Université de Montréal

Epigenetic reprogramming of imprinted genes in embryonic stem cells, fertilized and cloned embryos

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Faculté des études supérieures

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Epigenetic reprogramming of imprinted genes in embryonic stem cells, fertilized and cloned embryos

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Résumé

Mots clés: Empreinte génomique, Clonage, Reprogrammation épigénétique, Cellules souches embryonnaires, Méthylation

L'empreinte génomique est un processus épigénétique au cours duquel l'allèle maternel ou paternel est exprimé. La perturbation de la machinerie épigénétique qui reprogramme l'expression du gène soumis à l'empreinte conduit dans plusieurs cas à des anomalies de développement du fœtus, à un fort taux de mortalité et à une augmentation marquée de la taille des organes internes. Les animaux qui survivent présenteront des problèmes de croissance. Bien qu'on ne connaisse pas exactement l'assise moléculaire de la coordination du programme épigénétique des gènes soumis à l'empreinte, il a été démontré que la méthylation de l'ADN et l'acetylation de l'histone jouait un rôle dans la régulation de la répression allélique des gènes soumis à l'empreinte.

En dépit de certains succès récents dans le domaine du clonage animal chez différentes espèces, le mécanisme moléculaire dont dépend la réussite du clonage demeure largement obscur. Bien que plusieurs facteurs soient susceptibles de contribuer à la réussite de cette technique, dont la synchronisation des cycles cellulaires, l'âge et le type de cellules donneuses et les conditions de culture, bien peu a été fait au chapitre de l'optimisation du profil de l'expression génique particulièrement des gènes soumis à l'empreinte. Le traitement préalable des cellules donneuses en vue de reprogrammer le noyau a soulevé plusieurs problèmes pratiques tels que la capacité épigénétique d'une cellule donneuse à soutenir le développement à terme sans engendrer d'anomalies de croissance. Peu d'études ont porté à ce jour sur la question de savoir si le patron de l'expression d'un gène soumis à l'empreinte est affecté par la privation de sérum, les inhibiteurs de la

méthylation de l'ADN et la présence d'histone désacétylase dans les fibroblastes somatiques, fréquemment utilisés comme cellules donneuses dans le clonage animal. Étant donné que les cellules souches (ES) qui proviennent du bouton embryonnaire du blastocyste sont différentes des fibroblastes embryonnaires au chapitre de la méthylation de l'ADN et de l'activité de l'enzyme méthyletransférase, l'objectif général de ma thèse était de caractériser le patron d'expression d'une série de gènes soumis à l'empreinte dans les cellules ES de la souris de même que dans les embryons fécondés *in vitro* puis clonés, cellules et embryons ayant été préalablement traités avec des agents de reprogrammation epigénétique de la chromatine.

La première série d'expériences (article 1) portait plus particulièrement sur la détermination du profil d'expression de deux gènes de régulation de la croissance soumis à l'empreinte soit l'Igf2 et l'H19 dans des cultures de cellules ES dont la croissance était inhibée par privation de sérum et par confluence en plus du vieillissement in vitro (plusieurs passages). Nos données démontrent que la culture des cellules ES dans un milieu de culture exempt de sérum et où la densité cellulaire est élevée provoque une augmentation de l'expression des deux gènes soumis à l'empreinte et se traduit par un profil de méthylation aberrant d'une région de régulation cruciale de l'Igf2, la DMR2. En outre, après plusieurs passages, la culture des cellules ES a provoqué une réduction marquée de l'expression des deux gènes soumis à l'empreinte. Cette étude démontre donc que les contraintes de croissance imposées à des cellules ES mises en culture provoquent une altération de l'expression des gènes soumis à l'empreinte et du statut de méthylation d'un locus de régulation crucial.

