INT. J. BIOL. BIOTECH., 3 (3): 501-509, 2006.

SUCCESSES AND FAILURES IN MAMMALIAN CLONING

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ABSTRACT

Mammalian somatic cell cloning by nuclear transfer is relatively a new reproductive biotechnique with many potential applications. However, at the current stage of development, the reprogramming of epigenetic inheritance by nuclear transfer is still inefficient. The variable conception or birth rates currently associated with cloning in mammals cannot be cited as justification for not embracing this technology as the future use of therapeutic cloning and of embryonic stem cells in tissue and cell therapy will be determined by its help. Similarly the role of cloning in producing transgenic livestock is unequivocal. Therefore, it would be shortsighted to reject it out of hand until we learn more about its possible future role in therapeutic medicine and animal biotechnology. Further efforts and new paradigms are needed to perfect this biological tool and extend it to its fullest potential.

Keywords: Cloning, somatic cell nuclear transfer, mammals

INTRODUCTION

Somatic cell nuclear transfer (SCNT) is a process by which nucleus (DNA) is moved from a donor cell to an enucleated recipient cell to create an exact genetic match of the donor. If this happens to be a viable embryo that proceeds to term, the resulting offspring has the same genetic complement of the original donor, except for the mitochondrial donor, which is derived from the recipient (Wolf *et al*., 2001; Yang *et al*., 2004).

The success of cloning an entire animal, Dolly, from a differentiated adult mammary epithelial cell (Wilmut *et al.,* 1997) has created a revolution in reproductive biology. It demonstrated that genes inactivated during tissue differentiation can be completely re-activated by a process called nuclear reprogramming: the reversion of a differentiated nucleus back to a totipotent status. Somatic cloning may be used to generate multiple copies of genetically elite farm animals, to produce transgenic animals for pharmaceutical protein production or xeno-transplantation (Anderson and Seidel, 1998; Stice *et al.*, 1998; Robl, 1999; Polejaeva and Campbell, 2000; Paris *et al*., 2004), and to conserve rare or endangered species (Andrabi and Maxwell, 2006). With optimization, it also promises enormous biomedical potential for therapeutic cloning and allo-transplantation (Lanza *et al.,* 1999). In addition to its practical applications, cloning has become an essential tool for studying gene function (Capecchi, 2000), genomic imprinting (Solter, 1998), genomic re-programming (De Sousa *et al.*, 1999; Munsie *et al.,* 2000; Winger *et al.,* 2000; Surani *et al.*, 2001), regulation of development, genetic diseases, and gene therapy (Tian *et al.,* 2003).

The objectives of this paper are to review the successes of the nuclear transfer procedure in mammals since the production of Dolly, the sheep. In particular, we would review the major causes of lower efficiency of the procedure and discuss studies which have modified the procedure in ways which may impact on development of clones.

Efficacy of cloning procedure

The ultimate challenge of cloning using SCNT involves reprogramming a somatic nucleus in a manner conducive for embryonic development. A first landmark study in this regard was carried out by Wilmut *et al*. (1997) and cloning of a sheep triggered a wide interest in mammalian cloning followed by a number of mammalian clones created from adult or fetal cell nuclei. A summary of cloned mammals and the efficiency of the cloning technique in respective species are presented in Table 1.

For successful SCNT, Campbell *et al*. (1996) suggested that by inducing the donor cell to exit the growth phase, which in return can cause changes in chromatin structure, reprogramming of gene expression will be facilitated and that development would be normal if nuclei are used from a variety of differentiated donor cells in similar regimes. Later, transforming the above suggestion into reality, Wilmut *et al*. (1997) reported the birth of live lambs from three new cell populations established from mammary gland, fetus and embryo. The fact that a lamb was derived from an adult cell confirmed that differentiation of that cell did not involve the irreversible modification of genetic material required for development to term. The birth of lambs from differentiated fetal and adult cells also proved that by inducing donor cells to become quiescent it will be possible to obtain normal development from a wide variety of differentiated cells (Wilmut *et al*., 1997).

