THE KNOCKDOWN OF DNMT1 USING SMALL INHIBITORY RNA:

A METHOD TO ASSIST IN THE REPROGRAMMING OF A DONOR GENOME DURING NUCLEAR TRANSFER

By

ALLISON M. ADAMS

(Under the Direction of Steve Stice)

ABSTRACT

Increasing evidence has implicated the incomplete or aberrant reprogramming of donor nuclei as a contributing factor to the observed inefficiencies and outcomes inherent with the current technique of nuclear transfer (NT). The reprogramming of DNA methylation patterns is one of many events essential to convert a differentiated cell back into a totipotent cell using the donor eggs' ooplasm. DNA methyltransferase I (Dnmt1) is the enzyme responsible for maintaining methylation patterns. The somatic isoform of Dnmt1 has been shown to be aberrantly expressed in NT-derived embryos and is implicated in the improper reprogramming of the donor genome. Short inhibitory RNA (siRNA) is capable of post-transcriptionally depleting a cell of a specific gene transcript. Using Dnmt1-specific siRNA, the ability to reduce the supply of Dnmt1 transcripts was tested in murine and bovine primary cells. Results indicate the expression of Dnmt1 was successfully reduced in both cell types.

INDEX WORDS: Nuclear transfer, livestock cloning, reprogramming, genomic imprinting, DNA methylation, Dnmt1, RNA interference, RNAi, small inhibitory RNA, siRNA

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DEDICATION

To Mazzie.
How it all began.

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"The importance of somatic cell nuclear transfer is, without any doubt, beyond the scope of replicating superior animal genotypes. It is an invaluable experimental tool to address fundamental scientific issues such as nuclear potency, cell de-differentiation, chromatin structure and function, epigenetics, and genome manipulation. For these reasons the importance of cloning is not for what it can achieve but for the technical support it can provide to biomedical research and in particular to the study of epigenetics, cancer and stem cell biology, cell therapy and regenerative medicine."

K. H. Campbell
Nuclear equivalence, nuclear transfer, and the cell cycle
Cloning. Vol.1(1), p 3-15. 2001

CHAPTER 1

Livestock nuclear transfer

In animals, reproduction is a natural process occurring by sexual means whereby the female oocyte, or egg, is fertilized by the male-derived sperm. Fusion of these two gametes into a single unit or zygote initiates the development process resulting in the production of genomically unique offspring. However, prior to this occurring, the proper development of the sperm and egg in the parental organisms must take place. This involves the rearrangement of genomic information during meiosis and ultimately gives rise to gametes each with distinct genomes. Upon fertilization this genetic information, in the form of DNA, is contributed both by the sperm (the paternal genome) and the egg (the maternal genome) to produce a unique diploid organism. During development, cells making up the zygote and resulting embryo must grow and divide. Through this process of mitosis, the genome of each cell is meticulously copied whereby each resulting daughter cell contains an exact replica of this novel genome. Thus, offspring created through sexual reproduction are distinct in their genetic make-up from every other resulting offspring from the same parental units.

In contrast, a clone is defined as the process of asexually producing offspring that are genetically identical to a single parental organism – therefore containing a common identical genome. Asexual reproduction is carried out through an *in vitro* process known as nuclear transfer (NT). The technique of NT has an extensive history [4, 5] and was originally proposed over 60 years ago by Hans Spemann as a method to study cellular differentiation in amphibians. Until the mid-nineties, mammalian NT was limited to the use of donor genetic material from early staged embryos [6]. However, in 1997 a breakthrough was achieved when researchers at the Roslin Institute announced the birth of Dolly, the world's first animal derived through

somatic cell nuclear transfer (SCNT) using a cultured adult cell [7]. Since then, SCNT has successfully been used to produce mice [8], rats [9], cows [10], goats [11], pigs [12], rabbits [13], horses [14], a mule [15], a cat [16], an endangered species known as a Guar [17], and a calf produced using cells obtained from a two-day-old beef carcass [18].

The use of SCNT has tremendous applications in a host of fields and is not simply limited to the production of genetically elite farm animals. Since its discovery, NT has become an essential tool for studying gene function [19], gene targeting [20], genomic imprinting [21], genomic reprogramming [21], models for genetic diseases [22, 23], and gene therapy [24]. As a tool, NT may be used to produce transgenic animals for pharmaceutical protein production or to produce suitable organs and tissues for transplant needs (xenotransplantation) [25-29]. Currently, in the United States 87,000 patients are on the wait list for a donated organ with kidney requests reaching an astonishing all time high of 60,000 (Organ Procurement and Transplantation Network (OPTN) Press Release, October 14, 2004). With only a little under 16,000 transplants having taken place thus far this year, the need for alternative organ sources is clearly evident (data obtained from the OPTN website, http://www.optn.org, accessed October 18, 2004). NT also holds enormous potential in the biomedical field for therapeutic cloning and allo-transplantations - the transplant of organs, cells, or tissues between members of the same species [30, 31].

The nuclear transfer procedure

Although involving meticulously performed procedures, the method of SCNT will briefly be summarized – for a review see [32, 33]. Two raw materials are essential to carry out NT: a mature oocyte at the metaphase II stage of meiosis (often called a MII oocyte) and a donor cell – containing the DNA to be cloned. This cell, taken from a donor animal, contains the genome to

be duplicated. In a process called enucleation, all genomic material housed in the oocyte, located in the methaphase plate and polar body structures, is withdrawn and discarded using needle-like glass pipettes (Fig. 1.1a). The donor genome is supplied by transferring a single cell or nucleus, often propagated through cell culture techniques, into the perivitellin space where it lies in direct contact with the cytoplasm (Fig. 1.1b). The cell is then fused to the perivitelline membrane, with a short electrical pulse, followed by the activation of cell division through chemical or electrical means (Fig. 1.1c). The resulting embryo is subsequently transferred into a synchronized surrogate mother or allowed to incubate *in vitro* until the desired development stage is reached (Fig. 1.1b). In cattle, NT-derived embryos are transferred to the recipient at the blastocyst stage of development; whereas in other species, such as the pig, the NT embryos are often implanted into the surrogate mother on the day of reconstruction. The clone, who is identical in its nuclear genetics to the donor animal, is born to its surrogate mother. Depending on species, the nuclear transfer technique will slightly vary [34].

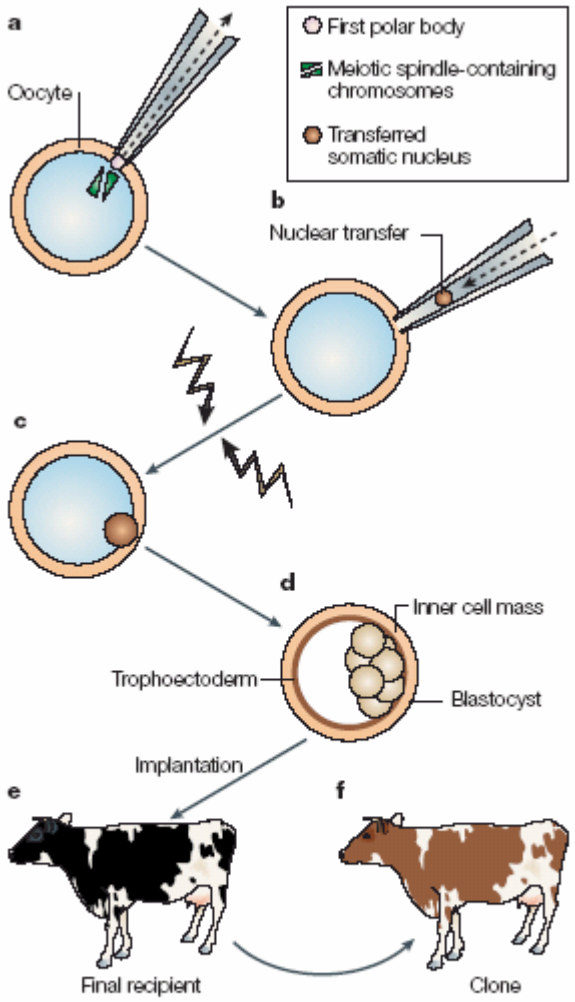


Figure 1.1: Overview of the somatic cell nuclear transfer procedure [2].

Although animals from several different species have successfully been produced through SCNT, it remains to be an inefficient process and can result in deleterious side effects. Table 1.1 provides a synopsis of the efficiencies associated with NT in five philogenetically divergent species. Typically, the efficiency of nuclear transfer is between 0 and 5% (# of live offspring/# of NT embryos), irrespective of the species, donor cell type, or technique used [35]. As seen in their low efficiency, pigs are notoriously more difficult to clone - perhaps due to the added difficulty of needing at least 4 viable embryos, and thus fetuses, to induce and maintain a pregnancy [36]. In essence, for every 100 NT embryos created, only 0-5 live births will occur.

Table 1.1: Key pathological phenotypes reported in species that have been cloned. Adapted from [2].

Organ	Cattle	Sheep	Goats	Pigs	Mice	References
Cloning efficiency (%)	0–5	0.4–4.3	0.7–7.2	0.1–0.9	0.2–5.8	*
Placenta	Impaired development, oedematous cotyledons‡, enlarged umbilical vessels, hydrallantois£	Reduced vascularity	-	-	Placentomegaly$^{\Delta}$	15–17,41,88,89
BW	Higher	-	-	Lower	-	41,90,91
Heart	RV enlargement	Hypertrophy	-	RV enlargement	-	42,92,93
Lungs	Hypertension$^{\neq}$	Hypertension, MPV	Pneumonia	-	Pneumonia	42,64,92,94,95
CNS	-	Pathology	-	-	-	96
Kidneys	Abnormalities (including size abnormalities)	Defects, hydronephrosis$^{\#}$	-	-	-	41,42,97,98
HLS	Lymphoid hypoplasia$^{\infty}$ anaemia	-	-	-	Immune impairment	95,99,100
Endocrine	Diabetes	-	-	-	-	101
Liver	Fibrosis, fatty liver	Enlargement, BDP, fibrosis	-	-	Hepatic necrosis	16,41,42,88,95
MS	Limb deformities	Body-wall defects	-	-	-	42, 97
Other	-	-	-	-	Obesity	19,102

Table Legend: The cloning efficiency is the number of live offspring expressed as a percentage of the total number of nuclear transfer oocytes. *Data were obtained from the amalgamation of many studies (see Somatic cell nuclear transfer (cloning) efficiency online at http://www.roslin.ac.uk/public/webtablesGR.pdf) all other references are as cited in Rhind *et al*., 2003. Rabbits, horses, mules, cats and rats have also been cloned, but no specific phenotypes were described in failed clones [9, 13-15]. ‡Cotyledons are focal zones on the placenta of apposition of maternal and fetal tissue. £Hydrallantois is the excessive accumulation of fluid in the allantoic sac of the placenta. ΔPlacentomegaly is enlargement of the placenta beyond its normal size. ≠Hypertension is high blood pressure, which causes the enlargement of vascular structures. #Hydronephrosis is dilation of the renal pelvis, which is caused by obstruction more distally in the urinary tract. ∞Lymphoid hypoplasia is an incomplete or underdeveloped lymphoid system. Abbreviations: BDP, bile-duct paucity (reduction in the number of bile ducts in the liver); BW, body weight; CNS, central nervous system; HLS, haemolymphatic system (the organs involved in the generation and function of red and white blood cells); MPV, misaligned pulmonary vessels (a condition in which there is abnormal alignment of the veins and arteries in the lungs); MS, musculoskeletal; RV, right ventricle.

Substantial loses are observed at every stage of NT embryo development, particularly in ruminants and mice, with abnormal phenotypes being seen in both fetal and placental tissues. In general, defects in the mouse, goat, and pig clones appear to be less severe than those found in failed sheep and cattle clones [2]. However, this may be due to researchers selectively reporting data for only surviving animals and not on pregnancies that were aborted or terminated in these species. Summarized in Table 1.1, problems associated with nuclear transfer fetuses and pregnancies include abnormal birth weights, placental aberrancies, altered organ growth and function, atypical skeletal formations, immunological defects and increased perinatal death (as reviewed by [2, 37-40]). The above mentioned abnormalities, however, are not exclusively observed in NT-derived embryos. As shown in Table 1.2, such phenotypes are also observed in embryos derived through the maturation, fertilization, and culture of *in vitro* produced (IVP) embryos [40] and are similar to those found in humans with Beckwith-Wiedeman syndrome (BWS) also as a result of IVP embryos [41]. In NT-derived pregnancies, the majority of losses occur during the first third of gestation in mice, cattle, and sheep. However, in the two ruminant species, a high rate of abortion also occurs during the last third of gestation as well as a significant amount of perinatal mortality (reviewed in [42]). Even apparently healthy surviving clones may suffer from immune dysfunction or kidney, brain, or heart malformations, all of which may contribute to their death at later stages [43, 44]. Thus, only a small proportion of reconstructed embryos are developmentally viable to produce live and healthy offspring.

Table 1.2: Summary of pathologies described in manipulated embryos derived from either nuclear transfer or other in vitro procedures [45]

Species	Type of embryo manipulation	Embryo loss	Gestation length	Placental abnormalities	Fetal size	Respiratory/cardiovascular dysfunction	Organ dysplasia	Perinatal mortality	Post-natal development
Cow	Nuclear transfer	High	Prolonged	Common	Increases	Common	Common	Raised	Altered
	Other	High	Prolonged	Common	Increases	Occasional	Occasional	Raised	Altered
Sheep	Nuclear transfer	High	Prolonged	Common	Increases	Common	Common	Raised	Altered
	Other	High	Prolonged	Common	Increases	-	Occasional	Raised	Altered
Goat	Nuclear transfer	High	Prolonged	None observed	Normal	No	No	Normal	Normal
	Other	High	Normal	None observed	Normal	No	No	Normal	Normal
Pig	Nuclear transfer	High	Prolonged	None observed	Reductions	Occasional	Occasional	Raised	Altered
	Other	High	Normal	None observed	Reductions	No	No	Raised	Normal
Mouse	Nuclear transfer	High	Caesareans	Common	Altered	Common	-	Raised	Altered
	Other	High	-	-	Reduced	-	-	-	Altered

There are a host of factors contributing to the inefficient nature of NT. In addition to the suitability of the donor genetic material (i.e., donor cell cycle, type, and passage number), the development of reconstructed embryos is influenced by many technical factors - the quality of the recipient oocyte, the chosen method of activation, and embryo culture conditions used by the researcher, to name a few [46]. Although an effort to increase the low rate of efficiency has actively been pursued, seven years after the birth of SCNT researchers continue to be plagued with substantial embryo losses throughout development as well as the production of unhealthy offspring. Thus far, tremendous emphasis has been placed on developing the NT technology with much less given to properly understand the precise mechanisms involved in the nuclear reprogramming of a differentiated cell back into that of a totipotent cell – begging the question, what epigenetic and genetic effects are responsible for driving the development of these pathologies? In order to increase the inefficiencies inherent in the current technique of SCNT, a conscious and cohered effort must be made to answer this question.

CHAPTER 2

Nuclear reprogramming and epigenetic modifications: A look at DNA methylation and genomic imprinting

Though inefficient, the successful production of live offspring derived from somatic cell nuclear transfer (SCNT) poignantly illustrates that genes, previously inactivated during tissue differentiation and specialization, can be re-activated through a process called nuclear reprogramming. This term is widely used and generally accepted to describe changes in gene activity and indicates the termination of one gene expression program (e.g., donor cell) and the initiation of another (e.g., embryonic) [4, 47]. Until recently, it has been difficult to exclude the possibility that stem cells, and not differentiated nuclei (as in a fibroblast cell), were responsible for producing the viable offspring created through SCNT. Arguments were made that stem cells or stem-like cells may be present, although at a low percentage, within the donor cell population used for NT [48, 49]. Setting out to prove or disprove this theory, researchers from the Jaenisch lab successfully produced cloned mice generated from a population of mature lymphocytes using tetraploid embryo complementation [50]. More recently, researchers within the same lab used post-mitotic, olfactory sensory neurons as nuclear donors to produce live murine offspring [51]. These studies provide clear genetic proof that through nuclear transplantation the totipotency of a once terminally differentiated cell can be restored to produce viable cloned mammalian progeny. However, the frequent anomalies and inefficiencies inherent in the NT procedure argue that events associated with the proper epigenetic reprogramming of a donor genome fail to be carried out efficiently and completely. The fact that phenotypically normal offspring are produced through the natural mating of clones with an abnormal phenotype, [52] provides direct evidence

that the inappropriate gene expression is not the result of genetic alterations, rather, it is the result of errors in epigenetic modifications [52].

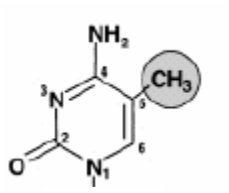
Epigenetics is the study of heritable, but potentially reversible, alterations in gene expression without a change in the DNA sequence [53, 54]; thus, no nucleotide alterations are involved in this process. An epigenetic trait is one that is transmitted independently of the DNA sequence itself. This can occur at the level of cell division — for example, daughter cells may inherit a pattern of gene expression from parental cells (so-called cellular memory) — or at the generational level, when an offspring inherits a trait from its parents [53]. Epigenetic modifications of the genome ensures proper gene activation during development and proliferation and involves (i) genomic methylation changes, (ii) the assembly of histones and histone variants into nucleosomes, and (iii) remodeling of other chromatin-associated proteins such as linker histones, polycomb group, nuclear scaffold proteins, and transcription factors [48]. Through these epigenetic modifications, a differentiated cell is capable of explicit genetic control – essentially having an on and off switch for the expression of genes specific for every cell type in the body. This ensures that while going through mitosis, a specialized cell is capable of perpetuating the 'molecular memory' of the developmental decisions that created it.

However, through NT this molecular memory can be defied. Immediately following the construction of a NT embryo, resetting these epigenetic gene modifications is pertinent to the proper reprogramming of the differentiated donor cell (as reviewed in [55-57]). The modifications which must occur during the initial development of a NT embryo include: DNA methylation, chromatin structure, telomere length adjustments, genomic imprinting, X chromosome inactivation, and epigenetic inheritance [48, 55]. Here, the process of

reprogramming DNA methylation patterns, their role in genomic imprinting, and the status of such epigenetic modifications in NT-derived embryos and offspring will be discussed.

DNA methylation

DNA methylation and its role in genomic imprinting is one of the most comprehensively studied epigenetic modifications of DNA in all unicellular and multicellular organisms and is recognized to be a chief contributor to the stability of gene expression states – for a comprehensive review see [58]. DNA methylation suppresses gene expression by recruiting methyl-CpG binding proteins, such as MeCP2, MBD1, MBD2, and MBD3, as well as associated histone deacetylases, co-repressor proteins, and chromatin remodeling machineries to the promoter of specific genes [59]. Figure 2.1 shows the structure of 5-methylcytosine (m^5C). The methyl group addition to cytosine residues causes an alteration in the appearance of the major groove in DNA to which the DNA binding proteins bind [60]. This post-replication modification is present in protests, plants, some fungi, virtually all vertebrates, but is absent in both budding and fission yeast, and only sparsely present in the nematode [61] and fruit fly [62]. DNA is methylated specifically at the C's that precede G's (CpG dinucleotides) in the DNA chain. In non-embryonic mammalian cells, m^5C accounts for ~1% of total DNA bases and therefore affects 70-80% of all CpG dinucleotides in the genome [54, 58, 63].



DNA

Figure 2.1: Structure of 5-methylcytosine as a modified nucleotide in DNA [3].

Amidst these methylated cytosine nucleotides are CpG islands – GC-rich short sequence domains stretching >200 to 500 bp [64]. These islands are highly present: of the estimated 30,000-40,000 genes in the human genome, approximately 29,000 are linked to CpG islands [65,

66] and are useful landmarks in identifying genes. All housekeeping and widely expressed genes have a CpG island which are frequently upstream of mammalian genes. These regions can be found near the promoter, untranslated regions, and the first exon but can also be found in regions more towards the 3' end [64]. In contrast, ~40% of non-housekeeping, or tissue-specific, genes or those with a limited expression are associated with such islands [64]. Researchers are uncertain as to how CpG islands remain unmethylated in a globally methylated genome [67, 68]. However, certain CpG islands do become methylated leading to long-term shutdown of the associated gene. This occurs during development specifically during X-chromosome inactivation and in imprinted autosomal genes (see below) where one of the parental alleles may be turned off [69, 70]. A significant fraction of all human CpG islands are prone to progressive methylation in certain tissues during aging (reviewed in [71]), in cancerous and abnormal cell types (reviewed in [72]) and in permanent cell lines such as NIH/3T3 [73].

The methylation of DNA plays a role in a number of important biological processes in mammalian cells. The primary function of this DNA epigenetic modification is to stably suppress gene expression, in which DNA methylation establishes or maintains a silent chromatin state in collaboration with the range of proteins that regulate nucleosomal structure (reviewed in [54]). This is particularly true in development and establishes the allele-specific expression status in many imprinted loci through differential DNA methylation of parental alleles (i.e. differential methylated regions or DMRs) [74]. During development, one of the X chromosomes in a female embryo is inactivated, called X-chromosome inactivation, and is dependent on methylation for both regulation of the Xist gene as well as for maintaining the inactive state [75]. It has been proposed that methylation is primarily a host defense against intragenomic parasites, such as viruses and transposons, with some of these being completely inactivated by the

methylation of their promoters (for a review see [76]). Methylation also targets sequences for assembly into the condensed state [77], which might suppress recombination. One clear role of methylation, and a heavily studied area, is in cancer where methylation plays a less defined role in genome stability [78, 79]. Cancerous tumors are often globally hypomethylated, but locally hypermethylated especially in tumor suppressor genes [80, 81] and has recently been reviewed by Baylin and Herman, 2000.

Genomic Imprinting

At a small number of mammalian loci (estimated to comprise 0.1-1.0% of all genes) only one of the two copies of a gene is expressed. Just which copy is expressed depends on the sex of the parent from which that copy was inherited. Such genes are said to be imprinted [82]. Genomic imprinting is a reversible epigenetic mechanism shown only to occur in mammalian species, so while a memory of parental origin is retained in all somatic cells, silencing is reversed when the allele passes through the germ line of the opposite parental sex [83, 84]. In its typical form, imprinting is the non-expression of a paternally or maternally derived gene in at least some tissues for some period of development. The best-known example is that of insulin-like growth factor II in humans [85] and mice [86] (IGF2 and Igf2, respectively): in most tissues, only the paternally derived gene is expressed and the maternally derived gene is silent. Currently, more than seventy imprinted genes have been catalogued in the mouse (http://www.mgu.har.mrc.ac.uk/imprinting/imprinting.html), with many (but not all) also being similarly imprinted in humans [87].

What are the genetic and epigenetic features that characterize imprinted genes? DNA methylation is a key molecular mechanism of imprinting; methylation marks the imprinted genes differently in egg and sperm, and inheritance of these epigenetic patterns leads to differential

gene expression throughout development [74, 88, 89]. One notable characteristic of imprinted genes is that they are rarely found on their own: around 80% are physically linked in clusters with other imprinted genes (see human chromosome 11p15.5 in [84]). When looking at the DNA sequence of such genes they are noticeably CpG rich: around 88% of imprinted genes in the mouse have CpG islands, compared with the average gene having 47% [84]. Most imprinted genes also contain differential methylated regions (DMRs) capable of having different properties independent of the expression, or lack thereof, of the imprinted allele. DMRs can show considerable changes in methylation during development and acquire tissue-specific methylation patters with the progression of time [90]. Although methylation of DMRs in most genes will result in their repression (e.g. H19), in some instances methylation is essential for gene activation (Igf2, Igf2r) [84, 91]. In general, DNA methylation governs gene expression: when a gene is methylated it is not expressed (turned off), when a gene is not methylated it is expressed (turned on). The importance of DNA methylation, at least in the maintenance of imprints, has been established genetically [74].

Established through DNA methylation patterns, genomic imprinting results in a developmental asymmetry in the function of parental genomes (for a review see [92, 93]). A number of theories have been proposed offering an explanation as to the need for such asymmetry between parental alleles [94]. In general, most hypotheses propose that control of fetal growth and the unique fetal-maternal relationship are selective forces in the evolution of imprinting [95]. The most widely held hypothesis, proposed by Moore and Haig, is the genetic conflict theory of genomic imprinting representing the clash between the interests of maternal and paternal genomes in the fitness of offspring and the demands on maternal resources [96]. Based on this hypothesis, paternal alleles for genes that increase fetal size by extracting more

nutrients from the mother should be selected to be as greedy as possible. Therefore it is in the mother's best interest to control the size of the fetus by conserving her resources since her total reproductive success may be compromised by giving a single offspring too many resources [96]. In other words, paternally expressed imprinted genes generally *enhance* fetal growth whereas maternally expressed genes primarily *suppress* fetal growth. Thus, growth-enhancing genes will be maternally inactivated and growth-suppressing genes paternally so. Since fetal growth depends on the availability of nutrients provided by the mother, an indirect way of monitoring such growth is to restrict nutrient transfer through the regulation of placental growth and function [84]. Therefore it is no surprise that the majority of imprinted loci are expressed in the placenta (Harwell imprinting web site, http://www.mgu.har.mrc.ac.uk/research/imprinting/). This 'tug-of-war' theory of imprinted genes, as shown in mammals, is particularly implicated in the regulation of fetal growth, development, and function of the placenta, as well as in postnatal behaviors [56]. This hypothesis is currently considered the most convincing explanation [94, 97], but it is not without problems [98-100].