L'objectif de la deuxième ronde d'expériences (article 2) était de caractériser le patron d'expression d'un groupe de gènes soumis à l'empreinte de même que le patron de méthylation de la région DMR2 du gène Igf2 et de les relier au statut de différenciation des cellules ES de souris exposées à la TSA, un inhibiteur de l'histone désacétylase et à la 5AzaC, un inhibiteur de la méthylation de l'ADN. Il s'est avéré qu'à l'exception du H19, l'expression des autres gènes soumis à l'empreinte (Igf2, $P57^{KIP2}$, Peg1 et Igf2r) subissait une régulation positive à la suite du traitement à la TSA des cellules ES. L'addition simultanée de la 5AzaC et de la TSA amenait une augmentation synergique de l'expression des gènes soumis à l'empreinte. Cependant, l'altération du patron d'expression génique imputable au traitement à la TSA n'était pas associé à la modification du statut de méthylation de la région DMR2 du gène Igf2. En outre, bien que les deux traitements entraînent une différenciation partielle des cellules ES et l'apparition de certaines colonies à la morphologie irrégulière, le traitement à la TSA et à la 5AzaC n'a pas provoqué une élimination complète des cellules indifférenciées dans les colonies ES tel qu'indiqué par la coloration à l'AP et l'expression de l'Oct4, qui n'ont jamais été complètement abolis par le traitement. Dans l'ensemble, ces résultats confirment que tout comme la méthylation de la séquence CpG, l'acétylation de l'histone joue un rôle crucial dans la machinerie épigénétique qui gouverne l'expression des gènes soumis à l'empreinte dans les cellules ES de souris.

L'objectif de la troisième ronde d'expériences (article 3) était de décrire les patrons d'expression relatifs de plusieurs gènes soumis ou non à l'empreinte pendant le développement embryonnaire en période de préimplantation de souris fécondées ou activées par parthénogenèse puis cultivées *in vitro* en présence de TSA et de 5AzaC.

Grâce au PCR quantitatif en temps réel, nous avons démontré que le patron d'expression des gènes soumis à l'empreinte, soit le Igf2, Peg1, P57^{KIP2} et Igf2r, était soumis à une régulation positive à la suite d'un traitement à la TSA et à la 5AzaC. De manière similaire, le traitement à la TSA a conduit, chez des embryons parthénogénétiques, à l'induction de gènes soumis à l'empreinte qui étaient présumément sous l'influence d'un silenceur. Bien que le traitement avec ces deux inhibiteurs ait causé la dégénérescence de plusieurs embryons, le traitement à la TSA a permis d'accélérer la division cellulaire et a hâté la différenciation comme le prouve l'expression en Oct4 chez certains embryons. Ces données indiquent que l'acétylation de l'histone joue véritablement un rôle dans la régulation des voies d'expression des gènes soumis à l'empreinte au stade de la préimplantation chez les embryons de souris.

Enfin, la dernière série d'expériences (article 4) avait pour but de déterminer si le clonage permet l'expression fidèle des gènes soumis à l'empreinte et également la reprogrammation de l'expression des gènes soumis à l'empreinte des noyaux donneurs (cellules somatiques et ES) exposés à des traitements qui modifient le niveau d'acétylation de l'histone et de méthylation de l'ADN. Il s'est avéré que certains gènes soumis à l'empreinte étaient partiellement programmés alors que d'autres n'étaient pas complètement corrigés en cours de développement avant le stade de blastocystes des embryons clonés. En effet, le passage du noyau ES dans le cytoplame de l'oocyte était incapable d'amener l'expression des gènes soumis à l'empreinte dans les cellules ES au nouveau biologique. En outre, les blastocystes clonés issus des cellules ES traitées avec la TSA et la 5AzaC présentaient des altérations supplémentaires de l'expression des gènes soumis à l'empreinte : le mauvais réamorçage du programme épigénétique après

transfert nucléaire serait donc directement relié aux changements des patrons de méthylation et d'acétylation.

