's subsequent independent analysis of all of the available data relevant to assessing the health of clones and their progeny (and other animals involved in the cloning process) or food consumption risks resulting from edible products from these animals. In order to make the Risk Assessment as transparent as possible, all of this information is available to the public, either by virtue of its publication in peer-reviewed journals, or by "publication" in this risk assessment. We have actively sought independent peer-review of these data by providing all of the data in raw form (not summaries) either in the text of the risk assessment or in appendices. In addition, we have also described the means by which the methodology was developed to facilitate peer-review by risk assessors.

[1] http://www.fda.gov/cvm/CVM_Updates/clones.htm

CVM has attempted to be as comprehensive as possible about identifying and using all of the relevant data in its analysis. We have performed extensive literature reviews, engaged in conversations with scientists involved in cloning animals, and requested data on animal health and food composition from scientists, breeders, and food producers. Unpublished data were provided to us in raw, unanalyzed form, which we subsequently analyzed. CVM determined whether a particular publication or dataset was relevant to the analysis. These judgments were framed by the two overarching objectives of the Risk Assessment: determining whether cloning poses any health risks to the animals involved in the cloning process, and whether any hazards arise during the development of clones or their progeny that may pose food consumption risks.

Literature searches for the draft version of the Draft Risk Assessment ceased in early 2006. For the final version of the Risk Assessment, we have conducted updated literature searches (through mid-year 2007), thoroughly reviewed hundreds of additional relevant papers from the peer-reviewed literature, and incorporated this information into the Risk Assessment. The final version also includes additional, unpublished data that were submitted to CVM after the release of the Draft Risk Assessment. We reviewed all of the public comments that we received on the Draft Risk Assessment and associated documents. Careful consideration was given to relevant, science-based information in the comments, and parts of the Risk Assessment have been revised in response to these comments.

In addition to understanding the Risk Assessment's goals, it is equally important to understand what it does not consider. It does not attempt to address the question of whether clones are "normal;" rather it concentrates on identifying the risks that cloning poses to animal health or to humans and animals consuming food derived from clones and their progeny. It also does not attempt to explore issues such as the influence of different donor cell types or cell cycle stages in the "success rate" for producing clones, or the degree to which clones are more or less identical at the phenotypic level. Studies addressing these questions have been used, however, when they provided data useful to the identification of hazards or risks. Similarly, the Risk Assessment does not attempt to parse out the relative effectiveness of different cloning techniques or different laboratories in generating live animals. Results of cloning in species not commonly used for food have been employed only as they have utility as model systems (*e.g.,* mice as models for livestock). Uncertainties associated with those models have been identified.

It is important to note that this Risk Assessment is a scientific document that provides a framework by which science-based questions regarding animal health and food consumption risks are evaluated. CVM recognizes that cloning raises many ethical and

economic concerns. These issues may be important to members of the public, however, they are not within FDA's mission and therefore not within the purview of this Risk Assessment.

Finally, the measures we will take to manage the risks associated with cloning and our recommendations regarding the use of clones or their progeny as food or feed are not included in the Risk Assessment, but are addressed in the accompanying Risk Management Plan and Guidance for Industry.

Chapter I:

Executive Summary

Chapter I: Executive Summary

Cloning is the colloquial term used to describe the process of somatic cell nuclear transfer (SCNT) that falls on a continuum of assisted reproductive technologies (ARTs) currently used in agriculture. In this Risk Assessment, the Center for Veterinary Medicine (CVM or the Center) at the US Food and Drug Administration (FDA) presents a science-based review of the available information on cloning in species traditionally used for food (*i.e.,* cattle, swine, sheep, and goats).

A. Overview

This Risk Assessment addresses SCNT technology, its impact on the health of animals involved in that process, and food consumption hazards that may arise in animal clones and their progeny[2] in the context of the use of ARTs in conventional animal agriculture. Chapter II is a summary of ARTs currently used in food animal breeding and a detailed explanation of SCNT. Chapter III describes the process of risk assessment, its application to animal cloning, and the nature of the hazards that may arise as the result of cloning. A synopsis of the processes involved in epigenetic reprogramming and their relevance to adverse outcomes noted in animals derived via SCNT and other ARTs is found in Chapter IV. Chapter V addresses potential health risks to animals involved in the process of cloning, including surrogate dams, clones, and their progeny. Chapter VI addresses potential food consumption risks that may result from edible products derived from animal clones or their progeny. Each chapter contains conclusions relevant to that subject; the Risk Assessment is summarized in Chapter VII, and our overall conclusions are presented there. In order to make this process as transparent as possible, all of our methodologies are presented in the text of the risk assessment; the information and data that CVM evaluated are publicly available, either in peer-reviewed publications, or in Appendices to this document. The process by which CVM drew its conclusions is presented in the Risk Assessment, along with explicit statements of potential bias and uncertainty. The document concludes with a complete bibliography, a glossary of terms, and appendices containing data and background information.

The Risk Assessment is the result of a qualitative analysis that identifies and characterizes the nature of hazards that may be introduced into animals as a result of cloning, and puts them in the context of other ARTs currently practiced in the United States. The strongest conclusions that

[2] For the purposes of this analysis, a clone is defined as an animal produced asexually from a single animal by somatic cell nuclear transfer. Clones are thus genetically identical to their nuclear donor animal. Progeny of clones have at least one animal clone as a parent (but could also result from mating two animal clones) and are produced by sexual reproduction. Clones of clones would be considered as clones (*i.e.,* directly arising from an SCNT process).

can be drawn regarding positive outcomes in risk assessments of this type are "no additional risk" because outcomes are weighed against known comparators. If a finding of "no additional risk" were to be applied to the health of animal clones, it would mean that the cloning process would not pose any greater risk to the health of the animals involved than other ARTs. Applied to the safety of edible products derived from clones, a finding of "no additional risk" would mean that food products derived from animal clones or their progeny would not pose any additional risk relative to corresponding products from conventional animals, or that they are as safe as foods that we eat every day. As with all risk assessments, some uncertainty is inherent either in the approach we have used or in the data themselves. Where uncertainties exist, CVM has attempted to identify the degree of uncertainty and the reasons for its existence.

B. Technology Overview (Chapter II)

Assisted reproductive technologies (ARTs) have been employed extensively in animal agriculture for over a century, and at least one (artificial insemination) has been practiced for several hundred years. These technologies form a continuum that ranges from the fairly minimal assistance provided to animals engaged in natural service through the more recent development of SCNT. ARTs have aided in the genetic improvement of domestic livestock species by the selection and propagation of desirable phenotypes, and accelerating the rate at which those characteristics have been incorporated into national herds. Artificial insemination, for example, permitted the propagation of valuable genomes without the sire being physically present, thereby allowing superior genetics to be spread beyond relatively small geographical areas.

Most commonly used ARTs rely on fertilization as a first step. This joining of egg and sperm is accompanied by the recombination of the genetic material from the sire and dam, and is often referred to as "shuffling the genetic deck." From a breeder's perspective, phenotypes resulting from sexual reproduction cannot be predicted—that is, the characteristics of the offspring from a mating may be estimated, but not predicted with certainty. Nuclear transfer, the most advanced of these technologies, does not require fertilization and allows for the propagation of known genotypes and phenotypes without the risk of genetic reshuffling. Thus, SCNT's greatest immediate impact on animal agriculture may be that it allows the propagation of genomes whose phenotypes are proven. It also allows the propagation of animals whose reproductive function may be impaired, or of very valuable animals that have died. SCNT, like the other newer forms of ARTs (*e.g., in vitro* fertilization, embryo splitting) results in some known adverse outcomes to the animals and possibly the dams bearing those pregnancies.

C. Risk Assessment Methodology (Chapter III)

Risk assessment is a science-based process used to identify hazards that may be present in predefined exposure scenarios, and to estimate the severity and chances of the outcome(s) occurring once that exposure occurs. Because many, if not all, of the individual steps that comprise a risk assessment contain various degrees of uncertainty, risk assessors should explicitly describe the sources of uncertainty and the effect(s) that the uncertainties may have on any judgment of risk. Risk assessment serves as the scientific underpinning from which risk managers may choose different options based on their understanding of, and responsibilities to, the broader contexts within which they operate.

Qualitatively, risk may be thought of as some function of the combination of exposure and the intrinsic properties of the substance or process under consideration by linking an exposure to the likelihood of an outcome. When performing a risk analysis, it is critically important to distinguish between a *hazard* and the potential *risk(s)* that may result from exposure. A *hazard* can be defined as an act or phenomenon that has the potential to produce an adverse outcome, injury, or some sort of loss or detriment. These are sometimes referred to as *harms*, and are often identified under laboratory conditions designed to maximize the opportunity to detect adverse outcomes. Thus, such observational summaries are often referred to as "*hazard identification"* or "*hazard characterization."* Risk, then, is the conditional probability that estimates the probability of harm given that exposure has occurred. In a qualitative assessment such as this, however, risks can be discussed only within a qualitative context, and no quantitative interpretations should be made.

In order to address the hazards and risks to animals involved in cloning and the food products derived from them four issues must be addressed: identifying hazards and risks; determining the degree to which existing data address the question of risk; characterizing residual uncertainties; and selecting the most appropriate definition of risk for the risk assessment.

This Risk Assessment explicitly excludes transgenic clones from the identification of hazards or risks experienced by "just clones" because of the inability to determine whether the transgenic event or cloning was causally associated with an adverse outcome. In addition, the Risk Assessment has assumed that, at minimum, animal clones, their progeny, and food products derived from them would be subject to the same laws and regulations as conventional animals and their food products.

Source of Hazards/Risks: Because no exogenous genes have been introduced into animals derived via SCNT, the underlying assumption regarding potential hazards that could arise is that anomalies observed in animal clones are due to incomplete or inappropriate reprogramming of

the donor cell nucleus. These anomalies may be macroscopic (*e.g.,* anatomical abnormalities, difference in size or growth rate, reduced fertility, morbidity, mortality) or they may be more subtle in nature. Potential *subtle hazards* would allow an animal clone to develop with apparently normal appearance and functions, but with sub-clinical physiological changes.[3] These include alterations in clinical chemistry, hematology, or changes in physiological set-points (*e.g.,* changes in hormone levels). For food consumption risks, relevant subtle hazards that might result from inappropriate or incomplete reprogramming include alterations in the expression of key proteins affecting the nutritional content of food, possibly leading to dietary imbalances. Similar hazards arise in animals generated via other ARTs or natural breeding. The goal of this risk assessment is to determine whether any unique hazards arise that are not noted in comparators, or have not been identified in cattle, swine, sheep, or goats produced via other ARTs or natural breeding.

To address animal health and food consumption risks associated with cloning, two complementary approaches were employed. First, information on the health of animal clones was evaluated within a framework developed by CVM called the *Critical Biological Systems Approach* (CBSA). For food consumption risks, the CBSA was applied in combination with a second approach referred to as *Compositional Analysis*. Following review of all of the available data using the CBSA and Compositional Analysis, a *weight of evidence approach* was then used to draw conclusions regarding risks to animals associated with cloning, and risks to humans from consuming foods produced by animal clones.

The CBSA: This approach divides the life cycle of an animal clone into five functional developmental nodes. Developmental Node 1 incorporates the initial technical steps involved in SCNT (cell fusion) and continues through fetal development. Developmental Node 2 encompasses the perinatal period, including labor induction in the dam, delivery, and the critical few days after birth. The third developmental node, Juvenile Development and Function, covers the period of rapid growth between birth and the onset of puberty. The Reproductive Development and Function Node (Developmental Node 4) includes puberty and reproductive function throughout the reproductive life of clones. The Post-Pubertal Maturation Node (Developmental Node 5) consists of all non-reproductive functions of sexually maturing or mature clones, including growth, weight gain, disease frequency, aging, and, where available, lifespan.

The nature of each component of the risk assessment (*i.e.,* animal health or food consumption risks) shaped the manner in which the available data were evaluated using the CBSA. For example, identification of adverse outcomes for animal health included both the animal clone

[3] Such subtle hazards are not typically included in standard food safety assessments.

and the surrogate dam carrying the pregnancy. Emphasis was placed on the clones' development and probability of normal development, compared with other ARTs such as artificial insemination (AI), *in vitro* fertilization (IVF), and blastomere nuclear transfer (BNT). In our assessment of animal health, we considered a wide range of hazards, ranging from macroscopic to biochemical changes (*e.g.,* changes in gene expression, differences in enzyme activity) that might affect the well-being of animal clones. For food consumption risks, animal clones bearing gross anomalies were excluded from the analysis, and emphasis was placed on identifying unique subtle hazards that could have arisen as the result of the SCNT process. The rationale for this approach is found in Chapter IV, and provides the molecular evidence for the role of epigenetic reprogramming as the source of these subtle hazards. Because of the assumption that hazards would be subtle, datasets were evaluated on as fine a level of resolution as possible, including individual animals or even individual analytes per animal in order to have as sensitive a screen as possible for adverse outcomes (and thus potential food consumption risks). In this risk assessment, the most detailed level of resolution used for evaluating animal health has been physiological and biochemical measures of individual animals. It is likely, as technologies mature, that molecular techniques such as genomics, proteomics, and their integrated metabolomic measures will assist in such determinations (NAS 2004).

Compositional Analysis: To reach conclusions about the risks of consuming food produced by animal clones, findings regarding animal health (derived from the CBSA) were considered in conjunction with results of the Compositional Analysis approach. In an attempt to find potential subtle hazards, the data considered in this part of the risk assessment included measurements of gross composition (*e.g.,* carcass composition, percent fat and protein) as well as detailed analyses of vitamins and minerals, fatty acid profiles, and protein characterization of meat and milk produced by clones. The composition of foods produced by clones was compared to the composition of foods produced by comparator animals, and also to published reference ranges for meat and milk. These comparisons formed the basis of our determination of whether meat or milk from clones differs materially from meat or milk from conventional animals, and thus contributed to the overall conclusions regarding food consumption risks.

Weight of evidence: Weight of evidence evaluations do not rely on a single study or even a subset of studies. Instead, they are based on expert judgments on all of the information gathered in the course of a risk assessment. This allows for variability in the amount of information on any particular aspect of the evaluation, as well as inconsistency in endpoints evaluated. Chapters IV, V, and VI contain detailed descriptions of studies that were considered relevant to the hazard identification and characterization, and subsequent risk evaluation. For each adverse outcome identified, the empirical evidence for the causal association of cloning with that outcome was weighed against the empirical evidence indicating that there were associations with other causal agents or processes.

D. The Implications of Epigenetic Reprogramming for Clones and their Progeny (Chapter IV)

Epigenetics has been defined as the study of stable alterations in gene expression potentials that arise during development and cell proliferation. In sexual reproduction, a new diploid genome is created by the fusion of two haploid genomes. The subsequent expression of that genome into a functional organism is governed by a "program." There are several examples of epigenetic control of gene expression, of which DNA methylation is likely the best characterized.

Mammalian embryos experience major epigenetic reprogramming primarily at two times in their development, both of which have significant implications for cloning. One of these takes place soon after fertilization, and is referred to as preimplantation reprogramming; the other occurs during gametogenesis (the development of cells that ultimately become the sperm and egg). Because preimplantation reprogramming occurs after fertilization, and in the case of nuclear transfer, after fusion of the donor nucleus with the oöplast, it is the most immediately affected by the cloning process, and may be most directly implicated in the development of clones with defects. Gametogenic reprogramming may also be involved in the abnormalities noted in clones, but it likely has more far-reaching implications for progeny, because it generates the gametes used for the sexual reproduction of clones.

The efficiency of producing clones (*i.e.,* the number of live offspring born compared to the number of embryos transferred) by SCNT is very low. The reasons for this low efficiency may be related to inappropriate epigenetic reprogramming. When cloning, the donor nucleus must be coaxed to direct embryonic development as if it were a fertilization-derived zygote. Most of the time, this is not successful. Anomalous epigenetic reprogramming is observed at the global genomic and individual gene level in clone embryos and fetuses, and in similar developmental stages of animals produced using ARTs with significant *in vitro* culturing components. Many of these are lethal, as demonstrated by the low success rate of IVF and the even lower success rate of SCNT. In the small number of successful cases that ultimately result in clones that appear normal and healthy, reprogramming in SCNT-derived embryos appears to be as successful as reprogramming in fertilization-derived embryos. Live and apparently healthy clones may exhibit some level of epigenetic differences relative to fertilization-derived animals, but these differences do not appear to have adverse effects on their well-being or ability to grow and develop normally.

The Center assumes that if clones were to pose food consumption risks, the only mechanism by which those risks could arise would be from inappropriate epigenetic reprogramming, similar to those observed for other ARTs. It is important to note that the genes that are being dysregulated are the "normal," naturally present genes that comprise the animal's genome, and have not been

introduced via recombinant DNA techniques from other sources (*i.e.,* clones are not transgenic or genetically engineered animals).

Inappropriate epigenetic reprogramming is not expected in the sexually reproduced progeny of animal clones at levels that exceed those observed in other ARTs or natural reproduction. Unlike their clone parent(s), the progeny of clones are produced by the union of male and female gametes. Production of these gametes *de novo* by the clone parents appears to reset any residual epigenetic reprogramming errors associated with nuclear transfer. Therefore, anomalies present in clones do not appear to be transmitted to the next generation, and the offspring that are produced are normal and healthy. Progeny of clones are thus not anticipated to pose any additional food safety concerns compared with other animals produced via sexual reproduction.

E. Risks to Animals Involved in Cloning (Chapter V)

To identify the potential hazards and assess any resulting risks to animals associated with cloning, Chapter V focuses on the health of clones at all five developmental nodes (pregnancy and parturition, perinatal, juvenile, reproductive, post-pubertal). Health risks to surrogate dams carrying clone fetuses are also considered, and the health outcomes of SCNT are compared with the outcomes of other ARTs. The overall conclusion of Chapter V is that animals involved in the cloning process (*i.e.,* cattle and sheep surrogate dams, and clones) are at increased risk of adverse health outcomes. The increased risks in cattle and sheep clones appear to be limited to the early stages of the life cycle. Although none of the adverse outcomes is unique to cloning, the incidence of these abnormalities observed in animals produced by SCNT is increased compared to animals produced by other ARTs.

Cows and ewes used as surrogate dams for SCNT-derived pregnancies are at increased risk of health problems during pregnancy and parturition. These problems include abnormal placental development and function and complications during late gestation such as hydrops (hydroallantois)[4] and dystocia (difficult birth) due to excessive fetal size. Overgrowth of the fetus and complications during late pregnancy are collectively referred to as large offspring syndrome (LOS). These conditions also occur with other ARTs that have a significant *in vitro* culturing component, but at a lower frequency. In contrast to cattle and sheep, surrogate swine and goat dams bearing clones do not appear to be at increased risk of complications during pregnancy.

Once clones are born, there are distinct differences between the species with respect to health

[4]The bovine fetus develops in a fluid-filled membrane called the amniotic sac. Surrounding the amniotic sac is a second fluid-filled membrane, the allantoic sac. Wastes from the fetus accumulate in the fluid contained in the allantoic sac. Hydroallantois, also referred to as hydrops, is excessive accumulation of fluid within the allantoic sac during pregnancy.

risks. In swine and goat clones, morbidity and mortality do not appear to be increased during the perinatal period. In calf and lamb clones, however, the incidence of both morbidity and mortality are increased during the perinatal period compared to calves and lambs produced using other ARTs. Clinical signs in perinatal clones associated with LOS include respiratory problems, prolonged recumbency,[5] enlarged umbilical cord, hyper/hypothermia, contracted flexor tendons, and symptoms associated with abnormal development of the major organs. Survival of these clones appears to be a function of both the severity of the clinical signs and appropriate post-natal management.

Similar to the perinatal period, the risk of morbidity and mortality in clones during the juvenile period varies among species. Compared with animals produced by natural service or ARTs, bovine clones continue to be at an increased risk of morbidity or mortality up to approximately six months of age. These risks appear to be sequellae of the abnormalities first noted in earlier stages of development that persist beyond the perinatal period. In contrast, swine and goat clones do not appear to be at increased risk of morbidity or mortality during the juvenile period. Swine and goat clones, as well as clone calves that are not adversely affected by congenital abnormalities, appear healthy throughout the juvenile period and exhibit normal patterns of growth and development.

As clones approach puberty, no increased risk of adverse health effects have been reported in any of the species evaluated. Clones of both sexes appear to have normal reproductive function, are fertile, and can produce normal offspring via sexual reproduction. Finally, the available information indicates that mature clones are normal and healthy, and there are no increased health risks at this developmental node relative to conventional animals.

Currently, it is not possible to draw any conclusions regarding the longevity of livestock clones or possible long-term health consequences associated with cloning due to the relatively short time that the technology has existed.

Sexually derived progeny of animal clones appear to be normal and healthy. As described in Chapter IV, any residual epigenetic reprogramming errors in clones are expected to be reset during gametogenesis, resulting in production of normal offspring by sexual reproduction. Consistent with these predictions, the data on the health status of clone progeny indicate that there is no increased risk of health problems in these animals compared with conventional animals.

[5] Respiratory problems and prolonged recumbency appear to be the most common problems associated with perinatal death in clone calves.

F. Food Consumption Risks (Chapter VI)

1. Two-Pronged Approach to Identifying and Characterizing Food Consumption Risks

In order to determine whether epigenetically-caused subtle hazards pose food consumption risks, CVM has developed a two-pronged approach. The first component, the *Critical Biological Systems Approach* (CBSA), incorporates a systematic review of the health of the animal clone or its progeny. Its role in the evaluation of food consumption risks is premised on the hypothesis that a healthy animal is likely to produce safe food products. It accepts that at this time, SCNT is a biologically imprecise and inefficient process, but recognizes that animals are capable of biological repair or adaptation. The cumulative nature of the CBSA allows for the incorporation of both favorable and unfavorable outcomes. The former, provided that all other measures appear to be normal, will result in the finding that the clone is likely to produce edible products that pose no food consumption risks; the latter implies that clones with anomalies are likely to be considered unsuitable for food. The second component, the *Compositional Analysis Method*, assumes that food products from healthy animal clones and their progeny that are not materially different from corresponding products from conventional animals pose no additional risks. It relies on the comparison of individual components of edible products, and the identification of appropriate comparators.