The establishment of genomic methylation patterns

How do the patterns of methylated and unmethylated mammalian DNA arise in development and how are they maintained? Mammalian development is characterized by bimodal DNA methylation reprogramming that occurs initially during germ cell development and then again during embryo development prior to implantation (for a recent review, see [57, 101]). The first phase occurs in the parental germ line. This phase of reprogramming is responsible for resetting, or erasing, imprints such that the mature gametes reflect the sex of the germ line – for example, as in X or Y bearing spermatozoa. The second reprogramming phase occurs upon sperm entry into the oocyte, whereupon the genome undergoes demethylation

without erasing genomic imprints by both active (paternal genome) and passive (maternal genome) mechanisms.

Reprogramming events in germ cells

As seen in the mouse, during development primary germ cells (PGCs) enter the germinal ridge at embryonic (E) day 11.5-12.5 where they begin to expand and differentiate [102]. Figure 2.2A depicts the reprogramming events in germ cells at this stage of development. The highly methylated PGCs begin to undergo rapid genome-wide demethylation and erasure of existing imprints in both male and female PGCs such that by embryonic day 13-14 most of the methylation is lost causing male PGCs to enter mitotic arrest and female PGCs to enter meiotic arrest [103]. Evidence indicates that all genomic imprints are erased during this stage of PGC development [104-106]; however, whether this phase of reprogramming occurs by passive or active demethylation has yet to be discovered [57]. The re-establishment of genomic imprints takes place several days later and occurs through *de novo* methylation beginning in both germ lines during late fetal stages, and continues after birth [102, 104]. This occurs foremost in male gametes (green line) during the prespermatogonia stage (E15 to E16 and onwards) [107], whereas in the female germ line (pink line) it occurs after birth during the growth of the oocyte [55, 57].

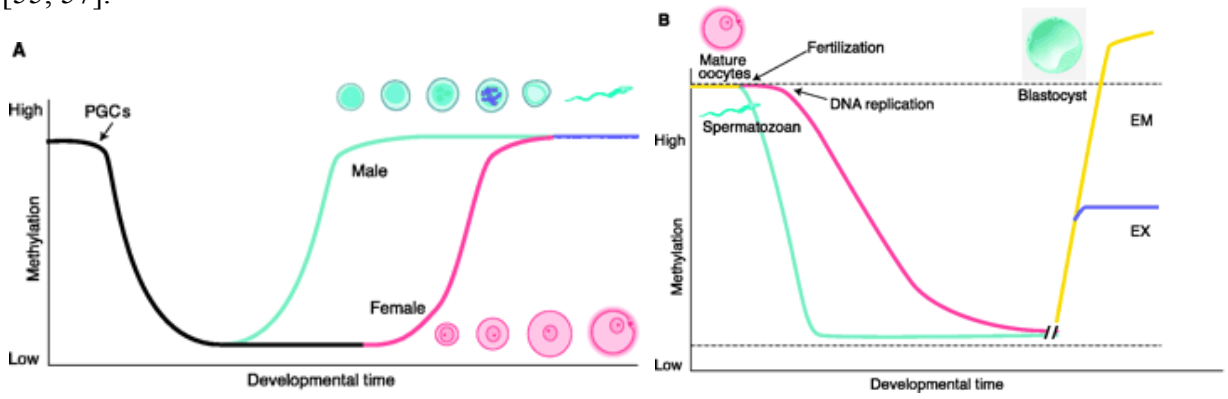


Figure 2.2: Reprogramming events in germ cells (A) and preimplantation embryos (B). (**A**) Methylation reprogramming in the germ line. Primordial germ cells (PGCs) in the mouse become demethylated early in development. Remethylation begins in prospermatogonia on E16 in male germ cells, and after birth in growing

oocytes. Some stages of germ cell development are shown. (**B**) Methylation reprogramming in preimplantation embryos. The paternal genome (blue) is demethylated by an active mechanism immediately after fertilization. The maternal genome (red) is demethylated by a passive mechanism that depends on DNA replication. Both are remethylated around the time of implantation to different extents in embryonic (EM) and extraembryonic (EX) lineages. Methylated imprinted genes and some repeat sequences (dashed line) do not become demethylated. Unmethylated imprinted genes (dashed line) do not become methylated. [57]

What is the function of reprogramming the germ line? This is primarily necessary for the resetting of imprints established in the parental lineages. Another likely purpose is the removal of acquired epigenetic modifications, which can be influenced by an individuals genetic and environmental background [108, 109]. Occasionally, epigenetic information can be inherited, and thus passed on, through the germ line [110] [111]. As it is not clear whether most or all epigenetic information is completely erased in germ cells, researchers have hypothesized that there might be a need to keep transposable elements silent by methylation, particularly in gametes [112]. Therefore a better understanding of this area is necessary.

Reprogramming events in early embryos

The second phase DNA demethylation, and thus reprogramming, in mammalian species occurs after fertilization and proceeds by both active (rapid loss of methylation without DNA replication) and passive (the lack of maintenance methylation following DNA replication and cell division) mechanisms (Fig. 2.2bB). As shown in the mouse, upon fertilization, the paternal genome undergoes a rapid transformation within the egg cytoplasm, where remodeling of sperm chromatin through removal of protamines and replacement by (acetylated) histones is closely followed by genome-wide demethylation (Fig. 2.2B - green line and Fig. 2.3A – lack of red male pronucleus 3 hpf) [113, 114]. This asymmetric loss of methylation takes place in the absence of transcription or DNA replication and is termed active demethylation resulting in a decline in methylation until the morula stage of embryo development [115].

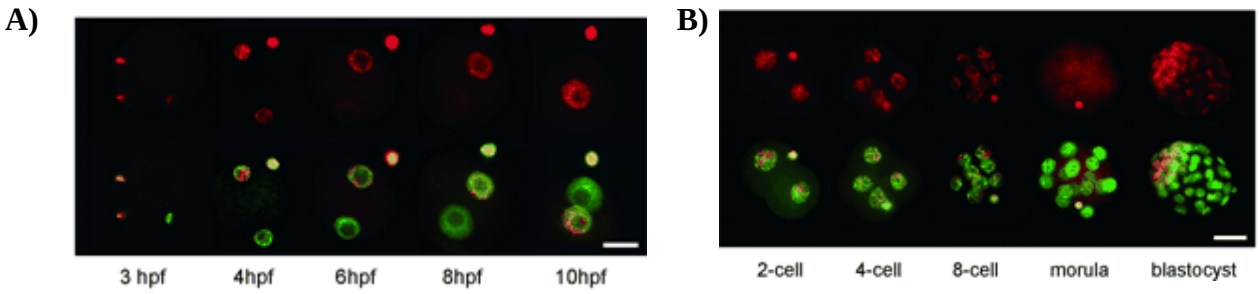


Figure 2.3: Genome-wide DNA methylation reprogramming is represented by indirect immunofluorescence of fertilized oocytes (A) and embryos (B) using an antibody to 5-methyl cytidine (5MeC). (A) Active demethylation: the first cell cycle. In the fertilized mouse oocyte a rapid and asymmetric loss of DNA methylation (red signal) can be observed in the male (green in lower merge panel) but not in the female pronucleus. Genome-wide loss of DNA methylation starts at sperm decondensation (left) and continues until it is undetectable in the paternal compartment (right), the process taking about 6 h. Lower panels show a merge (yellow) between the DNA methylation (red) and DNA stain (green). hpf, hours post fertilization. Scale bar 25 μm (Santos *et al.* 2002). (B) Passive demethylation phase. From the 2-cell stage (left) to the morula (right) the DNA methylation (red) is passively lost due to the exclusion of DNA methyltransferase 1 (Dnmt1) from the nucleus. By the blastocyst stage lineage-specific *de novo* methylation is apparent with the inner cell mass (ICM) being highly methylated (red) while the trophectoderm remains hypomethylated. Mouse embryos are depicted. Lower panels show a merge (yellow) between the DNA methylation (red) and DNA stain (green). Scale bar 25 μm (Santos *et al.* 2002). [101]

Although exposed to the same cytoplasm, the maternal pronucleus does not undergo rapid genome-wide demethylation prior to cleavage and remains highly methylated in both its DNA and histone structures (graphically depicted in Fig. 2.2B – pink line and visually in Fig. 6.3A – by the presence of red fluorescence in the female pronucleus). Rather, a passive loss in methylated CpG dinucloetides occurs until the morula stage of embryonic development due to the lack of maintenance methylation after each cell division and is therefore considered in a hypomethylated state [116] – as seen by the gradual loss of red fluorescence in Figure 2.3B. *De novo* methylation is not seen until the blastocyst stage (Fig. 2.3b) in which the first two cell lineages are established: the inner cell mass (ICM) and the trophectoderm (TE). The ICM, which gives rise to all the tissues making up the organism, becomes hypermethylated, while the TE, which forms most of the placental structures, is hypomethylated [115, 117]. In the mammalian zygote and resulting embryo, there is therefore a general (genome-wide) epigenetic asymmetry, with more heterochromatic (repressive) chromatin structures in the maternal genome

and more euchromatic (permissive) structures in the paternal one [92]. Although the overall epigenetic state of the mammalian genome changes dramatically during early development, imprinting remains relatively stable by an unknown mechanism. Thus, the erasure and resetting of imprints is specifically carried out during germ cell reprogramming [55]. If the cytoplasmic component of an oocyte has demethylase activity (see below), the maternal genome along with the genomic imprints must be structurally protected from such an enzyme [92].

The mechanism by which demethylation occurs is not well understood (for a review see [58, 101]). As shown in cancer cells [118], demethylation, or the removal of the methyl groups on DNA, can occur by the deamination of 5-methylcytosine followed by mismatch repair: possibly carried out by a methyl-CpG-binding domain (MBD) protein, MBD4, which has thymine/guanine mismatch glycosylase activity [119]. Another proposed mechanism for DNA demethylation involves the enzymatic removal of the methyl group from the cytosine base [120], or by the exchange of 5-methylcytosine for cytosine through a base excision mechanism involving RNA molecules [121]. A protein encoding a MBD, MBD2, was recently proposed as a candidate enzyme for the direct removal of the cytosine methyl group [120], however this has not been confirmed by others [8, 122]; reviewed in [123]]. Oocytes homozygous for a MBD2 knockout undergo normal demethylation patterns demonstrating that MBD2 is not required for demethylation in vivo [117]. Thus, the proper mechanism responsible for the removal of CH_3 groups from cytosine dinucleotides has yet to be determined.

Is the reprogramming mechanism evolutionarily conserved? The basic reprogramming events in the paternal genome: active demethylation in the zygote followed passive demethylation in the early cleavage stages, and maternal genome: passive demethylation initiated upon the first cell division, appear to be conserved in eutherian mammals. However, their

relative timing with respect to developmental stage can differ. Dean and coworkers compared the timing of such reprogramming events between mouse, rat, bovine, and pig *in vivo* and *in vitro* produced embryos [115]. Their findings confirmed that the genome-wide demethylation of the paternal genome is conserved in all four species. Consistent with the mouse, passive demethylation occurred from the two-cell to eight-cell stages in all species (Fig. 2.4A). However, discrepancies were noted in the bovine preimplantation embryo. In mouse embryos, for example, *de novo* methylation has been observed at the blastocyst stage (Fig. 2.4a, box f) and is predominately contained in the inner cell mass (ICM), whereas in bovine embryos *de novo* methylation occurs from the 8-cell to 16-cell stage (Fig. 2.4A, box k) [115]. Interestingly, this coincides with the transcriptional activation of the bovine embryonic genome [124], suggesting that a *de novo* methylase is functionally active at this time. As a result of *de novo* methylation occurring four cell divisions earlier in the bovine embryo, TE cells are considerably more methylated at the blastocyst stage than what is observed in their mouse counterparts (Fig. 2.4A and B) [115]. This perhaps is a function of evolution – as in cattle, extraembryonic tissues in the placenta are required for a greater length of time (more than 270 days) than in the mouse (15 days). The increased methylation state of the TE may confer added stability on the differentiated state of these cells [115]. Reprogramming discrepancies exist in other species as well. In sheep, for example, the rapid demethylation of the paternal genome has not consistently been observed [45] and has failed to be seen in zebrafish or frogs [125]. Thus, species-species variations do occur during genomic reprogramming of methlyation patterns in fertilized embryos.

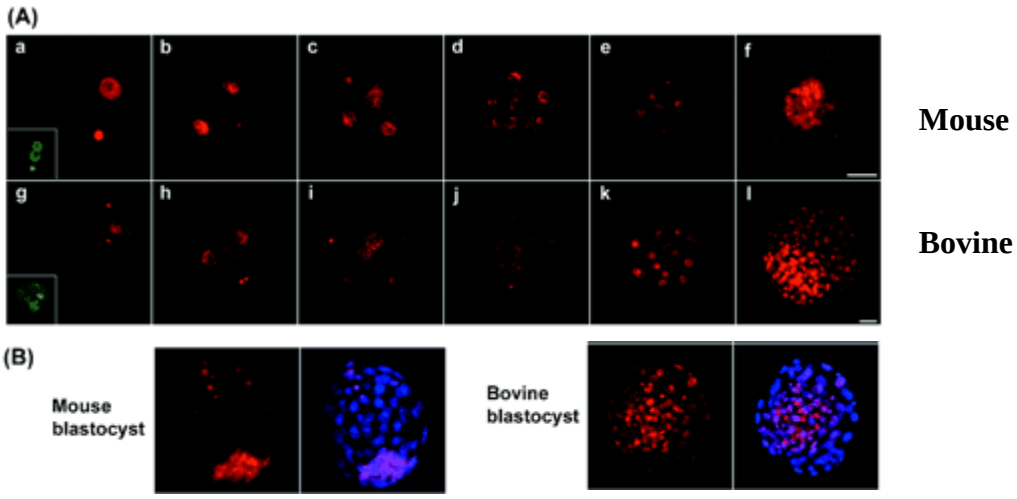


Figure 2.4: Demethylation and remethylation are conserved during preimplantation development. (*A*) Normal mouse (*a-f*) and bovine (*g-l*) embryos were stained for 5-methyl cytosine (red) from the zygote to the blastocyst stage. In the mouse (*a-f*), there is an initial loss of methylation specifically from the male pronucleus [(*Inset*) DNA stained to identify two pronuclei, green]. Thereafter the remaining decline in signal occurs in a stepwise fashion up to the morula stage (*e*). The ICM, but not the trophectoderm, has undergone *de novo* methylation by the blastocyst stage (*f*). Bovine zygotes also show loss of methylation from one pronucleus (*g*) followed by a further stepwise decline in methylation to the eight-cell stage (*h-j*). *De novo* methylation by the 16-cell stage results in heterogeneity with highly and moderately methylated nuclei (*k*) such that at the blastocyst stage (*l*) the ICM contains highly methylated nuclei and the trophectoderm moderately methylated ones. (*B*) To better define the location of the methylated nuclei images are presented with the methylation signal (red) and the merged image of the DNA (blue) superimposed on the methylation signal (pink). This superimposition of images clearly shows that in the mouse the ICM has become remethylated, but in bovine nuclei both ICM and trophectoderm are methylated. [115]

Epigenetic reprogramming through nuclear transfer

Through nuclear transfer, a once terminally differentiated cell can be reprogrammed back into a totipotent state suitable to produce live offspring [7, 51]. Although successful, the inefficiencies – between 0-5%, depending on species; persistent anomalies, collectively called "large offspring syndrome" [37, 40]; and perinatal death associated with the current technique of NT-derived offspring, highlight the lack of, or inadequate, epigenetic reprogramming of the donor genome [21, 48, 55, 126]. Realizing this, researchers have begun to unravel the reprogramming state of the donor genome following its transfer into the recipient cytoplast.

For cloned offspring to develop, it is thought that genes normally expressed during embryongenesis, but silent in the somatic donor cell due to epigenetic modifications, must be reactivated [48]. During SCNT, reprogramming of the donor nucleus is radically different from

that which occurs during gametogenesis and must take place in the brief period between nuclear transfer and when zygotic transcription becomes necessary for further development [48]. The reprogramming of a cells differentiated state through this mechanism must occur without also erasing essential genomic imprints. To do this, complex epigenetic alterations carried out in a temporal pattern of events must occur – including transcriptional silencing of the donor nucleus, erasure of differentiated cellular memory, appropriate activation of the reconstructed 'one-cell embryo', and proper embryonic gene expression at all later stages [126], for a review see Shi *et al*., 2003. Therefore, as stated by Rideout *et al*., there are three possible outcomes to this reprogramming process: (i) no reprogramming of the genome, resulting in immediate death of the NT embryo; (ii) partial reprogramming, allowing initial survival of the clone fetus but resulting in an abnormal phenotype and/or lethality at various stages of development; or (iii) faithful reprogramming producing normal animals [48]. The phenotypes observed in nuclear clones suggest that complete reprogramming is in fact the exception [48].

As reported, the majority of studied cloned embryos show a gross abnormality in the genome-wide DNA methylation level and patterns on repetitive sequences as compared to fertilized controls (Table 2.1). Immunofluorescence detection of 5-methylcytosine is commonly used to evaluate the genome-wide pattern of DNA methylation in embryos [115, 127]. Using this technique, Dean et al. has shown that, after fusion, fibroblast nuclei appear to go through a limited active demethylation stage followed by the precocious de novo methylation of the nuclei at the 4–8-cell stage in many cloned embryos [115], thus having a highly methylated genome characteristic of a differentiated cell at the blastocyst stage.

Table 2.1: DNA methylation status in cloned mammals [59]

Reference	Species	Donor cells	DNA sequence	Degree of DNA methylation[a]	Detection method
Kang et al., 2001b	Pig	Fetal fibroblasts	Centromeric satellite PRE-1 (euchromatic repeat)	Embryos: C=A Embryos C<A	Bisulfite
Dean et al., 2001	Bovine	Fetal fibroblasts	Whole genome	Embryos: C>A	Immunofluorescence[b]
Bourc'his et al., 2001	Bovine	Adult fibroblasts	Whole genome	Embryos: C>A	Immunofluorescence[b]
Kang et al., 2001a	Bovine	Fetal fibroblasts	Satellite I, Satellite II, SINE and 18S rRNA	In all sequences, Embryos D=C>A	Bisulfite
Kang et al., 2002	Bovine	Fetal fibroblasts	Tissue specific promoters	Embryos: C=A	Bisulfite
Cezar et al., 2003	Bovine	Genital ridge cells	Whole genome	Summary of 4 donor cell types	Reverse phase HPLC, restriction enzyme
		Fetal skin cells		Aborted fetuses: C<<A	
		Adult skin cells		Live fetuses: C<A	
		Fetal and adult cumulus cells		Adults: C=A	

[a] A: age-matched controls derived from fertilization, C: cloned animals and D: the donor animals.
[b] Immunofluorescence with anti-5-methylcytosine antibody.

In contrast, using the 5-mC antibody on methaphase chromosomes, Bourc'hi *et al*. found no evidence of active demethylation occurring in bovine somatic nuclei after fusion. However, in agreement with Dean et al., they found a limited passive demethylation of the donor genome in the cleavage stage embryo [127]. These observations suggest that NT-derived embryos fail to reproduce distinguishable parental-chromosome methylation patterns after fusion and maintain their somatic pattern during subsequent stages, mainly by a highly reduced efficiency of the passive demethylation process [127]. Studies also show an absence of asymmetry between the trophectodermal and ICM cells at the blastocyst stage with the methylation level being abnormally high in TE tissue [115, 128]. Using bisulfite analysis to observe the methylation status of several repeat and unique sequences, including satellite 1 DNA – a major component of centromeric heterochromatin, and the Bov-B LINE sequence – which is normally demethylated in bovine preimplantation embryos, researchers also found the occurrence of inefficient reprogramming with an absence of passive demethylation as compared to *in vitro* control embryos [128]. Notably, similar studies carried out on cloned porcine embryos show no signs of aberrant methylation patterns [129]. Studies investigating reprogramming in sheep SCNT embryos show comparable demethylation events also occur in fertilized controls. However, the NT embryos exhibit increased methylation levels up until the eight-cell stage with a substantial

difference in its distribution at the blastocyst stage [130]. In general, these studies present a picture of partial, but incomplete reprogramming of methylation patterns in cloned embryos.

When looking at the overall genome methylation state of individual cloned offspring, there is a high degree of variability [128] and researchers can not discount that extremely abnormal embryos may have died before such analyses were performed [59]. When observing the DNA methylation patterns of spontaneously aborted bovine clones, Cezar et al. noticed undetectable levels of methylation in six out of nine fetuses, whereas the methylation level was normal in the clones that survived to adulthood [131]. Taken together, the aberrant DNA methylation found in cloned embryos and fetuses is a probable contributor to the low efficiency of SCNT.

The gene expression profiles of cloned embryos have recently been documented by several groups and are summarized in Table 2.2. A properly reprogrammed somatic cell nucleus would result in an embryo having a gene expression profile resembling that of its *in vivo* or *in vitro* produced counterpart. Aberrant activation of developmentally regulated genes in embryos, including *IL6*, *FGF4*, and *FGFr2,* has been shown to fluctuate in cloned embryos derived using different construction and culture techniques [21, 132, 133]. Looking at genome-wide differences in gene expression patters of NT embryos, results from a DNA microaray showed that ~4% of over 10,000 genes were abnormally expressed in NT placentas as compared to controls [134], which may explain some of the placental anomalies observed in cloned pregnancies. In addition to the placentas, this study also investigated the gene expression in the livers of cloned mice – showing, although to a lesser extent, the abnormal expression of genes different than those affected in the placentas [134]. Favorably, and yet most astonishing, is the fact that more than 96% of the genes analyzed were properly silenced or activated in this

comprehensive genome-wide scan. However, this study examined RNA isolated from a whole tissue. In doing so, the gene expression in each cell may have been averaged and would therefore overshadow a population of improperly reprogrammed cells [59]. In a more recent study, Pfister-Genskow *et al*. removed this variable and prepared cDNA, after three rounds of amplification, from *individual* NT and IVF embryos and compared their differential gene expression using a cDNA microarray. After confirming the microarray results with real time PCR, they identified 18 genes aberrantly expressed in day 7 NT-derived embryos; including those coding for intermediate filaments (including cytokeratin 8 and vimentin – whose expression are required for post-hatching development of bovine embryos, thus leading to proper establishment and development of the chorio-allantoic placenta [135]), three metabolism proteins, lysosomal related proteins, a heat shock protein, mitochondrial 16s rRNA, and a few unidentified or novel proteins along with several others [136]. Unfortunately, they failed to mention differences in *Oct4* expression.

Table 2.2: Aberrant gene expression profiles of cloned mammals [59]

Reference	Donor	Cells	Number of the tissue	Aberrantly expressed genes[a]/total number of genes examined	Detection method
Daniels et al., 2000	Bovine	Granulosa cells	2-cell embryo to blastocyst (4–10)	3/7 (42.9 %)	RT-PCR
Daniels et al., 2001	Bovine	Fetal epithelial cells	Blastocyst (62)	1/4 (25 %)	RT-PCR
Wrenzycki et al., 2001	Bovine	Follicular cell line	Blastocyst	1/8 (12.5%) to 3/8 (37.5%)	RT-PCR
Humpherys et al., 2002	Mouse	ES cells	Placenta (12)	221/12,654 (1.7%)	Microarray
			Liver (13)	26/12,654 (0.2%)	
		Cumulus cells	Placenta (14)	286/12,654 (2.3%)	
Suemizu et al., 2003	Mouse	ES cells	Placenta (2)	Clone 1: 1,807/15,247 (11.9%) Clone 2: 1,964/15,247 (12.9%)	Microarray

[a]Gene expression is defined as aberrant in the microassay analysis when the gene expression level in the cloned mice is 2-fold higher or lower than in the controls derived from fertilization.

Looking at specific pluripotency markers, such as the transcription factor Oct4, could shed light on when and how effective the NT reprogramming process is. *Oct4* is exclusively expressed in germ cells and early embryonic cells [137], becomes downregulated after gastrulation, and is silent in somatic cells [138]. Therefore, *Oct4* must be reactivated soon after

NT when using somatic nuclei as donors [59]. Using *Oct4*-GFP transgenes, Boiani *et al*. showed the majority of cumulus cell-derived NT embryos initiated *Oct4* expression at the correct stage (80% of clones) but showed an incorrect spatial expression at the blastocysts stage. Their results demonstrated 54.7% of clones showed an aberrantly high level of *Oct4* expression in the TE at the blastocyst stage when it should be exclusively limited to the ICM [139]. Similar results were also obtained when the expression of *Oct4* and ten *Oct4*-related genes were analyzed in individual cumulus cell-derived cloned blastocysts [140]. Only 62% correctly expressed all tested genes, whereas in cloned blastocysts derived from ES cells and normal control embryos the same 11 genes were normally expressed [140]. The efficient development of clones derived from ES cells, as compared to those derived from somatic cells, suggests that the genes required for early post-implantation development are perhaps already actively expressed and need not be reprogrammed; in contrast with somatic donor nuclei which do not initially express such genes [141].