Globalement, ces études démontrent que l'expression des gènes soumis à l'empreinte est sensible à la privation de sérum, à la confluence et à l'altération de la méthylation de l'ADN et de l'acétylation de l'histone dans les cellules ES chez les embryons au stade de préimplantation. En outre l'oocyte n'arrive pas à reprogrammer correctement l'expression de tous les gènes soumis à l'empreinte chez les donneurs nucléaires soumis au traitement à la TSA et à la 5AzaC du moins pas avant le stade blastocyste. Des études supplémentaires devront être menées afin d'étudier la reprogrammabilité épigénégtique des gènes soumis à l'empreinte dans les cellules ES si on espère utiliser ces dernières comme source de noyaux pour le clonage animal ou de matériel thérapeutique pour la transplantation médicale.

Summary

Key Words: Genomic imprinting, Cloning, Epigenetic reprogramming, Embryonic stem cells, Methylation.

Genomic imprinting is an epigenetic process by which either the paternal or the maternal allele is expressed. Perturbation of the epigenetic machinery that reprograms imprinted gene expression in many cases leads to fetal developmental abnormalities, high mortality rate, disproportionate enlargement of internal organs and those animals that survive display growth disorders. Although the exact molecular basis which coordinates the epigenetic program of imprinted genes is unknown, it has been shown that DNA methylation and histone acetylation are involved in regulating the allelic repression pathway of imprinted genes. Despite recent success in cloning animals of various species, the molecular mechanism needed for successful cloning remains largely obscure. While many factors are likely to contribute to the success including cell cycle synchronization, donor cell (type and age) and culture conditions, little has been done to coordinate an optimum profile of gene expression, especially those that are imprinted. Prior treatment of donor cells for nuclear reprogramming purposes has raised many practical concerns such as the epigenetic suitability of a donor cell to support development to term without growth abnormalities. Few studies have investigated whether the pattern of imprinted gene expression is affected by serum starvation, inhibitors of DNA methylation and histone deacetylases in somatic fibroblast cells, which in many cases are used as a donor cell for animal cloning. Given the fact that embryonic stem (ES) cells, which are derived from the inner cell mass of the blastocyst, are dissimilar to embryonic fibroblast cells in

the level of DNA methylation and the enzymatic activity of methyltransferases, the general objective of my thesis was to characterize the expression pattern of a range of imprinted genes in mouse ES cells, fertilized and cloned embryos, all of which have been pretreated with nuclear reprogramming agents.

The first set of experiments (article 1) focused on determining the expression profile of two growth regulatory imprinted genes, namely Igf2 and H19 in ES cells grown under growth inhibitory conditions, specifically serum starvation and confluency in addition to in vitro aging (at several passages). Our data showed that culture of ES cells with serum-depleted media and at high cell density increased the expression of both imprinted genes and led to an aberrant methylation profile of a key Igf2 regulatory region (DMR2). Moreover, culture of ES cells for several passages resulted in markedly reduced expression of both imprinted genes. Thus, this study demonstrates that growth constrained cultures of ES cells are associated with alterations in imprinted gene expression in addition to the methylation status of a crucial regulatory locus.

The goal of the second set of experiments (article 2) was to characterize the expression pattern of a group of imprinted genes as well as the methylation profile of DMR2 of Igf2 and relate it to the differentiation status of mouse ES cells exposed to TSA, an inhibitor of histone deacetylases and 5AzaC, a global inhibitor of DNA methylation. In this experiment we report that with the exception of H19, other imprinted genes (Igf2, $P57^{KIP2}$, Peg1 and Igf2r) expression were upregulated following TSA treatment in ES cells. Simultaneous addition of 5AzaC and TSA synergistically increased the expression of imprinted genes. However, the alteration of gene expression pattern due to TSA treatment was not associated with modification in the methylation status of the

DMR2 of Igf2. Moreover, although both drugs caused partial differentiation of ES cells and the appearance of some morphologically irregular colonies, TSA and 5AzaC treatment did not fully eliminate undifferentiated cells within ES colonies as indicated by AP staining and Oct4 expression, which were never completely abolished after treatment. Taken together, these findings confirm that, like CpG methylation, histone acetylation plays a pivotal role in the epigenetic machinery that governs imprinted gene expression in mouse ES cells.