Assessing the safety of food products from animal clones and their progeny[6] is best accomplished by using both approaches: prospectively drawing on our knowledge of biological systems in development and maturation, and in retrograde, from an analysis of food products. Subtle hazards and potential risks that may be posed by animal clones must, however, be considered in the context of other mutations and epigenetic changes that occur in all food animal populations. No adverse outcomes have been noted in clones that have not also been observed in animals derived via other ARTs or natural mating that enter the food supply unimpeded.

2. Conclusions Regarding Potential Food Consumption Risks

Based on this review of the body of data on the health of animal clones, the composition of meat and milk from those animals and corresponding information on clone progeny, CVM has drawn the following conclusions:

a. Cattle Clones

[6] Although milk from clones might be marketed for human consumption, CVM anticipates that relatively few animal clones will enter the food supply as meat (*e.g.,* if culled from the herd due to injury or senescence). Relative to clones, it is more likely the progeny of clones will be used to produce meat and milk for human consumption.

Edible products from healthy juvenile bovine clones pose no additional risk(s) relative to corresponding products from contemporary conventional comparators.
The underlying biological assumption for this developmental node is that if anomalies were found in the youngest clones, the juvenile developmental node would be a period of equilibration and normalization as those animals proceeded toward adulthood. Animals experiencing severe developmental abnormalities are not expected to survive. The data are consistent with such a hypothesis.

Juvenile bovine clones that survive the perinatal period are largely healthy and normal. Although some younger clones in this developmental node may be more physiologically unstable than their conventional counterparts, most are able to equilibrate their physiological status and go on to exhibit normal patterns of growth and development. This normalization has been observed consistently in juvenile bovine clones except for those experiencing the sequellae of the developmental abnormalities present at birth. In some cases, these adverse outcomes can persist beyond the perinatal period, resulting in an increased risk to the health of these clones during the first six months of life. Animals bearing these problems are not expected to pass inspection and would not be allowed into the food supply, and therefore are not expected to contribute to food consumption risks. However, no additional subtle hazards that could pose food consumption risks were identified during the juvenile period, as demonstrated by the analysis of clinical chemistry and hematology data, demonstrating that healthy juvenile clones exhibit appropriate physiological responses to developmental signals.

Edible products derived from adult bovine clones pose no additional risk(s) relative to corresponding products from contemporary conventional comparators.
This conclusion is based on application of both prongs (CBSA and Compositional Analysis) of the risk assessment approach. The body of data comprising the CBSA approach is consistent with the biological prediction that there are no underlying biological reasons to suspect that healthy animal clones pose more of a food safety concern than conventional animals of similar age and species.

The data show that healthy adult clones are virtually indistinguishable from their comparators even at the level of clinical chemistry and hematology. These data also confirm the observation that physiological instabilities noted earlier in the lives of the clones are resolved in the juvenile developmental node (see previous conclusions regarding other developmental nodes), and do not reappear as the clones age. There are some reports of early deaths of clones; as these animals would be prohibited from entering the food supply, they do not pose a food consumption risk. Data on reproductive function in cows or bulls of this age cohort indicates that healthy bovine

clones surviving to reproductive maturity function normally and produce healthy offspring. These observations are consistent across studies. Given that reproduction is the most difficult "biological hurdle" placed on an organism, the observation of normal reproductive function provides an additional degree of confidence in the conclusion regarding the appropriate development of these animals.

All of the reports on the compositional analysis of meat or milk from bovine clones show that there are no biologically significant differences in the composition of milk derived from clone and non-clone cattle. Additionally, data from one report show no difference in allergenic potential for meat or milk derived from clone cattle compared to meat or milk from non-clone comparators, and neither meat nor milk from clone or non-clone cattle induced mutations *in vitro*. Finally, none of the reports identified an endpoint that would pose a hazard for human consumption.

b. Swine Clones

Edible products from adult swine clones pose no additional risk(s) relative to corresponding products from contemporary conventional comparators.

This conclusion is based on the same underlying biological assumption as cited for adult bovine clones. Because the data are more heavily weighted towards adult, market sized animals, judgments regarding the safety of food products from swine clones are provided in one aggregate set of comments.

Once piglet clones are born, they appear to be healthy. The most compelling argument for the normal health status of swine clones results from the evaluation of the behavior and physiological status of a small cohort of relatively young (15 weeks), and approximately market age (27 weeks) swine clones relative to closely related conventional pigs. No significant differences were observed in either behavior, epigenetic, or physiological measurements, indicating that these animals were not materially different from the comparators. Another small dataset on swine clones reared in very unusual settings (i.e., deprivation of colostrums, initial husbandry in pathogen-free conditions, switching to commercial settings) is confounded with respect to outcome. Nonetheless, these clones were able to respond appropriately to this stress, and their carcass characteristics, reproductive performance, including semen quality, farrowing rates and litter sizes were within normal reference ranges for conventional swine. No biologically relevant differences were observed in the composition of meat from these clones or their comparators.

c. Sheep Clones

Except by relying on underlying biological assumptions, and by inference from other species, there is insufficient information on the health status of sheep clones to draw conclusions with respect to potential risks that could be posed from the consumption of food products.
With the exception of reports on Dolly, CVM was unable to find any publicly available reports on the health status of live sheep clones. There are several studies addressing methodological issues for optimizing the generation of clones, but these do not address post-natal health. There are reports of anomalies noted in fetal sheep clones that have died or been terminated, and reports on the pathology associated with animals that do not survive. Although these are instructive for understanding the molecular and developmental pathways that may be perturbed during the process of SCNT, these studies have limited relevance to addressing food safety because the deceased animals would not have been allowed to enter the food supply. CVM was not able to find any reports on the composition of milk or meat from sheep clones.

d. Goat Clones

Edible products from goat clones pose no additional food consumption risk(s) relative to corresponding products from contemporary conventional comparators.
This conclusion is based on the same underlying biological assumption cited for the other livestock species, and a relatively small but compelling dataset. Once clone embryos are transferred to surrogate dams and pregnancies are confirmed, the "success rate" for live births is quite high. The animals appear to develop normally through reproductive age, and the available data indicate their physiological responses are appropriate for age and breed. The reproductive development and function of male Nigerian Dwarf goat clones demonstrate that those animals functioned appropriately relative to age- and breed-matched comparators. One male progeny goat was derived from the buck clones; this animal also appeared to function in an age- and breed-appropriate manner. No meat or milk composition data were identified for goat clones.

e. Clone Progeny

Edible products derived from the progeny of clones pose no additional food consumption risk(s) relative to corresponding products from other animals.
Relative to the amounts of meat and milk derived directly from clones in the U.S., it is likely that more edible products (both meat and dairy) will be produced by the progeny of clones. These progeny, unlike their clone parents, are produced by normal sexual reproduction. The underlying biological assumption for health of progeny animals (explained in Chapter IV) is that passage through the process of creating the cells that ultimately become ova and sperm naturally resets epigenetic signals for gene expression, and effectively "clears" the genome of incomplete or

inappropriate signals. This assumption has been supported by empirical[7] evidence in the mouse model system, which clearly indicates that phenotypic alterations noted in the parent clones are not passed to their sexually-derived progeny. Detailed observations of the progeny of bovine and swine clones demonstrate that these progeny are born healthy, develop normally, and do not exhibit any of the anomalies observed in clones. One extensive dataset on the progeny of swine clones providing direct data on the composition of their meat indicates that these animals are essentially indistinguishable from the comparable progeny of non-clone animals. These empirical data, together with our underlying biological assumption, support the conclusion that edible products from clone progeny pose no additional food consumption risk(s) relative to edible products from any other sexually reproduced animals.

We therefore concur with the high degree of confidence that the outside scientific community (NAS 2002 a,b) places in the underlying biological assumption, and conclude that consumption of edible products from clone progeny would not pose any additional food consumption risk(s) relative to consumption of similar products from sexually-derived animals.

G. Concluding Statements (Chapter VII)

For Animal Health: SCNT results in an increased frequency of health risks to animals involved in the cloning process, but these do not differ qualitatively from those observed in other ARTs or natural breeding. At this time, the overall efficiency of SCNT is low. Cattle and sheep exhibit a set of clinical signs collectively referred to as LOS that do not appear to be present in swine or goats. Surrogate dams are at risk of complications from birth if the fetus suffers from LOS, or from accumulation of fluid in the cavities of the placenta (hydrops). Risks to clones associated with LOS include increased incidence of fetal and neonatal death, and abnormalities that may require additional supportive care during the perinatal period. Clones affected by LOS can recover and mature into normal, healthy animals, but many succumb to complications of LOS during the juvenile period. The risk of morbidity and mortality appears to decrease with age, and after approximately six months of age most bovine clones are normal and healthy as determined by physiological measurements, behavior, and veterinary examinations. Progeny of animal clones also have been reported as normal and healthy.

For Food Consumption Risks: Extensive evaluation of the available data has not identified any subtle hazards that might indicate food consumption risks in healthy clones of cattle, swine, or goats. Thus, edible products from healthy clones that meet existing requirements for meat and milk in commerce pose no increased food consumption risk(s) relative to comparable products

[7] Empirical refers to that which can be seen or observed alone, often without reliance on theory. In the context of this risk assessment, conclusions drawn on empirical evidence are those that are drawn strictly based on the data. These conclusions may later be put in the context of underlying biological assumptions.

from sexually-derived animals. The uncertainties associated with this judgment are a function of the empirical observations and underlying biological processes contributing to the production of clones. There is less uncertainty about the health of clones as they age and have more time to exhibit the full range of functionality expected of breeding stock.

Edible products derived from the progeny of clones pose no additional food consumption risk(s) relative to corresponding products from other animals based on underlying biological assumptions, evidence from model systems, and consistent empirical observations.

The results of this comprehensive risk assessment agree with the preliminary findings of the NAS (2002a) conclusions that "The products of offspring of clone[s] … were regarded as posing no food safety concern because they are the result of natural matings," and "In summary there is no current evidence that food products derived from adult somatic cell clones or their progeny present a food safety concern."

Chapter II:
Technology Overview: Somatic Cell Nuclear Transfer and Other Assisted Reproductive Technologies

Chapter II:
Technology Overview: Somatic Cell Nuclear Transfer and Other Assisted Reproductive Technologies

Since the beginnings of livestock agriculture, selection criteria have been applied to foster the propagation of animals with traits more desirable to humans. The expansion of herds with desirable traits has been limited, however, by the reproductive capacity of the species or breed and the prevalence of particular versions of genes (or sets of genes) responsible for those traits in the available gene pool. (The gene pool can be considered all of the animals available for breeding.) The female contribution to reproductive success, for example, is limited by species-specific characteristics such as average litter size, frequency of estrus, and gestation length. In natural breeding, male contributions are restricted by the degree of proximity to fertile females and the ability to inseminate females with a sufficient number of normal sperm. Finally, individuals of both sexes are limited to the extent that they may carry the desired versions of genes or combination of genes.

To help overcome some of these complications, various forms of assisted reproductive technologies (ARTs) have been adopted in animal agriculture for over a century, and at least one (artificial insemination) has been used for several hundred years. These technologies form a continuum that ranges from the fairly minimal assistance provided to animals engaged in natural service through those containing components of significant *in vitro* manipulation such as *in vitro* fertilization and embryo splitting, to the more recent development of somatic cell nuclear transfer (SCNT), or what is colloquially referred to as "cloning"[8] (Faber et al. 2004; Sakai 2005; Oback and Wells 2007b). Beginning with the development and application of modern artificial insemination (AI) methodologies in the first half of the 20th century, ARTs have aided in the genetic improvement of domestic species, including selection of phenotypes such as behavioral and production traits in domesticated animals (Youngquist 1997, Faber and Ferre 2004). By accelerating the rate at which selective breeding goals can be met, improved genotypes have expanded rapidly into national herds in the United States and other countries (Faber et al. 2004; Wells 2005). In turn, this has resulted in lower costs for livestock producers and retail consumers, while simultaneously maintaining or improving the quality and consistency of foods of animal origin.

[8] The term "clone" originated before the late 1990s. The British biologist J.B.S. Haldane, in a speech entitled "Biological Possibilities for the Human Species of the Next Ten-Thousand Years," used the term in 1963. The Merriam-Webster dictionary, however, dates its use in a biological context to 1903.

Reproductive technology advances have also proven to be powerful tools in curbing the spread of vertically transmitted diseases (i.e., those that are passed from the dam to her offspring during the period immediately before and after birth, either across the placenta or in the dam's milk) (Youngquist 1997). For example, embryo transfer (ET) (see subsequent discussion for a description of this ART) has been used to prevent vertical transmission of *Neospora caninum* in cattle (Baillargeon et al. 2001; Landmann et al. 2002), scrapie in sheep (Wang et al. 2001), Bovine Virus Diarrhea (BVD) in cattle (Smith and Grimmer 2000), and *Brucella abortus* in an American bison (Robison et al. 1998). Embryo transfer is commonly used in laboratory animal research to re-derive valuable strains of gnotobiotic (i.e., animals in which all of the bacterial species are known) or specific pathogen-free research animals when colonies become infected with undesirable disease agents that cannot be controlled through more conservative means.

The following chapter begins with a brief overview of what cloning is, followed by an overview of the continuum of other ARTs commonly in use in current US agricultural practice, placing nuclear transfer technology into context of these breeding practices. Appendix B provides additional details on overall reproductive efficiency observed in current agricultural practice in the US, and Appendix C provides a comprehensive summary of the outcomes observed in ARTs, with particular emphasis on those technologies that contain a significant *in vitro* culturing component. Although all of these technologies are currently in practice, all are continually undergoing development and refinements with the goal of improving efficiencies. A reasonable expectation then, is that success rates (defined as the rate of production of healthy animals) will improve as expertise increases.

A. What is Cloning?

Cloning, or somatic cell nuclear transfer, is a process by which animals are reproduced asexually (embryo splitting and blastomere nuclear transfer are other ways of reproducing animals asexually and are discussed later in this chapter). In cloning, a differentiated somatic cell (a non-germ line cell from an existing animal) is introduced to an oöcyte (a cell that is the immediate precursor of a mature egg) that has had its nucleus (and thus its genome) removed, and then, following some manipulations, is induced to start replicating. If all goes well, the dividing cell is implanted into a female animal (dam), continues to develop normally, and is delivered just as any newborn.

Since the first report of a clone produced by SCNT (Wilmut et al. 1997), several other species have been cloned (Table II-1), although in some cases (e.g., companion animals) only a limited

number of animals have been generated. The reasons for this are multi-fold, but are largely driven by the relative difficulty in producing clones, and the various drivers, economic and technical, that affect the expansion of the technology. For example, the use of clones in expanding elite breeding stock in domestic livestock is perceived to have benefit for breeders and consumers. This risk assessment does not attempt to address those issues, however, and instead concentrates on those domestic livestock clones commonly consumed as food (e.g., cattle, swine, sheep, and goats).

Table II-1: Species of Animals that Have Been Cloned Using SCNT (Resulting in Live Birth)

Species	First Citation
Sheep	Wilmut et al. (1997)
Mouse	Wakayama et al. (1998)
Gaur	Lanza et al (2000)
Pig	Polejaeva et al. (2000)
Mouflon Sheep	Loi et al. (2001)
Cat	Shin et al. (2002)
Cow	Forsberg et al. (2002)
Goat	Keefer et al. (2002)
Rabbit	Chesne et al. (2002)
Deer	Texas A&M announcement (2003)
Horse	Galli et al. (2003a)
Mule	Woods et al. (2003)
Rat	Zhou et al. (2003)
Wildcat	Gomez et al. (2003)
Dog	Lee et al. (2005)
Banteng	Sansinena et al. (2005)
Ferret	Li Z et al. (2006b)
Swamp Buffalo	Suteevun et al. (2006)
Gray Wolf	Kim et al. (2007)

B. Continuum of Reproductive Technologies

1. Natural Service

Although many people who are not involved in intensive animal agriculture assume that most breeding occurs "naturally" [9] (*e.g.,* a male animal mates with receptive female), in fact, human intervention is the industry standard for many livestock operations (Youngquist 1997). In the US

[9] The process of non-assisted mating is referred to as natural "mating," "coverage" or "service."

dairy industry, for example, most reproduction involves some technological component, and swine producers rarely use natural mating for their production of offspring. Conversely, in the beef industry most reproduction occurs by natural service, and most of the world's sheep and goat production occurs under free range conditions and depends on natural mating.

Humans have assisted animals in natural mating by monitoring the reproductive status of females, introducing receptive females to the same location (*e.g.,* field, corral, or pen) as the male, and allowing nature to take its course. When this process does not result in sufficient offspring of the desired phenotype, or is otherwise compromised, assisted reproductive technologies can be called into play.

2. Artificial Insemination and Synchronized Estrus

The first ART developed was artificial insemination (AI), which in its simplest form involves the collection of semen from males and its subsequent human-assisted introduction into a physiologically receptive female. It is an important technique for the genetic improvement of animals, as a few select males can produce sufficient sperm to inseminate thousands of females per year, while natural service would provide for the insemination of only a fraction of those animals.

Reports of AI in horses as part of breeding programs have been traced to the Arabian Peninsula in the 14th century (Bearden and Fuquay 2000). AI of a beagle dog was first described by Spallanzani in 1780 (Hafez and Hafez 2000). In 1899, the Russian Czar Nicholas II commissioned I.I. Ivanov to develop an AI program for horses, and by 1933 Ivanov had developed methods for collecting semen and inseminating horses, cows, sheep, and pigs (Foote 2001). In 1931, 19,800 cows were bred by AI in Russia. By 1936, Denmark had established an AI cooperative association, and by 1939, the use of AI had spread to the United States. In 1970, it was estimated that 7,344,420 dairy cows were bred using AI (Webb 2003).

Although there are several methods for collecting semen, most involve training males to ejaculate into an artificial vagina. Semen is then diluted to maximize the number of services that one male can provide. A normal ejaculate from a dairy bull usually contains between 5 and 10 billion sperm; good conception rates generally require about 12-20 million sperm to be introduced. The diluting solution contains factors that help to stabilize and preserve the sperm, as well as antibiotics to inhibit bacterial growth and reduce the danger of spreading any potential disease or contamination. Most collected semen is stored in glass ampoules or plastic straws, and is generally stored either in dry ice and alcohol (-100^{o}F) or liquid nitrogen (-320^{o}F). To date, there appears to be no limit on the amount of time that bovine sperm can remain frozen and regain viability upon appropriate thawing. Since 1997, use of AI in swine breeding has increased

dramatically. A survey of swine producers conducted by the National Pork Board in 2003 indicated that even among small producers (1,000 to 3,000 swine marketed annually) as many as 60 percent of litters were sired by AI in 2003, while for large producers (> 50,000 swine marketed annually) 98 to 100 percent of litters were sired by AI.[10] Rams (male sheep) and bucks (male goats) can also be donors for artificial insemination.

In the US, AI of the female is usually performed either by trained technicians employed by breeding companies or large farms or by the producers themselves. The most common technique employed today for dairy cows involves the use of sterile, disposable catheters that are inserted vaginally and extended through the cervix into the body of the uterus of the recipient cow (whose estrous cycle has been documented). Thawed semen is warmed to the appropriate temperature, and sperm are deposited in the uterine/cervical regions.

The primary advantages of AI to farmers include the ability to use semen from bulls anywhere in the world rather than those that are more geographically proximate, and thus to have desirable genetics available for propagation. It also allows the farmer to use multiple sires in a herd without the attendant costs of maintaining animals that are often difficult to handle and in multiple breeding pastures. AI tends to be less expensive than natural service (a straw of semen generally costs less than transporting a female to the sire and the stud fee) and avoids the potential physical risks to either sire or dam as part of the mating process. The disadvantages of AI include the need to train personnel engaged in the breeding operations on how to detect estrus in females (see subsequent discussion of estrous synchronization), and training or retaining individuals to perform the insemination. Further, care needs to be taken not to rely excessively on a few apparently superior sires so as not to reduce the genetic diversity of the resulting herds.

Sperm collection and AI were further improved by the advent of sperm sexing, or selection of sperm carrying an X (female) or Y (male) chromosome.[11] Development of an effective and simple method for producing animals of the desired sex is economically desirable for livestock producers; sperm sexing is currently being used when available and economically feasible (Foote 2001; Faber and Ferre 2004). For example, in the dairy industry, females are desired because males do not produce milk; and excess males often become veal. In the beef industry, however, males are desired because they grow faster. Females can be the desired sex in the swine industry where leaner animals generally receive higher prices; young female pigs (gilts) tend to be leaner than castrated male pigs (barrows) when they arrive at market.

[10] http://www.pork.org/Producers/EconomicsMarketInfo/Production%20and%20Marketing2003.doc

[11] In normal mammalian sexual reproduction, the female always donates an "X" chromosome, and the male can donate either an "X" or a "Y" chromosome. XX yields a female animal; XY produces a male.

One method that shows the most promise for predetermining the sex of offspring is sexing semen using flow cytometry. This technique is based on the observation that in livestock species, sperm with X chromosomes have about 3 percent more DNA than those with Y chromosomes. Collected semen is diluted, and single sperm are passed through a laser beam that allows for the determination of the amount of DNA in each individual sperm. Based on their relative DNA content, sperm are sorted into "heavier" (female producing) and "lighter" (male producing) fractions. Another method sexes early embryos by removing one or two of the cells from the early embryo, arresting the further growth of the embryo by freezing, and identifying genes found only on the Y chromosome using the polymerase chain reaction (PCR) in the selected cells (Youngquist 1997). Semen sexing is more rapid, less invasive, and more economical, while embryo sexing is impractical at this time, as it is invasive, time intensive, and quite expensive. Further, the potential to damage the embryo by piercing the protective layer around it (*zona pellucida*), removing cells, and freezing the remaining cells in the embryo is quite high.