Genomic imprinting patterns are established during germ cell development and, although active demethylation occurs upon fertilization, are protected from being erased or methylated during development by an unknown mechanism in fertilized embryos [142]. If active demethylation occurs in the somatic nucleus in cloned embryos, this raises the question of whether imprinted genes will be protected against demethylation. Failure to do so would result in clones having altered patterns of imprinted genes [115]. This has in fact been observed in mice cloned from embryonic stem cells [143] as this cell type has been shown to be epigenetically unstable as reflected in the instability of DNA methylation during ES cell culture [144]. In this instance, the unstable ES cells were not properly reprogrammed indicating that the epigenetic state of the donor nucleus affects the gene expression pattern and presumably the

phenotype of a cloned animal [145]. There is clear evidence to show that the oocyte cannot initiate imprints if they are erased; clearly, imprints can only be initiated in the germ line [146]. This suggests the re-establishment of imprinted genes is not a function of the oocyte and thus aberrant imprinting patterns may not be corrected through NT. However, Humpherys *et al*. demonstrated that mouse ES-cell clones with widespread gene dysregulation are capable of surviving to adulthood despite their altered imprint patterns, indicating that mammalian development may tolerate some degree of epigenetic aberrations of the genome [144]. Although imprinted genes are expected to remain largely unaffected during the reprogramming of somatic nuclei, there are nevertheless instances where imprints may be erased which can lead to fetal and placental growth abnormalities [147]. Taken together, these observations demonstrate that faulty activation of developmentally important genes, like that of *Oct4* and other developmentally important genes (like those that are imprinted), can be detrimental to the outcome of a cloned pregnancy and may be responsible for the characteristic anomalies seen in cloned animals of all species.

Epigenetic modifications, specifically DNA methylation and genomic imprinting, are established in the germ line and are passed on to their offspring whereby involving two distinctly different phases of genomic reprogramming. To be successful, embryos generated through nuclear transfer must erase the differentiated cells memory through reprogramming events in order to establish embryo-specific gene expression profiles. Multiple studies have demonstrated this does not properly occur in such embryos, highlighted by the existence of aberrant epigenetic modifications in the resulting embryo and animal. The successful production of a live clone, albeit inefficient, clearly defies normal reprogramming events and is the exception to a developmentally established rule. If the goal of cloning animals is to produce normal offspring

flawless in their genetic, epigenetic, and phenotypic make-up, its imperative the technique of nuclear transfer be reevaluated to establish the feasibility to do so; but if the objective of cloning is to understand the mechanisms involved in reprogramming a differentiated cell into that of a totipotent cell – including the discovery of essential reprogramming factors contained in the cytoplasm of an oocyte, then the current process of nuclear transfer is an invaluable tool for such studies.

CHAPTER 3

A look at DNA methyltransferases: The roles Dnm1s and Dnmt1o play in maintaining methylation during embryonic development

Though the developmentally essential need for DNA methylation and its role in genomic imprinting has been discussed, the class of enzymes responsible for establishing these methylation patterns deserves equal consideration. DNA methyltransferases (MTases) are responsible for catalyzing the transfer of a methyl group ($-CH_3$) from *S*-adenosyl-$_L$-methionine (AdoMet) to the C5 position of cytosine residues in DNA (for a review on this mechanism see [148, 149]). In eukaryotes, the MTase family is composed of five independently encoded enzymes: Dnmt1, Dnmt2, Dnmt3a, Dnmt3b, and Dnmt3L [148]. Briefly, Dnmt1 is responsible for the maintenance of methylation patterns after DNA replication [150, 151]. Dnmt3a and Dnmt3b perform *de novo* methylation primarily during embryo development [117, 152, 153]. Although Dnmt3L is structurally similar to Dnmt3a and Dnmt3b, it has no *in vitro* catalytic activity, however, it has been shown to play a central role in the establishment of maternal genomic imprints [154-156]. Although Dnmt2 is the most conserved MTase gene in both eukaryotes and prokaryotes, it fails to methylate DNA *in vitro* and is not essential for *de novo* or maintenance methylation in ES cells; thus, at present its function is not known [157]. Here, a look at the maintenance MTase, Dnmt1, will be discussed. However, before its functional role in methylation is considered, a fundamental understanding of the MTase structure along with the mechanism involved in methyl group transfer is necessary.

The structure of eukaryotic DNA methyltransferases

The typical eukaryotic cytosine-5 DNA methyltransferase (C5-MTases) is a protein roughly three times larger than its prokaryotic counterpart [3]. Based on functional and

structural data, this enzyme may have arose from the fusion between three genes, with one identified as being an ancestral prokaryotic MTase [158]. C5-MTases are composed of 10 characteristic sequence motifs, 6 of which are strongly conserved [159, 160] (for a recent review, see Chen and Li, 2004). Based on x-ray crystallography data, researchers have determined the structural and functional domains comprising these enzymes [161-164]. As a whole, there are two distinct domains in MTases: a highly conserved catalytic domain near the C-terminal region linked by a stretch of repeated GlyLys dipepetides to a regulatory domain within the N-terminal region (Fig. 3.1). On the C-terminal end, motif IV and the AdoMet-binding pocket form the catalytic center which also comprises motifs I and X in addition to residues from motifs II-V. Motifs VIII and IX are responsible for DNA target recognition by forming a cleft suitable to harbor the DNA substrate. When bound, the minor groove faces the catalytic motifs, whereas the major groove is directed towards motifs VIII through IX. This causes the target cytosine to flip out of the DNA helix where it projects into the concave catalytic pocket. With the exception of Dnmt2, the C5-MTases have an N-terminal extension. The variable length of this extension (as seen in Fig. 3.1) gives rise to the distinct properties and biological functions of each Dnmt [165].

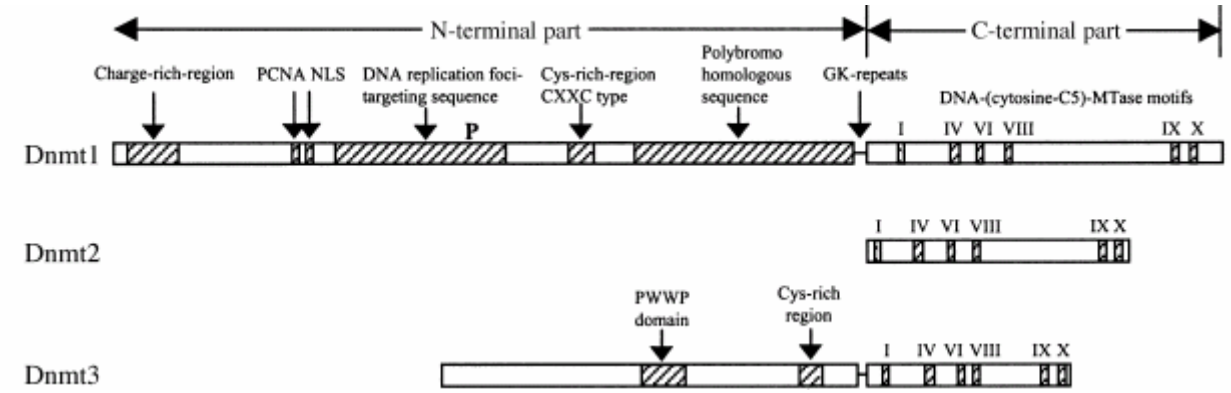


Figure 3.1: Overview of the general organization of the five DNA methyltransferase family members. Some of the functional domains or structural motifs recognized in the N-terminus of Dnmt1 and Dnmt3 include a PCNA binding domain (PCNA), a targeting sequence (TS), a cysteine-rich region, a polybromo homology domain, a tryptophan-rich region (PWWP) and another cysteine-rich region (C-rich). The vertical bars inside the C-terminus correspond to the highly conserved motifs found in most DNA methyltransferases. For simplicity, only motifs I, IV and X are

labeled. The linker region – a (GlyLys) 6 repeat – between the N- and C-terminus is represented by a short horizontal line. The scale bar (bottom right) indicates the length corresponding to 200 amino acid residues. Modified text from Chen and Li 2004. Figure adapted from Pradhan and Esteve 2003.

Catalytic mechanism of C5-MTases

DNA methylation is a post-replication modification involving an MTase along with a methyl-group donor – the cofactor adenosyl-$_L$-methionine (AdoMet). As seen in Figure 3.2, for catalysis to occur, the Dnmt enzyme (denoted as S⁻Cys in Fig. 3.2) must bind to a cytosine residue of the substrate DNA. Because cytosine is an electron-poor heterocyclic aromatic ring, the C5 position on the cytosine residue is not capable of making a nucleophilic attack. Thus, an enzyme must carry out this reaction [3]. Using the SH functional group of cysteine as a nucleophille, the Dnmt enzyme forms a covalent bond at position 6 of the nucleotide whereby forming an intermediate between the enzyme and DNA [166]. As a result, the C5 position on the cytosine residue is strongly activated and attacks the methyl group attached to AdoMet. The deprotonation at position 5 leads to the elimination of the Dnmt and reestablishes aromaticity [3]. This modification does not interfere with Watson/Crick base pairing, however, the added methyl group is positioned in the major groove where it can easily be detected by DNA interacting proteins [3].

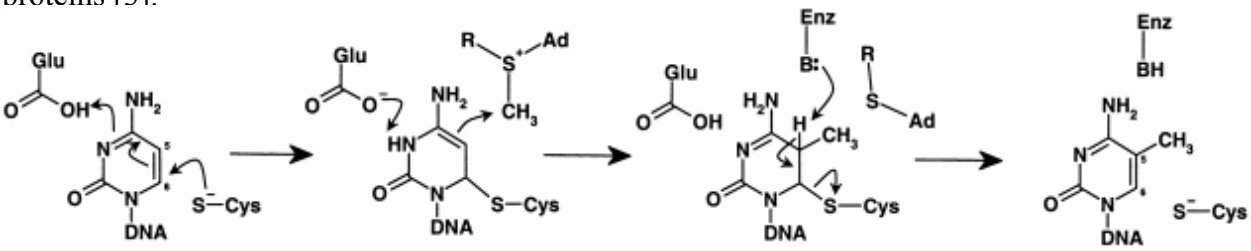


Figure 3.2: Catalytic mechanism of C5-MTases. The cysteine residue is from motif IV (PCQ), the glutamic acid residue is from motif VI (ENV) of the C5-MTase [3].

Now that a basic understanding of the functional structure and catalytic mechanism has been established for the C5-MTases, a closer look at the maintenance methyltransferase, Dnmt1, will be taken. Insight will be gained as to the role Dnmt1 plays in DNA methylation and

genomic imprinting, followed by an in depth look at the effects aberrantly expressed Dnmt1 has on mammalian cells and embryos.

The Dnmt1 Class of C5-MTases

The first eukaryotic DNA C5-MTase to be purified and cloned from murine cells was Dnmt1 [151]. The largest of all discovered DNA methyltransferases (Fig. 3.1), murine Dnmt1 consists of 1620 amino acid residues (an isoform lacking 118 N-terminal amino acids is found in oocytes and will also be discussed). Although Dnmt1 has been shown to methylate both unmethylated and hemimethylated CpG dinucleotides *in vitro*, it has a 5- to 50-fold preference for hemimethylated CpG dinucleotides [167-169]. Thus, it has been designated a maintenance methyltransferase. However, Dnmt1 can also play a role in *de novo* DNA methylation although a domain responsible for such a function has yet to be identified [165].

Dnmt1 homologs have been found in nearly all eukaryotes whose DNA bears m^5C modifications [148]. Between species, the Dnmt1 protein is nearly identical: human Dnmt1 consists of 1616 amino acids and is 78% identical at the amino acid level to its mouse counterpart [170]. Dnmt1 is the most abundantly expressed MTase in mammalian cells [171] and has been sequenced in five mammalian species: including mouse [151], rat [172], sheep (Fairburn et al. – not published but is registered with NCBI), cow [173], and human [170].

There are several distinguishable characteristics of the Dnmt1 protein. As shown in Figure 3.3A, the C-terminal domain of Dnmt1contains all the highly conserved motifs for catalysis and is more closely related to the bacterial DNA C-5 MTases than to the mammalian Dnmt2 and 3 enzymes [151]. Dnmt1 is lengthier than the other C5-MTases (Fig. 3.1) especially at the regulatory domain (N-terminal end), consisting of ~1100 additional amino acids [149]. Because of its added length, the N-terminal end is capable of housing several key domains:

including a nuclear localization signal (NLS), a replication foci-targeting (RFT) domain, a proliferating cell nuclear antigen (PCNA), a bromo-adjacent homology (BAH) domain, and a CXXC domain. Such domains are functionally responsible for its nuclear import [174], enzyme localization during S-phase [175], support during DNA replication and repair [176], protein-protein interactions [177], and the binding of DNA sequences containing unmethylated CpG dinucleotides [178], respectfully. As a nuclear protein, the localization of Dnmt1 changes dramatically during the cell cycle. During the G1 and G2 phases it is diffusely found in the nucleoplasm and is only localized to the replication foci during DNA replication [175]. Thus, Dnmt1-mediated methylation is coupled to DNA replication whereby ensuring that methylation patterns will be established on each daughter strand.

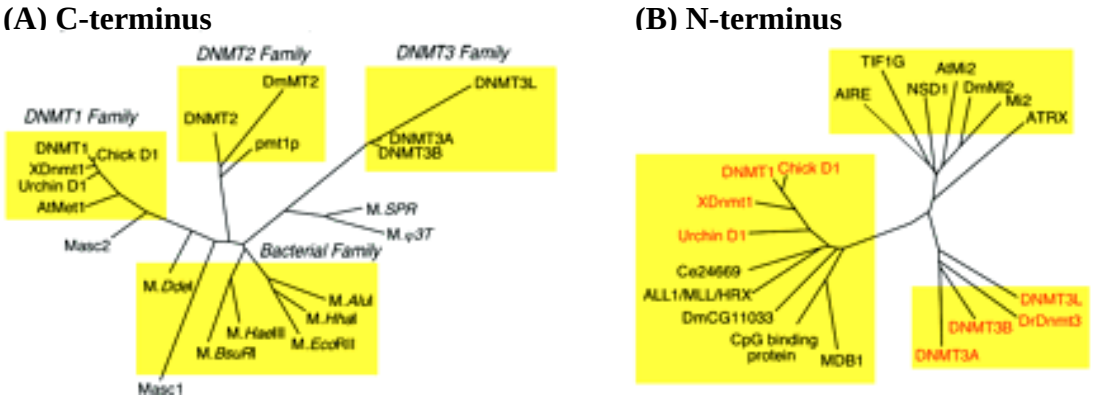


Figure 3.3: Phylogenetic trees comparing mammalian Dnmts to their non-mammalian counterparts (**a**) ClustalW analysis of the C-terminal end of DNA C5-MTases from bacteria, fungi, plants and metazoa. (**b**) Sequence comparison of the regulatory regions (N-terminal end) characteristic of metazoan DNA methyltransferases. Enzymes known to be active DNA methyltransferases are highlighted in red. [148]

The N-terminus has also been shown to interact with several other proteins. Some of which include the transcriptional co-repressor DMAP1 [179], the histone deacetylases HDAC1 [180, 181] and HDAC2 [179], the transcription factor E2F1 [179], and with the Rb tumor-suppressor protein [179]. Although not confirmed *in vivo*, it appears that the N-terminal domains of Dnmt1 serve as a platform for assembly of various proteins involved in chromatin

condensation and gene regulation [3]. Studies show that both termini are essential for catalytic activity as they are not capable of acting in absence of each other [158, 182, 183]. This implies that the catalytic domain of Dnmt1 is under tight allosteric control by its N-terminal neighbor. This contrasts the catalytic ability of the *de novo* MTases, Dnmt3a and Dnmt3b. When physically separated from its N-terminal counterpart, the C-terminus of Dnmt3a and Dnmt3b remains enzymatically active and has DNA methylation capabilities [184].

Specific promoter and exons at the Dnmt1 locus

The *Dnmt1* locus is subject to alternative splicing at sex-specific 5'exons which functions to control the production and localization of the Dnmt1 protein. Such splicing mechanisms reduce the amount of nuclear Dnmt1 protein at specific stages of gametogenesis [185]. The relative positions of the three alternate exons – including 1o, 1s, and 1p, of the *Dnmt1* gene are shown in Figure 3.4A. Briefly, exon 1s transcripts give rise to a somatic and germ cell-specific form of Dnmt1 and is composed of 1620 aa corresponding to a molecular weight of 190 kD [186]. Exon 1o transcripts are translated into a shorter, 1502 aa (175 kD), oocyte specific protein [186]. The third splice variant, exon 1p, is found in spermatocytes during the pachytene phase - the third stage of prophase in meiosis in which the paired homologous chromosomes are separated into four chromatids – and is the major or sole transcript at this stage of development [185]. Although transcribed, *Dnmt1p* however, does not associate with polyribosomes and is not actively translated into protein in spermatocytes at this stage [185]. Similar to Dnmt1s in cleavage stage embryos, *Dnmt1p* is a translationally down-regulated mRNA transcript [187]. The primary focus for the remainder of this chapter will be on Dnmt1s and Dnmt1o – their production, or lack there of, and localization within the cell or nucleus. For ease, in this section

the two Dnmt1 transcripts will be clearly defined as Dnmt1s – for the somatic isoform and Dnmt1o – for its oocyte counterpart.

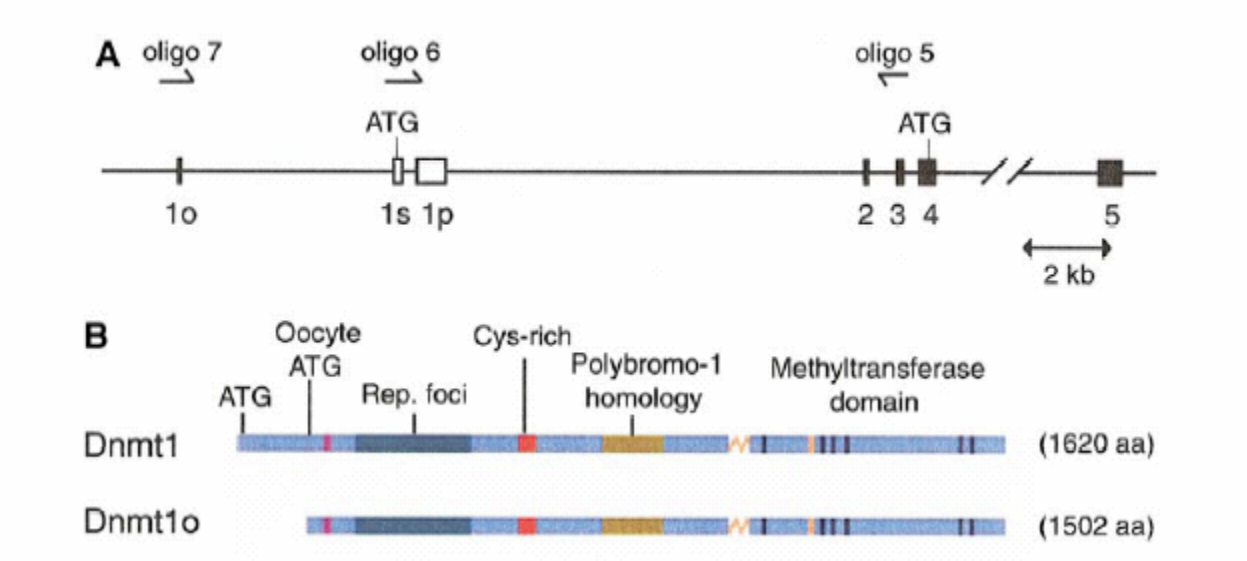


Figure 3.4: Sex-specific exons and oocyte-specific species of Dnmt1. (A) Organization of sex-specific exons in the 5' end of the Dnmt1 gene. The oocyte-specific Dnmt1 transcript initiates at exon 1o and is spliced to exon 2; Dnmt1o translation is initiated at the ATG in exon 4 (Mertineit et al., 1998). The resulting protein is 118 amino acids shorter than the somatic form, whose ATG codon is located in exon 1s (also spliced to exon 2). (B) Comparison of Dnmt1 and Dnmt1o [186].

The structure and function of Dnmt1s and Dnmt1o

Although on the same locus, *Dnmt1s* and *Dnmt1o* are transcribed from two different start sites [185]. The initiation codon for *Dnmt1s* is located in exon 1s, whereas the initiation codon for Dnmt1o is an internal methionine codon of *Dnmt1s* located upstream in the common exon 4 (Fig. 3.4A) [185, 188]. A larger protein than the oocyte isoform, Dnmt1s contains an additional 118 amino acids at its N-terminal end (Fig. 3.4B). The *Dnmt1* promoter responsible for transcribing the oocyte isoform is turned off immediately following fertilization as displayed by an absence in Dnmt1o transcripts after the one-cell stage [186]. Numerous studies have confirmed the presence of *Dnmt1s* transcripts in all adult tissues whereas the truncated *Dnmt1o* transcript is exclusively present in postnatal ovaries within the oocyte population [186]. In fact, in MII oocytes and in all preimplantation cleavage-stage embryos, there are large amounts of the

Dnmt1o protein, approximately 50,000-fold higher on a per-nucleus basis in the oocyte as compared to the amount of Dnmt1s protein present in a cycling somatic cell [186, 189]. Therefore, Dnmt1o is enzymatically active, is synthesized in the growing oocyte, and is stored to very high levels in the ooplasm of an MII oocyte [148, 186]. Thus far, the only known role Dnmt1o plays is to maintain methylation patterns on imprinted genes during a single S phase in preimplantation embryos [190]. Studies demonstrate an absence of a maintenance Dnmt present in the nucleus at all other S phases during preimplantation development [185, 186], thus explaining passive demethylation events during the early stages of embryo development.

Presence of Dnmt1s and Dnmt1o in the cleavage stage embryo

Dnmt1s and *Dnmt1o* transcripts are both present within the cytoplasm of the oocyte and fertilized embryo [186]. In order for normal development to occur, maintenance of the DNA methylation patterns requires both the expression and the correct stage-specific posttranscriptional and posttranslational regulation of the *Dnmt1* transcript and protein [74, 150, 186, 190]. Although *Dnmt1s* transcripts are present in the oocyte and embryo, they are never translated into a functioning protein [186]. Therefore, this leaves Dnmt1o as the only functional maintenance MTase to be expressed prior to implantation. Upon fertilization, *Dnmt1o* mRNA is immediately translated into protein at the one-cell stage [186]. Although translated, the Dnmt1o protein remains sequestered within the cytoplasm of each cell [191] possibly by binding to annexin V [186, 192], a calcium-sensitive phospholipid binding protein [190, 193]. As seen in Figure 3.5, Dnmt1o appears to be physically bound to or near the cytoplasmic membrane in one-, two, and four-cell embryos as well as in blastocysts. It is not until the eight-cell stage when Dnmt1o traffics into nucleus [189, 191]. It is only at this cleavage stage when Dnmt1o briefly

enters the embryonic nuclei to perform the essential function of maintaining the patterns of imprinted genes established during gametogenesis.

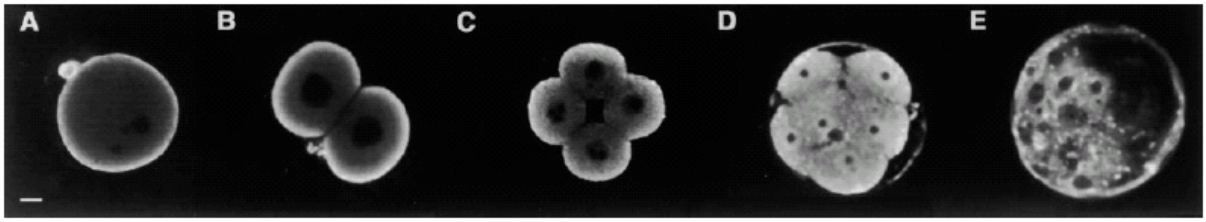


Figure 3.5: Localization of Dnmt1 mRNA in early mouse embryos. During preimplantation development, Dnmt1 localizes to the cytoplasm of one- (A), two- (B), and four- (C) cell embryos, it is partly imported into the nuclei with the remaining protein staying in the cytoplasm at the eight-cell stage (D), and it is again out of the nucleus at the blastocyst stage (E). Fertilized mouse embryos were collected and incubated in vitro. At different times, the embryos were fixed and stained with an anti-Dnmt1 polyclonal antibody and analyzed by confocal microscopy. The images show single confocal sections through embryos at different stages of development. Bar, 10 mm. [191]

It is interesting to note that when single blastomeres are taken from an eight-cell embryo and transplanted into a one-cell embryo, they are unable to reinstate normal gene expression [194]. In one such study, at least 50 proteins were aberrantly expressed [194]. This may be caused in part by the methylation of specific genomic DNA sequences around the eight-cell stage that are essential to propagate development through the initial cleavage stages [191]. In fact in such reconstructed embryos, the Igf2r gene locus is methylated at the eight-cell stage and could be one such candidate gene [191, 195]. Although the somatic Dnmt1 transcript is found in preimplantation embryos, it is not until the early stages of postimplantation development that Dnmt1s is localized and allowed to remain active in the nucleus [185, 186, 189] whereupon the oocyte specific *Dnmt1* locus (*Dnmt1o*) ceases to be transcribed. In oocytes and early staged embryos either the *Dnmt1s* mRNA is not capable of being translated, or, upon translation, Dnmt1s is not allowed to accumulate in the early embryo [196]. These findings establish that, in fertilized embryos, the expression and regulation of Dnmt1 is a function of posttranscriptional and posttranslational regulation which moderates the proper localization and accumulation of this enzyme.