The objective of the third experiments (article 3) was to establish the relative expression patterns of several imprinted and non imprinted genes during preimplantation development of fertilized and parthenogenetic mouse embryos cultured in vitro in the presence of TSA and 5AzaC. By the use of quantitative real time PCR analysis we showed that the expression pattern of the imprinted genes Igf2, Peg1, $P57^{KIP2}$ and Igf2r was upregulated following TSA and 5AzaC treatment. Similarly, TSA led to the induction of presumably silenced imprinted genes in parthenogenetic embryos. Although treatment with both inhibitors caused degeneration of several embryos, TSA treatment led to accelerated cell division and early differentiation evident by Oct4 expression of some embryos. These data indicate that histone acetylation is indeed involved in regulating the expression pathway of imprinted genes in preimplantaion stage mouse embryos.

Finally, the last set of experiments (article 4) was designed to determine whether the cloning procedure is capable of supporting faithful expression of imprinted genes and also whether cloning is able to reprogram imprinted gene expression of donor nuclei (ES and somatic cells) exposed to drugs that modify the levels of histone acetylation and

DNA methylation. In this experiment we report that, while some imprinted genes were partially reprogrammed, others remain not fully corrected during development to the blastocyst stage of cloned embryos. This was evident by the failure of the oocyte to bring about the high levels of imprinted gene expression in donor ES cells to biological levels. Moreover, cloned blastocysts derived from ES cells treated with TSA and 5AzaC show additional alterations in imprinted gene expression, suggesting that improper resetting of the epigenetic program after nuclear transfer is directly related to changes in methylation and acetylation patterns.

Collectively, these studies show that imprinted gene expression is susceptible to serum starvation, confluency and alterations in DNA methylation and histone acetylation levels in ES cells and preimplantation stage embryos. Moreover, the oocyte fails to correctly reprogram all imprinted gene expression of TSA and 5AzaC treated nuclear donors at least till the blastocyst stage. Further studies are required to investigate the epigenetic reprogrammability of imprinted genes in ES cells should they be used as a source of nuclear donor in animal cloning or therapeutic material in transplantation medicine.

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Abbreviations

5AzaC	5 Aza-Cytidine
cDNA	Complementary deoxyribonucleic acid
DMRs	Differentially methylated regions
DNA	deoxyribonucleic acid
FCS	Fetal calf serum
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase
Gas6	Growth arrest specific 6
h	Hour
hCG	Human chorionic gonadotropin
HDACs	Histone deacetylases
ICM	Inner cell mass
ICR	Imprinting control region
Igf2	Insulin like growth factor 2
kb	Kilobase
LIF	Leukemia inhibitory factor
LOI	Loss of imprinting
M	Mole
MeCP2	Methyl cytosine binding proteins
ng	Nano gram
Peg1	Paternally expressed genes 1
pg	Pico gram

PGC	Primordial germ cells
PMSG	Pregnant mare's serum gonadotropin
RT-PCR	Reverse transcriptase polymerase chain reaction
TRD	Transcriptional repression domain
TSA	Trichostatin A
UPD	Uniparental disomy

1. Introduction

Despite recent success in cloning animals of various species the molecular mechanisms needed for successful cloning remains largely obscure. While many factors are likely to contribute to the success, including cell cycle synchronization (Campbell et al., 1996), donor age, cell type (Hill et al., 2000; Kato et al., 2000; Shiota et al., 2002) and medium conditions (Betts et al., 2001), little has been done to coordinate an optimum profile of gene expression, especially those that are imprinted.

It is increasingly evident that the in vitro production of morphologically normal pre-implantation embryos does not guarantee that their post-implantation development and post-natal life will be normal. During early embryonic development, extensive modifications in global methylation take place. Therefore any perturbation of the methylation process can cause epigenetic deregulation of developmentally important genes. Such epigenetic alterations could affect in particular the expression of genes that are subject to genomic imprinting resulting in growth anomalies (Dean et al., 1998).