Estrous synchronization, or the timed induction of estrus (commonly referred to as heat), further permits the efficient use of artificial insemination (Hafez and Hafez 2000). Estrous synchronization was first practiced in the US in the 1960s and allows for the insemination of large groups of animals in a specified window of time when the female is capable of conceiving a pregnancy. The alternative to estrous synchronization is the time- and labor-intensive method of observing females' behavior to detect estrus, and then arranging insemination at the appropriate time on an animal-by-animal basis. Estrous synchronization is achieved by treating females with hormones (Hafez and Hafez 2000). These synchronization regimens mimic the natural hormonal patterns leading to estrus and ovulation, and allow the producer to control the timing of these patterns. Several FDA-approved hormones are available for estrous synchronization, such as progesterone and prostaglandin F2α.[12] These hormones are commonly used for conventional breeding in cattle throughout the U.S. According to a recent survey of large U.S. commercial dairies, 87 percent of herds use hormonal estrous synchronization methods (Caraviello et al. 2006). The advantage of linking AI to estrous synchronization lays in the ability of contained agricultural practices to operate on a more predictable schedule. For example, cattle breeders can avoid the reduced conception rates that occur during summer's heat by breeding animals during the cooler spring season. Predictability can benefit farmers by allowing them to allocate resources (*e.g.,* farm labor, veterinary visits) more efficiently, thus lowering production costs.

[12] See http://www.fda.gov/cvm/Green_Book/greenbook.html. During the natural estrous cycle, the ovaries produce progesterone. If pregnancy does not occur, the uterus secretes prostaglandin F2α which causes progesterone levels to decrease and initiates a series of events that culminate in estrus and ovulation. Estrous synchronization methods use exogenous progesterone and/or prostaglandin F2α to mimic the natural secretory pattern of these hormones, and thus control the timing of estrus and ovulation.

3. Embryo Transfer

It is impossible for a fertile female mammal to bear all of her potential offspring. Litter size, gestation time, and post-partum decreases in fertility all limit the potential number of progeny that she can produce. When the female animal reaches the end of her reproductive period, any remaining unfertilized eggs represent potential offspring that have been lost. One solution to this dilemma is to transfer embryos of genetically superior female animals to multiple surrogate dams. This technique, called "embryo transfer," is particularly useful in species in which a low number of progeny are produced per gestation.[13] In concept, then, embryo transfer (ET) is analogous to AI in that the total yield of offspring from a genetically superior, in this case, female animal can be increased (Youngquist 1997).

In 1890, rabbit embryos were first transferred from a donor female to surrogate rabbits. The experiment demonstrated that the surrogate's genetics would not influence the transferred embryo's genetics or development. In 1951, a successful live bovine ET was accomplished, but non-surgical methods of embryo collection did not succeed until the late 1960s (Hafez and Hafez 2000).

Currently, it is possible to flush large numbers of viable embryos from a superovulated cow with minimal stress to the animal (Hafez and Hafez 2000). Superovulation of the donor animal is generally accomplished by injecting the animal with follicle stimulating hormone or other exogenous gonadotropins before she enters estrus. The hormones induce production of a large quantity of ovarian follicles containing mature, preovulatory oöcytes. Insemination is performed at appropriate times relative to ovulation depending on the species and breed. Using standard synchronization methods, the estrous cycle in recipient surrogate mothers is synchronized in parallel with the cycle of the donor to be ready to accept embryos for implantation and gestation. When embryos are about a week old, they may be flushed out of the donor dam's uterus non-surgically[14] by guiding a catheter through the cervix and into the uterus. Saline solution is injected into the uterus via the catheter and then flushed, along with the embryos, back out of catheter into a collection vessel. Embryos are isolated from the flushing solution, and examined microscopically to determine whether they are of sufficiently quality to implant. If they meet the criteria for further use, embryos can be transferred immediately to a waiting synchronized recipient animal using the same non-surgical technique that was used to collect the embryos. Alternatively, embryos can be frozen for later use or split into halves (see embryo splitting

[13] Cattle usually produce one calf, with a twinning rate of approximately 1-4 percent depending on breed; sheep and goats generally produce one or two offspring, with an occasional triplet delivery. Swine, on the other hand, usually bear multiple piglets in a litter, and require multiple fetuses to maintain the pregnancy.

[14] Non-surgical techniques for collection of embryos are preferable to minimize risk to the health of the donor. In cattle, the transcervical (non-surgical) method is used routinely to collect and transfer embryos. In sheep, goats, and pigs, embryos are typically collected and transferred either transcervically or by laparoscopy, which involves insertion of two thin, lighted tubes into the abdominal cavity through small incisions (Hafez and Hafez, 2000).

discussion below). Fresh or thawed embryos are inserted into surrogate mothers, where they attach to the lining of the uterus. Following transfer of embryos, no additional hormones or special treatments are required and the pregnancy is allowed to progress normally.

This technique, referred to as MOET (multiple ovulation and embryo transfer), is often used in relatively intensive cattle breeding programs, but is less developed in other livestock species. Similar to fertilizing many females with sperm from one superior male, MOET provides the breeder the ability to expand genetic traits exhibited in superior females. Further, the ability to freeze embryos allows for the preservation of "genetic stock" to be used at a later time. Its prevalence in livestock breeding, however, is much lower than AI, as it is considerably more expensive (Wilmut et al. 2002).

The International Embryo Transfer Society (IETS), a professional society whose membership includes breeders and researchers, estimates that a total of approximately 612,178 *in vivo* derived bovine embryos were transferred worldwide in 2005 (Thibier 2006). This is an increase of approximately 62,000 embryos from the previous year. Most of those transfers occurred in North America (45.2 percent), with the rest taking place in South America (20.5 percent), Asia (18.9 percent), and Europe (~14 percent).

4. *In vitro* Fertilization

The first *in vitro* fertilized (IVF) offspring was a rabbit born in 1959 (Chang 1959a). Since that time, IVF offspring have been born to mice, rats, hamsters, cats, guinea pigs, squirrels, pigs, cows, monkeys, and humans (Bearden and Fuquay 2000). IVF allows for the production of offspring from animals where other ART methods fail due to difficulties with either the female (blocked oviducts, non-responsive ovaries) or male (marginal semen quality and/or quantity), or where disease is present. In cattle, it is also used for the production of embryos from sexed semen because of the low sperm counts resulting from current sexing protocols, and for the further extension of the semen of superior sires due to the relatively low level of sperm required for *in vitro* fertilization. (IVF procedures are also used to assist human couples with limited fertility.)

The overall technique for IVF is similar among species, and involves significant manipulations *in vitro*, or outside the body of animals. In livestock species, oöcytes are collected from the ovaries of either living or deceased animals whose genetic potential is desirable (Goodhand et al. 1999). Ovaries can be obtained by transvaginal aspiration from live animals, or from a deceased animal at time of slaughter. Slaughterhouse ovaries are cross-sectioned and the contents of all of the follicles are collected; mature oöcytes are collected, evaluated for quality, and used for fertilization. Immature oöcytes must be allowed to continue to develop in a maturation medium.

Either fresh or frozen-thawed semen can be used for fertilization. Sperm need to be capacitated *in vitro* in order to penetrate the zona pellucida and fuse with the ovum or to undergo the same maturation process that they would normally undergo in the female reproductive tract. Capacitation involves a series of cellular changes to the sperm including increased motility, calcium uptake and protein binding (binding to proteins produced by the female reproductive tract). *In vitro* capacitation is accomplished by creating a medium designed to simulate the female reproductive tract and allowing the sperm to incubate in it for a period of time. Sperm are then added to ova, incubated in culture medium for approximately 8-22 hours, and the resulting fertilized ova, called zygotes, are washed, examined for appropriate development, and allowed to continue to divide for up to seven days, again in culture. At that time, if embryos appear normal, they may either be frozen for future use or inserted into the uterus of a reproductively competent female.

The IETS reported that the number of *in vitro* embryos transferred in 2005 increased by 11 percent over 2004 numbers to more than 265,000. Almost all of these transfers were performed in South America (particularly in Brazil) and Asia (especially Korea and China).

The numbers of *in vivo* and *in vitro* embryo transfers for other species (sheep and goats) were considerably lower, with 34,458 sheep embryos transferred, mostly in Australia, New Zealand and South Africa, and approximately 5,000 goat embryos transferred, mostly in South Africa and Asia. According to IETS statistics, approximately 66,000 swine embryos were transferred in 2005 (a huge increase over the previous year's number of 16,016), with almost all occurring in Vietnam and Korea (Thibier 2006).

5. Embryo splitting

Genetically identical individuals derived from a sole embryonic source can arise naturally, as in the case of spontaneous monozygotic twinning, or *in vitro* via the manual separation (splitting) of early stage embryos. Embryo splitting may be considered the first true "cloning" procedure involving human intervention, and was first described by Willadsen and Polge in 1981, when monozygotic twin calves were produced.

Embryo splitting, or the mechanical separation of cells,[15] can be used in very early embryos. Briefly, two-cell embryos derived from either *in vitro* fertilization, or embryo rescue following *in*

[15] Common nomenclature for the early stages of development following fertilization include the **zygote**, which includes the fertilized egg contained in the ***zona pellucida***, through about the 8 cell stage of development (3 days in the cow, and 3-4 days in the sow). The ***morula*** refers to the time period (between about 4-7 days in cows, and 4-5 days in sows) following fertilization in which cells continue to divide within the *zona pellucida*, but there is

vivo fertilization (as described for embryo transfer) are held in place with micropipettes under a microscope. The *zona pellucida* (the clear layer of protein surrounding the oöcyte and fertilized ovum) of these embryos is opened, and the two-celled embryo is then split into individual cells with a finely drawn needle or pipette. One of the cells is left in the original *zona pellucida* and the other is either placed into an empty *zona pellucida* or allowed to develop without a *zona pellucida*. These so-called demi-embryos can be cultured *in vitro* for a few days, inspected for appropriate growth and then transferred directly to synchronized recipient dams or frozen for future use. Similar procedures can be used to multiply embryos that have developed beyond the 2-cell stage (Willadsen 1980). Each of the individual cells or blastomeres from a single early embryo is totipotent. That is, each cell retains the ability to generate a fully functional individual identical to the other individual(s) derived from the other cells of the original embryo.

Although commercial applications of embryo splitting have been tracked by breeders' associations, the technology has never gained significant market penetration for several reasons. It is a very expensive and time consuming procedure that has not provided the yield initially anticipated for the technology. For example, actual calf yield from blastomere splits is approximately 105 calves per 100 embryos, while direct transfer of intact embryos yields approximately 60 calves per 100 embryos (Wilmut et al. 2000). Unless embryos are sexed at the time of splitting, however, breeders may end up with half of their animals being of the undesired gender, thus incurring twice the cost for the desired offspring. In addition, even if the resulting calves are of the desired gender, their production potential is not known, making the procedure an expensive gamble.

6. Blastomere Nuclear Transfer

The next evolution of ART evolved from additional manipulations of the blastomere cell, and involved its fusion with an enucleated oöcyte. This method expands on the relatively simple early stage embryo cell separation procedure described previously by allowing the use of cells from later stage embryos. In this case, embryos of the eight to sixteen-cell stage, compact morulae, and the inner cell mass from blastocysts can be used as donor nuclei (First and Prather 1991a). Fusion of these later stage blastomere cells, which have lost their totipotency, with enucleated oöcytes, reprograms the blastomere nuclei to allow them to develop as zygotes. Blastomeres from bovine embryos up to the 64-cell stage can be fused with enucleated freshly

---

no discernable migration of cells into any particular region. At about 7-12 days in cattle and 5 days in swine, a group of cells migrates to a portion of the spherical mass, forming an ***inner cell mass***, with the remainder forming a ring of cells around a central hollow core (blastocoele). This is referred to as the ***blastocyst***. The inner cell mass continues to develop into most of the body mass that will constitute the fetus and the ring of cells around the perimeter, which is referred to as the ***trophoblast***, will eventually make up the placenta.

fertilized oöcytes and cultured to develop into genetically identical individuals (Keefer et al. 1994). Cell nuclei derived from the inner cell mass of expanded blastocysts transferred into enucleated host cells are also capable of development resulting in offspring (Sims and First 1994).

This technology, which may be considered the true antecedent of somatic cell nuclear transfer, had limited commercial applicability for the same reasons as embryo splitting: high cost, high loss rate, and the inability to predict phenotypic performance or the gender of the resulting offspring.

7. Somatic Cell Nuclear Transfer (SCNT)

In 1962, biologist John Gurdon of Oxford University pioneered the method of the two step "nuclear transfer" process in frogs: the enucleation of a recipient oöcyte and the subsequent transfer of a differentiated somatic cell nucleus to that oöcyte. Gurdon's experiments showed that despite the differentiated status of the donor nucleus, reconstituted cells appeared to reprogram, or dedifferentiate, the nucleus and enable it to function much as a naturally produced zygote. These zygotes successfully developed into viable embryos that hatched and grew into tadpoles. Because the tadpoles had all come from the gut cells of the same adult frog, they all had the same genetic material and thus were all clones. However, Gurdon's nuclear transfer tadpoles clones failed to metamorphose into frogs. When scientists attempted to apply this technology to other species such as mice, cattle, or other mammals, the developmental program could not be reset (Gurdon and Uehlinger 1966; Byrne et al. 2002).

Scientists continued to tackle the problem and in 1986, Randall Prather and colleagues, then working in Neal First's laboratory at the University of Wisconsin-Madison, cloned a cow from early embryonic cells using nuclear transfer (Prather et al. 1987). Although this was an example of blastomere nuclear transfer, it effectively set the stage for Dolly's birth a decade later, on July 5, 1996. Dolly the sheep, the first organism ever to be cloned from adult cells, was created by Ian Wilmut and Keith Campbell using a technique similar to that used to create the first sheep from differentiated embryo cells (*i.e.,* a blastomere clone) in 1995 (Wilmut et al. 1997).[16]

In July 1998, Ryuzo Yanagimachi, Toni Perry, and Teruhiko Wakayama of the University of Hawaii announced that they had cloned fifty mice from adult cells using the "Honolulu

[16] Blastomere clones differ from those produced by somatic cell nuclear transfer because they arise from cells taken from early embryos. In theory, these cells are less differentiated than somatic cells, and the expectation is that the dedifferentiation process would be less onerous than for cells that have terminally differentiated (*e.g.,* ear skin cells or kidney cells). Blastomere clones are also less attractive for animal agriculture in that their phenotypes have not been proven; that is, no actual animal exists whose characteristics can be evaluated and chosen for propagation. See Section C of this Chapter for further discussion.

technique" (Wakayama et al. 1998). This was particularly significant because mouse embryos begin to divide almost immediately after the ovum is fertilized, and scientists had believed that this would not allow sufficient time for reprogramming to occur. Sheep, on the other hand, because their ova do not divide for several hours after fertilization, were thought to be an "easier" species to clone, as the natural delay between fertilization and division might be replicated in SCNT, possibly giving the oöcyte time to reprogram its new nucleus.

SCNT is a relatively new technology described by many as complex, technically demanding and inefficient, that continues to be developed and improved. As such, there is no set "method" that is universally employed, although the basic steps outlined below are common to most SCNT procedures at the time that this overview was written.

a. Donor cell

For species in which the cloning process has been relatively well developed, the first step is identifying the animal to use as a nuclear donor. Animals to be used for breeding purposes are selected because they have been shown to be genetically superior to herd mates for the trait(s) to be propagated. Somatic cells can be collected from the ear (hole punch) or skin (surgical incision or needle aspiration), although many other cell sources have been used. Multiple factors may influence success or failure of the nuclear transfer process. Coordination of the cell-cycle stage of the donor nucleus and the recipient egg cytoplasm appears to be important for successful development of embryos. In general, the selection of a cell type for commercial cloning from an adult animal has evolved to choosing a collection method that is relatively noninvasive and minimizes stress to the live animal donor.

Several characteristics have been identified as contributors to the degree to which any given donor cell or type of cell will likely result in a successful cloning event (Sung et al. 2006; Oback and Wells 2007a). One example is the "replicative state" of the donor cell. In general, cells in culture accumulate nutrients, grow, and when they reach certain conditions, divide. Cells that adhere to a solid substrate, such as the bottom of a tissue culture dish tend to grow until there are so many of them that they begin to touch each other. Once that happens, they generally stop dividing, and go into a "resting state" with respect to replication (referred to as G0). Cells can also be directed into G0 by depleting the nutrients in their growth medium. Some laboratories have concluded that cells in G0 are the most effective donors (Wilmut and Campbell 1998, De Sousa et al. 2002). Conversely, other laboratories have found that actively dividing cells make good donors (Cibelli et al. 1998a, Lanza et al. 2001). Some laboratories find that cells from embryos or fetuses are the best donors (Batchelder 2005), while others are successful at cloning cells from aged or even deceased animals (Hill et al. 2000a, Tian et al. 2001). Another

characteristic that has been shown to influence the degree to which cells make good donors is how "inbred" the donor animal is (Rideout et al. 2000). These researchers have determined that "hybrid vigor" is important for the success rate of animal cloning and the more inbred the donor animal, the less likely it is that cloning will occur successfully. Further, some species appear to be more amenable to cloning than others (*e.g.,* goats compared with cattle, see Chapter V), and some species have not been cloned at all. At this time, the best conclusion that can be drawn with respect to the degree to which a cell (or animal) will serve as a "good" donor is that the technology is not sufficiently mature to predict with certainty which set of conditions will optimize cloning efficiency.

Once a cell has been isolated from culture, depending on the laboratory, either the entire cell or just its nucleus is transferred under the *zona pellucida* of the enucleated oöcyte using a very thin glass micropipette (Solter 2000) to await fusion.

b. Oöcyte

The cell type used as the recipient for the donor cell to be cloned is the mature oöcyte, the version of the ovum that participates in fertilization during sexual reproduction. The oöcyte contains all of the non-nuclear cellular components required for the early development of an embryo. Oöcytes can be obtained from ovaries collected at slaughterhouses or from live animals using aspiration techniques (see previous discussion of *in vitro* fertilization). Because the oöcyte donates only its cytoplasm (the oöplast), it must be enucleated prior to fusion with the donor. The nucleus is generally removed by microaspiration, using a finely honed needle (PIFB 2003[17]).

c. Fusion

In order to begin the development process, the membranes separating the oöplast and the donor nucleus (or cell) must be fused. This can be accomplished in two ways: (1) by the administration of a brief electrical pulse, or (2) chemical fusion. Electrical stimulation appears to be the more commonly used technique and involves the application of one to several microbursts of a mild electrical current in the vicinity of the cells. This induces the formation of pores between the somatic donor cell and oöplast which functionally makes the two cells one. This process also stimulates embryonic development,[18] which if successful, results in the development of blastocysts that are transplanted into surrogate mothers (Cervera et al. 2002).

[17] http://pewagbiotech.org/events/0924/proceedings2.pdf

[18] This step is also sometimes referred to as activation. During sexual reproduction, physiological activation of the oöcyte is stimulated by fertilization. Since sperm-mediated fertilization is subverted in SCNT, activation must be stimulated artificially *in vitro* by electrical current; addition of chemicals that increase intracellular concentrations of calcium (calcium ionophores) or inhibit protein synthesis; or a combination of electrical and chemical stimuli.

Technical modifications aimed at increasing the success rate of cloning by improving the efficiency of the enucleation and fusion approaches are steadily evolving. For example, Oback et al. (2003) have developed a method that removes the *zona pellucida* from the oöcyte, aligns the donor cells with enucleated oöplasts, and uses electrofusion and chemicals to activate the cells to begin dividing. The results of this technique seem to show similar success rates for generating cattle clones as the cloning techniques more commonly used, with the advantage of being faster to perform (in the authors' hands), and requiring less expensive equipment. Peura (2003) has also described a modified technique for preparing fused donor/oöplasts in which sheep oöcytes whose *zona pellucidae* had been removed were enucleated after fusion with donor cells, reversing the order in which those steps are usually performed. This technique appears to provide a higher rate of development of the blastocyst stage, implying that some factors present near the oöcyte chromosomes may be of assistance. Other factors that may influence the success rate of SCNT are the timing and method of embryo activation relative to fusion. For example, Sung et al. (2007) found that simultaneous electrical fusion and chemical activation of SCNT bovine embryos resulted in a higher rate of development to the blastocyst stage compared to embryos that were activated four hours postfusion. Compared to bovine SCNT embryos that were chemically activated, Schurmann et al. (2006) reported that postimplantation development of SCNT embryos was improved by using a non-chemical, more physiological method of activation (by transferring donor nuclei into enucleated oöcytes that had been fertilized *in vitro*).

Over the next few years other technical refinements may be developed, some based on improved technical practice, and others on increased knowledge about basic molecular mechanisms involved in the developmental process. These should increase the success rate of cloning, and decrease the potential for adverse events to occur.

d. Transfer to recipient

Just as the case for other ARTs with an *in vitro* phase, the developing clone is transferred at the blastocyst stage into a surrogate dam in which the estrous cycle has been synchronized using standard methods. After transfer of the embryo clone(s), the pregnancy is allowed to proceed normally; no additional hormones or special treatments are required to establish or maintain pregnancy.

In cloning's earliest days, the surrogate mother was often chosen to be distinctively different from the donor animal with respect to some clearly visible trait. For example, Dolly's donor animal was a Finn Dorset sheep, a breed with white faces. Dolly's surrogate mother, however, was chosen to be a black-faced sheep, so that if a white-faced sheep were born, it would be clear that it was not a genetic relative of the surrogate mother. In addition to choosing a distinctively

different embryo recipient, Dolly's identity was also confirmed by DNA fingerprint analysis of the donor cell line from which she was derived (Wilmut et al. 1997). DNA fingerprint analysis enables definitive confirmation that an animal clone was indeed derived from a specific cell type, and is now the method of choice for confirming genetic parentage of animal clones (First et al. 1994).