Though both Dnmt1s and Dnmt1o transcripts exist in MII oocytes and cleavage stage embryos, how is Dnmt1o the sole transcript to translocate into the nucleus? As mentioned above, the only structural difference between the two isoforms is found at the N-terminal end with Dnmt1s having an additional 118 aa. However, the absence of these amino acids does not impede with the catalytic function of Dnmt1o. When Dnmt1o is substituted for Dnmt1s in mutant mice, the oocyte form of Dnmt1 is capable of maintaining methylation in ES and somatic cells [197, 198]; thus, the two isoforms of Dnmt1 have equivalent maintenance methylation activity. The structural differences between the two *Dnmt1* transcripts may play a role in the inhibition of *Dnmt1s* translation in the oocyte and embryo. This may happen through negative translational control mechanisms including translational repressors that may bind to the 5' end of the mRNA and inhibit translation initiation of the *Dnmt1s* transcript [186, 190]. This could explain the absence of Dnmt1s protein in cleavage stage embryos. As proposed by Ding *et al*., perhaps there is a functional difference which lies in the stability of the two transcript isoforms [198]. As seen in its ability to accumulate in immature oocytes and be functionally active upon fertilization, Dnmt1o transcripts may be more stable as compared to the somatic transcript. This would allow Dnmt1o transcripts to persist in the ooplasm at high concentrations during cleavage, ultimately producing a high quantity of Dnmt1o protein to translocate to the nucleus at the eight-cell stage. Therefore, differences in the 5' UTRs of the two *Dnmt1* transcripts may not only be important for their conversion into protein, but it may also determine when translocation into the nucleus is permitted.

Aberrant expression of Dnmt1 in NT-derived embryos

As described in Chapter 1, NT-derived embryos are generally produced through the transfer of a somatic cell into an enucleated egg. In addition to the donor genome, the

transferred cell also donates countless other biological molecules including transcription factors and enzymes in addition to numerous housekeeping and regulatory proteins commonly found in the nuclear and cytoplasmic fractions of a cell. Transplantation of somatic nuclei into the ooplasm therefore introduces a nonnative form of Dnmt1 protein into preimplantation embryos. The ectopic expression of Dnmt1s in cloned embryos constructed using somatic nuclei may be responsible in part for the abnormal genomic methylation and developmental abnormalities common to SCNT offspring [57, 148, 186, 199]. A recent study in cloned mouse embryos revealed the aberrant expression of somatic Dnmt1 protein throughout embryo cleavage [196]. As a result, striking temporal differences are noticed with regard to the proteins cytoplasmic to nuclear translocation (Fig. 3.6). Using a common antibody, UPT82, specific for the unique region on the N-terminus of Dnmt1s protein, Chung et al. showed a mosaic expression of Dnmt1s protein in the cytoplasm and some nuclei of eight-cell stage blastomeres in cloned embryos (Fig. 3.6A). In contrast, Dnmt1s protein was absent at all stages in fertilized controls (Fig. 3.6B) [196]. As mentioned above, the eight-cell stage is precisely the time Dnmt1o translocates into the nucleus to maintain genomic imprints. Figure 3.7 compares and contrasts cloned versus fertilized embryos using an antibody (PATH52) which recognizes both Dnmt1s and Dnmt1o. Seen here, the localization of Dnmt1 near the cell membrane in 1-, 2-, and 4-cell embryos is consistent between NT and fertilized embryos. In agreement with Figure 3.6A, only a few nuclei in the cloned embryos (Fig. 3.7A) stained with PATH52 which contrasts that which occurs in fertilized embryos (Fig. 3.7B). Thus, the temporal localization and nuclear trafficking of Dnmt1o in SCNT embryos is properly carried out in some but not all blastomeres at the eight-cell stage. The aberrant expression and nuclear existence of the somaticically expressed Dnmt1 enzyme in cloned embryos could result from the lack of key regulatory functions such as gene

silencing and or activation of regulatory genes essential for proper embryo development [196]. Therefore, such a deviance in Dnmt1 expression could severely impede embryo development and may lead to problems in later fetal stages or upon birth.

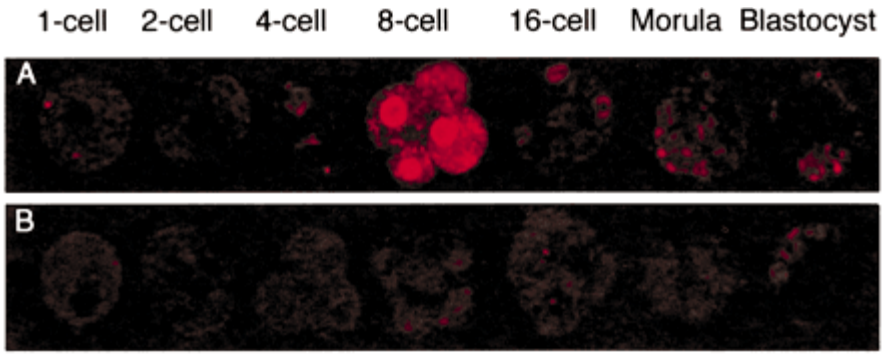


Figure 3.6: Immunostaining of normal and cloned preimplantation mouse embryos with the UPT82 anti-Dnmt1s antibody. **A**) Dnmt1s protein expression in an eight-cell-stage clone. UPT82 staining was absent in other cleavage stages. **B**) Absence of Dnmt1s expression in preimplantation-stage embryos derived from normal, fertilized eggs. [196]

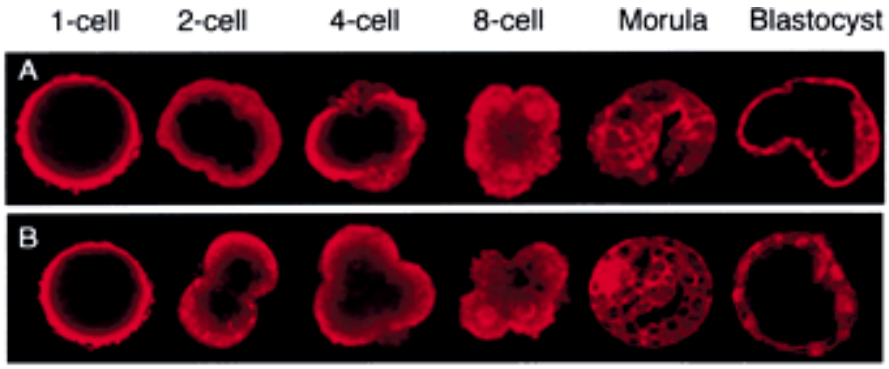


Figure 3.7: Immunostaining of normal and cloned preimplantation mouse embryos with the PATH52 anti-Dnmt1 antibody, which recognizes both Dnmt1o and Dnmt1s. **A**) Stage-specific nuclear staining for Dnmt1 in cloned embryos. **B**) Stage-specific nuclear staining for Dnmt1 in control embryos. **[196]**

Genetic and biological alterations to the expression of Dnmt1

In order to better understand the role Dnmt1 plays not only in the early stages of development but within cells in general, numerous genetic manipulations of DNA methyltransferase genes have been conducted in model organisms. Recently reviewed by Chen et al., 2004, deviant levels of Dnmt1 in embryos can alter DNA methylation patterns to either a hypermethylated or hypomethylated state [78, 200], can cause disruptions in gene regulation

[201], and can lead to death of the embryo [150]. In a classic experiment carried out by Li et al., a targeted mutation in the *Dnmt1* gene in the germline of mice resulted in abnormal development and embryonic death near the time of gastrulation [150]. However, DNA methylation was not shown to be essential for the survival of embryonic cells, and the effect of disrupting methylation patterns only becomes apparent during or after gastrulation when the pluripotent embryonic cells begin to differentiate [150, 152, 202]. This is in agreement with Dnmt1 knockout results in mouse ES cells. Such cells are completely viable with their DNA extremely hypomethylated; however, upon differentiation the ES cells undergo rapid cell death [200, 202, 203]. The demethylation of the genome through *Dnmt1* knockout causes aberrant activation of tissue-specific and stage specific genes, like those involved in imprinting, which may contribute to the lethality of mutant ES cells and embryos [74, 204]. Deleting *Dnmt1* in cultured fibroblasts gave rise to the aberrant activation of ~10% of all genes including those previously silenced through genomic imprinting [201]. In contrast to the above knockout studies, disruption of the *Dnmt1* gene by homologous recombination in human colon cancer cells resulted in a decreased cellular DNA methyltransferase activity, however there was only a 20% decrease in overall genomic methylation [205]. Therefore, excision of the *Dnmt1* gene in cells and embryos can result in altered gene expression states and can be lethal to the developing embryo upon differentiation events during gastrulation. However, the degree of such demethylation is variable and may depend upon the state of the cell.

As a contrast to the knockout of Dnmt1, the over-expression of this protein can lead to DNA being in a hypermethylated state [206, 207]. Increases in methyltransferase (MTase) activity and alterations in DNA methylation patterns are commonly seen in neoplastic cells and tumors of humans and mice [208, 209]. The oncogenic activity of over-expressed Dnmt1 can

lead to the *de novo* methylation of susceptible CpG island loci [210]. Similarly to *Dnmt1* knockout ES cells, overexpressing Dnmt1 using a BAC in ES cells has no obvious effects on ES cell proliferation [206]. When these overexpressing ES cells were injected into blastocysts, development of the chimeric embryo was severely impaired after implantation [206]. Taken together, these results demonstrate that pluripotent cells are capable of surviving with severely altered DNA methylation patterns. However, the proper level of Dnmt1 expression and the maintenance of established methylation patterns are crucial for embryo development past gasturlation.

In addition to manipulating the *Dnmt1* gene through genetic knockouts and knockins, chemical reagents can also be used to alter the methylation or histone acetylation status, and thus gene expression, of DNA [211-214]. Such reagents include 5-aza-2'-deoxycytidine (5-aza-dC) – a DNA methyltransferase inhibitor, and trichostatin A (TSA) – a histone-deacetylase inhibitor [212]. These reagents have been shown to induce DNA hypomethylation and hypoacetylation in treated cells. In a recent study Enright and Kubota *et al*. treated donor cells with TSA or 5-aza-dC prior to their use in NT [215]. Cells treated with TSA had less histone acetylation than control cells and resulted in improved embryo development to the blastocyst stage. Although 5-aza-dC treated cells resulted in global demethylation and histone hyperacetylation, there was a 6-fold decrease in blastocyst formation when compared to fertilized controls [215]. Similarly, Jones et al. found that supplementation of the donor cell culture medium with 5-aza-dC was not beneficial for increasing blastocyst rates and, although a pregnancy was established with such a blastocyst, no significant increase in establishing pregnancies resulted after NT reconstruction [216].

Researchers are now capable of using reverse genetic methodologies in mammalian cells to decrease the steady state levels of a targeted gene product (see Chapters 4-6). Primarily in cancerous cell lines, researchers have begun to investigate the knockdown of Dnmt1 expression employing the use of antisense [217-220] or RNA interference (RNAi) methods [218, 221-223]. Occurring with great specificity, antisense and RNAi induced silencing takes place at the post-transcriptional level – essentially depleting the cells supply of the targeted mRNA transcripts. Although primarily used as a method to study tumorigenesis, the outcomes of Dnmt1 knockdown experiments can further enlighten investigators about the effects aberrantly expressed Dnmt1 has on mammalian cells. The experiments described below were all carried out in human cancer cells. Studies indicate that Dnmt1 expression is regulated with the cell cycle [224, 225] and that antisense knockdown of Dnmt1 results in an intra-S-phase arrest of DNA replication [217]; which happens to be the cell cycle stage at which Dnmt1 activity peaks [226].

Using both antisense and small inhibitory RNA (siRNA) molecules, the knockdown of Dnmt1 has been shown to result in lower cellular maintenance methyltransferase activity [221], decreased cell proliferation [219], and global and gene-specific demethylation resulting in the re-expression of many silenced tumor suppressor and epigenetic genes [217-219, 222]. In one report, the methylation status of some promoters was decreased by 80% [227]. Recently, several knockout studies show Dnmt1 and Dnmt3b cooperate in performing maintenance methylation [222, 228-230]. This was confirmed using siRNA directed towards both Dnmt transcripts [222]. In this study, a greater decrease in DNA methylation was observed at specific promoter regions when both Dnmt1 and Dnmt3b were knocked down. This resulted in a 7-15 fold increase in gene expression at these sites [222]. These findings suggests that Dnmt3b acts as an accessory enzyme to support the maintenance methylation ability of Dnmt1 [222]. In addition to the

translational repression of gene products, specific siRNAs can be used to repress transcription by targeting the methylation of promoters at specific CpG islands [223].

Not all Dnmt1 knockdown studies have reported a loss in DNA methylation patterns. There are conflicting reports regarding the demethylation of CpG islands in human colon cancer cells [205, 218, 221]. Using siRNA (transient) and shRNA (stable) approaches, Ting *et al*. confirmed earlier *knockout* reports [205] that CpG methylation of tumor-suppressor genes are maintained and not re-expressed in human colorectal cancer cells [221]. Discrepancies between the knockout and knockdown of Dnmt1 may result from the differences in the methodologies and techniques used. The siRNA and antisense treatments transiently deplete Dnmt1 in cultured cells at the post-transcription phase. In knockout studies, such cells have undergone extensive genetic manipulations and multiple rounds of clonal selection procedures which may have induced unwanted and undetected genetic effects to these cells. Based on the results above, antisense and RNAi molecules are capable of inducing the post-transcriptional silencing of Dnmt1. These studies indicate that changes to the methylation status of DNA can occur resulting in the re-activation of previously silenced genes. Although these studies involving the knockdown of Dnmt1 were carried out in cancer cell lines, it is possible that similar results will occur in primary cells.

DNA methylation plays an essential role in the overall health and viability of cells as it allows for the proper expression of tissue-specific genes. Therefore, in addition to demethylating the cells DNA using the procedures and methods discussed above, potentially detrimental side effects can also unintentionally be induced. DNA methylation plays a role in maintaining genome stability [79] a role highlighted by DNA hypomethylation studies [78, 231]. Dnmt1 has been shown to interact with histone deacetylases 1 [180] and 2 [179] as well as histone

methyltransferase SUV39H1 [232], suggesting that Dnmt1 silences gene expression by recruiting chromatin-modifying enzymes [220]. Cells nullizygous for the *Dnmt1* gene exhibit significantly elevated mutation rates in which chromatin stability plays a role [78]. In addition, methylated cytosine residues also play a role in the suppression of transposon sequences within the genome [76]. Hypomethylation of such regions may encourage the expression of specialized intragenomic parasites which represent at least 35% of the genome [76, 233]. Therefore, when inducing DNA demethylation using any of the above mentioned techniques, researchers must take into account the role DNA methylation plays not only in gene expression but also in genome stability; thus, the outcomes of such studies should be carefully interpreted.

In summary, upon fertilization the maternal and paternal genomes undergo regulated DNA demethylation by both an active and passive process (reviewed in Chapter 2). This passive demethylation of the genome occurs through the exclusion of a maintenance methyltransferase. It is not until the eight-cell stage when Dnmt1o, the only maintenance methyltransferase protein in the pre-implantation embryo, enters the nucleus to maintain the methylation of imprinted alleles. Once completed, Dnmt1o is exported from the nuclei and once again sequestered in the cytoplasm of each blastomere. However, in NT-derived embryos this is not properly carried out. The results of numerous NT studies indicate variable DNA methylation patterns exist and may be related in part to the abnormal expression of developmentally important genes (imprinted genes) [57, 115, 127-129, 134, 143]. In contrast to fertilized embryos, cloned embryos aberrantly and ectopically express the somatic isoform of Dnmt. This altered expression may result from (1) a disruption in the post-transcriptional regulation of maternal Dnmt1s transcripts housed in the oocyte prior to NT or (2) the introduction of the enzymatically active Dnmt1s protein upon the transplantation of somatic nuclei into the ooplasm of an enucleated egg or (3) a

combination of the two. These explanations could account for the inefficient demethylation observed in cloned embryos. Regardless of its mode of arrival, the altered expression of Dnmt1s has been implicated to play a role in the abnormal genomic methylation and developmental abnormalities common to SCNT-derived embryos and offspring [57, 148, 186, 199].

In conclusion, several key points should be restated:

(1) There are three isoforms of Dnmt1: Dnmt1s, Dnmt1o, and Dnmt1p. The oocyte form, Dnmt1o differs from the somatic form, Dnmt1s, due to alternative promoter and first exon usage during mRNA processing. In the mouse, Dnmt1o briefly enters the nucleus only during the eight-cell stage (two-cell cycles after activation of the embryonic genome) when it is believed to maintain imprints for one round of replication. Dnmt1s is not expressed until post-implantation.

(2) Although *Dnmt1* and *Dnmt1o* transcripts are both present in the oocyte and embryo, differences in their N-terminal domain may account for the observed differences in oocyte-specific translation, localization, and translocation.

(3) Mammalian somatic cells are highly methylated and exhibit low MTase activity [234], whereas embryonic cells and gametes are less methylated with sperm being more methylated than oocytes [235].

(4) In NT-derived embryos, aberrant expression of Dnmt1s occurs in pre-implantation stage embryos where it has been observed to translocate into the nucleus at the eight-cell stage. The genomes of NT-derived embryos are often hypermethylated and stage-specific imprints have failed to be expressed. The ectopic expression of Dnmt1s in cloned embryos

constructed using differentiated nuclei may be responsible in part for the abnormal genomic methylation and developmental abnormalities common to SCNT offspring

(5) Through the knockout or knockdown (using antisense or RNAi methods) of Dnmt1, changes in DNA methylation can be induced resulting in the re-activation of previously silenced developmentally important genes including those involved with genomic imprinting.

CHAPTER 4

The discovery of RNA interference in mammalian cells

The specific degradation of mRNA via post-transcriptional gene silencing (PTGS) is one of the faster expanding fields in molecular biology. Without mechanically altering the rate of target gene transcription, PTGS is a regulatory process in which the steady-state level of a specific mRNA is reduced by sequence-specific degradation of the transcript [236, 237]. Once discovered, researchers rapidly employed the use of PTGS as a tool in model organisms, whereby making key observations allowing for the widespread use of this technique.

Initially discovered over a decade ago, PTGS was first discovered in petunia plants. Napoli and co-workers sought to overexpress chalcone synthase in an attempt to increase pigment production in these plants [238]. Instead of the expected deep purple color, they produced petunias with white or variegated flowers. This phenotype demonstrated the ability to suppress not only the introduced gene, or transgene, but also endogenous copies of the enzyme. This unexplainable event was termed cosuppression as the expression of both the introduced transgenes and the homologous endogenous genes were coordinately suppressed [239]. Cosuppression in plants happens not only at the post-transcriptional level but can also occur at the translational level as well [240]. In some plants, transcriptional silencing induced by transgenes can result in the epigenetic modification of homologous DNA through histone H3 methylation and the formation of heterochromatin [240-242].

A similar PTGS discovery was observed in the fungus *Neurospora crassa* in 1992 and was termed quelling [243]. Researchers were attempting to boost the production of an orange pigment made by the *al*1 gene. Using a plasmid containing a 1,500-bp fragment of the *al*1 gene, a few transformants displayed an albino phenotype and the native *al*1 mRNA was highly reduced

[244]. The gene silencing phenomena of co-suppression in plants and quelling in fungus, has since been observed in many evolutionarily divergent organisms (Table 4.1.) including a number of plant species [245, 246]. In addition to transgene induced RNA silencing, viruses are also capable of triggering this response in plants and has been termed VIGS (virus-induced gene silencing) [247]. Viruses can be either the source, the target, or both the source and target of silencing [248].

Table 4.1: Post-transcriptional gene silencing across kingdoms. [249]

Kingdom	Species	Phenomenon	Trigger	Genes implicated
Fungi	*Neurospora*	Quelling	Transgenes	*qde-1–3*
Plants	*Petunia*, *Nicotiana*, *Arabidopsis*, tomato, rice, potato, etc.	PTGS, co-suppression	Transgenes, viruses	*sgs1–3* *sde1–4* *ego1,2*
Animals				
Invertebrates	*C. elegans*	RNAi	dsRNA	*rde1–4* *mut6,7*
	Drosophila	RNAi	dsRNA	?
	Paramecium	Co-suppression	Transgenes	?
	Planaria	RNAi	dsRNA	?
	Hydra	RNAi	dsRNA	?
	T. brucei	RNAi	dsRNA	?
Vertebrates	Zebrafish	RNAi	dsRNA	?
	Mouse	RNAi	dsRNA	?

These initial studies using transgenes in fungi and various plant species set the precedent for the discovery of RNA silencing using double stranded RNA (dsRNA). Furthering the studies of Guo and Kemphues, who showed that sense RNA was as effective as antisense RNA in suppressing gene expression in worms [250], Andre Fire and Craig Mello described a new PTGS technique using dsRNA (containing both sense and antisense strands); a technology they called RNA interference (RNAi) [236]. Shown in the invertebrate species know as *Caenorhabditis elegans* (*C. elegans*), the presence of just a few dsRNA molecules was sufficient to almost completely abolish the expression of a gene exhibiting the same sequence identity to the foreign dsRNA molecule [236]. Testing the mutual effect sense and antisense RNAs had on the inhibition of gene expression, the exogenously delivered dsRNA mixture was ten times more

potent at silencing the targeted gene than with sense or antisense RNAs alone [236]. RNAi in *C. elegans* was initiated simply by soaking the worms in a solution containing dsRNAs or by feeding the worms Escherichia coli organisms that expressed the dsRNAs [251]. The offspring of these transiently treated worms also displayed a silenced phenotype [236] and cultured *C. elegans* cells treated with dsRNA showed signs of gene silencing for up to nine cell divisions [252].

Since its discovery, RNAi has rapidly developed into one of the most widely applied technologies in molecular and cellular research. With the advent of large-scale genome sequencing efforts, the discovery of RNAi has opened the door for researchers to discover the unknown function of their gene of interest [253]. Immediately following its discovery, researchers used RNAi to successfully knockdown various genes in a wide variety of organisms including *C. elegans* [254, 255], insects [255-261], planarian [262], *trypanosome brucei* [263], fungi [264], and various plant species [265-267].

Gene silencing in the form of cosuppression in plants, quelling in fungi, and RNAi in plants and animals is an ancient, highly conserved, endogenous mechanism thought to play a role in protecting the genome against dsRNA viruses [268] and genome-invading transposable elements [269, 270], as well as helping preserve genome stability in the germ line. Thus, the presence of foreign, or "non-self", RNA molecules alerts the cell of possible invaders and triggers the silencing of genes.

Although shown to be evolutionarily conserved, initially it was thought RNAi would have limited applications in mammalian and other vertebrate species [271-273]. Researchers observed severe physiological reactions as a result of introducing dsRNA into mammalian cells leading to the induction of interferon synthesis [274, 275]. It was noted that dsRNA > 30 bp

binds and activates the dsRNA-dependent protein kinase PKR [276] and 2',5'-oligoadenylate synthetase (2',5'-AS) [277]. When activated, PKR nonspecifically inhibits protein synthesis by phosphorylating a eukaryotic translation initiation factor (eIF2α). Whereas activated 2',5'-AS causes mRNA degradation by activating RNase L. Activation of this cellular pathway is in essence a global panic response which induces nonspecific gene silencing and can ultimately lead to cell death [274, 275].

Researchers were able to bypass this interferon response in mammalian cells by studying RNAi in mouse oocytes and embryos, as the immune system and the onset of PKR expression is not yet active at this early stage of development [278, 279]. Researchers were eager to show the same effects in zebrafish, however, a greater instance of nonspecific mRNA degradation was observed [280]. Although RNAi was shown to elicit a sequence specific degradation of targeted gene products in a wide variety of organisms, it seemed to not evoke the same response in all eukaryotic species.

Understanding the RNAi mechanism highlighted key observations in this pathway (Chapter 5). The activation of PKR by dsRNA was shown to be length-dependent; dsRNAs of less than 30 nucleotides are unable to activate PKR, and full activation requires ~80 nucleotides [276, 277]. Studies in *Drosophila* showed that long dsRNA molecules (>30 nts) are processed into 21-23-nt fragments [281]. Supported by studies showing targeted mRNA was cleaved into 21-23-nt intervals, researchers hypothesized smaller dsRNA molecules served as the guide RNAs for target recognition in various species [282, 283], [281]. Using the *Drosophila* in vitro system [284], researchers developed synthetic 21- and 22- nt RNAs, paired together in a Watson-Crick fashion, with 3'overhanging ends, which they termed small inhibitory RNA (siRNA) [260]. In their study, these siRNAs acted as a guide to degrade luciferase mRNA in *Drosophila*

embryo lysate; longer and shorter siRNAs were shown to be less potent than the short 21-23 nt sequences [260].

Following up on these discoveries, a breakthrough in the study of RNAi in mammalian cells was achieved three years after it was first documented in *C. elegans*. Two separate researchers exogenously delivered siRNAs into 293 (human embryonic kidney), HeLa (human epithelial cancer), and mouse embryonic fibroblast cells resulting in a transient knockdown of gene expression [272, 285]. These siRNAs proved to efficiently induce sequence specific degradation of target genes without initiating the antiviral/interferon response. Thus, for the first time RNAi was shown to occur in mammalian cells using siRNA molecules rather than long dsRNAs.

Now, just three years after the discovery of siRNA, the field of RNAi is moving at an accelerated pace. With well over 2,500 published articles on RNAi (PubMed), it has rapidly developed into one of the most widely applied technologies in molecular and cellular research, and although young, is now an essential experimental tool [286]. Nearly eight years after its discovery, RNAi is feasible in a plethora of organisms (Table 4.2). The versatility of RNAi has led to a number of exciting applications not only in model organisms but also in the study and potential therapy of human diseases and disorders. Undoubtedly, the extent to which RNAi can be used has yet to be recognized.