Table 1. List of cloned mammals

Species/ Breed	Donor cells	Cloning efficiency (Live birth/manipulated oocytes; %)	References
Sheep	Mammary epithelial cells	1/227 (0.4%)	Wilmut *et al*., 1997
Mouse	Cumulus cells	41/2468 (1.7%)	Wakayama *et al*., 1998
Bovine	Cumulus cells and Oviduct cells	5/99 (5.0%) and 3/150 (2.0%)	Kato *et al*., 1998
Bovine	Fetal fibroblasts	4/276 (1.4%)	Cibelli *et al*., 1998
Goat	Fetal fibroblasts	3/285 (1.1%)	Baguisi *et al*., 1999
Pig	Fetal fibroblast	1/210 (0.5%)	Onishi *et al*., 2000
Pig	Adult granulosa cells	5/183 (2.7%)	Polejaeva *et al*., 2000
Pig	Fetal cells	Not available	Betthauser *et al*., 2000
Rabbit	Cumulus cells	6/1852 (0.3%)	Chesne *et al*., 2002
Cat	Cumulus cells	Not available	Shin *et al*., 2002
Mule	Fetal fibroblasts	1/334 (0.3%)	Woods *et al*., 2003
Horse	Fibroblasts	1/841 (0.1%)	Galli *et al*., 2003
Rat	2-cell stage embryos	6/139 (4.3%)	Roh *et al*., 2003
Rat	Fibroblasts	Not available	Zhou *et al*., 2003
Dog	Adult fibroblast	2/1095 (0.1%)	Lee *et al*., 2005
Ferret	Fetal fibroblasts and Cumulus cells	890/1 (0.1%) and 878/3 (0.3%)	Li *et al*., 2006

The first successful cloning of mice by Wakayama *et al*. (1998), using the adult cumulus cell further accelerated the cloning research. Until then it was believed that the blastocyst is the last stage compatible as nuclear cloning donor but the mouse cloning enabled to investigate beyond like reprogramming of genetic imprinting and reactivation of the inactive X-chromosome.

Regarding cloning in bovines Cibelli *et al*. (1998) were the first to report the production of three cloned calves and later on Kato and colleagues (1998) were able to report a similar achievement (eight cloned calves). However, in the experiment of Kato *et al*. (1998) the percentage of nuclear transplants developing into blastocyst were quite high (72%) compared with that reported (12%) by Cibelli *et al*. (1998). The higher efficiency in the later study could be related to culture systems and the presence of more normal cell number (69-114 cells) in the nuclear transplant blastocysts (Kato *et al*., 1998).

As mentioned in previous paragraph that Kato *et al*. (1998) were able to produce eight calves cloned from somatic cells of a single adult. Looking into further details that enabled them to have a significantly higher percentage of nuclear transplant embryos developing to term might be due to:

-Populations of donor cells maintained an apparent normal karyotype culture during the *in vitro* culture before use of nuclear transfer, as even after 8-15 passages when they stopped dividing, most maintained normal diploid chromosomes.

-Nucleo-cytoplasmic interactions might be more compatible in this bovine study than in the previous mouse experiment by Wakayama *et al*. (1998) in which the genetic type of donor nucleus was critically important for later development.

-Donor cytoplasm of some somatic cell types might have interfered with the development of nuclear transplants (Wakayama *et al*., 1998), therefore, the cumulus cytoplasm used in this experiment may have been compatible with the oocyte cytoplasm (Kato *et al*., 1998).

Goats are also added to the list of cloned mammals, as first ever production of goats by SCNT was reported in 1999 (Baguisi *et al*.). All cloned offspring (three in number) born in this study were healthy with birth weights within the normal range for their breed. This contrasts with the perinatal morbidity/mortality with other demonstrations of nuclear transfers in ovine (Campbell *et al*., 1996; Wilmut *et al*., 1997; Wells *et al*., 1997) and bovine (Cibelli *et al*., 1998; Kato *et al*., 1998) systems. It was a speculated that whether this is due to a lesser susceptibility of caprine embryos to as ill defined complications caused by SCNT, *in vivo* sourcing of mature oocytes, or a reflection of the relatively low number of clones produced in the caprine SCNT system. Experimental protocol of Baguisi *et al*. (1999) also differed from that of ovine and bovine SCNT systems, as they transferred the cloned embryos to the oviduct of the recipient doe at the two or four cell stage with a minimal *in vitro* culture exposure.