C. Critical Biological Events in SCNT

Although SCNT is an inefficient process with a relatively low success rate, the extraordinary nature of the technology and the demands that it places on the biological system being manipulated should not be overlooked. Unlike the fertilized egg or early embryonic cells that may be considered totipotent (capable of becoming *any* cell in an organism) or pluripotent (capable of become many cells in an organism) "generalists," donor cells tend to be specialists. That is, they have differentiated to such a degree that their genomes have been "reconfigured" in ways that are, as yet, not fully understood in order to carry out the particular function for which they have been destined by their particular developmental fate. Kidney cells, therefore, do not transcribe the milk producing instructions of the mammary gland, yet they continue to carry those genes. The question then, is how to "reprogram" the full set of instructions contained in the genome such that "normal" development can occur. The following is a general overview of the events that are thought to occur during the SCNT process. There are several excellent reviews of the overall process or individual components that interested readers can reference for more details, and Chapter IV deals with some of these issues in more detail (Kikyo and Wolffe 2000; Sinclair et al. 2000; Solter 2000; Young and Fairburn 2000; Fulka et al. 2001; Rideout et al. 2001; Novak and Sirard 2002; NAS 2002a, b; Colman 2002; Dean et al. 2003, Santos et al. 2003; Morgan et al. 2005; Vajta and Gjerris, 2006; Meissner and Jaenisch, 2006; Oback and Wells, 2007a).

In principle, SCNT has demonstrated that cell differentiation can be reversed. Genetic reprogramming, the process of altering the gene expression pattern associated with the differentiated cell to one that is appropriate or early embryonic development, is normally carried out at two stages in the development of fertilization-derived embryos: after fertilization, and during the development of gametes (the sperm and ovum). The actual molecular events involved in reprogramming are not fully understood, although they may be categorized into a few overall steps. These include altering the way in which the chromosomes are packaged by changing the chemical nature of the proteins involved, and changing the chemical structure of the DNA in portions of the molecule that are not responsible for base-pairing (NAS 2002 a,b). A more complete description of these processes is found in Chapter IV.

The nucleus of a cell contains a complete copy of all of the genes required for life. This information is encoded in genes. Physically, genes are the linked nucleotides that comprise

DNA, or the "master molecule" of biology. The total genetic material of an organism is referred to as its "genome," and consists of long strands of DNA packaged in chromosomes, which come in pairs except for those specifying the sex of the resulting organism. The number of pairs of chromosomes differs among species. Cattle, for example, have 30 pairs, pigs have 19, sheep 27, and goats 30. (Humans have 23 pairs of chromosomes.)

Chromosomes can exist in different "conformations" depending on the stage of the cell cycle. When DNA needs to be moved, as in when a cell divides, or when a sperm needs to deliver the male genome, chromosomes are tightly condensed. During the rest of the cell's life cycle, chromosomes tend to exist in less tightly coiled conformations so the information encoded in the DNA is more accessible for processing. Specific proteins are responsible for holding chromosomes in different conformations. In all cells but sperm, these proteins are called histones; in sperm chromosomes are packaged by proteins referred to as protamines.

When an ovum is fertilized by a sperm, a complex series of molecular events ensues that is referred to as "chromatin remodeling" (chromatin is another term for the protein:DNA complexes that make up chromosomes.) Although the exact steps are not known, the overall process involves stripping away the protamines packaging the paternal DNA, removing histones from the ovum's DNA, and allowing the newly associated DNA molecules to reform chromatin in a way that allows the fertilized ovum and early embryonic cells to replicate and be "totipotent"(capable of developing into a complete organism). Many proteins are involved in this, only a few of which have been identified, and it is likely that there are chemical markers on the DNA bases that are altered (such as methylation). Chromatin remodeling is likely very different in SCNT. Disassembly of the tightly condensed sperm chromatids and the subsequent removal of protamines do not occur because there is no sperm present. Instead the oöplast must decondense and repackage the chromosomes of the donor somatic nucleus.

In order to perform the functions of life, cells have to convert the information in the DNA to ribonucleic acid (RNA) (a process referred to as transcription), and then to translate that RNA into proteins, which are the molecules that carry out life's functions. This coordinated set of activities is referred to as gene expression. Alterations in the expression of a given set of genes are often referred to as "epigenetic effects" (or "around gene effects") because they do not require changes in the base-pairing properties of the DNA that comprise genes. Instead, they reflect changes in the structure of the chromosome around the gene (such as control regions), or on the nucleotides, but outside the portion of the molecule involved in coding. (See Chapter IV for a more complete discussion). A classic example of the manifestations of epigenetic effects is the different fingerprint or freckle patterns observed in human twins. These individuals have exactly the same coding regions, but small changes in the non-coding regions of the DNA result

in different phenotypes. Other examples of epigenetic control of gene expression include the coat color or color patterns of many mammals.

D. Outcomes Observed in ARTs

As this risk assessment is being prepared, biologists are just beginning to understand the highly complex interactions that must occur to choreograph the millions of molecular interactions that signal the expression or silencing of genes in a particular cell or at any point in its life cycle. Although the exact mechanisms by which these effects occur are not fully understood, in all forms of reproduction, ranging from natural mating to SCNT, these processes may go awry in early development. Although most of the animals born following ARTs with significant *in vitro* components appear to be completely "normal," some of the outcomes are not so successful. In particular, some of the adverse outcomes noted in these "high *in vitro* component ARTs" appear to have common defects in gene expression, particularly in the overgrowth outcomes (Humpherys et al. 2002).

Published studies involving cattle, sheep, and mice demonstrate that embryos produced using *in vitro* systems may differ in morphology and developmental potential compared to embryos produced *in vivo* (Kruip and den Daas 1997; Young et al. 1998; Farin et al. 2004; Farin et al. 2006; see Appendix C for a more detailed discussion and additional references). For example, common abnormalities have been noted in fetuses (Farin and Farin 1995) and calves (Behboodi et al.1995; Sinclair 1999) associated with the transfer of bovine embryos produced using *in vitro* maturation (IVM), *in vitro* fertilization (IVF), *in vitro* culture (IVC) systems, and SCNT (Hill et al. 2000b). One set of reported adverse outcomes following transfer of embryos from cloning or *in vitro* production systems is often referred to as Large Offspring Syndrome (LOS). These include lowered pregnancy rates, increased rates of abortion, production of oversized calves, musculoskeletal deformities and disproportionalities, as well as hydroallantois (abnormal accumulation of fluid in the placenta) and other abnormalities of placental development.

The phenomenon of "large calves" was first described by Willadsen et al. (1991). The syndrome has also been identified in fetal and newborn lambs and in mice where the embryos were cultured *in vitro* (Eggan et al. 2001). Offspring with LOS tend to exhibit difficulties with placentation (Farin et al. 2004; Bertolini et al. 2004; Lee et al. 2004; Batchelder 2005). In cattle and sheep, the placentae of developing fetuses with LOS are unusually large for their species, and tend to have abnormal development of placentomes (the sites of attachment between fetal and maternally derived tissues of the placenta). LOS fetuses tend to have longer than usual gestation lengths, and often labor in the dams must be induced followed by Caesarian section

deliveries. The newborns tend to be large for their breeds, and often have abnormal or poorly developed lungs, hearts, or other affected internal organs (liver and kidney), which makes it difficult for them to breathe or maintain normal circulation and metabolism. LOS newborns may appear to be edematous (fluid filled), and if they are to survive, often require significant veterinary intervention. Problems have also been noted in muscle and skeletal development of animals with LOS. These animals also often have difficulty regulating body temperature. (For a more detailed discussion of LOS, see Chapter V).

Although the cause of LOS is not known with certainty, it is likely be related to changes in gene expression (*i.e.,* epigenetic changes) that result from the *in vitro* manipulation and culturing of embryos. A review by Young et al. (1998) suggests that *in vitro* culture alone is adequate to perturb the embryo. This hypothesis is supported by data from Sinclair et al. (2000) where *in vivo* matured and fertilized eggs recovered from superovulated sheep donors, cultured *in vitro* for 6 days, showed an 18-36 percent increase in mean birth weight at day 125 of gestation, depending on the culture system used (Sinclair et al. 2000, Young et al. 1998). Table C-1 (Appendix C) provides a more comprehensive summary of adverse outcomes noted in different ARTs.

This is an area of extensive research in the cloning and developmental biology communities. It is likely that advances in the understanding of these mechanisms will lead to significant improvements in the rates of successful outcomes of all ARTs that include a significant *in vitro* component, including cloning.

E. Future of Reproductive Technologies in Modern Agricultural Practice

Modern agricultural practices will likely continue to employ all of the reproductive modalities described in this overview. The factors that may influence which practices are used will likely be a function of the breeder/farmer/ranchers' needs and opportunities. Seidel (2006), in a foreword to a symposium on ARTs, emphasized that current differences in the reproductive management of cattle in different parts of the world are driven by multiple considerations. Some of these have to do with the nature of the differences in the husbandry of beef (mostly pasture based) and dairy (mostly intensively housed in the US, more pasture based in Australia and New Zealand) cattle. Some are economic (ARTs are much more expensive than natural matings), some are practical (the fertility of dairy cattle has declined significantly in the last 20 years, making ARTs more attractive; using ARTs in beef cattle is not practical for ranchers who look for replacement by natural coverage), and some are technological (the ability to choose genetics more precisely versus the developmental problems associated with ARTs with significant *in vitro* components).

Technological issues will be addressed by continued research and development in this field. To that end, several professional and scientific societies (e.g., the International Embryo Transfer Society, various animal science organizations and breeding associations) have been actively involved as clearing-houses for information and interaction.

SCNT has the potential to impact animal breeding in as fundamental a manner as artificial insemination. Given its current high costs (approximately $20,000 for a live calf) and relatively low success rates (< 10 percent), SCNT will likely be used to improve production characteristics of food producing animals by providing breeding animals, just as any breeding program would select the most elite animals for breeding, and not as production animals. In this way, cloning does not differ from any of the other ARTs that have been described in this chapter. Cloning has the relative advantage of allowing for the propagation of animals with known phenotypes to serve as additional breeding animals. This is critically important in breeding programs, especially when it may take years to "prove" the merit of a sire or dam. Second, it allows the propagation of animals whose reproductive function may be impaired. It has already been used to increase the available genotype of a particular dairy cow with low fertility; her clones appear to be exhibiting normal fertility (PIFB 2003). Third, it allows the propagation of valuable deceased animals from which tissue samples have been appropriately collected or preserved, which may have profound implications for species or breeds nearing extinction. Finally, for the first time, cloning allows for the careful study of the "nature-nurture" interactions that influence breeding programs by allowing a large enough sample of genetically identical animals to be raised in different environments, or with different diets. Such studies have been impossible to perform prior to the advent of SCNT and are likely to yield important information for developing livestock species to live in areas that have, until this time, been marginal for food animal production. This is of particular importance to the developing world, where even slightly increased wealth generally favors the incorporation of animal-based agriculture.

Regardless of the degree to which cloning may be adopted in animal breeding programs, FDA's role in performing this risk assessment is clear: the agency's responsibility is to determine whether cloning poses any risk to animals involved in the cloning process, and whether the consumption of food products from clones or their progeny poses any additional risk compared

with food from conventionally produced animals. This Risk Assessment presents the method by which we evaluated data on clones and their progeny, the data themselves, and the agency's conclusions, including discussions of uncertainty.

Chapter III:
Developing the
Risk Assessment Methodology

Chapter III:
Developing the
Risk Assessment Methodology

A. Charge

In July of 2001, the Food and Drug Administration's (FDA or the Agency) Center for Veterinary Medicine (CVM or the Center) issued an open letter (CVM Update 2001) to producers of animal clones to ask them to refrain from putting edible products from those animals into the food supply until the Center evaluated the safety of those foods.[19] This request had already been made to companies engaged in cloning food-producing animals during the previous year. The overall strategy chosen by the Center was to perform a risk assessment in order to determine what hazards might be introduced into animals as the result of the cloning process, to characterize the resulting potential risks, and to develop risk management proposals commensurate with the identified risks.

B. General Discussion of Risk/Safety Analyses

1. Risk and Safety

Risk and safety can be thought of as two sides of the same coin. In general, the answer to the question of "Is it safe?" is addressed scientifically by determining the conditions under which the substance or action in question is not safe, and then limiting exposures to conditions outside those limits. Because knowledge is always incomplete, and not every circumstance can be controlled, there is no such thing as "absolute safety" or "zero risk." Risk assessors attempt to identify conditions under which risks are estimated to be as low as possible, and risk managers use that information in developing policies to protect human or animal health. The methodology used to characterize potential risk is referred to as risk assessment.[20] One of the real values in performing a risk assessment is that in addition to arriving at an outcome, the process of arriving at an answer provides a framework by which data can be organized, analyzed, and interpreted. By dividing the risk assessment process into discrete steps, and then reintegrating them into an

[19] http://www.fda.gov/cvm/CVM_Updates/clones.htm

[20] Appendix A provides an overview of risk and safety assessments, especially as they have evolved to address issues relevant to cloning, and may be useful background reading for individuals not familiar with the processes.

overall characterization of potential risk, risk assessment allows both the details and the "big picture" to be addressed for complex problems.

Discussion of uncertainty must accompany every risk assessment. Uncertainties may stem from a lack of fundamental understanding of biological processes and/or from data gaps that may be filled with the appropriate empirical studies; they may be exacerbated by intrinsic variability in datasets. Given that the process of risk assessment identifies data gaps and helps direct the acquisition of data that decrease uncertainties, it should not be thought of as a process that is performed just once, but rather a recursive process in the responsible development of research programs, new products, and science-based regulatory strategies.

2. Risk Assessment vs. Risk Management

Risk management can be defined as the set of activities of identifying and evaluating alternative strategies (often regulatory) to deal with the risks characterized in the risk assessment, and then selecting among them based on social, economic, ethical, and political conditions or criteria (NAS 1996a). Risk managers choose among different options based on the risk assessment, which is generally regarded to be relatively value-free compared to the risk management phase,[21] and their understanding of and responsibilities to the broader social or economic constructs within which they operate. Risk-benefit or risk-risk decisions are risk management, as they involve an active choice between two or more possible courses of action. A risk management plan based on this Risk Assessment is presented in the accompanying document.

C. Risk/Safety Assessment of Cloning

In order to address the hazards and risks to animals involved in cloning and the food products derived from them (and their progeny[22]) four issues must be addressed: identifying hazards and risks; determining the degree to which existing data address questions of safety; characterizing residual uncertainties; and selecting the most appropriate risk metric for the Risk Assessment:

[21] Although risk assessment is based on science and relatively value-free, it generally contains a few policy-based judgments such as the selection of health protective (conservative) defaults when data are incomplete or when choosing among datasets of equal quality. The selection of policy-driven alternatives should be explicitly discussed in the risk characterization, and the implications of such choices should be described in a risk assessment.

[22] An animal clone is one arising directly from a somatic cell nuclear transfer event. A progeny animal is one derived from sexual reproduction that has at least one animal clone as a parent (but could result from two animal clones mating).

(1) **Identifying hazards and risks**. As there are no existing risk paradigms for animal clones and the food products that may be derived from them, this assessment attempts to identify hazards and risks based on the available data and consideration of the biological processes affected by cloning.

(2) **Determining the degree to which existing data address questions of animal health or food consumption risk.** Many of the peer-reviewed publications on cloning report on the ability to generate live animal clones from various donor cell sources and culture conditions; the frequency of successful outcomes (where success is defined as a surviving dam and a live offspring with no apparent abnormalities); and the nature and frequency of developmental errors. The nature of the published reports, with some exceptions, reflects the institutions producing them: academic laboratories tend to report the development of new technologies and the observation of abnormalities, while corporate entities tend to report successful implementation of the technology, including summaries of the health status of animal clones. These studies are useful in identifying potential hazards to the health of animals involved in the cloning process and characterizing any risks that may stem from those hazards. The number of reports directly addressing food safety is much smaller than the number of reports reviewed for animal health.

Reports from the peer-reviewed literature likely suffer from "publication bias," an artifact of the criteria used to determine the "attractiveness" of publication in leading peer-reviewed journals. In general, investigators tend to submit to journals, and journals tend to publish, novel findings or hypothesis-testing results rather than surveys of the health of cohorts of animals. With a few notable exceptions, the literature on animal clones tends to consist of reports of studies of the role of various technical manipulations on the success of cloning procedures, descriptions of initial successes of cloning in species that have not yet been cloned, or descriptions of adverse outcomes. Much of the work in which cloning has been refined (and is therefore more likely to be successful) is being performed by the private sector. Given the competitive nature of the breeding and biotechnology industries, as well as the need to maintain business confidential information, much of the important information on more recent cloning outcomes has not been published or made publicly available. In order to keep the current analysis transparent to the public, however, this assessment only cites information that has been published in peer-reviewed journals, or otherwise made available to the agency by companies engaged in cloning, with explicit permission for release to the public.

(3) **Characterizing residual uncertainties persisting following a review of the existing data.** Due in large part to the novelty of the technology, the concentration of data at the earliest stages of clone development, and limited data directly addressing food safety,

uncertainty will persist in any estimates of risk associated with animal cloning. As with all science-based uncertainties, additional data may increase the confidence with which judgments are made. The decision as to "how much is enough," however, is a function of the nature of the risk(s) (*i.e.,* its severity), the quality and consistency of the data (*i.e.,* the weight of the evidence), and the tolerance of the risk management policies for uncertainty.

(4) **Selecting the most appropriate risk metric for this risk assessment.** The most appropriate standard to apply to the potential risk(s) associated with the consumption of foods derived from animal clones and their progeny is whether such food poses any additional risk relative to that derived from sexually-derived animals. For the purposes of this risk assessment, conventional animals are defined as those animals derived by any reproductive means other than SCNT.

D. Transgenic Animal Clones

This risk assessment addresses "just clones," that is, animals derived via SCNT whose donor genomes have not intentionally been modified by molecular biology techniques. Transgenic clones, on the other hand, are clones whose donor cells contain exogenous heritable DNA inserted by molecular biology techniques. They are considered to occupy a different "risk space" from "just clones" because the transgenic event (the insertion of a heritable DNA sequence) is intrinsically accompanied by a series of potential risks. These include those associated with the DNA construct and those associated with the product of the gene (if there is a gene product). Organisms derived from transgenic cells will have risks specific to the inserted construct, its insertion site, and its subsequent expression. Although it is entirely possible for transgenic clones (or any transgenic organism) to be produced safely and to be a safe source of edible products, the risks associated with each animal must be determined separately on a case-by-case basis, because of the added genetic material.

Nonetheless, much of the literature on animal clones reports on experiences with transgenic clones. In some cases, the transgenic nature of the animals is explicit (*e.g.,* Hill et al. 1999), but in many others, only careful reading or tracing back references cited in the methods section of the papers allows the reader to learn the transgenic status of the clones (*i.e.,* Lanza et al. 2001, Cibelli et al. 2002). The question is whether any information from the transgenic clone reports can inform the identification of hazards and characterization of risks associated with "just clones."

After a careful review of the key papers addressing transgenic clones, CVM has decided that it is not possible to determine with certainty whether any particular adverse outcome is due to the process of cloning, the transgenic nature of the donor cell, or some combination of the two. Clearly, the insertion of exogenous DNA introduces a set of hazards not present in non-transgenic clones, and by inference, the creation of a different set of risks. If transgenic animals appear to be normal, the logical inference is that neither cloning, nor transgenesis (or the combination of cloning and transgenesis) has perturbed the animals' development. This is the case of transgenesis and cloning posing no significant (or apparent) risk. In either case, this risk assessment puts greatest weight on reports of outcomes from non-transgenic animal clones, and uses studies of transgenic clones for secondary or corroborative purposes. Nonetheless, given the large proportion of the peer-reviewed literature that reports on transgenic clones comprise, these studies have been cited with the preceding caveats. A more complete discussion of this topic is found in Appendix D: Transgenic Clones.

E. Methodology Development

When considering how to develop a risk assessment methodology for animal cloning, it became apparent the need to develop a framework that could be applied to both animal health and food consumption risks. Early in the development of this risk assessment, there were no studies explicitly evaluating the safety of food products from animal clones. Therefore, our initial analyses of both animal health and food safety were based largely on the health status of the animals producing food. As development of the risk assessment progressed, data on the composition of edible products from clones became available, and these data were included in our analysis of risks related to human consumption of foods produced by animal clones.

Interpreting hazard and risk from the same dataset but for different sets of receptors (animal health - the animals involved in producing clones; food safety - the consumers of the food products) requires shaping the manner in which the data are evaluated to suit the ultimate outcome of the assessment. For example, identification of adverse outcomes for animal health requires evaluating data on both surrogate dams carrying pregnancies and resulting clones. For food consumption, however, animal clones that would be condemned at slaughter, as currently practiced with conventional food animals, were excluded from the analysis, and emphasis is placed on the identification of unique hazards to food consumers that could arise as the result of the cloning process. As described in the following section, and Chapter VI, this requires evaluating the dataset at a finer level of resolution than for animal health outcomes.

The net effect of the different ultimate outcomes of the animal health and food consumption risk assessments is that although the datasets considered by both assessments may overlap

considerably, the manner in which they are evaluated differ, and the conclusions generated from the same (or largely overlapping) datasets vary with respect to the amount of risk present.

1. Hazard Characterization

Identifying and characterizing potential hazards is the first step in characterizing the nature of risks due to cloning (see Appendix A). CVM therefore sought to develop a framework in which adverse outcomes associated with cloning could be presented in a systematic manner that would facilitate interspecies comparisons of outcomes.

For food safety purposes, the scientific and regulatory communities have traditionally operated under the principle that domestic animals (*i.e.,* cattle, swine, sheep, and goats) commonly consumed for food have not developed specialized organs producing toxicants to kill prey or avoid predation (*e.g.,* venom producing glands). Further, because the components of animal tissues are necessary for life, and closely resemble the processes in humans, it is highly unlikely that "silent" pathways to produce intrinsic toxicants exist. Thus, "it is convention that animal metabolites are not considered to be natural toxicants" (Watson 1998).