Table 4.2: Eukaryotic organisms exhibiting RNAi-related phenomena [287]

Kingdom	Species	Stage tested	Delivery method
Protozoans	*Trypanosoma brucei*	Procyclic forms	Transfection
	Plasmodium falciparum	Blood stage	Electroporation and soaking
	Toxoplasma gondii	Mature forms in fibroblast	Transfection
	Paramecium	Mature form	Transfection and feeding
	Leishmania donovanii		Tried but not working
Invertebrates	*Caenorhabditis elegans*	Larval stage and adult stage	Transfection, feeding bacteria carrying dsRNA, soaking
	Caenorhabditis briggsae	Adult	Injection
	Brugia malayi (filarial worm)	Adult worm	Soaking
	Schistosoma mansoni	Sporocysts	Soaking
	Hydra	Adult	Delivered by micropipette
	Planaria	Adult	Soaking
	Lymnea stagnalis (snail)	Adult	Injection
	Drosophila melanogaster	Cell lines, adult, embryo	Injection for adult and embryonic stages, soaking and transfection for cell lines
	Cyclorrphan (fly)	Early embryonic stages	Injection
	Milkweed bug	Early embryonic stages	Injection
	Beetle	Early embryonic stages	Injection
	Cockroach	Larval stage	Injection
	Spodoptera frugiperda	Adult and cell line	Injection and soaking
Vertebrates	Zebra fish	Embryo	Microinjection
	Xenopus laevis	Embryo	Injection
	Mice	Prenatal, embryonic stages, and adult	Injection
	Humans	Human cell lines	Transfection
Plants	Monocots/dicots	Plant	Particle bombardment with siRNA/transgenics
Fungi	*Neurospora crassa*	Filamentous fungi	Transfection
	Schizosaccharomyces pombe	Filamentous fungi	Transgene
	Dictyostelium discoideum		Transgene
Algae	*Chlamydomonas reinhardtii*		Transfection

CHAPTER 5

The mechanism of RNA interference

Although the history of PTGS, whether it is cosuppression in plants, quelling in fungi, or RNAi in animals, is fascinating, the underlying ancestral mechanism and its innate role in these organisms is all the more intriguing. Although designated by different names, the mechanism induced by dsRNA molecules is similar among all organisms and will universally be referred to as RNAi in the remaining text of this chapter – however discrepancies between the mechanisms will be noted. Since its discovery, huge efforts have been made to further examine the mechanism of RNA silencing, therefore enabling researchers to improve the stability, uptake efficiency, and selective target binding capacity of these double stranded nucleotides. As first established by Fire *et al*., RNAi occurs through a sequence-specific posttranscriptional gene silencing mechanism carried out by double-stranded RNA [236].

The RNAi mechanism has been extensively reviewed [1, 286-292] and is highlighted in Figure 5.1. RNAi is triggered when a cell encounters double-stranded RNA (dsRNA), which can be introduced by a transgene, a virus, or a foreign genetic element. An enzyme called Dicer cleaves the long dsRNA into a small inhibitory RNA (siRNA) duplex, around 21 to 23 nt in length each with 2-nucleotide 3' overhangs and 5'-phosphate and 3'-hydroxyl termini [260, 293]. An RNA-induced silencing complex (RISC) then distinguishes between the two siRNA strands. The sense strand is simply degraded in the cytoplasm. The antisense strand is used to target specific genes for silencing and, depending upon the organism, may affect mRNA degradation through two distinct mechanisms (Fig. 5.1).

In fruit flies and mammals, the antisense strand is incorporated directly into RISC to target a complementary mRNA for destruction. In the absence of siRNA, RISC lacks sequence-

specific mRNA-binding properties; but when bound to the antisense strand, the now activated RISC can participate in repeated cycles of mRNA degradation, such that no protein is made — effectively silencing the gene from which the mRNAs are produced [281, 283, 284, 293].

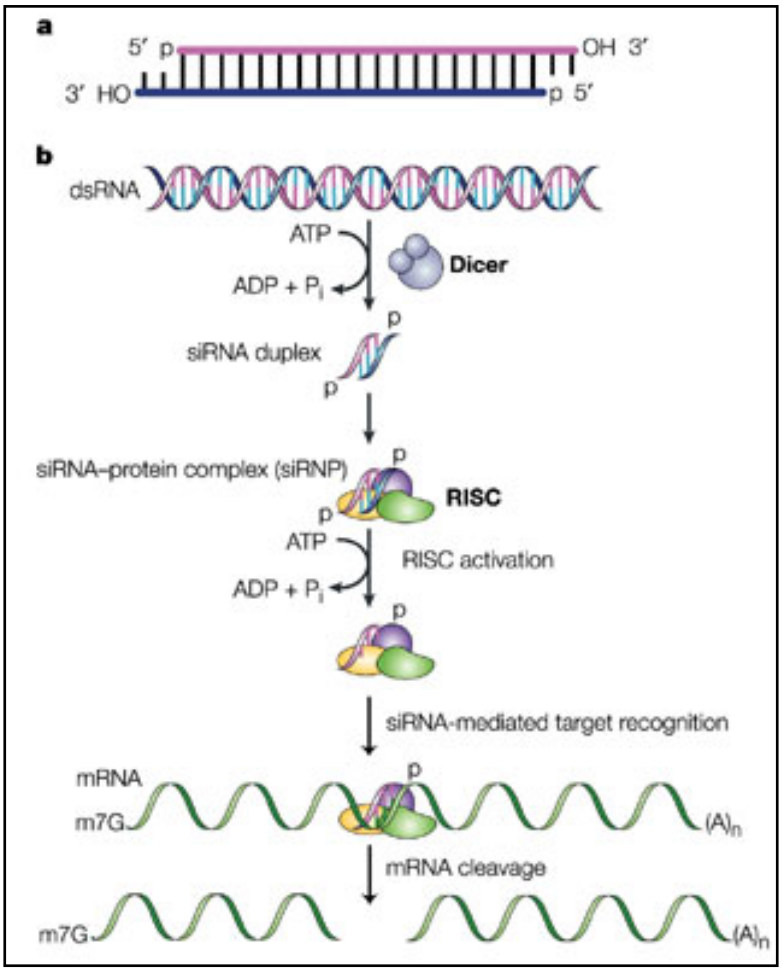


Figure 5.1: Proposed mechanism of RNAi.

In worms and plants, the antisense strand appears to act as a primer to be used in an amplification process. The antisense strand, bound by an RNA-dependent RNA polymerase (RdRP), pairs with its complementary mRNA and acts as a start point for the synthesis of a new dsRNA, a phenomenon called transitive RNAi [294]. This not only results in the elimination of target mRNA, but also the generation of a new population of siRNAs by Dicer [268]. These newly synthesized siRNAs are specific to different sequences on the same mRNA [291]. As

with mammals and fruit flies, siRNAs associate with RISC to target homologous mRNAs for destruction by nucleolytic cleavage [260, 295].

Although both mechanisms result in the destruction of target mRNA, the varied biology of dsRNA-induced silencing suggests that the core machinery probably adapted to meet specific biological needs in different organisms. However, the common critical features underlying this shared mechanism include (1) a dsRNA inducer, (2), the necessary machinery – including Dicer and RISC (Table 5.1) and (3) the recognition and homology-dependent degradation of target RNA.

Table 5.1: Genes associated with RNAi in various species. [286]

Name (species)	Structure and function
Initiation step	
Dicer: human Dicer, DCR-1 (Ce), Dicer-1 and 2 (Dm), DCL1 (At)	dsRNA-binding, 2 RNaseIII-; ATP-binding-, PAZ-; DEAD/DEAH box helicase domain; dsRNA processing into siRNAs; pre-miRNA processing into miRNAs. Essential in all organisms studied. DCL1 is not essential for PTGS in plants.
Drosha (Dm)	2 RNaseIII domains, dsRNA binding domain; involved in generation of pre-miRNAs
R2D2 (Dm)	dsRNA binding motif; transfer of siRNAs into RISC complexes
RDE-4 (Ce)	dsRNA binding motif, RNA helicase
RDRPs: SAD1 (Nc), QDE-1 (Nc), EGO-1 (Ce), RRF-1 (Ce), RrpA (Dd), Spn-E (Dm), SGS2 (At), SDE1 (At)	RNA-dependent RNA helicases use siRNAs to prime dsRNA synthesis. Secondary siRNAs are produced by Dicer. Putative amplification mechanisms required for systemic and heritable RNAi. No homologues identified in humans.
Execution step: components of RISC and miRNP	
Argonaute proteins:	PAZ and C-terminal Piwi domains; function unknown; protein-protein interaction
eIF2C-related: eIF2C1,2,3 (Hs); Ago1-5 (Mm), ALG-1 (Ce), AGO1 (At), ZWILLE (At)	Human eIF2C proteins are found in miRNPs; AGO1 and ZWILLE are developmental regulators
QDE-2 (Nc), RDE-1 (Ce)	Essential for RNAi in *Neurospora crassa* and *C. elegans*, resp.
Piwi-related: Piwi (Dm), Aubergine (Dm), HIWI (Hs), Miwi (Mm)	Piwi: nuclear protein, regulates germline stem cell fate in *Drosophila* Aubergine: cytoplasmic, required for RNAi in *Drosophila* oocytes
Others:	
Gemin 3 (Hs)	RNA helicase domain; complexes with eIF2C2 and Gemin 4
Gemin 4 (Hs)	Function unknown
FMRP (Hs, Dm)	Human FMRP: RNA binding protein involved in translational control; regulates axon guidance and neuronal plasticity; mutated in patients suffering from fragile X-syndrome
VIG (Hs, Dm)	RNA binding domain
Tudor-SN (Dm)	Nuclease found in FMRP and VIG containing RISC complexes
Systemic and inheritable RNAi	
MUT-7 (Ce)	RecQ helicase domain, related to Blooms' and Werner's syndrome helicases.
RDE-2 (Ce)	Function unknown
SID-1,2,3, (Ce)	SID-1: transmembrane protein, which facilitates dsRNA uptake; homologues in humans

At, *Arabidopsis thaliana*; Ce, *Caenorhabditis elegans*; Dd, *Dictyostelium discoideum*; Dm, *Drosophila melanogaster*; Hs, *Homo sapiens*; Mm: *Mus musculus*; Nc, *Neurospora crassa*.

The Common Components of Gene Silencing

To gain insight into the components involved in RNAi, genetic screens have been carried out in model organisms including the fungus, *N. crassa*; the alga, *C. reinhardtii*; the nematode, *C. elegans*; and the plant, *A. thaliana* to search for mutants defective in RNAi. These screens have identified proteins essential to the gene silencing process and have also highlighted the common factors involved among these organisms; therefore demonstrating the existence of a common genetic base for RNAi (Table 5.1).

Dicer

As an initiator of the RNAi response, dsRNA can be introduced into the cell via experimentally expressed dsRNA, aberrantly expressed transgenes, RNA viruses, transposons, or as short hairpin RNAs (shRNA) [1]. Once incorporated into the host cell (in *C. elegans* possibly through SID-1, an RNA transporter [296, 297]), the large dsRNA is cleaved into smaller 21-23 nt siRNAs by a dsRNA-specific endonuclease called Dicer [293, 295] (for a recent review on the discovery and function of Dicer see [288]). Homologues of Dicer have been shown in *C. elegans* (termed *dcr*-1), *Arabidopsis*, mammalian cells, and in *Drosophila* (Table 5.1) supporting the notion this RNAi related enzyme is evolutionarily conserved [293, 298].

Figure 5.2: Domain structure of mouse Dicer. Helicase (DexH/DEAH RNA helicase/ATPase) domain/56–544; PAZ (Piwi Argonaute Zwille)/912–1037; RNAse III (catalytic) domains/1278–1380+1637–1860; and a DSRM (double-stranded RNA binding motif) 1849–1906. [288]

Dicer has five specific domains (Fig. 5.2) including an N-terminal helicase domain, a dsRNA binding motif (DSRM), two RNAse III motifs at the C-terminus, along with a PAZ domain and is thought to work as a dimeric enzyme [299]. Interestingly, Dicer contains two

RNAse III domains. In bacteria, proteins with single RNAse III domains cleave dsRNA at 11-nt intervals. In Dicer, the presence of two structural but only one functional RNAse III domains (Fig. 5.3 a & b) could explain the generation of 21-23 nt long siRNA molecules with 3' overhanging ends [300]. Once the dsRNA is cleaved by Dicer, as depicted in Figure 5.1 and 5.3a, the resulting siRNAs serve either as a primer (plants and worms) for further amplification and siRNA synthesis or as a guide sequence (flies and animals) to induce target-specific mRNA cleavage by the RNA-induced silencing complex (RISC) [281-283].

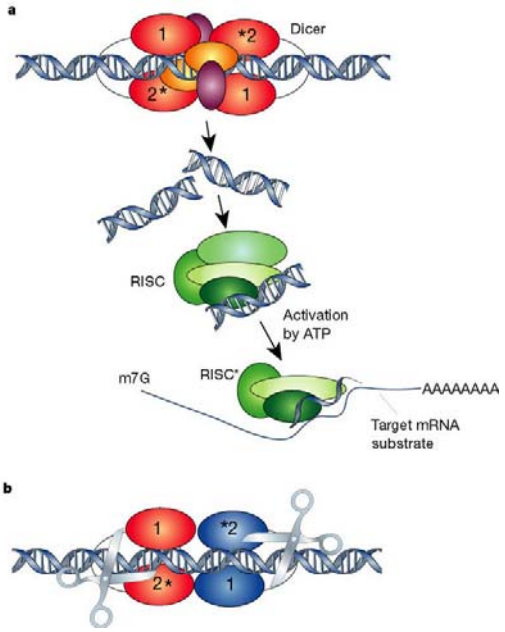


Figure 5.3: Dicer and RISC activity on dsRNA and siRNA respectively. [1]

a. Dicer (red), a dimeric enzyme cleaves dsRNA into 21-23 nt fragments. Although having two RNAse III sites, only one is functionally active to cut dsRNA (indicated by asterisks). The siRNAs are incorporated into RISC (green) where it unwinds to allow the antisense strand to act as a guide to locate the target mRNA to cleave.

b. Representation of Dicer binding and cleaving dsRNA (for clarity, not all the Dicer domains are shown, and the two separate Dicer molecules are colored differently). Deviations from the consensus RNase III active site in the second RNase III domain inactivate the central catalytic sites, resulting in cleavage at 21 to 23-nt intervals.

RNA-induced silencing complex (RISC)

Once generated by Dicer, the siRNAs are incorporated into RISC, a large multi-component ribonuclease protein complex [283, 295]. The siRNA structure, with its 2 nt 3' overhangs and 5'- phosphate termini, is functionally important for incorporation into the inactive RISC complex [295, 301]. Located in the cytoplasm [302], the siRNA-RISC complex acts on mature rather than nuclear precursor mRNA [237]. Strand separation of the siRNA duplex must occur prior to target RNA recognition. In order to do so, studies occurring in Drosophila embryo

extracts show RISC is activated during the ATP-dependent unwinding of the siRNA duplex [295]. In the absence of ATP, the antisense RNA strand remains bound to the activated RISC complex and cleaves the cognate mRNA sequence [303] This is done by specific base pairing whereby causing an endonucleolytic cleavage of the mRNA 11 nt downstream from the 3' end of the guide siRNA [260, 301, 304]. Although siRNAs 21-23 nt in length are generated by Dicer and are more efficient at mRNA degradation, two separate researchers have demonstrated single stranded antisense RNAs ranging from 19 to 29 nt can also enter the RNAi pathway and bind to the RISC complex [303, 304].

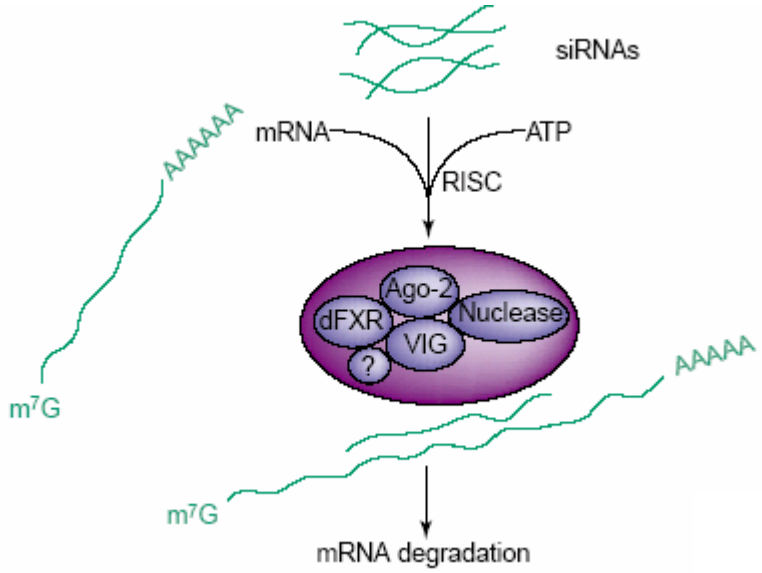


Figure 5.4: RNA-induced silencing complex (RISC). VIG – (Vasa intronic gene) RNA binding; dFXR – (fragile x syndrome) RNA binding; Ago2 – (Argonaute 2) facilitates siRNA incorporation into RISC; Nuclease – unwinds the siRNA. [292]

Researchers characterizing the function and general makeup of the RISC complex in multiple organisms have discovered several conserved proteins (Fig. 5.4). RISC is a ribonucleoprotein complex at times containing both siRNA and protein. Isolated in *Drosophila* Schneider 2 (S2) cells, one protein component associated with RISC was identified as Argonaute-2 (AGO2) [305]. Along with Dicer, RISC also contains a PAZ domain and is housed

within the AGO2 protein. As hypothesized, it was recently shown in human that AGO2 is responsible for the endonucleolytic cleavage of target mRNA [306]. All characterized RNA silencing effector complexes contain at least one Argonaute protein and comprises the largest protein family specifically involved in dsRNA-triggered gene silencing [254, 304, 305, 307]. Taken together, these similarities add additional support to the hypothesis that gene silencing, either by PTGS, quelling, or RNAi, are derived from a common ancestral mechanism that controls expression of invading nucleic acid molecules at the post-transcriptional level.

RNA-dependent RNA polymerases

One distinctive difference between RNAi in plants and the response triggered in animals is that in plants gene silencing is heritable and acts in a systemic fashion [1]. The gene silencing effect is potent and systemic. This has led researchers to propose mechanisms in which RNA-dependent RNA polymerases (RdRps) play a role in triggering and amplifying the silencing effect. Homologous genes essential to RNAi have been identified including the *Arabidopsis* SDE1/SGS2 gene [308], the *C. elegans ego*-1 gene [255] and the *qde*-1 gene in *N. crassa* [309] which is homologous to a tomato gene encoding a protein with RdRp activity [310].

RdRps are enzymes characteristically involved in RNA-virus replication by synthesizing complementary RNA molecules using RNA as a template. Studying its activity in tomato leaves, RdRP catalyzes in vitro the transcription of short single-stranded RNA and DNA molecules into precisely complementary RNA copies [311]. Although it has been proposed that RdRp plays a role in the amplification of the RNAi pathway in plants, mutants of these genes have varying phenotypes making the role of RdRP in RNAi difficult to discern [239, 312].

Although the presence of these RNA-dependent RNA polymerases have been displayed in several eukaryotic species, RdRp homologues have yet to be identified in humans or flies.

Though several of the same key components are involved, different RNAi mechanisms may exist in different species, with one being primarily RdRp-dependent and one RISC-dependent (see Fig 3.1) [287, 288]. Thus, it continues to be important to identify the biochemical steps involved in the RNAi pathway throughout all organisms.

Numerous genetic and biochemical studies have been performed to help elucidate the RNAi mechanism in a number of organisms, however, many questions remain. As seen in Table 5.1 and discussed above, orthologous proteins essential to gene silencing have been identified in divergent species; thus underscoring the conserved nature of many aspects in RNA silencing. Though varied in its mechanism, the core machinery involved in RNAi has no doubt adapted over time to meet the needs of each individual organism.

CHAPTER 6

Small inhibitory RNA:
Their design, delivery, and efficiency in mammalian cells

First documented by the Tuschl lab at the Max-Planck-Institute in Germany, siRNAs are the effectors of RNAi in mammalian cells [285]. Originally, it was thought mammalian cells were incapable of conducting the specific inhibition of gene expression, as observed in lower organisms through RNAi. In mammalian cells, the introduction of long dsRNA induces an antiviral interferon response, ultimately leading to non-specific gene silencing and cell death [274]. Researchers were able to overcome this by delivering short synthetic dsRNAs (<30 nt) whereby successfully bypassing the detrimental interferon response. Since its discovery in 2001, the use of siRNA to perform gene-silencing studies in mammalian species has revolutionized the area of functional genomics and, when used as a tool, has tremendous potential in the field of biomedical research as therapeutic agents and in the area of drug target discovery.

Inducing gene silencing using siRNA in mammalian cells involves the following variables: (i) selecting the siRNA sequence in the target gene, (ii) the synthesis of siRNAs or construction of plasmids bearing the DNA sequence encoding the siRNA, (iii) optimizing transfection of the siRNAs or the plasmids into target cells, and (iv) monitoring the efficiency and/or cell morphology in response to gene silencing. This chapter looks at the methods used in the design of siRNAs and their subsequent transfection/transduction into the host cell along with tools used to detect the knockdown phenotype(s).

Designing siRNAs

The design and production of synthetic siRNA is now common practice for researchers wanting to perform gene knockdown studies in mammalian species. Many well-known research

supply companies offer services to aid in the design and production of these oligonucleotides including: Qiagen (http://www.qiagen.com), Ambion (http://www.ambion.com), Dharmacon (http://www.dharmacon.com), Invitrogen(http://www.invitrogen.com), Oligoengine (http://www.oligoengine.com), Mirus (http://www.mirusbio.com), Proligo (http://www.proligo.com), and Pierce (http://www.piercenet.com) among others. The majority of companies have developed siRNA design programs using proprietary algorithms based on the design recommendations established by the Tuschl lab [313].

As an alternative to the services offered by these commercial companies, publicly available siRNA design tools are available on the web which incorporates additional selection parameters and automatically performs BLAST searches on selected sequences. Research centers offering such programs include: The Whitehead Institute of Biomedical Research at MIT; The Center for Genomics and Bioinformatics, Stockholm, Sweden (http://sonnhammer.cgb.ki.se/siSearch/siSearch_1.6.html) [314]; The Wistar Institute, Philadelphia, Pennsylvania (http://bioinfo.wistar.upenn.edu/siRNA/siRNA.htm) [315]; and Scionics Computer Innovation in Dresden, Germany (http://cluster-1.mpi-cbg.de/Deqor/deqor.html) [316]. There are several methods for generating siRNAs: including chemical synthesis, in vitro transcription, plasmid and viral vectors, and PCR expression cassettes. Irrespective of the method used, the first step in designing a siRNA is to choose the targeted site on the mRNA transcript. The recommendations summarized below have set the standard followed by researchers and companies alike.

To properly design siRNAs, knowledge of its structure and function is a necessity. As discovered by Bernstein *et al*., long dsRNA is cleaved into ~21 nucleotide siRNA duplexes that contain 2 nt 3' overhangs with 5' phosphate and 3' hydroxyl termini (Fig. 6.1) [293]. To

efficiently incorporate into the RISC complex, the thermodynamic stability of the siRNA duplex is essential. Due to the relatively low thermodynamic stability in the 5' end of the antisense (red) strand compared with the high thermodynamic stability in the 5' end of the sense strand, the incorporation of the antisense strand into the RISC complex is favored. As few as 11 to 14 continuous base pairs from the 5' end of the siRNA have been observed to target gene silencing [317]. Thus, the 5' half of siRNAs have a more significant role in target recognition than the 3' half. Cleavage of the target mRNA occurs in the middle of the siRNA duplex (marked with a triangle) and is defined by the 5' end of the guide siRNA [301]. Although still functional, 1 to 2-bp mismatches between the siRNA and target RNA reduces the rate and extent of mRNA cleavage 2 to 4 fold with the 3' end being more sensitive to these mismatches [301].

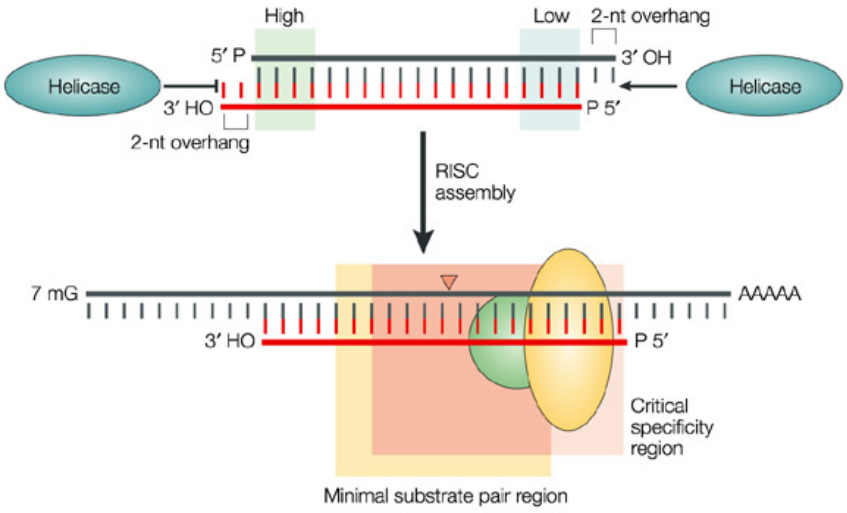


Figure 6.1: Structure and characteristics of siRNA duplexes. **[317]**

Based on the above mentioned structure, function, and kinetic stability of siRNAs along with the established literature recommendations (see [313] for an extensive list of methods), a few guidelines have been established for the synthesis of these molecules. First, knowledge of the accurate target sequence is required and, if sequenced, can be found at http://www.ncbi.nlm.nih.gov/Entrez/index.html. As a general rule, the 21 nt sequence should

have the nucleotide composition of AA(N19), where N denotes any nucleotide, at least 100 nt downstream from the AUG start codon. This ensures the presence of 3' overhanging Uracil dinucleotides on the antisense strand and is more effective at silencing the targeted transcript [301].