The first successful pig cloning by SCNT was reported by Onishi *et al*. (2000), and in the same calendar year two more studies by Polejaeva *et al*. (2000) and Betthauser *et al*. (2000) on SCNT in pigs were published. Comparing all the three studies it appears that the results obtained were almost similar in terms of ratio of cloned embryos transplanted and number of piglets born (success 1-2%). Onishi *et al*. (2000) got only one piglet born after transfer of 110 cloned embryos to four surrogate mothers, while Polejaeva *et al*. (2000) and Betthauser *et al*. (2000) got seven and four piglets born, respectively, with proportion of embryos transferred to recipients like that of Onishi *et al*. (2000). However, a major difference in research methodology between studies of Onishi *et al*. (2000) plus Polejaeva *et al*. (2000) as compared with that of Betthauser *et al*. (2000) was the use of *in vivo* oocytes derived from gilts rather than *in vitro* matured oocytes from sows. Considering the overall efficiency of SCNT in pigs it appears that comparatively a large number of good quality embryos are required to induce and maintain a pregnancy, and as fully developmentally competent embryos are rare in SCNT procedures (Polejaeva *et al*., 2000), there is every chance of squandering those good embryos unless very large numbers of reconstructed embryos are transferred back into recipients or cloning can be carried out more successfully by taking into account of physiological features of their oocytes and embryos (Niemann and Rath, 2001).

Rabbits are also cloned at about the same frequency of success as most of the other mammalian species (Chesne *et al*., 2002). The difficulty in cloning the rabbits as reported by Chesne *et al*. (2002) was overcome by taking into account both the rapid kinetics of the cell cycle of embryos and the narrow window of time for their implantation after transfer into foster recipients. In this regard findings of Hoffman *et al*. (1998) and Ozil and Huneau (2001) further suggest that the maximization of developmental response of rabbit oocytes to external activating stimuli through controlled Ca^{++} stimulation regimes and characterization of the embryonic signals that regulate rabbit uterine epithelial responsiveness at implantation could help to improve term survival rates of cloned embryos.

A cloned cat has also been produced by nuclear transfer from adult somatic cells. An interesting observation in this study was that the cloned kitten's colour patterning was not exactly the same as that of the nuclear donor. According to Shin *et al*. (2002) this is because the pattern of pigmentation in multicoloured animals is the result not only of genetic factors but also of developmental factors that are not controlled by genotype.

More recently there are reports of successful cloning of a horse (Galli *et al*., 2003) and a mule (Woods *et al*., 2003). The cloning procedure adopted by Galli *et al*. (2003) for horse seems to be comparatively efficient as one live foal was produced from four pregnancies, although there was high developmental failure from the cleavage stage to blastocyst and early implantation. The remarkable birth of foal by the SCNT is thought to be due to advances in ART in horse, particularly at the oocyte activation stage, when protein synthesis and phosphorylation must both be inhibited, and the refinement in zona-free manipulation technique (Galli *et al*., 2003). While Woods *et al*. (2003) attributes the successes of cloning a mule by SCNT to elevated extracellular calcium concentration, which is believed to be associated with development of activated oocytes of other animals.

Recent animal to be cloned is a rat (Roh *et al*., 2003; Zhou *et al*., 2003). The salient findings of Roh *et al*. (2003) were that by using nuclear karyoplasts from the 2-cell embryos as the nuclear donors and reconstructing them with enucleated 2-cell embryos, healthy pups were developed in rats. While Zhou *et al*. (2003) highlights the importance of adapting the SCNT procedure to oocyte physiology for successful cloning.

The most recent mammals to be cloned are a dog and a ferret (Lee *et al*., 2005; Li *et al*., 2006). The successful cloning in dog is linked to maturing canine oocytes *in vivo* and transfer of very early-stage nuclear-transfer constructs, that is, less than 4 hours after oocyte activation, as this transfer of early-stage embryos is believed to be a crucial factor in successful ART dogs (Lee *at al*., 2005). Regarding the successful SCNT protocol for the ferret (Li *et al*., 2006) was the finding that hormonal treatment, normally used for superovulation, adversely affected the developmental potential of recipient oocytes. The onset of Oct4 gene expression was delayed and incomplete in parthenogenetically activated oocytes collected from hormone-treated females relative to oocytes collected from females naturally mated with vasectomized males. Stimulation induced by mating and *in vitro* oocyte maturation produced the optimal oocyte recipient for SCNT. Although nuclear injection and cell fusion produced mid-term fetuses at equivalent rates (~3–4%), only cell fusion gave rise to healthy surviving clones. Single cell fusion rates and the efficiency of SCNT were also enhanced by placing two somatic cells into the perivitelline space.