Culture conditions such as serum starvation of donor cells is a common practice in embryo cloning (Cibelli et al., 1998; Jones et al., 2001; Shiga et al., 1999; Wilmut et al., 1997), whereby such a process could interfere with components of the cell cycle resulting in improper maintenance of epigenetic tags and chromatin imprints (Hayashida et al., 1997; Rideout et al., 2001). Similarly, addition or removal of serum in the culture of preimplantation mouse embryos was associated with an aberrant expression pattern of imprinted genes, improper methylation of key imprinting control regions (DeChiara et al., 1990; Doherty et al., 2000; He et al., 1998) and reduced developmental potential after embryo transfer (Khosla et al., 2001a). Moreover, it has been shown that levels of DNA

methyltransfereses 1 and 3b were downregulated when cells were arrested (serum deprivation) at G_0/G_1 stage of the cell cycle (Robertson et al., 2000b). Furthermore, studies monitoring the differences in the epigenetic profile between various cell origins has revealed that DNA mathylation patterns in ES cells are different than those observed in somatic tissues (Shiota et al., 2002), not to mention dissimilarities in the levels of DNA methyltransferase activity between ES and somatic cells (Jahner et al., 1982; Lei et al., 1996; Li et al., 1992; Palmiter et al., 1982; Robertson et al., 1999; Stewart et al., 1982). These dissimilarities between somatic versus ES cells were more evident in cells deficient of Dnmt1 which showed that unlike somatic cells, ES cells are viable in culture although they contain minimal levels of genomic methylation (Lei et al., 1996; Nan et al., 1996). Similarly, ES cells lacking MeCP2, a methyl cytosine binding protein whose activity is associated with histone deacetylases, have no effect on survivability (Tate et al., 1996).

Prior treatment of cells as source of donor nuclei has raised many practical questions such as, increased abortion rates and aberrant phenotype. These abnormalities are characterized by placental defects, cartridge and skeletal deformation, respiratory difficulties in addition to fetal overgrowth and disproportionate enlargement of internal organs (organomegaly). Together, these symptoms were designated the “large offspring syndrome” (LOS), which has been observed in cattle, sheep (Farin and Farin, 1995; McEvoy et al., 1998; McEvoy et al., 2000; Schnieke et al., 1997; Sinclair et al., 2000; Thompson et al., 1995; Young and Fairburn, 2000; Young et al., 1998) and mice (Tanaka et al., 2001; Wakayama et al., 1998; Wakayama et al., 1999). Several studies have suggested that these deformations are a consequence of improper/insufficient genetic and

epigenetic reprogramming of donor DNA (Humpherys et al., 2001; Lanza et al., 2000; Tanaka et al., 2001). In fact, epigenetic errors of the donor cell were carried to a later stage of development and was exerted in the placenta and the skin of cloned animals which again revealed inappropriate reestablishment of methylation profiles in these tissues (Humpherys et al., 2001; Ohgane et al., 2001).

Other donor cell pre-conditioning agents might be required to epigenetically reprogram the nuclei. For example, 5AzaC, a demethylation compound, which leads to reduced methylation levels of donor cells so as to "simulate" genomes found in gametes or blastomeres. Similarly, trichostatin A (TSA) an inhibitor of histone deacetylases, can give rise to positive epigenetic alterations by rendering the chromatin easily reprogrammable by the oocyte environment.

Taken together, it seems that a proper genomic "ingredient" of the donor cell is a prerequisite to maximize survivability and minimize fetal abnormalities associated with cloned embryos. Therefore, new methods are required to epigenetically synchronize ES cells should they be used in animal cloning or in transplantation therapy applications.