In order to generate a viable clone, the differentiated genome of the donor cell or nucleus must be reprogrammed by the recipient oöplasm. Because no additional genes are being added, and the presumption is that there are no silent pathways to produce intrinsic toxicants, the only method by which hazards may arise in animal clones is from the incomplete or inappropriate reprogramming of the genetic information from the donor somatic nucleus (*i.e.,* epigenetic effects). These phenomena are described in more detail in Chapter IV.

Where, then, would the potential hazards in clones arise? As outlined in Chapter II, during the development of an embryo, a complex series of molecular events are responsible for balancing gene expression from the maternal and paternal genomes, and directing the appropriate expression of genes in the developing embryo and mature mammal. This process is referred to as "reprogramming." Alterations in gene expression due to those changes are referred to as "epigenetic" variability, and are present normally in conventional animals, including humans.

The most severe errors in reprogramming will result in death, obvious malformations, or metabolic derangements, and are reflected in the low "success rate" of cloning, the perinatal difficulties observed in some newborn clones, and occasional examples of altered metabolic pathways in very young animals (see Chapters V). These are clearly the subject of the animal health risk assessment. Because animals found to have a disease or condition that would render them adulterated (e.g., unfit for consumption, unhealthful, unwholesome) are prohibited from

entering the human food supply, however, the only remaining food consumption hazards arising from gene dysregulation would be those that allow an animal clone to develop with apparently normal functions, but with sub-clinical physiological anomalies.

These *subtle hazards* are outside the conventional range of hazards commonly the subject of food safety analyses, and can be divided into three overall classes:

(1) Alterations in gene expression that lead to phenotypic variability such as coat color, size, behavior, longevity;

(2) Disruption of immune function; and

(3) Alterations in metabolism leading to changes in physiological "set-points" such that the animal has apparently compensated and appears to be normal on gross inspection, but whose physiology may be aberrant.

It is important to note, however, that changes in gene expression in individuals sharing identical genotypes have been observed in conventional animals and in humans. This phenomenon is often referred to as phenotypic variability, and can be seen at the human level in the different fingerprint and freckle patterns that identical (monozygotic) twins possess. Non-clone mice of identical genotypes fed different levels of certain nutrients can have different coat colors, and exhibit significant differences in body weight and lifespan (Cooney et al. 2002).

2. Potential Risks

Risk is defined as the probability of an adverse outcome given that exposure has occurred. This concept is often presented in the format of the "risk equation" that may be expressed as

$$\text{Risk} \propto f_{outcome}\,(exposure,\ hazard)$$

or, stated more simply, risk is some function of exposure and hazard.

The "risk equation" can be run in the forward or reverse direction. Characterizing risks from a set of hypothetical hazards is a case of running the equation in the forward direction: it allows the estimation of the probability that adverse outcomes might occur once changes that create hazards have occurred. Such approaches are useful when there is some understanding of the underlying biological processes being evaluated. For example, if incomplete genetic reprogramming (a change that may result in a hazard) were to result in animals with altered

calcium transport mechanisms, a possible animal health risk could be bone weakness or malformation, and a possible food consumption risk (a probability of an adverse outcome) could be compromised human nutrition resulting from a diet of milk containing lower than expected calcium levels (the adverse outcome). In the case of the animal health risk, the degree of risk could vary from insignificant, in which no physical symptoms were present, to severe, in which the animal could experience misshapen or fragile bones leading to difficulties in walking. Because animals found to have a disease or condition that would render them adulterated (e.g., unfit for consumption, unhealthful, unwholesome), only the animals without obvious visible anomalies (and therefore less severe calcium transport anomalies) would be sources of edible products. The food consumption risks then could possibly arise from a lower available calcium pool accessible to milk production, and thus a potential nutritional risk to individuals consuming milk from such animals.

Analysis of end products such as milk constituents is an example of running the risk equation in the reverse direction: it captures the potential outcome(s) of the biological changes, and allows for the identification of exposures and hazards responsible for the risk(s). The nutritional hazard identified in the preceding example might be detected more efficiently by a compositional analysis of milk. Compositional analyses, however, are limited by available analytical methods and comparators. As far as CVM is aware, no complex food (*e.g.,* bacon, beef steak, milk, cheese) has been fully characterized with respect to its chemical composition.[23] The organisms that are or make up foods are comprised of hundreds of thousands of chemical substances that can be influenced qualitatively and quantitatively by diet, environmental conditions, and genetics. Attempts to characterize all of the chemical constituents of "milk" or "meat," then, are neither practicable nor desirable (NAS 2004). Instead, milk and meat analyses have tended to be limited to characterizing proximates (*e.g.,* water content, proteins, fats, carbohydrates, minerals, ash), or, when necessary or desired, to profiles of particular nutrients, anti-nutrients, or individual components of interest (*e.g.,* vitamin content, fatty acid profiles, or protein composition).

3. Proposed Approaches

a. Animal Health Risks

The Center determined that at this point in the development of the technology, risks to animal health are best characterized using a retrospective approach. In other words, CVM approached this issue by recording and cataloguing adverse outcomes in a biological context, rather than by elucidating specific examples of gene dysregulation and searching for their physiological

[23]The International Life Science Institute (ILSI) is currently coordinating an effort to generate a database of the known chemical constituents of major food crops (*e.g.,* corn, soy, wheat).

sequellae. The *Critical Biological Systems Approach* (CBSA), described below, provides a framework in which this may be accomplished (Figure III-2). In general, the Center has relied on integrated physiological measurements to survey animal health, although it is likely that genomics, proteomics and metabolomics will see increased use for such purposes in the future. At the time that this risk assessment was prepared, however, these methods had not been sufficiently developed and validated to allow them to be used as survey tools.

b. Food Consumption Risks

Determining the safety of food products from animal clones and their progeny, at least in its earliest stages, is likely best accomplished by using both approaches: prospectively drawing on knowledge of biological systems in development and maturation, and in retrograde, from an analysis of food products. An intrinsic and valuable part of this analysis is cataloging the available information, and identifying data gaps and uncertainties that may in turn suggest research that could serve to decrease the identified uncertainties. The following sections describe the methodology CVM has proposed to accomplish a rigorous, science-based analysis of potential hazards and risks associated with the consumption of food products derived from animal clones and their progeny.

Prior to undertaking such an analysis, however, subtle hazards and potential risks that may be posed by animal clones must be considered in the context of other mutations and epigenetic changes that occur in all food animal populations. Some are considered beneficial, and have been selected for by animal breeders when a desirable phenotype is obtained. For example, not-so-subtle genetic mutations that have occurred at least twice in nature are the development of double-muscled beef breeds such as the Belgian Blue and Piedmontese, which arose from different mutations in the myostatin gene (McPherron and Lee 1997). These animals appear to be healthy, although sexual maturity appears to be delayed relative to other breeds, and female fertility appears to be somewhat lower. Nonetheless, these animals are used in selective beef breeding programs in several countries as they have 20-30 percent more muscle mass than cattle with the wild-type myostatin gene, feed efficiency is increased, and the meat is considered to be more tender although lower in fat content. Meat from these animals is presumed to be food, and as such enters the food supply with no additional regulatory scrutiny. Epigenetic changes that occur on a regular basis include variations in pigmentation patterns (*e.g.,* coloration patterns on Holsteins) and are perhaps most easily thought of as those differences observed in identical twins, such as different fingerprints and freckle patterns.

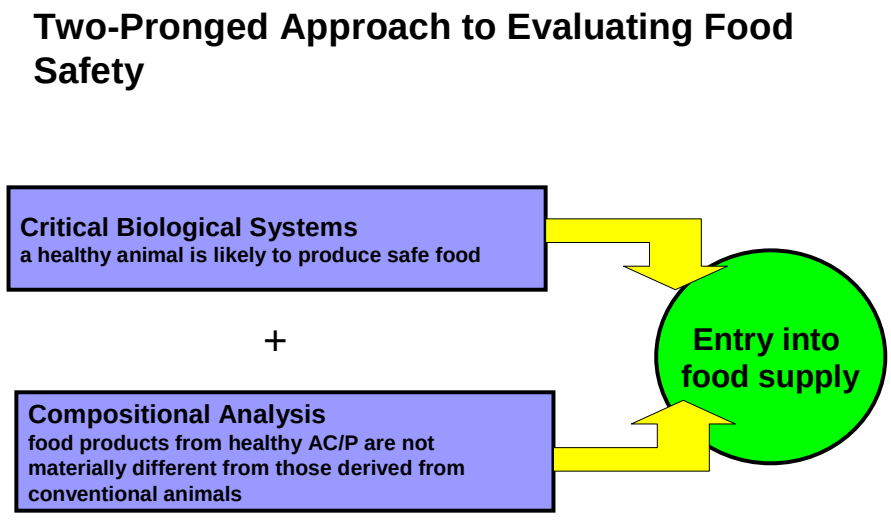
Finally, it is important to remember that any discussion of subtle hazards and potential risks associated with the products of animal clones is not conducted in a regulatory vacuum. All food,

including that from animal clones, must meet existing regulatory requirements in order to be marketed lawfully in the United States.

F. Two-Pronged Approach to Assessing Food Consumption Risks

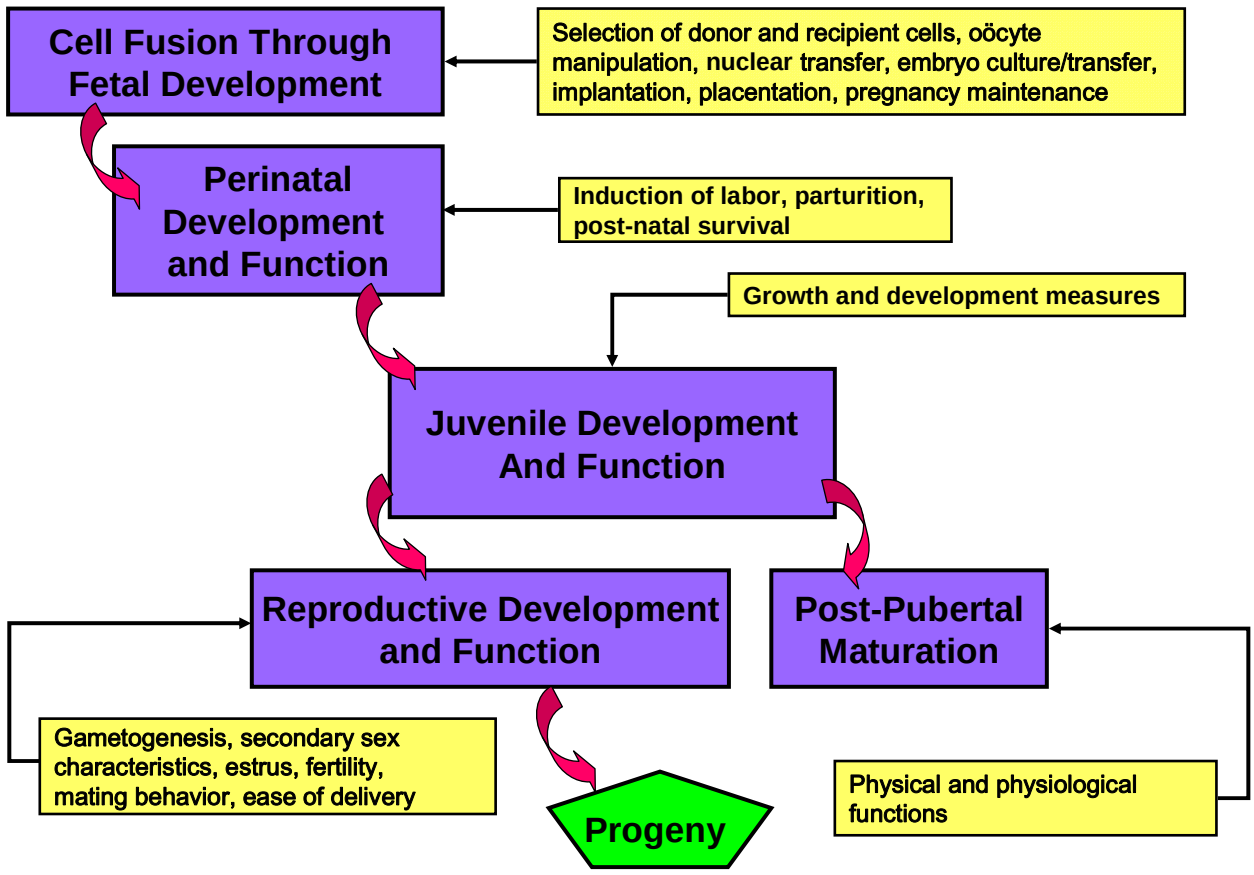
Given the assumption that food derived from clones will be in compliance with existing regulatory requirements for food products from conventional animals, CVM proposes a two-pronged approach for evaluating the potential risks associated with the food products of animal clones and their progeny (AC/P) (Figure III-1). The first component, the *Critical Biological Systems Approach* (CBSA) is based on the hypothesis that a healthy animal is likely to produce safe food products, and incorporates a systematic review of the health of the animal clone or its progeny. The second component, or the *Compositional Analysis Method*, is based on the operating hypothesis that food products from healthy animal clones and their progeny that are not materially different from corresponding products from conventional animals are as safe to consume as their conventional counterparts. It relies on the comparison of individual components of edible products, and the identification of the appropriate comparators.

Figure III-1:



1. Critical Biological Systems Approach

Figure III-2: Critical Biological Systems Approach



a. Overview

The CBSA (Figure III-2) is based on a cumulative evaluation of health status indicators of animal clones. Mechanistically derived, it considers SCNT and the subsequent development of the animal clone from a biological "systems analysis" perspective, and thus may be thought of as being "HACCP[24]-like." It accepts that at this time SCNT is a biologically imprecise and

[24]HACCP is the Hazard Analysis Critical Control Point approach adopted by USDA and FDA for assuring the safety of certain food products undergoing some degree of processing. http://www.fsis.usda.gov/Science/HACCP_Models/index.asp

inefficient process resulting in few live births relative to the number of implanted embryos, and that some animals are born with obvious defects or subtle anomalies. It also assumes that biological systems are capable of repair or correction, either intrinsically or following human intervention. For example, animals that may have difficulty surviving on their own immediately after birth may develop into healthy, reproducing individuals if provided support in the form of respiratory assistance and warmth during the period immediately after birth. Alternatively, these animals may not recover, and may remain "sickly" or unthrifty until they are culled.

The cumulative nature of the CBSA allows for the incorporation of both favorable and unfavorable outcomes. The former, provided that all other measures appear to be normal, will result in the judgment that the animal will produce food that is safe for consumption; the latter implies that animals with anomalies may be unsuitable for food.

b. Evaluation Nodes

The CBSA selects five key developmental stages of an animal clone's life, analogous to the "critical control points" of the HACCP analysis. These stages provide biologically-based developmental "collection nodes" (Developmental Nodes) (indicated in Figure III-2 by periwinkle-colored boxes) that also serve as agronomically appropriate points at which to collect data. Examples of the types of data that could be collected are illustrated in Figure III-2 as yellow boxes. It is important to note that these Developmental Nodes address functionality and not necessarily discrete time points, as the latter will vary among species and breeds.

Developmental Node 1 incorporates the initial technical steps involved in SCNT, including cell fusion through implantation, and subsequent embryo and fetal development. Chapter IV covers many of the early common molecular events that occur during this time period common to mammals; Chapter V reviews these issues as they impact on the health of clones and their surrogate dams; and Chapter VI reviews these steps from the perspective of identifying food consumption risks.

Developmental Node 2 encompasses the Perinatal period, including late gestation, labor induction in the dam, delivery, and the critical time period of approximately 0-72 hours after birth. This developmental node allows for the analysis of animal health data relevant to both the surrogate dam and the clone, although few food consumption risks are anticipated to occur at Developmental Node 2 because clones of that age would not be consumed as food.

The third developmental node (Developmental Node 3), Juvenile Development and Function, encompasses the period of rapid growth between birth and the onset of puberty, and may vary in duration among the species considered.

The Reproductive Development and Function Node (Developmental Node 4) encompasses puberty and reproductive function throughout the reproductive period of the animal. Food consumption risks arising from milk production may first be encountered at this point of the animal's life. Because of the complex integration events that must occur for effective reproduction to take place, this developmental node is critically important for evaluating the health and functionality of animal clones. Proper reproductive function indicates that the complex and inter-related physiological systems required for the development and delivery of functional germ cells (and, in the case of females, viable offspring) are functioning appropriately.

The Post-Pubertal Maturation Node (Developmental Node 5) encompasses all non-reproductive functions of sexually maturing or mature animals, including growth, weight gain, disease frequency, aging, and lifespan, where available.

Table III-1: Summary of Developmental Nodes and Implications for Food Consumption Risks

Developmental Node	Types of Observations/Data	Potential for Entry into Food Supply
1: Cell fusion through implantation, embryo and fetal development	Selection of donor and recipient cells, oöcyte maturation and activation, fusion method, days in culture, culture conditions, number of fusions, number of blastocysts formed (if measured). Number of implantations, early and late gestation losses, placentation, pregnancy maintenance, morphological anomalies.	None
2: Perinatal period including immediate pre-partum, delivery, and up to 72 hours post-partum	Number of animals delivered with/without assistance, survival, morphological abnormalities, post-parturition survival, physiological/biochemical characterizations of surviving/dead animals.	Minimal, due to low likelihood of entry into food supply as meat, except for injured animals.
3: Juvenile Development (cattle: pre-weaning; swine, sheep, goats: post-weaning period)	Survival rate, measures of growth, physiological and biochemical markers of health status.	Relatively low, but possibly as meat (*e.g.*, veal, lamb, suckling pig).
4: Reproductive Development and Function	Development of secondary sex characteristics, spermatogenesis, oögenesis, gender appropriate behavior, age of pubertal onset. Fertility measures for males and females. For females, mothering behavior, milk production.	Milk
5: Post-Pubertal Maturation	Growth, weight gain, muscle/fat ratios, milk production.	Meat, Milk

Because the value of clones lies in their use as breeding stock (and is reflected in their relatively high cost), "founder" animal clones are not likely to be slaughtered initially for meat. It is anticipated that most of the food products, especially meat, from clone lineages will enter the food chain as the progeny of animal clones, or their subsequent offspring. Milk from dairy clones could enter the food supply, following breeding and delivery of offspring. Meat from clones could enter the food supply if, for instance, conditions outside the producer's control forced herd culling (*e.g.,* loss of funding), or when older animals reach the end of their functional utility (*e.g.,* loss of fertility in breeders). Table III-1 summarizes the Developmental Nodes, the types of data likely to be collected at each node, and the potential for the entry of clones into the food supply.

G. The Weight of Evidence Approach

The final step in the Risk Assessment was to consider the information derived from the CBSA and the Compositional Analysis approach as a whole, and then draw conclusions regarding risks, if any, to the health of animals involved in the cloning process and humans consuming food from clones and their progeny. With respect to animal health, individual statements of risk were derived for each species and at each of the five developmental nodes. For food safety, individual statements of risk were derived for meat from each species and for milk from clone cattle.

Weight of evidence evaluations do not require a balanced (or minimum) number of studies from each component of the assessment because they look across all of the studies to determine the degree to which results are consistent, and if not, what potential sources of differences may be. In this Risk Assessment, the weight of evidence approach consisted of four steps:

(1) *Evaluation of the empirical evidence* (*i.e.,* data on molecular mechanisms, physiological measurements, veterinary records, and observations of general health and behavior) for the species being considered;

(2) *Consideration of biological assumptions* predicated on our growing understanding of the molecular mechanisms involved in mammalian development;

(3) *Evaluation of the coherence of the observations* with predictions based on biological mechanisms; and

(4) *Evaluation of the consistency of observations* across all of the species considered, including the mouse model system.

When drawing conclusions in this risk assessment, empirical evidence was critically evaluated not only within an experimental context (*i.e.,* compared to observations and data from comparator animals and foods), but was also evaluated for *coherence*. The concept of coherence is derived from Hill (1965), and refers to the degree to which the observations are compatible with known biological knowledge and principles.

Consistency, a concept also described by Hill (1965) requires close conformity between the findings in different samples or populations, or in multiple studies conducted by different investigators. In this risk assessment, empirical data derived from clones and foods from clones were evaluated for their consistency across all of the species for which data were available. Like the criterion of coherence, the degree of consistency of the data influenced the strength of the conclusions reached in this Risk Assessment, both for risks to animal health due to cloning and food consumption hazards associated with meat and milk from clones.

The availability of empirical evidence available on each topic was variable and, in many cases, the observations of multiple studies on the same hazard were not consistent. For each hazard, the empirical evidence demonstrating a risk associated with cloning was weighed against the empirical evidence (if any) demonstrating the absence of a risk. This comparison was both quantitative (*i.e.,* based on the number of studies supporting presence vs. absence of risk) and qualitative, putting results into the appropriate physiological context and placing more weight on well-designed studies that provided the most useful information. In some cases, there was insufficient information available to draw meaningful conclusions.

H. Limitations of the Risk Assessment

This is a qualitative, comparative risk assessment that does not attempt to assign a quantitative value to estimates of risk or safety. The strongest conclusions that can be drawn regarding positive outcomes in risk assessments of this type are "no additional risk" because outcomes are weighed against known comparators. If a finding of "no additional risk" were to be applied to the health of animal clones, it would mean that the cloning process would not pose any greater risk to the health of the animals involved than other ARTs. Applied to the safety of edible products derived from clones, a finding of "no additional risk" would mean that food products derived from animal clones or their progeny would not pose any additional risk relative to corresponding products from non-clones, or that they are as safe as foods that we eat every day. As with all risk assessments, some uncertainty is inherent either in the approach we have used or in the data themselves (for a more complete discussion of the uncertainties in this Risk Assessment, see Chapter VII).