Despite the long list of animals, which are successfully cloned to date through SCNT, the technique still remains not quite effective (Eckardt and McLaughlin, 2004; Piedrahita, *et al*., 2004). In this context it is obvious from the Table 1 that most of the cloned embryos failed after implantation, as overall success of cloning procedure is not more than 5% in terms of live births/cloned embryos transplanted. Also abortions are observed at various stages of pregnancies after transfer of cloned embryos in number of species (Tsunoda and Kato, 2002). Furthermore, clones surviving the term frequently exhibit phenotypes such as enlarged or abnormal placenta (Hill *et al*., 2000; Tanaka *et al*., 2001; Ogura *et al*., 2002), as well abnormally large birth weight (large offspring syndrome; Wakayama *et al*., 1998; Young *et al*., 1998; Wakayama and Yanagimachi, 1999; Eggan *et al*., 2001), respiratory and

circulatory problems (Cibelli *et al*., 2002; Lee *et al.,* 2005). It is also documented that adult clones often suffer from obesity (Tamashiro *et al*., 2002), tumour formation as well as immunological and metabolic defects (Ogonuki *et al*., 2002). It is relevant to mention that many of the problems associated with SCNT embryos particularly large offspring syndrome have also been found with conventional IVF and ET procedures (Young *et al*., 1998), however the frequency and severity of the syndrome appears to be much higher with cloning (Wells, 2003).

Potential factors contributing to low efficiency of cloning

A wide variety of nuclear donors have been found to be successful in a number of different mammalian species, they all share the problems of low efficiency and high levels of embryonic mortality (early and later), post natal and adulthood complications, suggesting that the errors that arise are fundamental and systematic in nature. The most likely factor interpreted to be involved is insufficient epigenetic reprogramming of the somatic cell nucleus (Eckardt and McLaughlin, 2004; Piedrahita *et al*., 2004; Tamada and Kikyo, 2004).

Epigenetic reprogramming can only be measured by the functional outcome of normal development. Since term development of clones remains a rare exception but a number of other factors have been analyzed in clones at various stages of development as possible indicators of reprogramming and as means to explain the low efficiency of cloning. The mostly like factors influencing reprogramming are gene expression pattern, DNA methylation, histone modifications and telomere length regulation (Mitalipov and Wolf, 2000; Renard *et al*., 2002; Eckardt and McLaudglin, 2004; Latham, 2004; Piedrahita *et al*., 2004; Tamada and Kikyo, 2004).

Gene expression

Studies to date indicate that pre-implantation stage clones have a high incidence of gene expression making the reprogramming a highly error prone (Eckardt and McLaughlin, 2004). Analyzing expression of eight developmentally important genes (DNMT, Mash2, Glut-1, Hsp, Dc II, E-cad, IF and Igf2r) in cloned blastocysts of bovines using RT-PCR (real time-polymerase chain reaction), Wrenzycki *et al*. (2001) reported that several genes were properly activated in the blastocysts but with a marked difference in the gene expression levels. These differences were found to be dependent on parameters in SCNT procedure, including the activation protocol, the cell cycle of the donor cells and the passage number of the donor cells. Daniels and colleagues (2000, 2001) also reported a similar finding again in bovines but based on the study of a different set of genes (Oct4, IL6, FGF2, FGF4, FGfr2, and gp130) specific to early embryonic development.

To understand the genome-wide difference in the gene expression patterns between wide cloned mice and fertilization-derived controls, a DNA microarray was employed using RNA isolated from placentas and liver of these mice (Humpherys *et al*., 2002). The results showed that less than 3% of over 12000 genes were expressed abnormally in the clone's placenta and the liver showed a less conspicuous abnormality in gene expression then placentas. However, it is important to note that this study examined RNA isolated from a whole tissue, and by doing so, an irregularity of the gene expression in each cell may have been averaged (Tamada and Kikyo, 2004).