2. Literature Review

2.1. Preimplantation embryo development and embryonic stem cells

Embryonic development starts with fertilization of the egg by the sperm. After fertilization, the embryo divides slowly with the absent of size increase. Up until the 2-cell stage, the embryo relies exclusively on the oocyte recourses for development. By the end of the 2-cell stage (species specific) the embryo starts activating local genes required for early growth and development. As cleavage proceeds, compaction of the embryo is evident by the late 8-cell stage embryo and the formation of the morula. The following stage (blastocyst) is characterized by the gradual formation of two distinct cell lineages, namely, the trophectoderm (TE) and the inner cell mass (ICM). Eventually, the embryo starts to expand at 4.5 days post fertilization in the mouse, whereby hatching occurs, enabling the embryo to be implanted in the uterus.

ES cells are derived from the undifferentiated inner cell mass (ICM) of the 3.5 day blastocyst, and are capable of maintaining an undifferentiated state indefinitely in culture in the presence of leukemia inhibitory factor (LIF). ES cells express markers of pluripotent undifferentiated cells such as Oct4, SSEA1 and alkaline phosphatase (Berstine et al., 1973; Solter and Knowles, 1978; Wobus et al., 1984). Upon LIF withdrawal, they underexpress these markers, lose their pluripotent capacity and differentiate into various cell types. ES cell pluripotency has been manifested by the production of chimeric, tetraploid and cloned mice (Bradley et al., 1984; Wakayama et al., 1999).

Recent work showed the possibility of directing the fate of these undifferentiated ES cells *in vitro* into a variety of highly specialized differentiated cell types including neurons, islet cells, hepatocytes and cardiac muscle cells (Fuchs and Segre, 2000; Wobus, 2001). Moreover, gene targeting in ES cells through recombinant mutation has been widely used to create genetically modified ES cell lines (Capecchi, 1989; Ramirez-Solis et al., 1993) and to repair spontaneous mutations (Doetschman et al., 1987). Together, these possibilities made ES cells a useful source of therapeutic material in transplantation medicine/therapeutic cloning to treat diabetes, Alzheimer and Parkinson disease patients (Colman and Kind, 2000).

The controversial use and derivation of human ES cells (hES) from *in vivo/in vitro* produced embryos led scientists to develop a new concept whereby ES cells are derived from cloned embryos of somatic donor cell, designated as ntES cells. In fact mouse ntES cells have been derived from cloned blastocysts generated by somatic donor cells (Kawase et al., 2000; Munsie et al., 2000). Importantly, mouse ntES cells have been shown to differentiate into several cell lineages (Wakayama et al., 2001) and lately, genetic defects in mice have been corrected by the combination of ntES cells and gene therapy (Rideout et al., 2002). As of today, few hES cell lines are established (http://escr.nih.gov/index.html), however, it remains to be seen whether therapeutic cloning can be applied with the use of hES cells.

2.2. Genomic Imprinting

Imprinting in mammals is a unique epigenetic process, whereby either the paternal or the maternal allele of the gene is expressed. This phenomenon was observed as early as 2 decades ago through elegant pronuclear transplantation experiments by McGrath and Solter, which demonstrated that the parental genomic contribution to the embryo are non-equivalent (McGrath and Solter, 1984; Surani et al., 1984). This can be manifested by the failure of parthenogenetic and androgenetic embryos to develop to term. So far a total of 66 imprinted genes (31 maternally and 35 paternally expressed; Fig. 1) have been identified in the mouse (http://www.mgu.har.mrc.ac.uk/imprinting/imptables). Imprinted genes are estimated to constitute 1% (around 150-200) of total genes in the mammalian genome, they generate considerable interest due to their unique profile of expression and the critical role they play in prenatal (DeChiara et al., 1991) and postnatal (Itier et al., 1998; Lefebvre et al., 1998) growth and development. An additional role for imprinted genes has emerged in live animals lately, whereby inactivation of some imprinted genes in female mice caused abnormal maternal behavior (Lefebvre et al., 1998; Li et al., 1999). Not to mention that deregulation of imprinted genes leads to several growth disorders and abnormalities. Although the imprinting mechanism has not been defined completely, the accomplishment of monoallelic expression requires each gene to be "tagged" and subjected to germ line-specific epigenetic modifications such as DNA methylation, which are propagated subsequently from sperm and egg to the fertilized embryo then to the fetus and throughout adulthood.