As a rule, it is desirable to choose sequences having approximately a 50% G/C content. However, researchers have experienced success with siRNAs containing anywhere from 32 to 79% G/C's [313]. Other recommendations include avoiding long stretches of any one nucleotide (for example AAA or GGG) as well as having a more G/C rich 5' end and a more A/T rich 3' end (Qiagen, personal communication). As a general rule, inverted repeats and UTR regions should be avoided. Once a target sequence has been identified, a BLAST search should be performed against the appropriate genome database (http://www.ncbi.nlm.nih.gov/BLAST). To ensure that only a single gene is targeted in the respective organism, sequences having more than 16-17 contiguous base pairs of homology should be eliminated. Although expensive, multiple sequences meeting the above mentioned criteria should be tested. Research has shown individual siRNAs directed against different sites on the same target mRNA can show striking differences in their silencing efficiency, shifting the target site 2-3 nt in either direction can have a drastic effect [318-320]. Though these recommendations and computer based design tools exist, no consensus on choosing effective siRNA sequences has evolved and remains to be a trial and error process.

Exogenous delivery of siRNA

The exogenous delivery of siRNAs (method II in Fig. 6.2) has classically been achieved using liposome-mediated transfection reagents. Currently, cationic lipid-based reagents are the most popular non-viral method to serve as siRNA carriers into mammalian cells. First used to

shuttle antisense oligonucleotides into cells [321], cationic lipids bind to oligoribonucleotides through anion-cation and hydrophobic interactions. The efficiency of siRNA uptake is dependent upon the cell type and the phospholipid composition. However, since high concentrations of cationic lipids may be toxic to the target cells, their application must be optimized [289]. Future development of liposomes with less cell toxicity and higher stability could enhance their utility to deliver siRNAs to a broader range of cells.

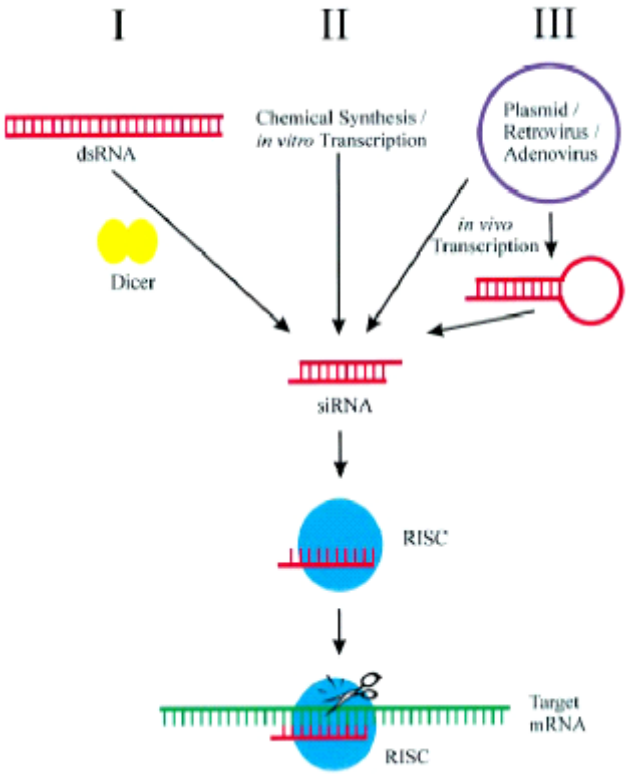


Figure 6.2: Three methods of siRNA delivery. [322]

Alternative, and perhaps less toxic, methods to exogenously deliver siRNAs into the cytoplasm involve the use of electroporation. Performed on cell suspensions, this technique uses high-voltage pulses to produce transient pores in the cell membrane through which siRNAs, and other molecules, can enter the cells. This method of delivery can be used on a variety of cell types by varying the voltage, capacity, and shape of the electric pulse. Limitations of this

method include the non-specificity of molecule uptake and the cell damage resulting in low cell viability [289]. Although both cationic liposomes and electroporation delivery methods result in negative side-effects, with their use the successful knockdown of specific genes has been realized in a whole host of cell types. In both cases, the optimization of cell transfection parameters is necessary.

Endogenous delivery and short hairpin RNA (shRNA)

Due to the exorbitant cost of synthesizing siRNAs, their lack of amplification and transient nature, and their low transfection efficiency in mammalian cells (see below), investigators were compelled to explore alternative strategies to generate siRNAs (method III in Fig. 6.2). Several groups have devised methods to synthesize short RNAs *in vitro* using the T7 RNA polymerase [323] or by introducing plasmids with the ability to make de novo siRNAs inside the cell [324-327]. The most promising alternative to synthetic siRNAs is the endogenous delivery of these duplexes within the cell. Table 6.1 provides a comparison of the advantages and disadvantages of vectors versus synthetic siRNAs.

Table 6.1: Endogenous versus exogenous delivery of siRNAs in mammalian cells. [317]

Approach	Advantages	Disadvantages
Vector-based	Delivery to non-transfectable cells. Stable silencing for non-essential genes. Inducible expression. Enzymatic preparation of hairpin libraries using cDNAs and cDNA libraries. Flexibility of shuttling of hairpin insert between different vectors (for example, between lenti-, retro- or adenoviral expression vectors; variation of promoters). Stable positive-readout screening using complex polyclonal libraries.	Prone to nonspecific interferon-response-related effects caused by high expression of hairpin RNA. Difficult to select and construct highly effective hairpin RNAs. Decreased potential for systemic delivery in therapeutic applications.
siRNA-based	Less prone to induce nonspecific side effects due to greater control over amount of transfected reagent. Ease of chemical synthetic production and quality control. Small size and chemical modifications hold best potential for therapeutic applications. Useful for structural functional studies of RNAi machinery.	Duration of silencing is dependent on rate of cell division.

The overall costs for both approaches are similar if arrayed libraries are produced targeting individual genes. RNAi, RNA interference; siRNA, small interfering RNA.

Currently, two vector based approaches have been developed to endogenously deliver siRNAs (Fig. 6.2) (for a review, see [328]). One strategy involves the independent transcription of the sense and antisense strands, whereby they hybridize within the cell to form a functional siRNA duplex (Fig. 6.2A). The second strategy involves the simultaneous transcription of the sense and antisense strands separated by a short loop sequence (Fig. 6.2B). This loop forms a short hairpin RNA (shRNA) and is processed by Dicer into a functional siRNA [324, 325]. Vectors of this design most commonly are generated to use the RNA polymerase III (pol III) promoter. The pol III promoter normally transcribes small RNAs such as tRNAs and does not allow tissue-specific siRNA expression. Employing the use of a pol III promoter enables the selective delivery of a relatively large supply of siRNA transcripts to either the nucleus or the cytoplasm. A program for constructing shRNA cloning primers, along with detailed protocols, is available from the Hannon lab at http://katahdin.cshl.org:9331/portal/scripts/main2.pl.

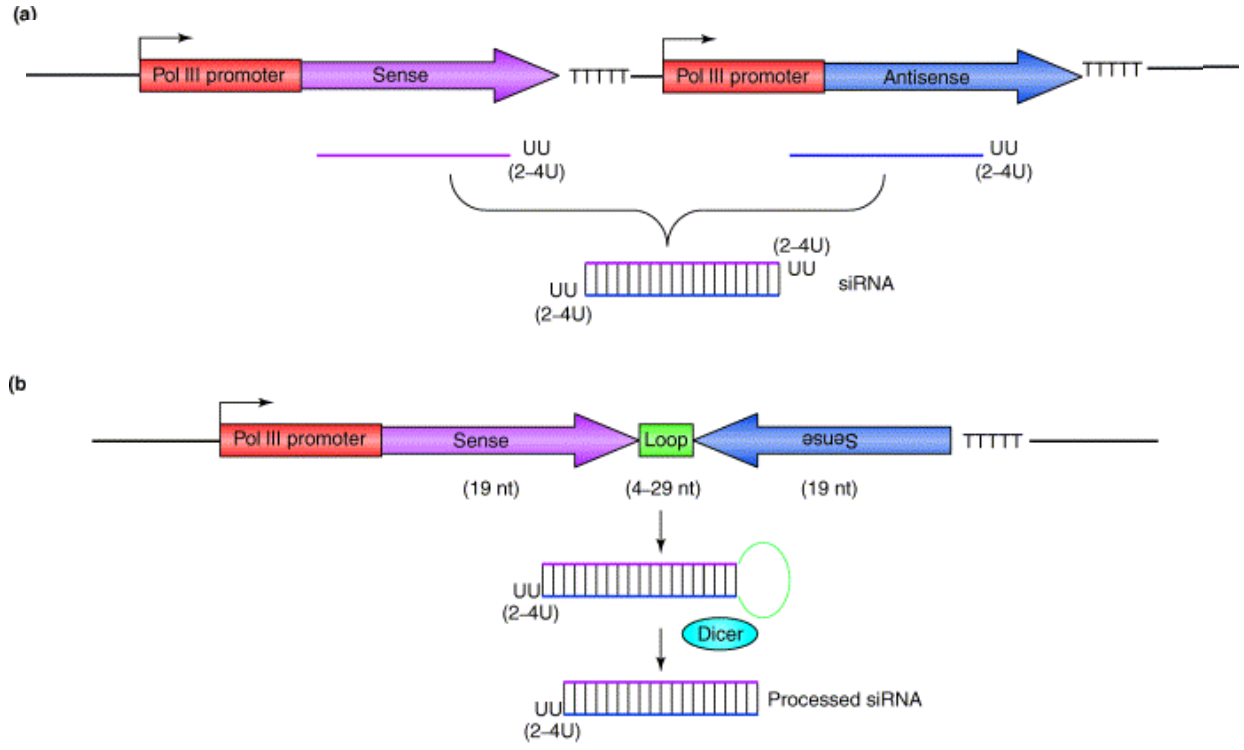


Figure 6.2: Two methods to endogenously express siRNAs using a pol III promoter. [329]

In addition to the above mentioned plasmid-based delivery of siRNA, virally delivered vectors have been proven useful. Several groups have developed adenoviral [330, 331], adeno-associated viral (AAV) [332], retroviral [333], and lentiviral vectors [334-336] that initiate RNAi in transduced cultured cells. Extending the utility of RNAi in functional genomics and potentially in therapeutic applications are selectable and inducible vectors, whereby cells expressing siRNAs can easily be turned on and off in cultured cells [337-340]. The endogenous expression of siRNA or shRNA transcripts using vectors allows for the stable, selectable, and inducible silencing of essential and non-essential genes.

Several commercially available plasmids have been developed offering this mode of delivery to the every-day researcher. Ambion's pSilencer (available at http://www.amibon.com) is available in a wide variety of forms to suit almost every RNAi application in mammalian cells. Researchers have the ability to choose vectors constructed with the U6, pol III, or CMV (recognized by adenovirus) promoters with three different selectable markers to deliver shRNA into cultured cells. Another company, OligoEngine (http://www.psuper.com), markets the vector first presented by researchers in the Bernards lab in which they named suppressor of endogenous RNA (pSUPER) and is under the direction of a pol III promoter [324]. The first vector designed to deliver siRNA endogenously into a mammalian cell, pSUPER is also marketed with selectable markers, tags, antibiotic resistance and, as available in the pSUPERIOR model, an inducible vector for on/off control.

Summarized in Figure 6.2, three modes of siRNA delivery exist. Although resulting in the same outcome - the knockdown of a gene product, the stability, efficacy, efficiency, and potency of each method is variable in mammalian cells. Thus, researchers must optimize the transfection/transduction conditions to best suit their needs.

Efficiency and detection of gene silencing

A host of factors plague the efficiency of transfection and achievable knockdown when using siRNAs in mammalian cells. The limiting factor in such studies is the transfectability of each cell type. Regardless of the transfection reagent or method used, the efficiency of transfection is typically higher for siRNAs than for plasmid DNA [322]. Other factors to take into consideration are the passage number and confluency of the cells at the time transfection or transduction is carried out. In RNAi, the steady-state level of target mRNA is determined by the rate of two independent reactions: the rates of target gene transcription and mRNA depletion [289]. In every knockdown study, the half-life of the targeted gene product, its abundance, and the regulation of its expression must be considered. In most cases, the amount of gene product available in the cell is reduced for ~ 3-5 cell doublings, that is, 3-5 days for most cell lines with normal gene expression resuming within ~7-10 cell doublings [322]. The most potent siRNAs are capable of generating >90% reduction in target RNA and protein levels. Thus, differences in siRNA effectiveness, transfection efficiencies, cell type and culture state along with protein stability are all factors affecting the efficiency of siRNA gene knockdown.

Since the basis behind RNAi is the specific knockdown of a targeted gene product, detection methods highlighting the presence or absence along with the quantity of mRNA and protein should be employed. Prior to this, the need for proper screening methods to determine if the siRNA is in fact inside and functional within the cell is of precedence. Methods using the luciferase assay or fluorescently labeled siRNAs have been used to do this. Once determined, the absence of the targeted gene product, at both the transcriptional and translational levels, will determine the knockdown efficiency. Techniques including immunofluorescence, Western

blotting, and Real Time PCR analysis have all been used to detect the extent to which knockdown has occurred in a given cell population.

Small interfering RNAs have become powerful tools for triggering gene-specific silencing in mammalian cultured cells. These short dsRNA molecules can be used to assess the functionality of essential and nonessential genes as determined by common molecular biology techniques. There are multiple ways to deliver siRNAs into mammalian cells, through endogenous and exogenous mechanisms, although proper optimization steps must be performed to increase knockdown efficiencies. Silencing can persist for several cell doublings with maximum mRNA knockdown being around 90%. siRNAs are a novel tool for genome wide analysis of gene function and will no doubt become useful for therapeutic applications and drug target discovery in mammalian cells.

CHAPTER 7

Knockdown of Dnmt1 transcripts using small inhibitory RNA in primary donor cells to be used in somatic cell nuclear transfer studies

Introduction

Since its discovery, nuclear transfer (NT) has become an invaluable method for the study of gene function [19], gene targeting [20], genomic imprinting and reprogramming [21], and genetic diseases [22, 23]. When used as a biological tool, NT has tremendous potential to generate tissues for gene therapy [24, 31, 341] and to produce biopharmaceuticals for human use [27-29, 342]. Thus, NT is not simply a method to propagate genetically elite farm animals. However, in order for the biomedical benefits of cloning to be realized, it is imperative that this technique develop to a level devoid of errors and inefficiencies. Since its conception [7], SCNT has successfully been used to produce mice [8], rats [9], cows [10], goats [11], pigs [12], rabbits [13], horses [14], a mule [15], a cat [16], an endangered species [17] along with a calf produced using cells obtained from a two-day-old beef carcass [18].

Although successful, the inefficiencies – between 0-5% depending on species, persistent anomalies [37, 40], and perinatal death associated with the current technique of NT-derived offspring are thought to result from the lack of, or inadequate, epigenetic reprogramming of the donor genome [21, 48, 55, 126]. In support of this, studies demonstrate that normal offspring are produced from the mating of abnormal cloned mice – suggesting that abnormalities associated with cloned animals are not passed on to their offspring [52]. This implies that the inappropriate gene expression seen in cloned animals may arise due to errors in epigenetic rather than genetic modifications [52]. Epigenetic modifications – most notably in the form of DNA methylation and histone acetylation, establish explicit genetic control over gene transcription [48, 54, 55].

For SCNT offspring to develop properly, genes normally expressed during embryogenesis, but silent in the somatic donor cell due to epigenetic modifications, must meticulously be reactivated [48].

DNA methylation plays a key role in maintaining genomic imprints in preimplantation embryos [74, 101]. Therefore, aberrant methylation during this stage of development, as seen in cloned embryos [115, 127, 128], could substantially inhibit proper development. When compared to fertilized controls, the majority of bovine SCNT-derived embryos undergo altered demethylation and precocious de novo methylation primarily resulting in hypermethylated blastocysts – resembling the methylation status of the donor cell [115, 127, 128, 343]. Methylation discrepancies between NT and control embryos have also been seen in mice [134, 143] and sheep [130], however such aberrancies have not been observed in the pig [129]. Irregular genomic methylation may contribute to SCNT associated gestational abortions and perinatal deaths and anomalies observed in non-viable offspring [131].

DNA methyltransferase 1 (Dnmt1) is the enzyme responsible for maintaining DNA methylation and is a possible contributor to the aberrant methylation patterns and resulting gene expression failures inherent in the current technique of SCNT [57, 148, 186, 199]. In fertilized embryos, transcripts of the somatic isoform of Dnmt1 (Dnmt1s) are present within an oocyte and cleavage stage embryo. However, this gene is not actively being transcribed nor are the maternal transcripts translated into protein; further more, Dnmt1s protein is not found within the nucleus until post-implantation [186, 191, 344]. This leaves the oocyte-specific isoform, Dnmt1o [151], as the only active maintenance methyltransferase protein in the embryo [186, 191]. Accounting for the passive demethylation events during early development, Dnmt1o is sequestered within the ooplasm where it is bound until the eight-cell-stage [191]. It is only at this cleavage stage

when Dnmt1o enters the embryonic nuclei for one round of replication where it is thought to maintain genomic imprints [186]. However, this does not properly occur in NT-derived embryos. In contrast to fertilized embryos, cloned embryos aberrantly express Dnmt1s where it has precociously been found in the nuclei [196] and may account for the loss in passive methylation observed in these embryos [115, 127]. An increase in methyltransferase activity and alterations in DNA methylation patterns are commonly seen in neoplastic cells and tumors in mice and humans [209, 345]. In these cell types, elevated levels of Dnmt1 has been shown to cause genomic hypermethylation, loss of imprinting, and embryonic lethality [206, 207, 210]. Therefore, an abundance of Dnmt1 could account for the hypermethylation and lack of proper demethylation observed in cloned embryos.

Using both antisense and small inhibitory RNA (siRNA) molecules, the cellular level of Dnmt1 can be knocked-down resulting in lower maintenance methyltransferase activity [221], decreased cell proliferation [219], and global and gene-specific demethylation and therefore re-expression of many silenced tumor suppressor and epigenetic genes [217-219, 222, 223]. In one report, the methylation status of some promoters was decreased by 80% [227]. However, not all Dnmt1 knockdown studies have reported a loss in DNA methylation patterns [205, 221]. It is essential to note that the studies mentioned above were all performed in transformed cell lines which have abnormalities in their genomes, with deletions, duplications, and alterations in chromosome number and may not represent that whicht may occur in primary cells. Therefore, knockdown studies using siRNA, like those described above, must be confirmed in primary cells and eventually in animals.

Aberrant expression of Dnmt1 in cloned embryos may result from (1) a disruption in the transcriptional or post-transcriptional regulation of Dnmt1s [196] or (2) the introduction of a

non-native and enzymatically active form of Dnmt1s upon the transfer of a somatic nucleus into an enucleated egg [57, 148, 186, 199] or (3) perhaps a combination of the two. In order to develop a means to explore this, the ability to knockdown the level of Dnmt1s using identical siRNAs in primary murine and bovine cells was investigated. First, the transfection conditions were optimized using a non-silencing siRNA control. Using the optimized conditions, the knockdown potential of two siRNAs specific for different areas on the Dnmt1 transcript were transiently transfected into the cells. Using real time RT-PCR, the expression level of Dnmt1 transcripts was significantly reduced in all three cell lines with differences occurring with regards to which siRNA and at which time point the greatest knockdown was achieved. There was no observable reduction in Dnmt1 protein across all groups and time points as determined using Western blot analysis. Once the depletion of protein and/or mRNA stocks of Dnmt1s has been achieved in a somatic donor cell we can test whether the transfer of this cell into an enucleated egg can enhance the reprogramability of a donor genome and may result in a more efficient method of SCNT.

Materials and Methods

siRNA Design: *si*RNAs were synthetically produced corresponding to the DNA Methyltransferase 1 (DNMT1) transcript. Target sight were selected by aligning the murine (Locus# NM_010066), bovine (Locus# NM_182651), and ovine (Locus# 29536010) cDNA sequences (found at http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=unigene) using CLUSTLW (Biology Workbench; http://workbench.sdsc.edu/). Two homologous regions between the aligned sequences were selected following standard siRNA design recommendations [313]. A BLAST search (http://www.ncbi.nlm.nih.gov/BLAST) was performed on the selected sequences to ensure only the specified gene is targeted for knockdown by the specific siRNA.

The siRNA duplexes were synthesized by Xeragon/Qiagen, Inc. (Valencia, CA) with 5' phosphate, 3' hydroxyl, and two 3'-TT overhangs on the sense and anti-sense strands. The following sequences were generated for DNMT1: sequence #1 sense 5'-CCAAGCAGGCAUCUCGGAA and antisense 5'-UUCCGAGAUGCCUGCUUGG; sequence #2 sense 5'-GAGAUGCCAUCACCCAAAA and antisense 5'-UUUUGGGUGAUGGCAUCUC. A non-silencing (negative) control was used in all transfections: sense 5'-UUCUCCGAACGUGUCACGU and antisense 5'-ACGUGACACGUUCGGAGAA (Qiagen, cat.#1022077) and during optimization experiments a 3'-Fluorescein labeled non-silencing (negative) control (Qiagen, cat.#1022079) was used.

*Cell Lines and Media Composition: Pr*imary cultures of fibroblast cells were isolated of a murine (E13 outbred mice) and bovine (E30 Angus fetus) origin. As a positive control culture, the compromised NIH/3T3 cell line (ATCC #CRL-1658) was used. All cells were cultured at 37^{o}C under 5% CO_2 in a humidified incubator. The mouse embryonic fibroblasts (MEF) and NIH/3T3 cells were cultured in high glucose DMEM (Hyclone, #SH30081.02), 10% FBS (Hyclone, #SH30071.03), 1% penicillin/streptomycin (Gibco, #15070-063), and 2mM L-glutamine (Gibco, #25030-081). The bovine fetal fibroblasts (BFF) cells were cultured in medium containing DMEM/F12 (Gibco, #11320-033), 10% FBS, and 1% penicillin/streptomycin.

siRNA Transfection Optimization and Dnmt1 Knockdown: Transfection conditions were optimized for all three cell lines as described by the manufacturer of the RNAiFect transfection reagent (Qiagen, #301605). Frozen cell vials were thawed and seeded into a 75 cm^2 tissue culture flask (BD Falcon, #353136). Cells were grown to 75-85% confluence and subcultured into 24-well culture plates (Costar, #3524) at a density of 3 x 10^4 cells per well. Transfection

efficiency was evaluated using one of three siRNA concentrations (0.5, 1.0, and 1.5 μg/ per well) of a 3'-Fluorescein labeled non-silencing (negative) control and four ratios of siRNA concentration to RNAiFect reagent (1:3, 1:6, 1:9, and 1:12). Media containing the siRNA-transfection reagent complexes was added drop-wise into each well 36-48 hrs after subculture and incubated undisturbed for six hours. For visualization, medium was aspirated and the cells were rinsed and overlaid with 1ml DPBS. Transfection efficiency was determined using fluorescence microscopy with representative pictures taken of each treatment group.

For Dnmt1 knockdown experiments, all cells were treated using the transfection conditions as determined in the optimization experiments described above. In these experiments fresh media was applied six hours post-transfection and was preceded by three washes with culture medium to remove any remaining siRNA complexes. Transfected cells were allowed to incubate in standard culture conditions with RNA and/or protein being collected at 0, 24, 48, and 72 hours post-transfection. Transfections were carried out in triplicate.

Flow Cytometry: The NIH/3T3 and MEF cells were plated, transfected, and cultured as described above in the optimization step. Cells were exposed to the siRNA complexes for six hours, whereupon they were harvested and counted using trypan blue (Sigma #T8154). Prior to flow cytometry analysis, 100μl of propidium iodide (Roche #1348639) was added to a 1 ml cell suspension containing 10^6 cells (final concentration, 50 μg/ml). Cells were analyzed using a MoFlo High-Performance Cell Sorter (DakoCytomation, Ft. Collins, CO).

RNA Isolation, cDNA Preparation, and Real Time RT-PCR: At the specified time points, culture medium was aspirated and cells were washed 1x with DPBS (Hyclone, SH30028.02). The cells were harvested with total RNA being isolated using the RNeasy Mini Kit (Qiagen, #74104) per the manufacturer's instructions. Samples were quantified using an RNA 6000 Nano Assay

(Agilent Technologies) and the Agilent 2100 Bioanalyzer which also verifies the integrity of each isolated product. RNA samples were treated with DNase (Promega, #M6101) and 0.75 µg cDNA was prepared using Applied Biosystems High-Capacity cDNA Archive Kit (#4322171) with the addition of 1U/µl of RNase Inhibitor (Ambion, #2694) per reaction. Reaction conditions for reverse transcription were 25°C for 10 min followed by a 120 min incubation at 37°C.