Chapter IV:
Epigenetic Reprogramming:
Implications for Clones and their Progeny

Chapter IV: Epigenetic Reprogramming: Implications for Clones and their Progeny

The previous Chapters of this Risk Assessment have introduced the concept that incomplete or inappropriate epigenetic reprogramming appears to be one of the primary underlying causes for the relatively low success rate of cloning, and the source of potential subtle hazards for the consumption of food from animal clones. Although a complete discussion of the rapidly emerging field of epigenetics is beyond the scope of this risk assessment, readers are directed to a series of excellent reviews for more details (Reik and Walter 2001b; Surani 2001; Bird 2002; Li E 2002; Davidson et al. 2003; Kelly and Trasler 2004; Santos and Dean 2004; Tian 2004; Allegrucci et al. 2005; Holliday 2005; Morgan et al. 2005; Eilertsen et al 2007; Reik 2007). An overview of the topic, however, is useful to put the issue of the source of potential subtle hazards in clones into context.

Briefly, epigenetics has been defined as the study of stable alterations in gene expression potentials that arise during development and cell proliferation (Jaenisch and Bird 2003), or alterations in DNA function without alterations in DNA sequence (Jones and Takai 2001). The central idea behind the concept of epigenetics is that although the DNA sequence of almost all the nucleated somatic cells in the body of an adult mammal is identical (except some very specialized cells whose development requires DNA rearrangements), the phenotypes of those cells can be quite different because alternate subsets of genes are expressed at different times in development and during cellular differentiation. In other words, each cell type in an organism has its own epigenetic profile or signature (Morgan et al. 2005).

Epigenetic changes have been implicated as the source of the anomalies noted in clones and other ARTs. The primary biological assumption is that as no exogenous genes are being introduced into the genome being copied and expressed (as in the case of clones) or being expressed following the union of two gametes (as in the case of the other ARTs), alterations in gene expression are responsible for the adverse outcomes noted in the resulting animals. Although much of the focus of the ongoing research in this rapidly expanding field is directed towards gathering and understanding observations of epigenetic changes in early development, epigenetic changes also occur later in life. They are part of the normal and necessary way that organisms adapt to their environments.

For example, Fraga et al. (2005) have demonstrated that monozygotic or “identical” human twins begin life with very similar epigenetic patterns. Over time, however, they accumulate epigenetic differences so that their epigenetic profiles become quite different. Smoking, diet, and other life

experiences are proposed as exerting influence of the epigenetic differences observed between genetically identical twins, with more differences in life experiences correlated with more different epigenetic profiles later in life. Epigenetic changes have also been associated with various disease states that arise from the dysregulation of normal genes (reviewed in Egger et al. 2004, Jiang et al. 2004).

Epigenetic differences are also noted in conventional animals, and may reflect the status of the uterine environment. Cooney et al. (2002) investigated the effects of maternal methyl food supplements[25] prior to and during pregnancy on the epigenetic control of various health outcomes using an experimental system based on the expression of an epigenetically-regulated mouse coat color. The genome of mouse strain employed in this study includes an endogenous retrovirus containing viral genes and promoter enhancer sequences referred to as long terminal repeats (LTRs), which can drive the expression of retroviral genes and murine genes in their vicinity. When the LTR is active (relatively demethylated), it overpowers the endogenous mouse promoters, and allows the constant transcription of the genes giving rise to the "yellow" phenotype. This phenotype exhibits a solid yellow coat color, obesity, predisposition to cancer, diabetes, and a relatively short lifespan. When the LTR is suppressed (relatively methylated), the agouti[26] gene locus is regulated by its own promoters, and is expressed cyclically and only in hair follicles. The phenotype of these mice is lean, healthy animals of normal lifespan and agouti-patterned coats. In this study, pregnant dams were fed diets containing three levels of dietary 1-carbon sources or cofactors. The lowest consisted of typical laboratory mouse chow; the intermediate level was supplemented with choline, betaine, folic acid and vitamin B-12, while the highest supplementation included three times the supplement level of the intermediate diet plus methionine and zinc. In addition to evaluating coat color, Cooney et al. (2002) also determined the relative degree of methylation of the LTRs driving the agouti gene locus. They observed that as the level of methyl donors in the diet increased, phenotypes of progeny animals shifted towards the agouti phenotype. Corresponding changes were observed in the methylation status of the LTR, with increasing methylation in the animals whose dams received higher levels of methyl donors in their diets.

Another example of altered phenotype dependent on methylation status has been identified in inbred sexually-derived mouse strains. In this case, mice that carry a particular allele (specifically axin-fused which is referred to as $Axin^{Fu}$) variably express a "kinked tail" phenotype rather than a normal tail morphology. The variability in heritability of the phenotype at this locus

[25] Methylation of DNA is performed by specific enzymes (methylases) that obtain methyl sources from either the diet (as folates or folic acid) or from endogenous one-carbon metabolism. The latter requires essential dietary components such as methionine, zinc, and vitamin B-12 to act as cofactors in the synthesis of intermediates that give rise to 1-carbon donors

[26] The agouti coat color is a continuous spectrum of variegated coat color patterns on a yellow background. In cats, this coat color pattern is referred to as "tortoiseshell."

has also been shown to be a result of epigenetic inheritance (Rakyan et al 2003). Similar to the variable coat color phenotype described by Cooney et al. (2002, above) the variable expression of the kinked tail phenotype has been shown to correlate with differences in methylation status of a particular type of transposable element at the locus (intracisternal A particle). Additionally, the variability of expression is influenced by the parent that contributed the *Axin*^{Fu} allele. The authors hypothesize that the variability in the phenotype is related to resistance of this type of retrotransposon to epigenetic reprogramming and further influenced by differing levels of reprogramming of the genome contributed by the male or female parent. The two models are not mutually exclusive in that the retrotransposons at the loci are similar and reprogramming may be influenced by maternal methyl donor status and different for maternally and paternally derived alleles (Cropley et al 2006).

Because the field is relatively new, and the scientific community has not identified all of the mechanisms involved in epigenetic remodeling, with few exceptions (*e.g.,* X-chromosome inactivation), the direct links between any one mechanism (or a series of mechanisms) and the health outcomes in live animals are not clear. Animals produced by non-SCNT ARTs, including natural mating, may have different epigenetic profiles, and even exhibit developmental abnormalities, but are not considered to pose unique food consumption risks.

A. Overview of Epigenetic Reprogramming in Early Embryonic Development

In conventional breeding, a new diploid genome is created by the fusion of two haploid genomes; one each from the sperm and the egg. The subsequent expression of that newly formed diploid genome to generate a functional multicellular organism is governed by a "program." This term was first used by the genetic pioneers Jacob and Monod, who in 1961 proposed that "…*the genome contains not only a series of blueprints, but a coordinated program…and a means of controlling its execution*." More than a half-century later, researchers are still trying to understand how that control is exerted.

Multiple mechanisms respond to the cell's developmental stage or its environment by acting as positive (more transcription[27]) or negative (less transcription) control elements. Transcriptionally active regions of DNA (or heterochromatin) may be considered to be "open" so that various molecules involved in DNA processing can gain access to certain regions, whereas "euchromatin" is physically tightly condensed, or "closed" with respect to access by other molecules, and transcriptionally silent. The picture emerging through current research (see

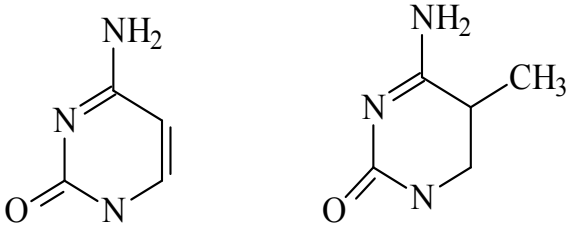
[27] Information encoded in DNA is converted into RNA by a process referred to as transcription. Those RNA molecules (messenger RNA) that encode information for protein synthesis are converted to proteins by the process of translation.

citations above) suggests that the overall system is extremely complex, with many degrees of "openness" existing.

One of the examples of this complexity is manifested via the extent and variety of modifications that can occur to DNA itself and its associated histones (positively charged proteins that are responsible for maintaining chromosome structure). These modifications include DNA and histone methylation at a number of positions, acetylation, phosphorylation, and ubiquitination of histones (Kanka 2003; Quivy et al. 2004; Cheung and Lau 2005; Fuks 2005; Verschure et al. 2005). Although histone modification seems to be important for fully appreciating the complete range and stability of regulation possible as well as the subtleties of the system, the methylation state of the DNA is central to the epigenetic regulation of gene expression. DNA methylation has been the subject of considerable research (reviewed by Holliday 2005, Scarano et al. 2005), as scientists begin to understand its role in gene regulation. The bulk of the discussion of epigenetics in this chapter centers on DNA methylation primarily because, to date, most of the studies of epigenetic changes in animal clones examine changes in methylation states.

DNA methylation refers to the addition of methyl groups to the 5 position of cytosine, a non-coding portion of the nucleoside, to regulate the appropriate expression of genes (see Figure IV-1). Methylation tends to occur in areas of the chromosome that are rich in sequences that contain stretches of repeating cytosine-guanosine residues (CpG islands), which tend to be positioned at the 5' ends of genes.[28] Most of these regions are unmethylated regardless of developmental stage, tissue type, or gene expression level. DNA methylation in somatic cells is generally faithfully restored at each replication cycle (for dividing cells), although changes in methylation levels are often associated with aging, or occur in abnormal cells (Bird 2002, Jaensich 2004). Methylation may affect gene transcription by physically impeding the access of cellular transcriptional machinery to coding regions, or by attracting proteins that bind specifically to the modified CpG sequences, thus impeding the transcriptional machinery (Cezar 2003).

Figure IV-1: Cytosine and 5-methyl cytosine Addition of a methyl group at the 5 position of the base is shown.



[28] The 5' end of a gene is often considered to be at the start of the coding sequence on the DNA molecule. The nomenclature is derived from the position of a hydroxyl group in the deoxyribose sugar ring at the beginning of the strand of the DNA.

Mammalian embryos experience major epigenetic reprogramming primarily at two times in their development, both of which have significant implications for cloning. One of these takes place soon after fertilization, and is referred to as preimplantation reprogramming; the other occurs during gametogenesis (the development of cells that ultimately become the sperm and egg). Because preimplantation reprogramming occurs after fertilization, and in the case of nuclear transfer, after fusion of the donor nucleus with the oöplast, it is the most immediately impacted by the cloning process, and may be most directly implicated in the anomalous development of clones with defects. Gametogenic reprogramming may also be involved in the anomalies noted in clones, but it likely has more far-reaching implications for progeny, because it generates the gametes used for the sexual reproduction of clones, (although, by definition, the absence of gametogenic reprogramming in the somatic cell donors used for SCNT poses a high biological burden for the preimplantation reprogramming (Jaenisch et al 2004)). Most of the literature on epigenetic dysregulation in clones and animals produced using other ARTs addresses preimplantation reprogramming; the literature on gametogenic reprogramming often evaluates endpoints related to the sexual reproduction of clones (Yamazaki et al. 2003).

1. Preimplantation Reprogramming

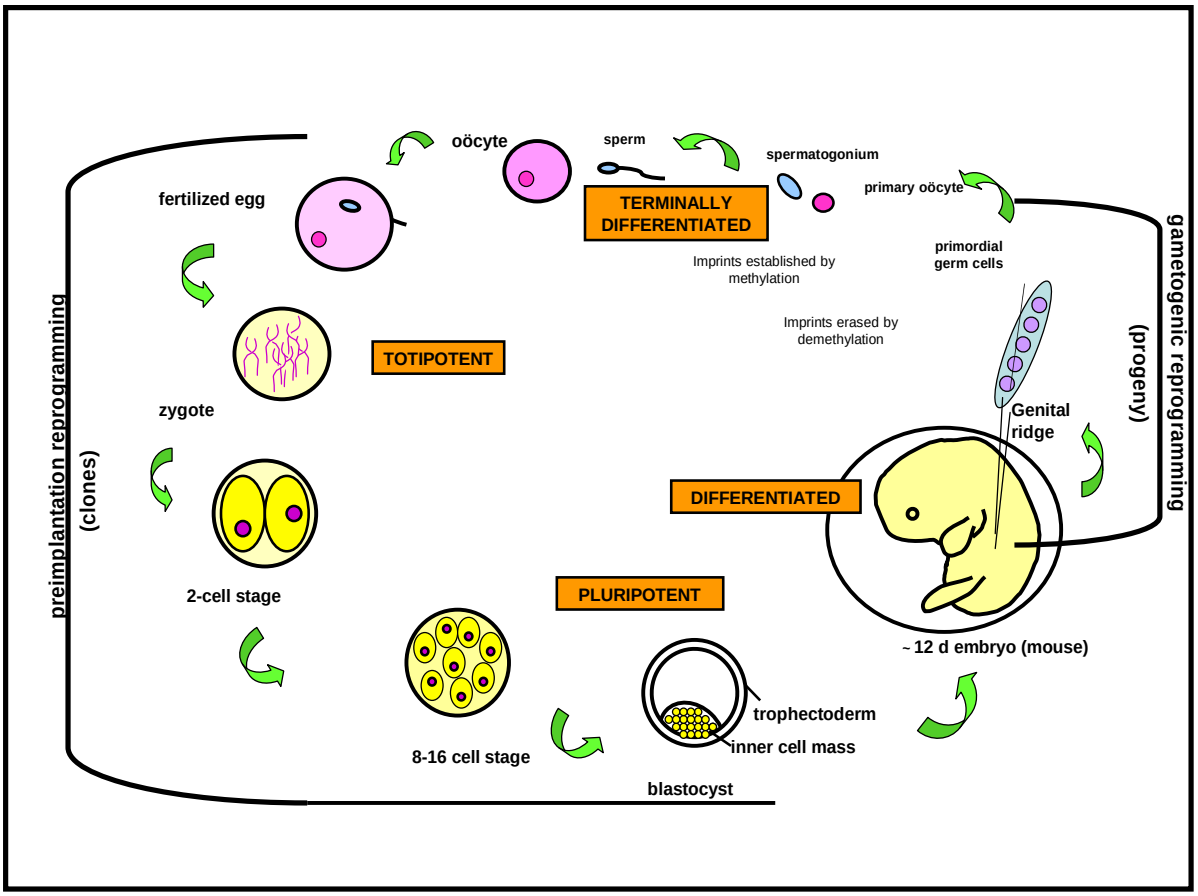
a. Fusion and Cleavage

In sexual reproduction, mammals use cells of highly different morphology and function to deliver haploid genomes. Sperm are small relative to the oöcyte, and package their highly condensed DNA by tightly coiling the DNA around a set of proteins called protamines. The oöcyte's genome is packaged more loosely around a different set of proteins called histones, also found in somatic cells (Cezar 2003). In order for the embryo to form a unique genome, the two chromatin structures must be resolved into one that is capable of coordinated gene expression. A number of factors present in the oöplasm of the ovum, only a few of which have been identified, are thought to aid in this remodeling (Kang et al. 2003a; Chen et al. 2006; Fulka and Fulka, 2007). In the first hour after fertilization, the sperm head swells, the nuclear envelope of the sperm breaks down, and protamines are replaced with histones (Santos and Dean 2004). The chromatin then decondenses, and the male pronucleus[29] forms (Mann and Bartolomei 2002). The female genome completes its second meiotic division, expels the resulting polar body, and then forms the maternal pronucleus. Both the male and female pronuclei begin to replicate DNA, and depending on the species, some transcription may ensue. In mice, transcription occurs in the male pronucleus in the first cell cycle, followed by a larger burst in the second cell cycle (Aoki et al. 1997), while in bovine embryos, transcription is delayed (Mann and Bartolomei 2002).

[29] The pronucleus is the structure that contains the haploid genome of the sperm or ovum after fertilization occurs, but before they fuse to make the nucleus of the zygote, or the single-celled diploid organism. Once the zygote has undergone the first division (or cleavage), it is referred to as an embryo.

During SCNT, however, different initial events must take place. SCNT begins with the removal of the nucleus of the oöcyte that contains the meiotic metaphase II chromosome-spindle complex, followed by microinjecting or fusion of the donor cell or nucleus into the enucleated oöplast. The presence of oöcyte factors probably causes the breakdown of the nuclear envelope of the donor cell (similar to swelling and breakdown of the sperm head). Following oöcyte activation (usually by electrical stimulation), the chromatin from the donor nucleus decondenses, and a pseudopronucleus is formed. If successful, DNA replication and cellular division follow.

Figure IV-2: Epigenetic Reprogramming and Embryonic Development



Legend to Figure IV-2. *The top of the diagram illustrates the terminally differentiated sperm and egg (oöcyte). These gametes fuse to form the fertilized egg or zygote and begin preimplantation reprogramming (emphasized by the bracket at the left). Following the steps counter-clockwise around the figure sequential cell divisions are illustrated with corresponding differentiation from totipotent through pluripotent to differentiated. The right half of the figure represents gametogenic reprogramming (emphasized by the bracket at the right), the epigenetic marking of the primordial germ cells that will become the sperm or eggs of this new individual at sexual maturity and setting up another cycle.*

Information from either terminally differentiated gametes (fertilization-derived zygotes) or a terminally differentiated somatic cell (in SCNT) must be reprogrammed so that the resulting zygote is totipotent (capable of developing into any cell type). Totipotency appears to be lost early in development and almost certainly after the blastocyst is formed, when the trophectoderm and inner cell mass begin to separate (see Figure IV-2). At this point in development, the cells are pluripotent—no longer capable of being *any* cell type, but retaining the ability to become *many* cell types. The end process is referred to as "terminal differentiation," in which cells acquire a set of characteristics that allows them to perform a specific function (*e.g.,* muscle cells contract, neurons transmit electrical pulses, and gametes serve as genome donors for subsequent generations). One of the ways that this overall process is accomplished is by resetting the epigenetic marks of the gametes. At this time, the signals that determine "lineage allocation" are not clear. Fujimori et al. (2003) have noted that each of the two cells in the early blastomere is completely totipotent; some lineage bias is observed when the developmental potency of four-cell stage blastomeres is evaluated. Cells inside the eight and 16 cell stage of the morula appear to be more likely to become committed to the inner cell mass lineage (which becomes the embryo), while those outside appear to be directed to the trophectoderm and the development of placental tissues (Morgan et al. 2005).

The next sections provide an overview of events as they are understood in the development of fertilization-derived embryos, followed by examples of observations noted in clones and, when available, other ARTs with significant *in vitro* culturing components. The examples are intended to be illustrative and not comprehensive, as an encyclopedic review is beyond the scope of this discussion. The important points to be made are that

- Mechanisms of epigenetic reprogramming are complex and not fully understood, even in "normal" fertilization-derived embryos;
- SCNT-derived embryos often do not develop normally, and all the available evidence indicates that this is due to incomplete or inappropriate epigenetic reprogramming;
- Genomes are "plastic" and can accommodate some errors in epigenetic reprogramming, regardless of whether those embryos are derived via fertilization or nuclear transfer; and that
- Some SCNT-derived embryos go onto full gestation and delivery; clones that are born can range from those exhibiting some epigenetic dysregulation to no detectable differences.

Some have suggested (Wilmut 2002b, Jaenisch 2004) that no clone is completely "normal" with respect to its epigenetic profile. Although this is an important point for assessing the overall safety of the cloning process for any particular species, and for determining risk to animals involved in the cloning process, the relevance of "epigenetic normality" to food consumption

risks is unclear. This is particularly true when considering the degree to which epigenetic changes are observed in other ARTs with a significant *in vitro* culturing component, and the accumulation of epigenetic changes expected during the aging process. The most compelling conclusions that can be made about food consumption risks are drawn from assessments of the health status of the animals and the composition of food products derived from them, and not from gene expression studies.

b. Demethylation and Remethylation in Early Embryos

Dean et al. (2001) and Morgan et al. (2005) have outlined how the process of demethylation and epigenetic resetting occur in various mammals. Hours after fertilization, but prior to DNA replication and cleavage, the paternal genome of mice, rats, pigs, cattle, and humans, but not sheep, is actively stripped of the epigenetic methylation markers by mechanisms not fully understood, but that likely require the activity of a demethylase enzyme present in the oöcyte (Morgan et al. 2005). This genome-wide methylation erasure appears to be conserved among cattle, swine, and rats, but is not observed in sheep (Wilmut et al. 2002; Beaujean et al. 2004; Young and Beaujean 2004). In mice and cattle, the maternal genome retains its methylation markers during this period, and does not undergo demethylation until the zygote undergoes the first cleavage to yield the two-celled embryo. Demethylation of the maternal genome is thought to be passive, that is, diluted by the lack of remethylation on newly replicated DNA (Cezar 2003). In two to eight cell bovine embryos, Dean et al. (2001) observed a further reduction in methylation, consistent with the passive demethylation occurring during DNA replication seen in the mouse. In contrast, mouse 16-cell embryos continued to remain demethylated, and genome-wide *de novo* methylation did not occur until approximately four cell divisions later, and appeared to occur preferentially in the inner cell mass (ICM). Thus, although the overall process of demethylation and *de novo* methylation appears to be conserved in the species evaluated, the timing of these phenomena may differ among species (Morgan et al. 2005). The more important observation, however, is that the first differentiation event in mammalian embryos (the differentiation of the trophectoderm and ICM and the resulting loss of totipotency of the ICM cells) is accompanied by genome-wide *de novo* methylation.

Fertilization-derived bovine embryos begin to demonstrate global genomic *de novo* methylation in the eight- to 16-cell stage, what is often referred to as the maternal to embryonic transition (MET). During this time, the developmental program that is initially directed by components within the egg (maternal) is replaced by a new program directed by the expression of new genes (Wrenzycki et al. 2005), and is accompanied by different rates of demethylation of maternally and paternally derived genes to give rise to a new methylation pattern for the embryo.

Although the early demethylation described above is global (occurring over the entire genome in general), methylation marks on imprinted single copy genes tend to be protected from demethylation so that parental imprints are preserved in the resulting somatic cells of the developing mammal (Li E 2002). It is unknown whether the extensive global demethylation of the genome during pre-implantation development is essential for normal development.

DNA-methylation patterns unique to the developing mammal are established in the embryo after its implantation in the uterus through lineage-specific *de novo* methylation that begins in the inner cell mass. DNA methylation increases rapidly in the primitive ectoderm, which gives rise to the entire embryo. Conversely, methylation is either inhibited or not maintained in the trophoblast and the primitive endoderm, from which the placenta and yolk-sac membranes develop, respectively (Li E 2002). The net effect is that extra-embryonic tissues appear to have a lower methylation state than embryonic tissues. These global differences in methylation status between the embryonic and extra-embryonic tissues appear to be conserved in mice, cattle, sheep, and rabbits (Morgan et al. 2005).