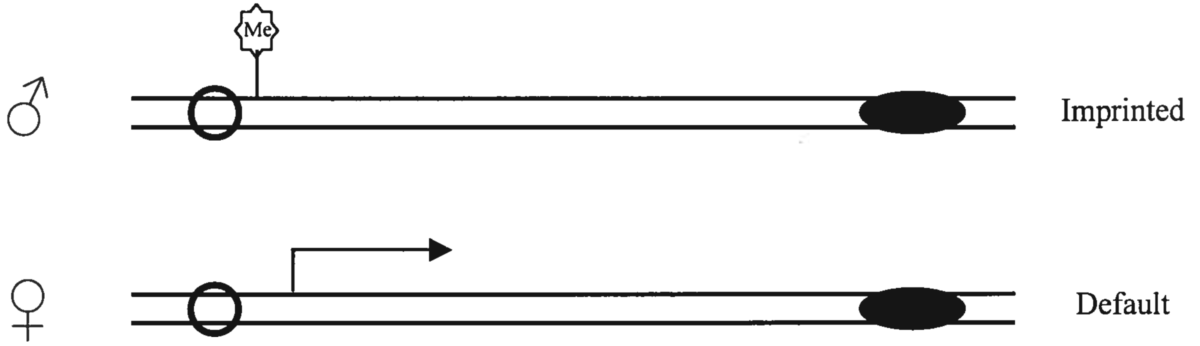
Fig. 1 (Beechey, 2002)

Fig. 1. List and chromosomal location of all imprinted genes identifies as of August, 2002. Adapted from {Beechey 2002}

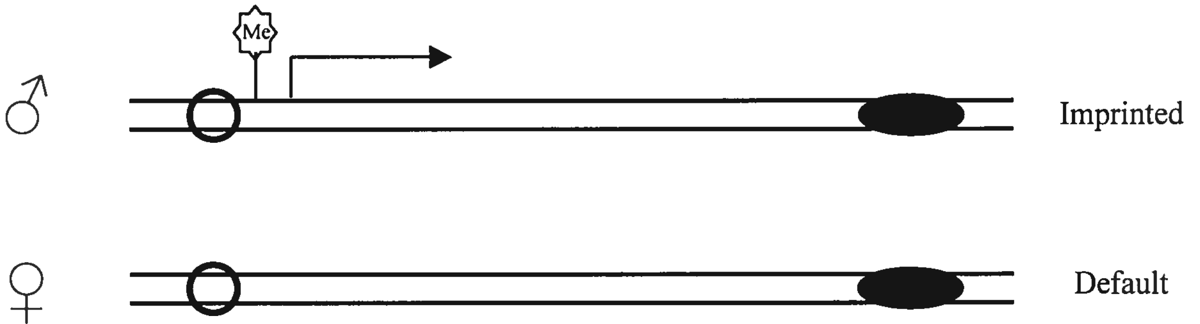
2.2.1 Epigenetic characteristics and the role of imprinted genes