Real-time RT-PCR was performed using an ABI Prism 7900HT detection system (Applied Biosystems, Foster City, CA). All primers and fluorescent probes for exonic regions were purchased from Applied Biosciences (Foster City, CA). The following TaqMan Gene Expression Assay (ABI) ID numbers, with the corresponding gene locus ID in brackets, were used to detect the murine transcripts: DNA methyltransferase(cytosine-5)1 - Mm00599763_m1 (NM_010066) and beta-glucuronidase (used as an endogenous control) - Mm00446953_m1 (NM_010368). To amplify the bovine Dnmt1 sequence, a Custom TaqMan Gene Expression Assay was developed using locus ID NM_182651 imported into ABI's File Builder Software (http://home.appliedbiosystems.com/support/software/filebuilder). Eukaryotic 18S rRNA (ABI, #4319413E) was used as an endogenous control for all bovine real time RT-PCR reactions. Amplification was carried out in duplicate using the TaqMan Universal PCR Master Mix (ABI, #4304437), at 50 °C for 2 min (1 cycle), 95 °C for 10 min (1 cycle), and 40 cycles at 95 °C for 15 sec and 60 °C for 1 min with fluorescent probe and appropriate primer pairs.

Protein Isolation and Western Blot: Nuclear protein was isolated using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Pierce, #78833) according to the supplier's directions at 0, 24, 48, and 72 hrs post-transfection for each treatment (siRNA#1, siRNA#2, and non-silencing control) by pooling cells from three wells of a 24-well plate. The concentration of each protein

sample was determined using the Micro BCA Protein Assay (Pierce, #23235). SDS-PAGE was performed using the NuPAGE Bis-Tris Gel and Buffer system (Invitrogen; Carlsbad, CA) on 4-12% SDS polyacrylamide gels with proteins being transferred onto a PVDF membrane. Immunoblotting was conducted to detect Dnmt1 and β-actin using commercially available antibodies (Dnmt1, Imagenex Corporation, San Diego, CA; b-actin, Santa Cruz Biotechnology, Inc., #sc-1616). Secondary antibodies were conjugated with HRP (Santa Cruz Biotechnology, Inc) and chemiluminescence detection was carried out using the ECL Plus Western Blotting Detection System (Amersham Biosciences, #RPN2132) and captured using a ChemiImager 4400 (Alpha Innotech Corp.; San Leandro, CA). The membranes were probed first for Dnmt1 then stripped for 35-55 minutes at 50°C in a solution containing 62.5mM Tris (pH6.7), 2% SDS, and 100mM β-mercaptoethanol and re-probed for β-actin.

Data Analysis: Real Time PCR data was analyzed using the SDS software package supplied with the ABI Prism 7900HT in which the threshold cycle (C_T) and baseline settings are automatically determined for each investigated transcript. The C_T values were normalized to the expression level of the endogenous control, beta-glucuronidase (GUS) or 18s (target gene C_T – calibrator gene C_T), and the relative expression of Dnmt1 was calculated using the $2^{-\Delta\Delta CT}$ method [346] based on the expression of the non-silencing control group within the same time period. For statistical analysis, delta C_T values of non-silencing control and treatment groups within the same time period were subjected to a one-tailed T-test ($p< 0.05$).

Results

This study investigated the knockdown potential of siRNAs designed for homologous regions on the DNA Methyltransferase 1 (Dnmt1) transcript between the murine, bovine, and ovine species. Based on homology and sequence recommendations set by the Tuschl lab [313],

two candidate siRNAs were produced and tested in murine and bovine fetal fibroblast cells. Using a fluorescently labeled control siRNA, transient transfection conditions were optimized following the suppliers (Qiagen) recommendations for cell confluency, siRNA concentration, and the ratio between siRNA concentration and transfection reagent (siRNA/TR). Based on a visual assessment of fluorescence six hours post-transfection, within each well there was a noticeable correlation between an increase in the siRNA:TR ratio and the amount of background fluorescence. In all treatment groups, diffuse fluorescence within the cells cytosol could be discerned as reflected by a localized and prominent punctate fluorescence within the cell (Fig. 7.1A). Morphologically, some cells had the appearance of vacuole-like structures within the cytoplasm (Fig. 7.1B). Cell viability was not severely affected since culture confluency increased over time.

Using fluorescence microscopy and a 3X3 factorial allotment of treatments, the optimization experiments revealed a high number of transfected cells in the 1.5 µg siRNA/1:9; 1.0 µg siRNA/1:12; and 1.5 µg siRNA/1:12 groups in the murine cell populations (NIH/3T3 and MEF) with little or no transfected cells in the remaining six groups (see Fig. 7.2 A,B). The BFF cells had a decrease in cell viability and proliferation and abnormal morphology in cells transfected with a siRNA:TR ratio of 1:6 or higher. Based on visual fluorescence levels, the 1.5µg siRNA/1:3 group displayed the greatest transfection efficiency when taking into account the observed morphology (as described above) of the cells (Fig. 7.2 C). Therefore, all subsequent siRNA transfections in the BFF cell line utilized this siRNA concentration and ratio.

In order to obtain a more accurate assessment of the transfection efficiency in the murine cultures, flow cytometry was performed on the transfected NIH/3T3 and MEF cell lines using the 1.5 µg siRNA/1:9, 1.0 µg siRNA/1:12, and 1.5 µg siRNA/1:12 treatment groups. As to not

count the fluorescent punctate particles unincorporated within the cell and which failed to be washed away, an inclusive gate was set to exclusively count fluorescent events corresponding to the diameter of a control population of cells. The 1.5 µg siRNA/1:12 treatment group showed the highest level of transfection resulting in 99.6% (NIH/3T3) and 54.8% (MEF) fluorescent cells with less than a 2% death rate (Fig. 7.4). However, at this high levels of transfection reagent ratio (1:12), the cells morphology in these optimization groups (observed using fluorescence microscopy) was altered and would be classified as a non-viable cell for future studies. Thus, the two 1:12 treatment groups was not chosen to carry out further transfection studies in the murine cultures. Based on cell morphology, the transfection optimization rates, and the flow cytometry data, the optimized treatment groups were determined to be 1.5 µg siRNA/1:9 (murine) and 1.5 µg siRNA/1:3 (bovine). All further experiments were carried out using these transfection conditions.

Real time RT-PCR was used to quantitatively determine the knockdown achieved using siRNAs designed for two independent regions on the Dnmt1 transcript in the murine and bovine cells. Cells were transfected in triplicate with either siRNA#1, siRNA#2, or a non-silencing control siRNA in a 24-well plate format as in the optimization experiments. Real time RT-PCR was run using cDNA generated from mRNA extracted 24 and 48 hrs PT from all three siRNA groups. In order to compare results between siRNA treatments, Ct values were normalized to either beta-glucuronidase or 18s rRNA and were compared to a non-silencing control within the same time period. Results are expressed in percent knockdown (%KD) of Dnmt1 with a 100% expression level being assigned to the non-silencing control group. Results indicate a significant knockdown was achieved in the all three cell lines ($p<0.05$) (Fig. 7.5). A 56.5% reduction (or knockdown) in Dnmt1 occurred in both murine cultures (NIH/3T3 and MEF) whereas a 15.4%

reduction was observed in the bovine primary cell lines as compared to the non-silencing control within the same time period. However, this reduction occurred 24 hrs earlier in the NIH/3T3 and bovine transfections than those carried out in the MEF cells. (Fig.7.5). A significant difference in the knockdown capability between siRNA#1 and siRNA#2 was only seen in the 48 hr NIH/3T3 transfected cells ($p<0.05$). Although not significant, transfections with siRNA#1 in the NIH/3T3 culture and siRNA#2 in the MEF culture resulted in an overall greater Dnmt1 knockdown. Using these designed siRNAs, the real time RT-PCR results indicate murine Dnmt1 can be reduced by >50% not only in a compromised cell line but in a less easily transfected primary culture as well. These same siRNAs did not reduce the expression of Dnmt1 to the same degree in bovine cells.

In order to determine whether mRNA knockdown correlated with protein knockdown, nuclear protein extracts were generated from all three treatment groups (siRNA#1, siRNA#2, and non-silencing control) at 24, 48, and 72 hours post-transfection. Western blotting and immunoprobing for Dnmt1 and β-actin (Fig. 7.6) revealed the presence of Dnmt1 across all treatments and time periods along with a proteolytic cleavage fragment at 145 kD. The presence of a very faint β-actin band at 43 kD suggests minimal cytoplasmic protein contamination in the nuclear protein lysates. These results indicate the reduction of Dnmt1 protein was not observed at these time points and under these conditions in all three cell types.

Discussion

This study attempted to decrease the amount of somatically expressed maintenance methyltransferase (Dnmt1) using siRNAs designed based on the sequence homology between three phylogenetically divergent animals (ovine, bovine, and murine). Using siRNAs designed against two different sites on the Dnmt1 transcript, our results indicate that the steady-state level

of Dnmt1 mRNA was depleted in both a compromised and primary murine cell line. Although a significant knockdown of Dnmt1 was achieved using these same siRNAs in primary bovine cells it was not to the same degree as seen in the murine cultures. However, this is the first report of Dnmt1 being kocked-down in a primary cell type in any species.

The reason for designing siRNAs from homologous regions in the murine, bovine, and ovine Dnmt1 sequences was based in part on the concept of finding a universal siRNA to knockdown Dnmt1 in murine and bovine cells. Eliminating a compounding factor, obtaining a universal siRNA would allow comparative studies to be performed across species. This is of interest when performing cross species nuclear transfer studies [347]. Successfully knocking-down Dnmt1 in both bovine and murine cells using a universally designed siRNA could test the effects of genomic reprogramming in our previously established cross species nuclear transfer model [347]. However, the results obtained in this study suggest a universal siRNA is not equally effective within intra- and interspecies. Thus, a specific siRNA should be designed and tested in order to obtain a maximal level of knockdown in differing cell types.

In RNAi studies, differences in siRNA effectiveness, transfection efficiencies, cell type and culture state along with protein stability are all factors affecting the efficiency of an siRNA-induced protein knockdown [322]. An initial concern in this study was the transfection potential of our primary cells. The NIH/3T3 cell line was used as a positive control due to its increased transfection potential. In this study, based on flow cytometry data, the transfection efficiency of the primary murine cells was 45-50% lower than that observed in the compromised NIH/3T3 cells. Thus, a clear difference in the transfection capability of our two murine cell cultures existed. Surprisingly, this difference did not carry over into the observed level of Dnmt1 knockdown: within 48 hrs of being transfected the greatest decrease in Dnmt1 expression was

56.5% for both murine cell cultures. Notably, the specific siRNA resulting in the greatest level of knockdown differed between the two murine cell lines. Studies indicate that regardless of optimized transfection conditions, there are cell-type-dependent global effects – in regards to transfection efficiency, and cell-type-independent positional effects – in regards to knockdown efficiency [320]. Thus, regardless of transfection efficiency, siRNA knockdown can depend on positional effects, and shifting siRNAs by only one or more nucleotides may significantly influence their silencing efficiency [318, 320, 348].

Such positional effects may explain why there was only a 15.4% significant knockdown in the primary bovine cells when using these same siRNAs. Because the bovine and murine Dnmt1 sequences are not 100% homologous, the secondary and tertiary structures of the target mRNA may slightly differ and may also affect the location of associated proteins at any given region on the transcript. Similar to problems associated with antisense and ribozyme approaches, for an siRNA to be effective, site accessibility is essential for proper base pairing to occur [349, 350]. The selection of siRNA target regions on an mRNA transcript is currently a trial-and-error process; thus, alternate regions on the bovine Dnmt1 transcript might produce different results. In addition to this, the co-transfection of two or more siRNA duplexes targeting different sites on the same mRNA transcript has been used to enhance gene silencing [351].

Morphologically, within six hours of transfection there was a discernable difference between the transfected and non-transfected groups in all three cell types as noted by vacuole-like structures within the cells cytoplasm. The possibility these morphological changes resulted from the knockdown of Dnmt1 can be ruled out as these vacuole-like structures were seen with both the custom and non-silencing control siRNA. It is plausible that the vacuole-like structures observed in the cytoplasm may result from the cells internalization of the siRNA-transfection

reagent. Most non-viral vectors exploit the endocytic pathway for uptake and subsequent processing within the cell [352]; thus, the vacuole-like structures may be the siRNA complexes housed within the endosomal or lysosomal compartments [353].

Of essential consideration in any knockdown experiment is the proteins half-life, its abundance, and the regulation of its expression [313]. The level of Dnmt1 expression significantly differs during the cell cycle with it being high in the S phase and low in G1/G0 [225, 226]. Notably, when cells enter into G1/G0 by the stimulus to differentiate or during low serum conditions, Dnmt1 mRNA and protein levels decrease while the transcription of *Dnmt1* remains relatively active like that of a cycling cell [354, 355]. In essence, Dnmt1 is a post-transcriptionally regulated enzyme [224, 354]. Although a significant knockdown of Dnmt1 mRNA was achieved in the murine cultures, no reduction in protein was observed up to 72 hours following siRNA transfection. Thus, a 50-60% decrease in Dnmt1 transcripts was not sufficient to decrease the protein levels of Dnmt1 within the transfected cells. This indicates the Dnmt1-specific siRNAs were not transfected at an adequate concentration to overcome the steady-state level of Dnmt1 transcripts within these cells to show a decrease at the protein level. In studies showing the successful knockdown of Dnmt1 protein in human colorectal cancer cells, transfections were carried out every day and the cultures were split every other day [218, 221]. In contrast, in our attempt to transiently knockdown Dnmt1, the cells were subjected to a single round of transfection and, due to our timepoints, were never sub-cultured.

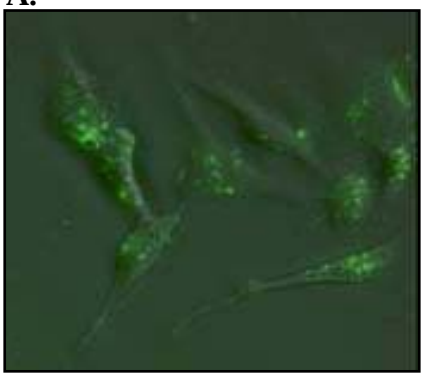
The purpose behind this study was to test our ability to knockdown the level of somatically expressed Dnmt1 in normal murine and bovine karyoplasts to be used as donor cells in future nuclear transfer studies. Research indicates cloned embryos aberrantly express the somatic form of Dnmt1 where it precociously associates within blastomere nuclei [196]. This

may account for the high incidence of inefficient genomic reprogramming observed in NT-derived embryos [57, 148, 186, 199]. Although the antisense or siRNA knockdown of Dnmt1 can result in DNA demethylation [217-219, 222, 223], this was not the underlying goal of our study: our primarily interest was in the elimination of Dnmt1 from within a donor population of cells. The results presented here indicate that on average a knockdown in Dnmt1 mRNA was achieved to a similar degree not only in a murine compromised cell line but also in a more difficult to transfect primary culture. However, these same siRNAs did not produce a Dnmt1 knockdown to the same degree when used in bovine primary cells indicating one limitation of identifying an interspecies universal siRNA. We cannot discount the fact, however, that a significant level of Dnmt1 knockdown was achieved; to our knowledge this is the first report to do so in primary cells.

Although ten species have successfully been produced, cloning by SCNT is highly inefficient with the average number of live births ranging from less than 1% to more than 5% depending on the species [45]. Although an effort to increase this low efficiency has actively been pursued using various reagents along with altering the methods of activation and fusion [356], no substantial breakthroughs have been made to increase this efficiency. In the current method of SCNT, researchers have been relying on the innate reprogramming capability, or lack there of, within the eggs ooplasm to de-differentiate a specialized cell into that of a totipotent cell. However, this is not properly being carried out in the majority of NT embryos. In order to substantially increase the efficiency while decreasing the observed anomalies seen in SCNT, novel methods, such as the one proposed, are needed to assist the oocytes ability to reprogram a differentiated genome. Until this occurs, the current field of SCNT is best suited for

fundamental studies in the discovery of reprogramming molecules essential to cell de-differentiation and plasticity

A.



B.

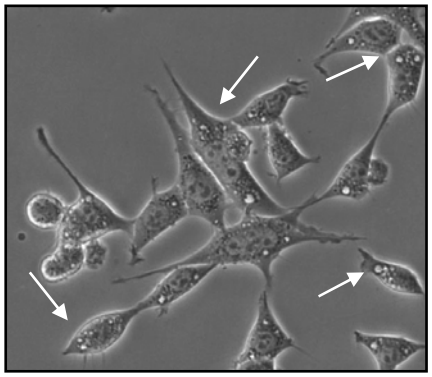
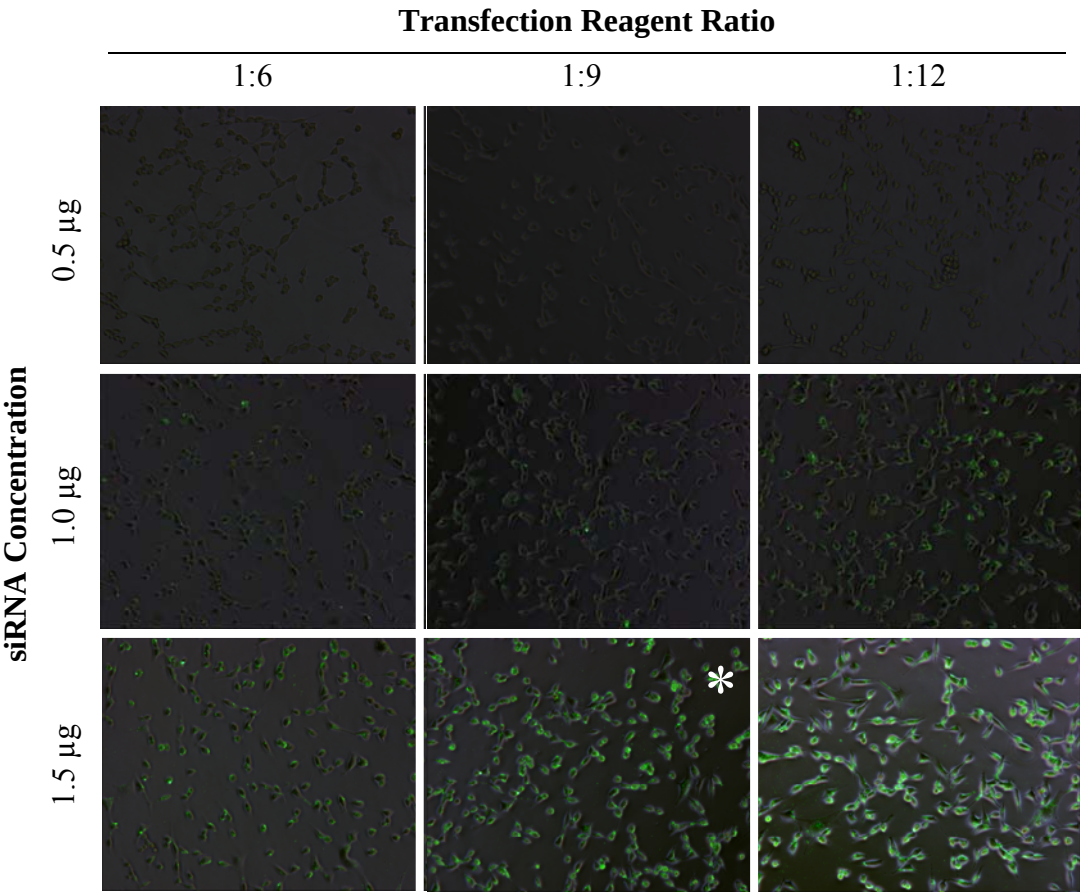
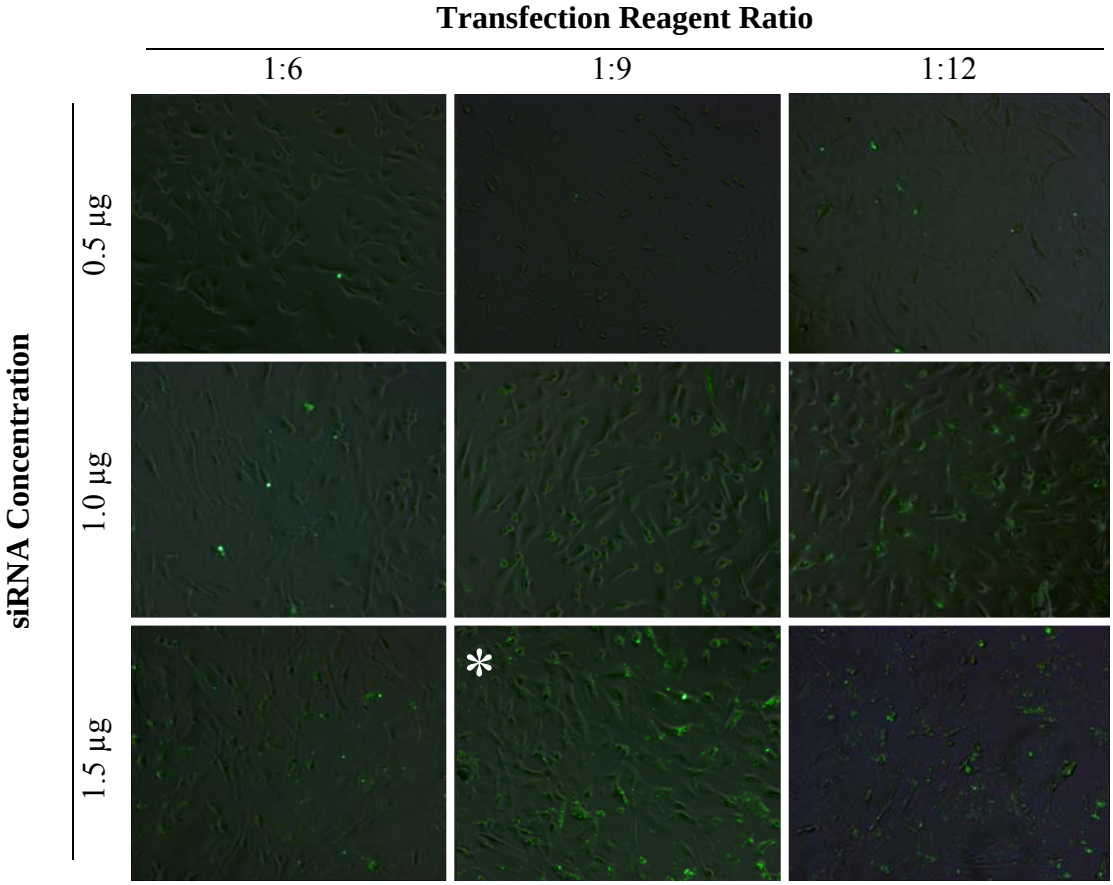


Figure 7.1: Specific morphology traits of transfected cells. (A) Peri-nuclear punctate staining of fluorescently labeled siRNA in MEF cells. (B) Regardless of siRNA (Dnmt1 specific or a non-silencing control), vacuole-like structures (arrows) were observed in all three treated cell lines.