Reprogramming the donor nucleus in SCNT or the nucleus of the early fertilized embryo has been the subject of considerable investigation over the past few years. Much of this research has been summarized in reviews by Rideout et al. 2001; Jaenisch et al. 2002; Mann and Bartolomei 2002; Cezar 2003; Han et al. 2003; Jouneau and Renard 2003; Smith and Murphy 2004; Young and Beaujean 2004; Wrenzycki et al. 2005; Armstrong et al. 2006; and Eilertsen et al. 2007.

These reviews and the studies contained in them have come to approximately the same conclusions: although some clones may develop into healthy animals, the low success rate of SCNT is likely associated with the inability of clones to reprogram the somatic nucleus of the donor to the state of a fertilized zygote. Similarly, the rates of successful embryo formation resulting in term gestation and live births in ARTs that have a high degree of *in vitro* culturing are likely also due to difficulties in reprogramming (Gardner and Lane 2005, Wrenzycki et al. 2005). The sources of the stresses on the embryos that cause these difficulties may be a reflection of the intrinsic biological differences between fertilization- and nuclear transfer-derived embryos (e.g., the need to fully reprogram a differentiated nuclear donor), or technological (e.g., the *in vitro* environment in which early embryos are cultured prior to introduction into the uterus). The following discussion briefly outlines the current state of knowledge of how this is accomplished in fertilization- or nuclear transfer-derived embryos.

In embryos derived via nuclear transfer, epigenetic modification, such as the waves of demethylation and *de novo* methylation observed following fertilization must also occur, but may be hampered by both the nature of the donor DNA and the partially depleted oöplasm. There are reports of both aberrant and “normal” demethylation and remethylation in clones and

fertilization-derived embryos. Differences may be reflections of different methodologies, source cells, species differences, or may reflect unexplained phenomena. The following discussion summarizes the key observations that contribute to the body of knowledge regarding epigenetic remodeling in SCNT- and other ART-derived embryos.

Some species-specific responses in the degree of methylation reprogramming have been observed, although in general, the overall processes appear to be relatively conserved among the clones of different species. Dean et al. (2001) found that somatic nuclei of mouse, rat, pig, and bovine embryos undergo the genome-wide reprogramming described previously, but that reprogramming occurred aberrantly in many cloned preimplantation embryos. Bourc'his et al. (2001), using a similar method, did not observe active demethylation in bovine SCNT zygotes, although they did observe that the somatic pattern of methylation from donor nuclei was preserved through the four cell stage.

Ohgane et al. (2001) compared the methylation status of CpG islands (CG-rich sequences located at promoter regions) in placenta and skin cells of sexually reproduced mice to similar regions in normal-appearing mouse clones. Most of the methylated regions in fetal clones (99.5 percent in the placenta and 99.8 percent in the skin) were identical to those of the controls, but different methylation patterns were observed in the two different tissues. The sites of discordant methylation were located in regions responsible for expression of tissue-specific genes, despite the absence of grossly observable abnormalities. In bovine preimplantation embryos, however, Kang et al. (2001a) noted that bovine clone embryos failed to demethylate satellite regions of the genome (certain repetitive sequences), and instead maintained methylation levels similar to the donor cell. In a subsequent study, however, Kang et al. (2001b) were able to "rescue" the inefficient demethylation of bovine embryos by providing an additional "dose" of oöcyte factors to the early embryo. This work confirms the presence of an active element in the oöcyte for erasure of paternal epigenetic methylation, and implies that this component, which may be removed or diluted during the process of preparing an enucleated oöplast, is involved in the appropriate epigenetic modeling observed in zygotes and early embryos derived from fertilization. In a third study, Kang et al. (2001c) investigated demethylation in swine clone embryos relative to those derived by *in vitro* fertilization (IVF). They observed that, unlike mice and cattle, the sequences investigated (centromeric satellite DNA) were negligibly methylated in swine oöcytes, and hypermethylated in swine sperm. (Sperm satellite DNA sequences in cattle and mouse tend to be undermethylated.) The satellite sequences of the donor pig fibroblast cells were hypermethylated, and retained that status until the 4-8 cell stage. Demethylation began at that time, and the methylation status of the clone embryos decreased significantly in the blastocyst, just as it did in the blastocysts of *in vitro* or *in vivo* fertilization-derived embryos. Their finding thus indicated that satellite sequences of SCNT-derived pig embryos undergo preimplantation demethylation in a manner similar to fertilization-derived embryos. Analogous

results were observed when another sequence, PRE-1 (from the euchromatin) was evaluated. These results are comparable to the pattern observed in mouse embryos by Dean et al. 2001.

Whether the results obtained from these two DNA sequences studied by Kang et al. can be extrapolated to global DNA methylation or other single-gene sequences in the pig remains unknown. Additionally, the reasons for the interspecies differences between mice and pigs on one hand, and cattle on the other, also remain unknown. Nonetheless, one of the key implications of these observations is that global demethylation soon after fertilization appears to be a prerequisite for successful reprogramming later in embryonic development, and possibly for successful SCNT outcomes.

Kang et al. (2003) have also demonstrated that at least some SCNT-derived bovine embryos are capable of normal remethylation during early embryogenesis. They evaluated the methylation status of a 170 base pair fragment of single copy gene in IVF and SNCT-derived bovine embryos. This sequence is negligibly methylated in both sperm and oöcyte DNA, and moderately (approximately 37 percent) methylated in the fibroblasts that served as nuclear donors for SCNT. In single celled zygotes, as well as the 4-to 8-cell stage embryos derived via IVF, the low methylation levels of the sperm and oöcyte genomes were observed. No significant changes in methylation status of the IVF-derived embryos were observed at the 8-16 cell stage, but by the time a blastocyst had formed, *de novo* methylation appears to have taken place. In SCNT-derived embryos, the methylation pattern of the donor cell was nearly completely lost by the 4-8 cell stage, and demethylation appeared to be complete by the 4-8 cell stage. At the blastocyst stage, the methylation stage of the SCNT-derived embryo was exactly that of the IVF blastocyst, with the same CpG sites exclusively methylated in both sets of embryos. The authors claim that this study is "the most elaborate example of recapitulation of normal embryonic process[es] occurring in [SC]NT embryos." Although this study clearly demonstrates the ability of somatic cells to be epigenetically reprogrammed in an accurate manner relative to an IVF comparator, and that these molecular results are consistent with observation of apparently healthy and normal animal clones being generated from somatic cell donors, the predictive value of this particular gene for other single copy genes, or the entire genome has not been demonstrated.

In another study showing differences in methylation states among species, Beaujean et al. (2004) evaluated the global methylation status of fertilized and SCNT-derived sheep embryos. They observed that unlike mice and cattle, sheep oöcytes do not appear to demethylate the sperm-derived pronucleus after fertilization. *In vivo*-derived sheep embryos demonstrated that a partial demethylation of the global genome occurred up to the 8-cell stage, with similar qualitative findings in SCNT-derived embryos (fibroblast cell nuclear donor), but to a lesser extent. Interestingly, between the 8-cell and blastocyst stages, both *in vivo*- and SCNT-derived embryos

showed comparable overall levels of methylation, but the distribution of methylation patterns differed among the SCNT-derived embryos and between some of the SCNT-derived embryos and those derived from fertilization. The authors attributed these differences to differences in the overall high-order chromatin structure, rather than simply to changes in methylation. They suggested that many SCNT-derived embryos do not undergo the rapid reorganization of the DNA prior to first cleavage that successful *in vivo*-derived (and a small proportion of SCNT-derived) zygotes do. Further, they suggest that perturbations in methylation (and possibly remodeling) correlate with the lack of appropriate trophectodermal development and subsequent placental development in later embryos and that these alterations may contribute to the high observed levels of placental defects and embryonic loss during SCNT-pregnancies. Beaujean et al. conclude that although "DNA methylation appears to be marker of reprogramming in all mammalian species examined to date, it is not yet clear to what extent it is a determinant."

In a recent attempt to more directly address the role of methylation status in reprogramming, Blelloch and colleagues (2007), used cells in a mouse model system containing an allele (variant) of a DNA methyltransferase (Dnmt1) that has reduced levels of activity. As a consequence of the mutation the cells have reduced DNA methylation. They found that fibroblasts containing the mutation could be reprogrammed into embryonic stem cell lines three times more efficiently than comparators whose DNA was relatively more methylated, consistent with the hypothesis that decreased global DNA methylation yields a less differentiated state.

c. Epigenetic Reprogramming in Later Development

This summary covers studies of epigenetic reprogramming from the fetal through adult developmental nodes (as described in more detail in Chapter VI).

In a study of genome-wide epigenetic reprogramming in bovine clone embryos and adults, Cezar et al. (2003) measured the amount of 5-methyl cytosine in DNA from various tissues in fetuses and term pregnancies generated via SCNT and fertilization. Their results showed that the amount of methylation was lower in spontaneously aborted fetal clones, fetal clones sacrificed as part of the study, and tissues collected from pregnancies that had experienced hydroallantois relative to controls. These results are in contrast to others that have found hypermethylation in clones relative to fertilization-derived controls (Bourc'his et al. 2001; Dean et al. 2001; Kang et al. 2001b). Adult clones, however, had similar levels of DNA methylation as adults derived via fertilization. Cezar et al. (2003) concluded that there may be an epigenetic reprogramming threshold that is met by a subset of animal clones. They also proposed that clones surviving into adulthood have the ability to overcome epigenetic challenges determined by their somatic cell origin. These hypotheses are consistent with the observations by Chavatte-Palmer et al. (2002),

Chapters V and VI, and the Cyagra dataset, described in Appendix E, in which early physiological instabilities appear to resolve as the clones mature.

Similar to Cezar's observations, Chen et al (2005) also noted that aberrant methylation likely plays a role in the poor development noted in clones and other forms of assisted reproduction. In their study, the methylation status of aborted bovine clone fetuses, aborted fetuses generated by artificial insemination (AI), and adult cattle generated via AI or cloning was studied. Three genomic regions were evaluated: a repeated sequence and the promoter regions of two single copy genes (interleukin 3 and cytokeratin). All of the aborted fetuses (AI- and SCNT-derived) were females between 60 and 90 days of gestation; adult animals were all classified as "healthy" and between 18 and 24 months of age. The adult animals all had approximately the same level of methylation at all of the loci examined, regardless of method of production. The aborted AI-derived fetuses all had similar, but lower levels of methylation than the healthy adults, as well as different methylation patterns. For the single copy genes, methylation could be classified into two groups: one group had very low methylation patterns in the promoter regions, while the other group had methylation patterns similar to the aborted AI-derived fetuses. One of these fetuses also showed low methylation patterns in the satellite region. Although this study is not conclusive, it does provide evidence that at least for certain regions of the genome, appropriate methylation appear to be correlated with normal development.

Dindot et al. (2004) developed a unique bovine hybrid interspecies model (*Bos gaurus* x *Bos taurus*) to study epigenetic markings and imprinting in gestation day 40 female SCNT-derived fetuses and placentae (derived from cumulus cell donor cells) that were genetically identical to fetuses derived by fertilization. Previously, Hill et al. (2000b) had shown that more than 80 percent of bovine clone pregnancies were lost between days 30-60 of gestation, and attributed the losses to placental anomalies including a reduction in the number of expected cotyledons and a decrease in chorio-allantoic blood vessels. These observations were similar to those of Stice et al. (1996) who reported that no placentomes had developed in NT fetuses that died between gestation days 33-55. Mouse clone pregnancies have also shown increases in placental size (Tanaka et al. 2001). These abnormalities have been hypothesized to arise from anomalies in nuclear reprogramming of the trophectoderm, which gives rise to placental structures including the chorion. By using the hybrid *Bos gaurus/Bos taurus* model, Dindot et al. were able to discriminate between parental alleles by following single nucleotide polymorphisms (SNPs) (changes in the nucleotide sequence of the DNA at only one site that allow for the cleavage or the lack thereof by enzymes that recognize specific DNA sequences). In particular, three genes associated with epigenetic reprogramming were selected including *IGF-2*, Gene trap locus 2 (*GTL2*), and the X chromosome inactivation specific transcript (*Xist*). Clone fetuses and placental tissues were isolated from surrogate dams at gestation day 40; none of the clone placentae developed cotyledons, unlike the fertilization-derived fetuses, which had 4, 16, and 25

cotyledons per pregnancy. Although appropriate allelic expression of *IGF2* and *GTL2* relative to fertilization-derived fetuses was observed in both chorionic and fetal liver tissues of the clones, disruptions of genomic imprinting of the *Xist* locus was found in the chorion, but not the liver tissues of any of the clones. Further analysis of two other regions of the genome in the chorion of the clone fetuses, the satellite I repeat element and epidermal cytokeratin promoter, indicated that the trophectoderm-derived tissues of the clones had higher levels of methylation relative to fertilization-derived controls. No differences in methylation levels were observed in the livers of clones or fertilization-derived embryos. In this study, at least, there were differences in the degree of epigenetic reprogramming between ICM-derived tissues (the fetus proper) and those derived from the trophectoderm, consistent with the observation by Hill et al. (2000b), that clones with aberrant placental structures can survive gestation and be born alive and apparently healthy.

To understand epigenetic reprogramming over the lifetime of mice and to identify differences between the methylation status of animal clones and sexually derived comparators, Senda and colleagues (2007) have extended their previous studies (Ohgane et al. 2001) using a system that allows them to simultaneously examine ~2,000 methylation sites. In this study, they compared the methylation patterns in kidney tissues of newborn, adult (8-11 months old), and aged (23-27 months old) mouse clones with age matched comparators. The study also includes up to third generation sequential clones (animal clones that were generated with SCNT of nuclei from animals that are themselves clones).

This study had three main findings. In the first, the authors determine that the methylation patterns for newborn mouse clones differed from age matched comparators in only three of ~2,000 methylation sites, indicating that these clones did not differ markedly from comparator mice derived from IVF. The second finding indicated that the differences in the methylation pattern between clones and sexually reproduced comparators disappeard by 23-27 months of age. Clones at the intermediate time point measured (11 months) differed from their sexually reproduced counterparts in only one point of the methylation pattern. Finally, sequential cloning (that is, using a cell from a clone as a nuclear donor for a subsequent round of cloning), did not appear to either increase or perpetuate differences in methylation patterns in this study.

Although the extent to which these data from this mouse model system is a compelling indication that the epigenetic dysregulation assumed to be responsible for adverse outcomes in clones may indeed resolve with age. These results may also be useful for observation that although animal clones are at greater risk for health problems at birth, those that reach adulthood are often healthy (See Chapters V and VI).

d. Studies of Gene Expression and Development in Clones and Animals Produced by Other ARTs

The previous sections summarized studies of alterations in methylation associated with cloning; the following section summarizes reports of gene expression and phenotypic observations in similar clone populations. The overview is intended to be more illustrative than comprehensive as the literature on this subject is large and growing rapidly. The studies indicate that for non-viable clone embryos, fetuses, or neonates, key genes are inappropriately expressed. In some cases, viable clones have differences in expression compared to fertilization-derived counterparts, leading investigators to speculate that genomes are plastic and that a certain level of gene dysregulation can be tolerated. In other studies of healthy, live clones, no significant differences can be observed between the expression profiles of animals generated via SCNT or other fertilization-based ARTs. Finally, it should be noted that studies comparing embryos generated via various ARTs (including SCNT) with significant *in vitro* culturing components, appear to be sensitive to the culture environment, with developmental success often being a function of the culture medium used.

Most of the earliest studies of gene expression in clones were performed in mice. Boiani et al. (2002) and Bortvin et al. (2003) evaluated patterns of gene expression in mouse blastocysts derived from SCNT to identify which critical genes were involved in the inability of most of those blastocysts to develop further. In particular, they evaluated the expression of *Oct4* and *Oct4*-related genes in these embryos. (*Oct4* is a transcription factor specifically expressed in stem and primordial germ cells, and appears to be required for maintaining pluripotency and the self-renewal ability of stem cells.) Boiani et al. (2002) compared *Oct4* expression in blastocysts cloned from somatic cell nuclei and germ cell nuclei to that observed in synchronous blastocysts produced by IVF and intracytoplasmic sperm injection (as the control groups independent of cloning but involving micromanipulation). Their results demonstrated that mouse blastocysts derived from clones had abnormal *Oct4* expression, and that the failure of mouse clones embryos to develop beyond the blastocyst stage was related to incorrect lineage determination by the inappropriate expression of *Oct4*. Bortvin et al. (2003) identified 10 candidate genes with expression patterns similar to *Oct4* and compared their expression in preimplantation embryos derived from fertilization to embryos whose SCNT donors were somatic cumulus and pluripotent embryonic stem cells. They demonstrated that successful reactivation of the full set of 10 genes correlated with the development of embryo clones, but also noted that almost 40 percent of the cumulus cell-derived blastocysts failed to reactivate these genes faithfully, even though the blastocysts were morphologically normal. Thus, some other factors were required to maintain the pluripotency of the inner cell mast cells. Marikawa et al. (2005) found that the DNA methylation status of the *Oct4* regulatory element in mouse embryos directly influences the level of gene expression. They further noted that the methylation status of the *Oct4* regulatory element was

highly heterogeneous among alleles in a population of adult somatic cells, and hypothesized that that the degree to which *Oct4* can be reactivated in SCNT may be a function of the methylation status of the donor cell(s).

Boiani et al. (2005) further evaluated *Oct4* expression in early post-activation SCNT-derived zygotes, fertilization-derived early embryos and parthenotes in six different culture media. (Lack of expression of *Oct4* precludes further development beyond the blastocyst). Among their first observations was that similar to fertilization-derived embryos, progression to blastocyst did not ensure further development of the embryos, and that some of the primary influences on whether such development occurred could be environmental in origin. They also noted that nuclear transfer embryos appeared to be more sensitive to environmental conditions than the other two types of embryos. They concluded that not only was the ability of mouse clone embryos to progress through development contingent on the nature of the donor nucleus and recipient oöplasm, but that culture conditions could have a significant impact on the expression of key genes required for reprogramming (and subsequent development), and the ability of the blastocyst to continue to develop successfully.

To study the correlation between gene expression, survival, and fetal overgrowth (*e.g.,* LOS-type symptoms), Humpherys et al. (2001) examined imprinted gene expression in mice cloned by nuclear transfer and in the embryonic stem cell donor population from which they were derived. They determined that transcript levels of selected imprinted genes varied widely in placentae from animal clones relative to non-clones, although alterations in the expression of one imprinted gene did not correlate with abnormal expression of other imprinted genes. They also observed that changes in DNA methylation levels at one imprinted locus did not necessarily predict changes at other loci. Certain genes (*e.g., H19* and *Igf2*) were largely silenced in the heart and kidney, and their expression reduced in the livers of animal clones relative to conventional animals. No correlations were observed between changes in gene expression and birth weights, placental weights, or neonatal mortality. Culturing the embryonic stem cells *in vitro* resulted in highly variable levels of gene expression; gene expression in the animals resulting from those cells was even more variable than in the cells in culture, implying that culturing early embryos may contribute to the degree of embryonic gene dysregulation. Furthermore, mice derived from the cells of the same cellular lineage differed in their expression of imprinted genes. Given that viable animals were generated with variable expression of imprinted genes, the authors concluded that "mammalian development may be rather tolerant to epigenetic abnormalities and that lethality may only result from the cumulative effects of a stochastic loss of normal gene regulation at multiple loci....even apparently healthy animal clones can have gene expression abnormalities that are not severe enough to impede development to birth but that may cause subtle physiological abnormalities which could be difficult to detect." The degree to which such subtle abnormalities could exist within conventional populations of animals is not discussed.

Humpherys et al. (2002) evaluated expression of more than 10,000 genes in the placenta and liver tissues of mouse clones derived from embryonic stem cells and cumulus cells using microarray analyses. The expression of 286 genes was altered in clones derived from cumulus cells compared to the fertilization controls, with a similar level of altered expression detected in the embryonic stem cell-derived clones. The general concordance in the expression differences between the mouse clones from different donor cell types suggested to the investigators that most of the expression abnormalities were common to all placentae of mouse clones rather than specific to animals derived from one particular cell type. Consistent with their previous summary (2001), the authors concluded that differences in gene expression, even those that are highly variable, may be tolerated during differentiation and even in clones that survive.

Several groups have recently extended this type of microarray technology to analysis of bovine embryos derived via nuclear transfer and other ARTs (Smith SL et al. 2005; Herath et al. 2006; Beyan et al, 2007a). Although there are procedural differences among the studies, the results are complementary and consistent. They include the observations that the gene expression patterns in cell lines from which embryos were derived were strikingly different from the patterns observed in the resulting embryos. In addition, gene expression patterns in the embryos were relatively similar overall regardless of the cell type and methods used for derivation of the embryos. Finally, a small fraction of the overall number of messages examined was differentially expressed in embryos derived using different methodologies. Similar to the studies using mouse embryos discussed above, these studies show that significant reprogramming also occurs in bovine embryos. These studies are also consistent in that the reprogramming is currently imperfect and there are a number of messages that are differentially expressed in SCNT derived embryos compared with embryos derived using other ARTs. Additionally, numerous studies have shown that many SCNT embryos will not reach term. The embryos used in these studies were early in gestation, so we do not know if the expression differences reflect significant abnormalities or tolerable variation. However, these studies are useful because they contribute to a better understanding of both development and technical approaches to improving SCNT technology.

Sebastiano et al. (2005) noted that in single cells derived from early preimplantation embryos of mice developed via SCNT and *in vitro* fertilization, a series of genes important to appropriate embryonic development began transcription at approximately the same time in both types of embryos. Different levels of expression, however, were found in the nuclear transfer-derived embryos, particularly as the embryos progressed through development. They concluded that reprogramming was initially quickly shifted towards embryonic development, but that reprogramming was incomplete and inaccurate, particularly in the latest stages of preimplantation.