Based on another microarray study by Suemizu *et al*. (2003), gene expression profiling for mouse placentas demonstrated that there was inappropriate expression of imprinted genes, altered expression of regulatory genes in global gene expression such as DNA methyltransferase and histone acetyltransferase, increased expression of oncogenes and growth promoting genes, over expression of genes involved in placental growth such as Plac1 and identification of many novel genes over expressed in nuclear transfer mouse placentas such as Pitrm1. Therefore, this study indicates that placentomegaly in cloned mice is associated with large-scale deregulations of normal gene expression.

DNA methylation

DNA methylation of cytosine at the CpG dinuclleotides plays vital roles in the regulation of gene expression in mammalian development (Bird, 2002; Li, 2002). 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 remodelling machineries to the promoter of specific genes. A majority of the cloned bovine embryos show a gross abnormality in the genome wide DNA methylation level and DNA methylation patterns on various respective sequences when compared with fertilization-derived controls. The DNA methylation level in clones can be higher or lower than that in the control embryos depending on the donor cell type, target DNA sequences, examined embryonic stages and detection methods (Kang *et al*., 2001a) and extremely abnormal embryos may have died before the analysis was done. Indeed DNA methylation was undetectable in six out of nine spontaneously aborted bovine cloned embryos, but methylation level was normal in the clones that survived to adulthood (Cezar *et al*., 2003).

Bovine somatic nuclei are resistant to the erasure of DNA methylation in early embryogenesis and the clones have tendency to preserve the DNA methylation patterns inherited from the donor cells (Bourc'his *et al*., 2001; Dean *et al*., 2001). Re-establishment of DNA methylation is potentially deregulated by precocious *de novo* methylation in clones (Dean *et al*., 2001). This abnormal methylation transition in cloned bovine embryos could be due to the specific features of the somatic chromatin structure and/or defective regulation of DNMTs.

Results from the investigation of Piedrahita *et al*. (2004) indicate that the methylation patterns in cloned bovine in mid-gestation differ drastically in the chorion but not in the fetus proper when compared with non-cloned controls. They have interpreted this as being a result of a rapid differentiation of the cell line, with a concomitant reduction in the activity of the demethylases and methylases.

Conversely, Kang *et al*. (2001b) found during typical demethylation events in cloned pig embryos that there pattern of repetitive sequences were similar to the ones detected in fertilized counterparts. They concluded that the species-specific differences occur in modification of DNA methylation and imprinting.

Histone modifications

Global release and uptake of linker histone H1 is another challenge for the donor nuclei during the nuclear reprogramming. Alteration of histone modification is an important aspect of chromatin remodelling in cloning. Histones receive a number of covalent modifications including acetylation, methylation, phosphorylation, ubiquitinqtion and ADB-ribosylation at the amino termini protruding from the chromatin core. A specific combination of these histone modifications on a given gene provides a recognition site for interacting molecules and thus contributes to regulating the gene activity (Strahl and Allis, 2000; Jenuwein and Allis, 2001). Bovine oocytes and early embryos express several histone acetylases and deacetylases with some variability in he transcript levels depending on the development stages (McGraw *et al*., 2003). In mouse oocytes, histone H3 and H4 are globally deacetylated on several lysine at the metaphase II of the second meiosis, which was reproduced in somatic nuclei transferred into the same stage of oocytes (Kim *et al*., 2003). This genome wide decrease of histone acetylation may contribute to the erasure of the previous gene expression patterns specific to the donor cell differentiation. Also detailed enzymology responsible for these transitions of acetylation and methylation in early embryos is not yet available, but these histone modifications should almost certainly affect expression of a number of genes.

Telomere restoration

Telomeres are DNA-protein complexes at the ends of eukaryotic chromosomes essential for chromosomal integrity and normal cell growth (McEachern *et al*., 2000; Blasco, 2002). Results of Betts *et al* (2001) and Miyashita *et al*. (2002) in bovines indicate that shortened adult cell telomeres can be restored during early developed of cloned animals but the degree of telomere elongation remains quite variable. Also there was significant variation in telomere length among individual clones and among different tissues isolated from a single clone, thus underscoring the complexity and difficulty of telomere length control in clones.