A. NIH Cells


Transfection Reagent Ratio
1:6
1:9
1:12
siRNA Concentration
0.5 µg
1.0 µg
1.5 µg
*

B. MEF Cells


Transfection Reagent Ratio
1:6
1:9
1:12
siRNA Concentration
0.5 µg
1.0 µg
1.5 µg
*

C. BFF Cells

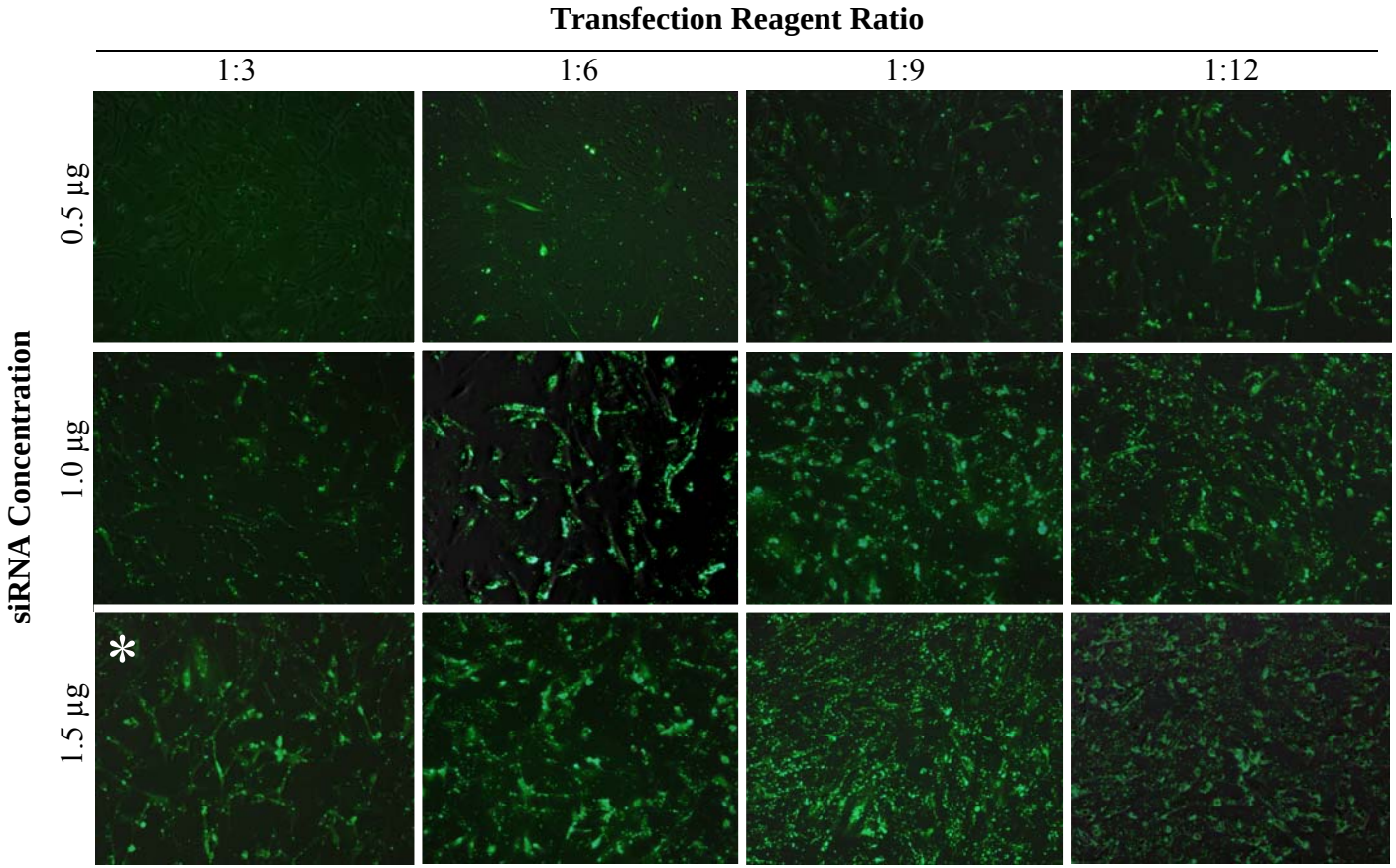
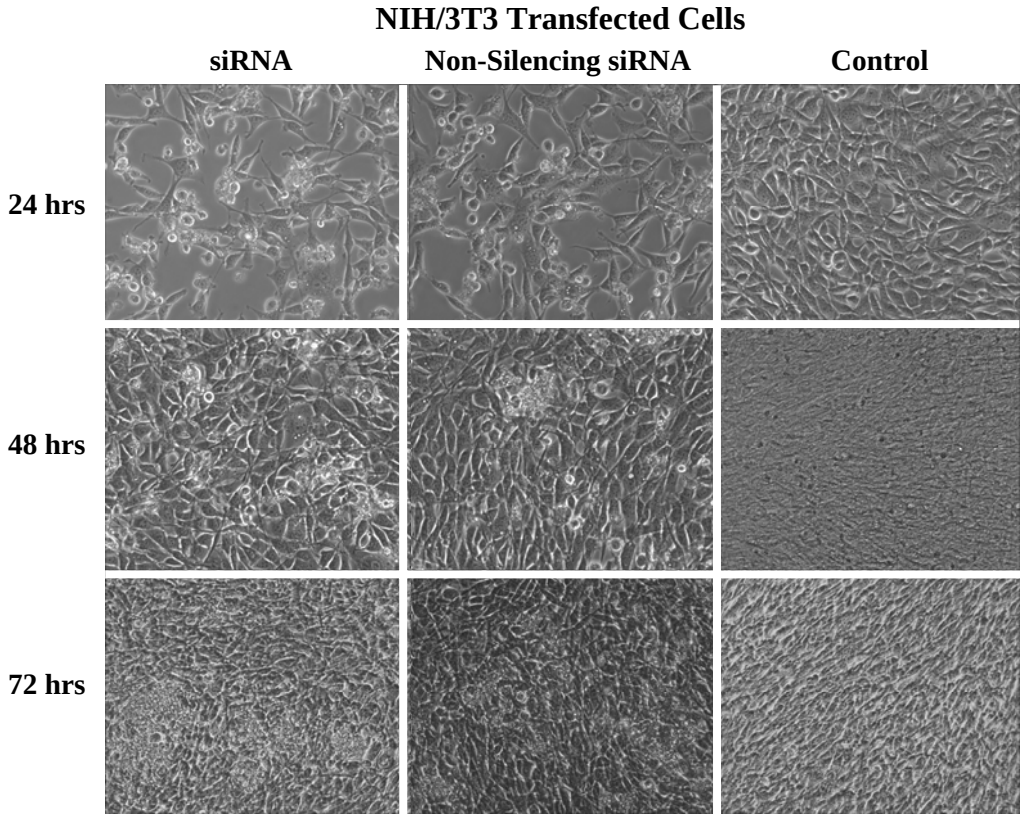
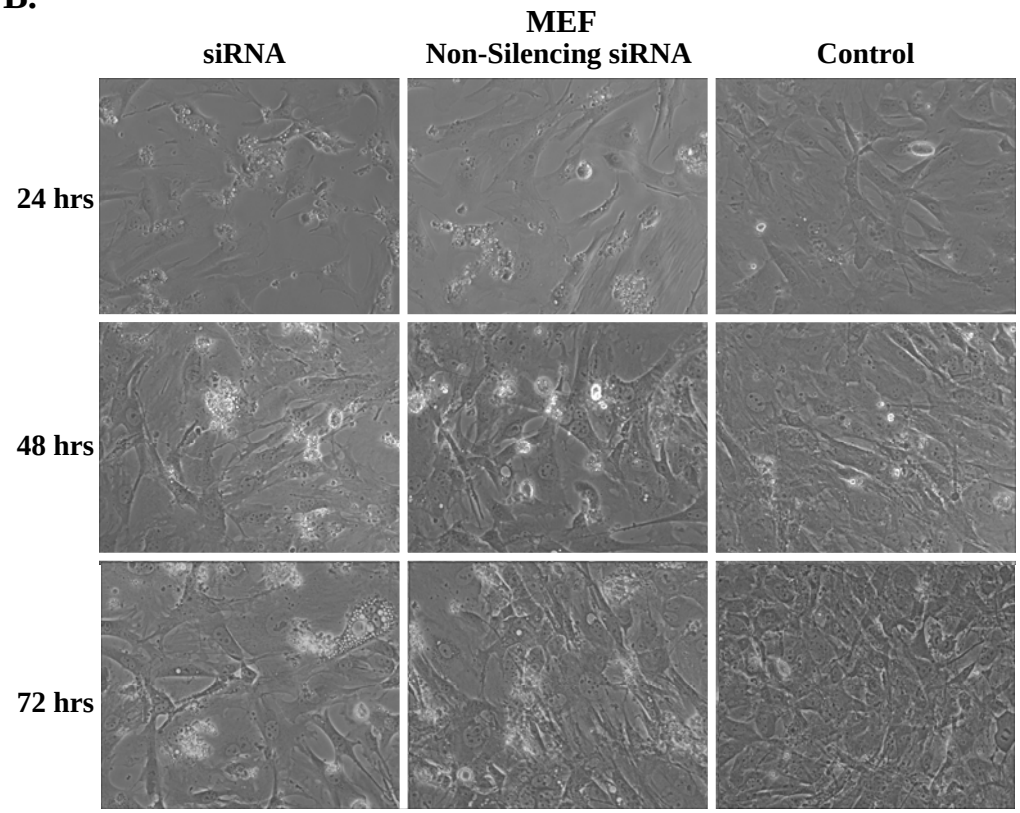


Figure 7.2: Transfection optimization using a fluorescein labeled non-silencing control siRNA. Merged phase contrast and fluorescent images for the (A) NIH/3T3, (B) MEF, and (C) BFF cell lines. Asterisks designate the treatment group used for Dnmt1knock-down.

A.


NIH/3T3 Transfected Cells
siRNA
Non-Silencing siRNA
Control
24 hrs
48 hrs
72 hrs

B.



C.

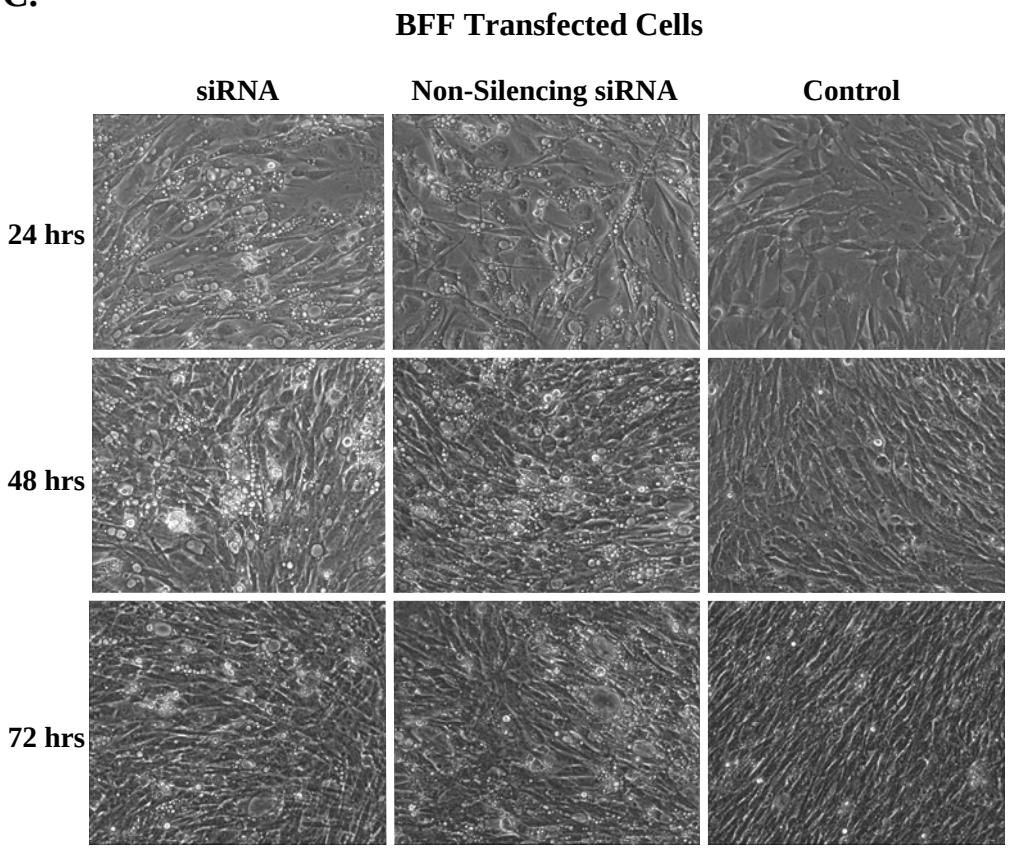


Figure 7.3: Morphology comparisons of transfected cells 24, 48, and 72 hrs post-transfection. Treatment groups include: Dnmt1-specific siRNA, non-silencing control siRNA, and non-transfected control for the (A) NIH/3T3, (B) MEF, and (C) BFF cell lines.

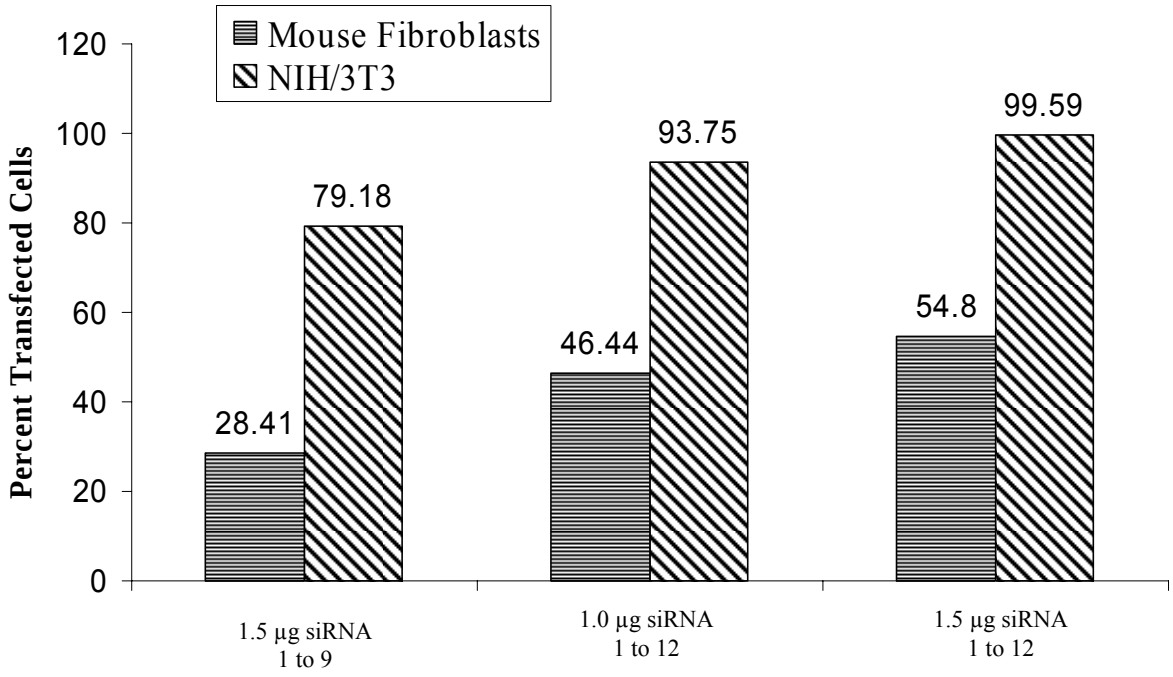


Figure 7.4: Percentage of transfected NIH and MEF cells using flow cytometry on three optimization groups. Less than a 2% death rate was observed in all three treatment groups as determined using propidium iodide.

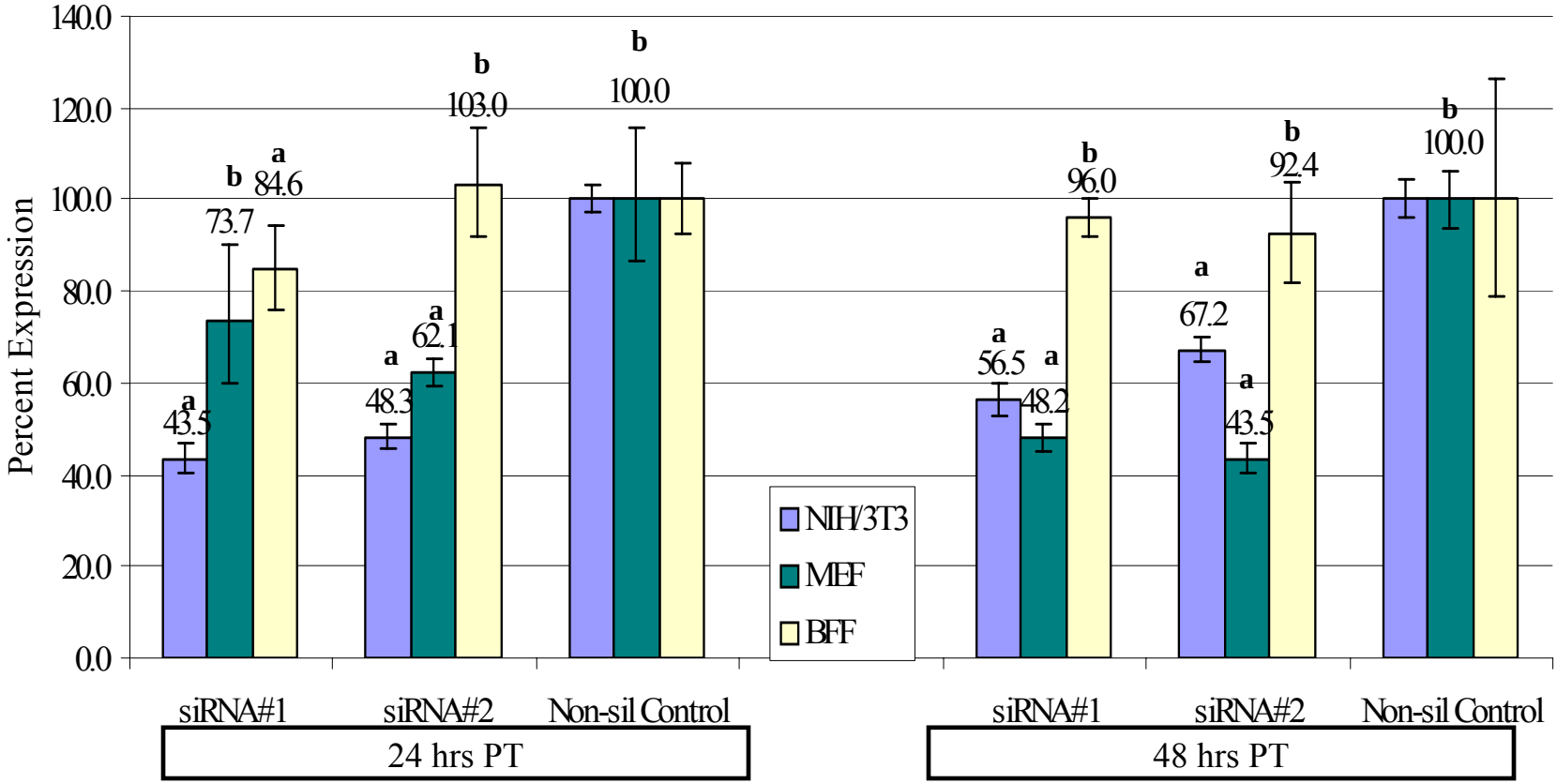


Figure 7.5: Real Time RT-PCR results reflecting the percent expression of Dnmt1 following siRNA transfections. Cell lines and treatment groups include: NIH/3T3, MEF, and BFF cells transfected with either siRNA#1 or siRNA#2 or a non-silencing control siRNA. Groups with different letters are significantly different ($p<0.05$).

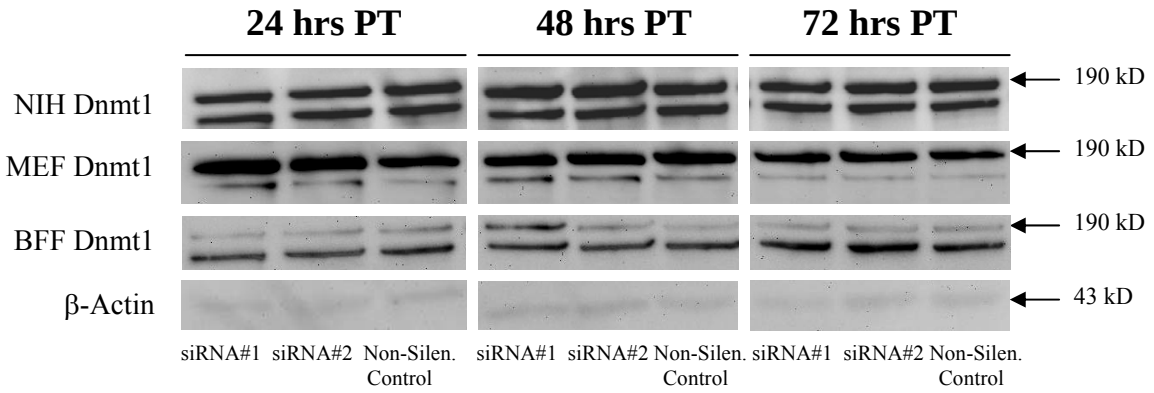


Figure 7.6: Western blot analysis depicting Dnmt1 band at 190 kD and its proteolytic cleavage fragment at 145 kD for all three cell lines. Time points of 24, 48, and 72 hrs post-transfection (PT) are shown for cells transfected with siRNA#1, siRNA#2, and the non-silencing control siRNA. β-actin band appeared at 43 kD.

CHAPTER 8

Conclusion

The current technique of NT is inefficient at producing viable embryos and offspring. It is therefore essential that multi-disciplinary techniques, like that of RNAi, be investigated to assist the oocytes ability to reprogram a differentiated cell. Based on the information discussed in this text, it is plausible that depleting protein stocks of Dnmt1s within a somatic cell prior to its introduction into an enucleated egg could enhance the efficiency of SCNT. If properly carried out, a decrease in the aberrant methylation and imprinting patterns typically observed in NT-derived embryos and fetuses may occur. At present, no published reports have attempted to combine the use of RNAi with that of SCNT – be it in the donor cell or in the NT-derived embryo. However, there are reports of siRNA molecules being injected into fertilized pre-implantation stage embryos resulting in the successful knockdown of the targeted mRNA [357-359]. In a recent report, siRNA expression vectors were injected into the nucleus of preimplantation stage embryos resulting in a reduction of maternally-derived mRNA [358].

With regards to the study discussed in this text (Chapter 7), injecting such a vector specific for Dnmt1s directly into SCNT-derived embryos would perhaps be a better delivery method than the one selected. A vector designed to generate shRNAs specific for the unique 118 aa region on the N-terminus of the Dnmt1s transcript would ensure exclusive knockdown of the somatically expressed isoform whereby leaving the oocyte specific Dnmt1 (Dnmt1o) transcript intact. To ensure the temporal expression of the Dnmt1s-shRNA, an inducible promoter could be incorporated into this vector for exclusive on/off control of its transcription. If this route proves unsuccessful, such siRNA expression vectors could also be introduced directly into the

somatic cell. Recently, the controlled knockdown of Dnmt1 in human cancer cells was carried out using conditional vectors for shRNA and are freely distributed to the public [340].

A significant problem associated with the transfection of exogenously delivered siRNAs is the toxicity associated with the transfection reagent (as experienced in the Chapter 7 study). In the present study, expressing such siRNA molecules directly into the embryo would bypass the need to pre-treat somatic donor cells; thus eliminating the toxicity problems associated with the transfection of cultured cells. To reduce the occurrence of cytotoxicity, multiple transfection reagents should be tested to determine the optimal reagent for each cell type being used. However, this was not carried out in the Dnmt1 knockdown study performed in Chapter 7. The transfection reagent used was selected based on it being specifically marketed for primary cells. Perhaps the cytotoxicity and low transfection efficiency associated with the present study would have been alleviated or reduced with a more optimal transfection reagent. An alternative to the exogenous delivery of our siRNA molecules using a non-liposomal lipid transfection reagent would have been via viral induction by any of the adenoviral [330, 331], adeno-associated viral (AAV) [332], retroviral [333], and lentiviral vectors [334-336] that initiate RNAi in transduced cultured cells. In future knockdown studies such methods should be looked into.

In conclusion, there is no doubt that the NT technique – as performed by researchers around the world – is inefficient at properly reprogramming a differentiated genome. Although still in its infancy, the SCNT technique has tremendous potential in a wide array of fields. In order to realize these applications, it is essential that researchers make a conscious and cohered effort to develop novel approaches directed towards alleviating this problem.

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APPENDIX A

PRODUCTION OF A CLONED CALF USING KIDNEY CELLS OBTAINED FROM A 48-HOUR COOLED CARCASS

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The ability to produce cloned livestock using postmortem tissue could incorporate an additional application into the field of nuclear transfer. This study examined the feasibility of producing cloned cattle using a primary cell line established from a postmortem beef carcass. A market beef heifer processed at a USDA certified slaughterhouse was used to develop a primary somatic cell line. Tissue samples were taken from the kidney and forelimb regions either 1) immediately following slaughter (fresh) or 2) 48-h post slaughter (cooled) where the carcass was housed at 2 to 4.0ºC. Tissue was removed and placed on ice in PBS + 5.0% (v:v) penicillin/streptomycin. A primary culture was established using standard techniques and cultured in supplemented DMEM F-12 medium. Once established, cells were trypsinized and either frozen or continually passaged. Cells used for nuclear transfer (NT) were passaged (48 h before use) and cultured with 15 μM roscovitine roughly 24 h prior to nuclear transfer. Cells were approximately 80% confluent and between passage number 1 and 11 at the time of NT. Selected slaughterhouse-derived oocytes were matured in supplemented TCM 199 medium for 18-20 h at 39^{o}C in 5.0% CO_2 and air. Mature Metaphase II oocytes were vortexed and stained with Hoechst 33342 to help with chromatin removal. Following enucleation, roscovitine-treated carcass cells were placed in the perivitelline space of the oocyte. Reconstructed NT embryos were fused in Zimmermann's medium and pulsed using needle-like electrodes. This was followed by activation using a combination of calcium ionophore (5 μM), cytochalasin D (5 μg/mL), and cycloheximide (10 μg/mL) in TCM + 10% FBS. Fused NT embryos were cultured in 50 μL drops of BARC medium (USDA; Beltsville, MD) for 7 days at 39 ^{o}C in a 5% CO_2, 5% O_2 and 90% N_2 environment. Embryo development for all four groups (Table 1) was assessed with blastocysts (grade 1 or 2) being transferred into recipient cows 7 days post estrus. Cleavage rates were not significantly different between groups and the source of cells (fresh or cooled) did not impact blastocyst formation. However, there was a significant difference ($P<0.05$) in % blastocyst based on the source of the donor cell. Overall, one live calf resulted from 34 transferred NTs produced using kidney cells taken from a 48-h cooled carcass. These results display the feasibility of producing cloned calves from cells collected post mortem, which ultimately could be used as a tool to select breeding bulls based on their own steer carcass characteristics.

Table 1. Embryo development and pregnancy data for the production of beef carcass clones.

				Embryo Development				
Cell Source - Status of Carcass	**# Oocytes**	**# Mature (%)**	**# NTs Cultured**	**# Cleaved (%)***	**# Blasts (%)***	**# ETs**	**Initial Pregnancies**	**Live Calves**
Forelimb - Cooled	543	432 (80± 6)	203	138 (68±18)	18 (9±8)[a]	5	1	0
Kidney - Cooled	811	638 (79±8)	409	301 (74±13)	75 (18±10)[b]	34	6	1
Forelimb - Fresh	438	336 (77±7)	175	112 (64±20)	27 (15±10)[a]	0	0	0
Kidney - Fresh	520	414 (80±5)	268	196 (73±19)	36 (13±7)[b]	0	0	0

Medians with different superscripts are significantly different between cell source (P=0.05). * Percentages calculated based on # NTs cultured.