Several studies have attempted to determine whether the expression of any particular gene(s) could be used as a marker to determine the developmental success of embryos produced via SCNT or other ARTs. Camargo et al. (2005) evaluated differences in gene expression in individual preimplantation bovine embryos produced via SCNT (same donor cell line), *in vitro* fertilization (IVF), or *in vivo* derived embryos obtained following superovulation, artificial insemination, and harvested, and cultured *in vitro* to reach the same degree of development as the nuclear transfer or IVF embryos. Using real time PCR, they studied a panel of 11 genes (including *Oct4*) preferentially activated at the maternal-embryo transition (~ the 8-12 cell stage in bovine embryos), during which demethylation of parental genes (or donor cell genes) largely has been accomplished and *de novo* methylation, in which transcription of embryonic genes becomes predominant. Also evaluated was the expression of a fibroblast gene expressed in the donor cells to determine whether cessation of expression of donor genes was also appropriate. The results indicated that the expression patterns of the 11 genes common to the IVF and SCNT-derived embryos were virtually indistinguishable. Further, the expression of the donor cell gene was appropriately turned off in the SCNT-derived embryos. Compared to expression levels in the *in vivo* derived embryos, however, all transcripts except one, lactate dehydrogenase, in both the IVF and SCNT-derived embryos were found at lower levels. They attributed the differences in expression between the *in vivo*- and *in vitro*-produced embryos to differences in culture conditions. To support this hypothesis, the investigators noted that the IVF and SCNT embryos exhibited similar variability in expression among individual embryos, but different from their *in vivo* counterparts.

Miyazaki et al. (2005) compared the expression of a different set of genes from SCNT-and intracytoplasmic sperm injection (ICSI)-derived 2-4 cell and blastocyst stage porcine embryos. The genes selected have previously been suggested as candidates as markers for identifying embryos that would successfully develop (Daniels et al. 2000) included two genes from the fibroblast growth factor family, *Xist* (important in X-chromosome inactivation), genes encoding interleukin-6 and its receptor, and *c-kit* ligand (another gene important in early embryonic development). Donor cells for the SCNT-derived embryos came from two different cell lines, with different degrees of success at developing blastocysts. Additionally, SCNT-derived embryos were developed using two different activation protocols. Although the percentage of embryos in which expression of these genes was similar between the SCNT- and ICSI-derived embryos, actual levels of transcripts of two of the genes (*FGFr72IIIb*, one of the fibroblast growth factor genes, and interleukin 6 receptor gene) were lower and higher, respectively, in SCNT-derived versus ICSI-derived embryos in one of the SCNT-activation protocols, while *FGFr72IIIb* and *Xist* transcripts were lower than ICSI-derived embryos when evaluating the other method of SCNT activation. No significant differences in gene expression were noted at these early developmental stages between the two different SCNT donor cell sources. No

comparisons were made to *in vivo* derived embryos. It is not clear whether the differences between the results observed by Miyazaki et al. and Camargo et al. are due to experimental design, species, or the genes assayed.

Both appropriate and inappropriate gene expression have been observed later in the development of fetuses, neonates, or more mature clones. Yang L et al. (2005) used real-time PCR[30] to compare the expression levels of three imprinted genes associated with growth regulation (*Igf2r* and *Igf2)* or imprinting regulation (*H19*) in eight tissues from deceased newborn calf clones, three tissue sources from apparently healthy, genetically identical adult bovine clones, and cattle obtained from a slaughterhouse. The deceased clones all exhibited signs of LOS, and exhibited abnormal and highly variable expression of the genes, despite being produced from one nuclear donor. The decreased levels of expression of *Igfr2* (which inhibit fetal growth) in the deceased clones compared to controls were consistent with the decreased expression of the same gene noted by Young et al. (2001), in LOS sheep clones, but interestingly, these levels were not correlated with increased birth weights of the deceased clones. Expression of the three genes in the healthy clones was largely normal, except for *Igf2* in the muscle tissue of adult clones, which was found to be highly variable, although lower than the reported levels for the newborn controls. These results are consistent with the idea that significant dysregulation of imprinted genes results in embryonic or neonatal death, but that those animal clones surviving to adulthood can be epigenetically similar to control animals.

A similar study, this time using real-time PCR to study the expression of IGF binding proteins and IGF receptors in Japanese Black beef bovine embryos derived using via SCNT, BNT, IVF, and *in vivo*, production was performed by Sawai et al. (2005) to determine whether the rates of developmental failure seen in nuclear transfer embryos could be related to changes in this complement of genes. The results indicated that the amounts of *Igf-1* receptor mRNA did not differ significantly among the types of embryos; in contrast, the amounts of mRNA of the Igf-2r differed depending on how the embryos were derived. In general, the proportion of embryos exhibiting Igf-2r receptor mRNA was more variable in embryos derived via SCNT. This is in contrast to the observations of Wrenzycki et al (2001), who found that the levels did not differ significantly. There are multiple reasons to account for the differences including the source of donor cells, their cell-cycle, etc. Heyman et al. (2002a) also reported that there were no significant differences in the proportion of embryos expressing IGF-2r transcripts among nuclear transfer, IVF and in vivo produced embryos. These apparently contradictory observations serve to imply that it is not some process associated with cloning per se (e.g., electrical pulses for cell fusion or other treatments) that adversely affect development, but rather provide further evidence

[30] Real-time PCR is a technique that allows for the rapid and precise identification and quantification of genetic material (in this case, RNA) during the actual time that the reaction is running.

that epigenetic dysregulation (i.e., failure of appropriate gene expression) that appears to be responsible for growth dysregulation.

Li S et al. 2005 also used real time PCR to compare expression levels of eight developmentally important genes in six organs from bovine clones that within 48 hours of birth relative to control animals produced by artificial insemination and also slaughtered within 48 hours of birth. Organs that were evaluated included the heart, liver, kidney, spleen, lung, and brain. Aberrant and highly variable gene expression in the clones occurred in a tissue-specific pattern, with the heart most (five of eight genes), and the kidney, least (two of eight genes) again indicating the role of gene expression in the ability of particular tissues and organs to develop appropriately in clones. They also noted that organ systems could be affected independently of others, implying a stochastic process at work. No mention was made of whether a similar study had been performed on live, healthy clone births in this report.

Finally, Archer et al. (2003a) have performed the most comprehensive study of the correlation between epigenetic reprogramming and live clone outcomes in a cohort of female swine clones. (More detailed discussions of the results of this study are found in Chapters V and VI). In addition to evaluating methylation in two different regions of the genomes of these animals and half-sibling comparators, the investigators studied the growth, clinical chemistry, and behavior (Archer et al. 2003b) of these animals. The overall degree of methylation between clones and their half-siblings was the same, with a small random variability in the PRE-1 SINE regions, and one CpG site in the centromeric satellite region. Further, the clones exhibited two patterns in specific phenotypic traits: one set of traits exhibited variability similar to the comparators, and another set showed less variability than the comparators. CpG methylation was measured in PRE-1 SINE (repeat sequence in a euchromatic region) and centromeric DNA (repeat sequence in a heterochromatic region) obtained from skin punch samples. Finally, the clones appeared to have grown and developed normally: no differences were observed between clones and their comparators with respect to growth rates, physiological measures of health, or behavior.

e. Studies of Technical Contributions to Epigenetic Variability in Clones and Other ARTs

In the previous discussion, we have repeatedly referred to the contribution of the methodology used to generate embryos (particularly the culture environment) as playing a critical role in developmental success for cloning and other ARTs. The continuing reports of relatively low efficiency of SCNT have stimulated a wide interest in how a variety of factors affect the efficiency of SCNT in particularly but including various ARTs with an eye to improving the process (Oback and Wells 2007a, Collas and Taranger 2006) particularly, but not exclusively, by improving nuclear reprogramming. A number of factors affecting the status of the cells used for

nuclear transfer seem to be important considerations for improving the efficiency of the process and recent reports identify the morphology, proliferative characteristics, chromosome stability (Giraldo et al. 2006; Mastromonaco et al. 2006), cell type, culture conditions (Bosch et al. 2006; Inoue et al. 2006; Beyhan et al. 2007b), and stage of the cell cycle (Bordignon and Smith 2006) as important considerations for improving efficiency of SCNT.

Others reports are more directed at identifying the technical conditions that influence cloning efficiency with most focusing on improving reprogramming. Several investigators have examined the contribution of the oöcyte to reprogramming in SCNT (Chen et al. 2006, Fulka and Fulka 2007) in the hope of identifying oöcyte factors that increase reprogramming and consequently cloning efficiency. Preparation of the oöcyte (Li GP et al. 2006) as well as timing (Sung et al. 2007) and method (Schurmann et al. 2006) of activation after fusion of the oöcyte and transferred nucleus have been examined for their contribution for the process. Reprogramming may be improved by the addition of exogenous "remodeling factors" such as nucleoplasmin (Betthauser et al. 2006) or even caffeine (Lee and Campbell, 2006). Although all of these studies (which use a variety of species and most of which harvest the embryos before term) identify factors in the process that are important, the efficiency of SCNT remains low.

Two studies by Wolf and colleagues (Hiendleder et al. 2004a, 2006) also provide insight into the relationship between reproductive methodology, methylation status, and fetal characteristics. The 2004 study compares a number of anatomic parameters to the "global" DNA methylation status of a variety of tissues from embryos produced using AI, IVF, and SCNT. In general, they correlate increased DNA methylation in fetal tissues and increased fetal size. In this study, 80 day AI fetuses were smallest and contained the least methylation, IFV fetuses of the same age were intermediate for both traits and clone fetuses, also 80 days old, were largest and had the most DNA methylation. The 2006 study extends these observations by focusing on the culture conditions used for IVF. This latter study demonstrates that the authors can manipulate the culture conditions so that under one set of culture conditions the fetuses are physically comparable to *in vivo* fertilized comparators while other culture conditions result in consistently large offspring as reported for IVF and SCNT derived fetuses as reported in their 2004 study. However, even in the "normal" size IVF fetuses, global methylation status is not identical to the comparators, indicating that further work needs to be performed to identify and characterize key parameters related to fetal overgrowth syndromes.

2. Gametogenic Reprogramming

The development mechanisms involved in gametogenic reprogramming were initially studied most extensively in the mouse; conservation of mechanisms involved in sexual reproduction are

similar in all species examined to date, although the timing of events differs depending on the length of gestation.

Germ cells (those developmentally destined to become gametes) are first detected as founder population cells at about embryonic day (E) 6.5 in the mouse. By E 7.2, approximately 45 primordial germ cells can be counted in the mouse embryo (Hajkova et al. 2002). These cells begin migration into the genital ridge (the portion of the embryo destined to become the reproductive organs) about 10 days after embryo formation (Hajkova et al. 2002, Yamazaki et al. 2003) (See Figure IV-2). Their epigenetic methylation status at this point resembles that of the rest of the embryo: they contain genomic imprints from the maternal and paternal genomes, and one of the two X chromosomes in female gametes has been inactivated in the somatic tissues (Surani 2001). Once the primordial germ cells migrate into the genital ridge (the thickening near the kidneys of the embryo that gives rise to the ovaries and testes), however, profound changes in their methylation status occur. A period of rapid demethylation ensues, in effect "erasing" all of the epigenetic modifications that were present on the cells prior to their migration (Yamazaki et al. 2003, 2005). This demethylation appears to be selective by affecting single copy imprinted and non-imprinted genes (*e.g.,* coding sequences), whereas the reprogramming of repetitive elements (whose function in the cell is not fully understood but is thought to be structural and regulatory) is more protected and incomplete.

In describing this phenomenon, Surani (2001) states that this "mechanism also erases any aberrant epigenetic modifications, so preventing the inheritance of epimutations, which consequently occurs very rarely." The mechanism by which erasure of the epigenetic markings, including demethylation, in primordial germ cells is not yet understood. Other "resetting" mechanisms also occur in primordial germ cells, including the restoration of telomere length, and repair of lesions to the coding regions of the DNA (Surani 2001).

Random X inactivation in XX (female) germ cells also occurs during the migration phase of PGCs, coinciding with the timing of X inactivation in somatic tissues (reviewed by Avner and Heard 2001; Heard 2005). Inactivation of one X chromosome in female mammals is absolutely essential to compensate for the potential doubling of the "gene dosage" that a XX genotype would present. Although not fully understood, the process by which this occurs involves coating one of the X chromosomes by an RNA molecule itself encoded by a gene (*Xist*) on the X chromosome, followed by DNA methylation, and covalent modifications of the histones associated with the inactive chromosome. In mice, X inactivation first occurs in the placental trophoblast cells, where the paternal X tends to be inactivated by a mechanism thought to involve the expression of a maternal gene at the blastocyst stages that exclusively inactivates the paternal X chromosomes in the trophoblast cells. The end result is that the structure of the chromosome is

altered from an active, relatively loosely coiled state to a highly condensed and transcriptionally silent DNA molecule (Avner and Heard 2001).

Restoration of epigenetic modification in primordial germ cells in mice appears to take place several days later when the male germ line appears to acquire methylation at 15-16 days after conception. Remethylation of the female germ line in mice does not appear to occur until after birth during the growth of the oöcytes, and probably continues until the first meiotic division (a stage in the maturation of the cells destined to become gametes in which the chromosome number is reduced from 2n to n) (Davis et al. 2000; Surani 2001). This overall process appears to be conserved in other mammals, although the exact timing may differ according to species.

Although the preceding discussion has focused on methylation as the primary marker of imprinting, it is important to remember that there are other modifications that may contribute to the retention of "epigenetic memory" in germ cells whose identity and mechanism remain to be characterized (Davis et al. 2000; Fazzari and Greally 2004).

3. Mitochondrial Heteroplasmy

In addition to incomplete or inappropriate epigenetic reprogramming, the relatively low success rate of cloning has been hypothesized to be related to changes in the pattern of mitochondrial DNA (mtDNA) transmission following SCNT (Hiendleder 2007; St. John et al. 2005; Spikings et al. 2006). Because sperm deposit very few of their own mitochondria[31] during sexual reproduction, mtDNA in developing embryos tends to come almost exclusively from the oöcyte and tends to be maternally inherited. During the SCNT process, if intact donor cells are used as nuclear donors, following fusion with the enucleated oöcyte, the resulting embryo may have mtDNA from both the donor and recipient cells *i.e.,* mitochondrial heteroplasmy. If the nuclear and mitochondrial DNA originate from different sources, the normal coordination of expression of nuclear and mtDNA may be altered, resulting in altered or impaired energy production in the cell or developing organism.

The extent to which mtDNA heteroplasmy is observed in animal clones is inconsistent (see reviews by St. John et al. 2005; Bowles et al. 2007; Hiendleder 2007). Bowles et al. (2007) reported that donor mtDNA is deleted by normal cellular regulation of mtDNA transmission during embryonic development in both NT embryos and clones themselves. Hiendleder (2007)

[31] Mitochondria are the only organelles in animals that contain their own DNA, and are considered to be the "powerhouses" of eukaryotic cells. They are the site of the electron transport chain, which is the cell's major source of energy. Proteins in the electron transport chain are encoded by chromosomal or mtDNA, necessitating tight coordination and regulation of expression between the nuclear and mtDNA. In sexual reproduction, these interactions, which govern mtDNA copy number, mitochondrial morphology, and the number of mitochondria per cell are tightly controlled to optimize energy production (St. John et al. 2005).

observed that most of the sheep, cattle, and swine SCNT clones investigated appeared to be homoplasmic (have nuclear and mtDNA from the same source) or display only mild heteroplasmy. Hiendleder (2007) further noted that factors involved in the process of nuclear transfer (*e.g.,* embryo culture conditions, the choice of donor cell types, and the quality of the oöcyte recipient) may affect the level of heteroplasmy,

Even if mtDNA heteroplasmy were present at a significant level in clones, the extent to which it could affect clone health or food consumption risk is difficult to determine. Clones may show little or no heteroplasmy, or may have considerable levels of mtDNA diversity but be phenotypically normal (Bowles et al. 2007). Heteroplasmy may result in impaired mitochondrial function and energy production, contributing to the poor success rate of cloning, but the empirical demonstration of that possibility has not yet been proven. Smith LC et al. (2005) noted that "To date, there is no clear indication that heteroplasmy caused by nuclear transfer procedures in farm animals is detrimental to development."

4. Conclusions from Studies of Epigenetic Reprogramming

- Inappropriate or incomplete epigenetic reprogramming is the source of the frank adverse outcomes and subtle anomalies that pose animal health risks in animals developed by SCNT or other ARTs.

- SCNT-derived embryos must demethylate the differentiated and generally relatively highly methylated nuclear donors to restore totipotency. The high rate of failure to progress beyond the early stages of cleavage of SCNT-embryos may be a function of the inability to carry out that demethylation, and likely involves other mechanisms, some of which may involve higher-order chromatin remodeling.

- In studies evaluating the differential reprogramming of trophectoderm- and ICM-derived tissues, more dysregulation is observed in the trophectodermally-derived tissues (placental tissues) than in the somatic tissues derived from the ICM. Whether this disparity is a function of the more stringent requirement of appropriate reprogramming of the ICM-derived tissues for survival (embryos and fetuses with significantly altered epigenetic reprogramming simply do not survive) is not known.

- Live and apparently healthy clones can exhibit some level of epigenetic differences relative to fertilization-derived comparators. Many of these differences appear to resolve as the animals age, consistent with the adaptation observed in clone populations studied for physiological and growth parameters. It is not known whether these animals are

tolerant of these differences, or whether a "threshold" of epigenetic differences exists that has not been exceeded in the live and apparently healthy animals.

B. Phenotypic Evidence for Gametogenic Reprogramming

The initial observations confirming the biological assumption that phenotypic expression of underlying inaccurate epigenetic reprogramming observed in clones disappear in the progeny due to gametogenic reprogramming come from the studies of Shimozawa et al. 2002a and Tamashiro et al. 2003, who demonstrated that a phenotype observed in mouse clones was not transmitted to their progeny. These studies have led to the conclusion that "Progeny of animal clones, on the other hand, are not anticipated to pose food safety concerns, as natural mating resulting from the production of new gametes by the clones is expected to reset epigenetic reprogramming errors that could persist in healthy, reproducing clones" (NAS 2002a). Or stating a similar conclusion *"*. . . epigenetic rather than genetic aberrations are the cause; epigenetic changes, in contrast to genetic changes, are reversible modifications of DNA or chromatin that are usually erased in the germ line*"* (Hochedlinger and Jaenisch 2002). This postulate can be further summarized as: "All epigenetic problems in the parents seem to be erased when cell nuclei go through the germ line" Yanagamichi (2002), and, ". . .the progeny of cloned animals will be normal" Fulka et al. (2004b).

In the following section, the studies that have led to these conclusions, as part of a summary of the utility of the mouse model for estimating risks in livestock clones are reviewed. It is organized by Developmental Nodes, as in the Critical Biological Systems Approach to evaluating the health status of livestock clones, although several nodes are combined to better reflect the existing mouse dataset.

1. Phenotypic Anomalies Observed in Mouse Clones

a. Utility of Mouse Model

Although the subject animals of this assessment are domestic livestock clones, the use of the mouse as a model system provides some insights into the underlying biology of the cloning process and its implications for food safety, particularly for understanding the role of sexual reproduction in resetting residual epigenetic reprogramming errors. SCNT in mice was first reported by Wakayama et al. (1998) using the "Honolulu technique" at approximately the same time as publication of the "Dolly" paper (Wilmut et al. 1997). Since that time, mice have been cloned from a range of cells from embryonic and adult sources (reviewed by Yanagimachi

2002). The mouse model is useful because of its well-characterized genotypes, small size, short generation period, and shorter life span than larger animals.

b. Pregnancy (Developmental Node 1)

The key measure of the success of SCNT is the normal development, maturation, and reproduction of the animal clones. As with livestock, the efficiency of this process in mice is very low, and in the same range as livestock: approximately 0.2-3.4 percent when calculated from the total number of reconstructed embryos resulting in live offspring (Yanagimachi 2002). In mice, the rate of embryo survival is most reduced early in development, particularly in the days immediately before and after implantation (Yanagimachi 2002). Yanagimachi (2002) also found that more than 90 percent of mouse embryos cloned with cumulus cells had normal chromosomal constitutions, indicating that the poor survival rates are not due to chromosomal problems, again pointing to epigenetic reprogramming as the determining factor in cloning efficiency.

Placental enlargement has been observed in almost all of the studies of mouse clones reported to date (Wakayama and Yanagamichi 1999; Humphreys et al. 2001; Ono et al. 2001b; Tanaka et al. 2001; Ogura et al. 2002; Yanagimachi 2002). Tanaka et al. (2001) performed histological examination of term placentae from mouse clones and evaluated the expression of a number of genes relevant to fetal development. Placentae from these animals were larger than from conventional controls, and exhibited histological changes in all three layers of the placenta (*i.e.,* the trophoblastic giant cell, spongio-trophoblast, and labyrinth layers). Most of the anomalies appeared to be related to the expansion of the spongio-trophoblast layer, which exhibited an increased number of glycogen cells and enlarged spongio-trophoblast cells. Despite these morphological changes, there were no critical disturbances in regulation of gene expression in the placentae associated with term clone placentae. Unlike cattle and sheep, in which clone fetuses tended to be larger than comparators, the average weight of the mouse clone fetuses appeared to be lower than that of comparators, suggesting that a "latent negative effect from somatic cell cloning may occur on fetal growth, potentially due to incomplete placental function" (Tanaka et al. 2001). Despite the morphological changes observed in their study, Tanaka et al. (2001) noted that the placentae "could support full development of the fetus, suggesting that their functions are adequate for apparently normal fetal development" similar to the observation of Hill et al. (2000b) for cattle clones.

Both Ono et al. (2001) and Ogura et al. (2002) reported morphological changes in the placenta of mouse clones similar to those observed by Tanaka et al. (2001). Ono et al. (2001) observed that increased placental size was caused by proliferation of the trophoblastic cells, endometrial