To understand the functional consequences of telomere restoration, two groups of scientists examined whether nuclear cloning could elongate the reflective lifespan of senescent cells. Lanza *et al*. (2000) found in bovines that clone derived fibroblast cells, which contained fully restored telomeres comparable to the age-matched controls, showed longer proliferative lifespan than the senescent donor fibroblast cells. However, when Clark *et al*. (2003) tested the cloned fibroblast cells in ovine that harbored partially restored telomeres, the proliferative lifespan of the cells was not extended. Therefore, it remains to be examined to what extent the restored telomeres can influence the proliferative lifespan of these cells. It is also unclear whether the resetting of proliferative lifespan of isolated cells has something to do with the lifespan of cloned animals (Xu and Yang, 2003).

Influence of donor tissues/cells on cloning procedure

Many somatic cell types, including mammary epithelial cells, ovarian cumulus cells, fibroblast cells, fibroblast cells from skin and internal organs, Sertoli cells, and macrophages have been utilized for nuclear transfer. Almost all cell types tested so far have resulted in live offspring, although with great differences in their cloning efficiency (Di Berardino, 2001).

In murine species, at least eight types of fetal and adult origin from males and females and different genetic backgrounds have been tested. Live offspring were obtained with similar efficiency only with fibroblasts, undefined fetal gonad and cumulus cells (Wakayama and Yanagimachi, 2001). Many cell types like macrophages, spleen, brain and mature Sertoli cells repeatedly failed to develop after implantation (Lai and Prather, 2003). The success with the types of cells for cloning in murines is mostly attributed to the reactivation of Oct4 gene at the correct stage, but the pattern and levels of expression are still doubtful (Boiani *et al*., 2002). Regarding the use of embryonic germ cells in the form of primordial germ cells, which were considered to be efficient for mouse model (Matsui *et*

al., 1992; Resnick *et al*., 1992), they have been recently proved to be inadequate nuclear donors for cloning because they either erase or reset the epigenetic pattern (Yamazaki *et al*., 2003).

In bovine, ovine and caprine species, at least fifteen somatic cell donors of fetal, newborn and adult origin from male and female, and different genetic backgrounds were tested. Interestingly all these supported development *in vitro*, and live offspring were obtained from cumulus, fibroblast, oviduct, skin and liver cells. A more recent study by Tian and colleagues (2003) suggests that cumulus cells are the most effective cell type in comparison with mammary epithelial and skin fibroblast cells for somatic cloning in farm animals according to both the *in vitro* development test as well as full term survival. They further suggest that DNA from cumulus cells is more effectively reprogrammed following nuclear transfer. Similarly, Kato *et al*. (1998, 2000) compared cells from the liver, testis, skin, ear, along with cumulus and oviductal cells and concluded that cumulus and oviductal epithelial cells are the most suitable for nuclear donors. Evidence of supporting the superiority of cumulus cells for nuclear transfer also comes from the study of Forsberg *et al*. (2002), who conducted large number of ET in cattle, and it was demonstrated that cumulus cells gave an overall 15.2% calving rate, while fetal genital ridge cells and fibroblast cells produced a 9% calving rate. Adult fibroblast cells in this study gave the lowest calving rate of only 5%.

In pigs' fibroblasts and cumulus cells have been clonable (Lai and Prather, 2003). The use of isolated precursor cells of adipocytes from the subcutaneous adipose tissue of adult pigs for nuclear transfer has also been documented (Nagashima *et al*., 2003). However, the problem with all Somatic cells is that they tend to become senescent before sufficient rounds of gene transfer and/or targeting and selection can be performed. This problem can be overcome by isolation of readily transfectable and selectable cells with high proliferative potential and long-term karyotypical normalcy. Thus further development is needed to create cells that are developmentally competent and able to proliferate for pig cloning (Lai and Prather, 2003).

The most recent study on cloning in ferret by Li *et al.* (2006) also highlights the influence of the type of donor cells, with cumulus cells proving to be more effective than fibroblasts for SCNT.

Conclusions

For many years, researchers cloning mammals experienced little success, but recent advances have led to the successful cloning of several mammalian species. However, cloning by the transfer of nuclei from adult cells is an inefficient procedure, and this has been attributed to incomplete reprogramming of the somatic nuclei by the cloning process. Learning more about basic mechanisms involved in SCNT will teach us much about the control of gene expression and the genetic control of development.

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(*Accepted for publication May 2006*)