While CpG islands in non-imprinted housekeeping genes are hypomethylated, hyperacetylated and transcriptionally active (Bird, 1986; Stein et al., 1983; Tazi and Bird, 1990), imprinted genes contain highly methylated CpG islands (Surani et al., 1990b) often located on the repressed allele thereby designating them as differentially methylated regions (DMRs), and in many cases, configured heterochromatically (Jones, 1999). Almost all imprinted genes contain DMRs, while some of these DMRs are established either in the oocyte or the sperm and are called primary DMRs, secondary DMRs are usually produced after fertilization and may result from methylation spreading from a primary DMR (Bartolomei et al., 1993; Bird, 1999; Brandeis et al., 1993; Eversole-Cire et al., 1993; Feil et al., 1994; Ferguson-Smith and Surani, 2001; Neumann and Barlow, 1996; Olek and Walter, 1997; Plass et al., 1996; Razin and Cedar, 1994; Stoger et al., 1993; Surani, 1998; Tremblay et al., 1997). Although DNA methylation is attributed to the allelic repression observed in imprinted genes, some imprinted genes however have their DMRs on the active allele (Fig. 2) (Bestor, 2000; Constancia et al., 1998; Reik et al., 2001). Furthermore, imprinted genes are physically linked in clusters that contain both maternally and paternally expressed genes (Wutz et al., 1997; Zemel et al., 1992). Interestingly, many of these clusters contain at least one imprinted gene that encodes an untranslated RNA such as H19 and Snrpn (Brannan et al., 1990; Ripoche et al., 1997; Wutz et al., 1997). Moreover, imprinted genes are often enriched with CpG islands (Paulsen et al., 2000) and similarities in specific short repetitive sequences in DMRs between numerous imprinted genes has been well established (Bartolomei et al., 1993;

A



B



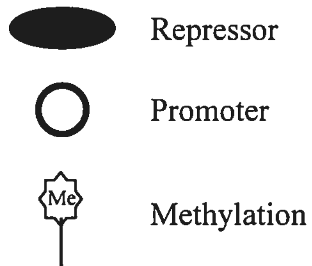


Fig. 2. Examples of imprinted gene expression in association with DNA methylation. Imprints (methylation) can be located on the repressed allele (A) or on the expressed allele (B). Adapted with modifications from Sleutel et al., 2000.

Hatada et al., 1995; Neumann et al., 1995; Stoger et al., 1993). Furthermore, it seems likely that differences in chromatin structure as an epigenetic modification, influence the expression of imprinted genes (Feil and Kelsey, 1997). These modifications include asynchronous replication also known as replication timing between the maternal and paternal allele (Izumikawa et al., 1991; Kitsberg et al., 1993; Knoll et al., 1994; Ripoche et al., 1997) and frequencies of meiotic recombination rates during male and female meiosis in germ cells (Paldi et al., 1995; Robinson and Lalande, 1995; Simon et al., 1999). However, it is worth noting that asynchronous replication also occurs in non-imprinted genomic regions (Chess et al. 1994). Other chromatin structure modifications, such as differential histone acetylation levels between parental alleles has been reported as well (Hu et al., 1998; Khosla et al., 2001b; Saitoh and Wada, 2000), this is manifested by the fact that the heavily methylated inactive X chromosome is depleted of the acetyl moiety on histone H3 and H4 and similar to heterochromatic regions (Jeppesen and Turner, 1993).

A substantial number of imprinted genes have been functionally implicated in the regulation of embryonic, fetal and postnatal growth and development (Barton et al., 1991; Itier et al., 1998). Usually, paternally expressed genes are growth enhancers while maternally expressed genes are growth repressors. Moreover, whereas embryos carrying only maternal contribution such as parthenogenetic embryos poorly develop extraembryonic tissues, androgenetic embryos which contain solely paternal genomes, negatively contribute to the formation of the embryo proper. The Igf system is the most documented feature of all imprinted genes that has been correlated with embryonic development. This system include insulin-like growth factor II (Igf2), and embryos

deficient of this paternally expressed gene show fetal lethality and are significantly smaller than control littermates (DeChiara et al., 1990). Similarly, conversely however, mutant mice lacking Igf2r/mannose-6-phosphate receptor, a maternally expressed gene, are approximately 30% larger at birth than controls (Barlow et al., 1991; Lau et al., 1994; Wang et al., 1994) and this overgrowth can be equilibrated by combining Igf2 and Igf2r mutations (Wang et al., 1994). It is worth noting that the main function of Igf2r is to degrade extra-cellular Igf2 via receptor-mediated endocytosis (Czech, 1989; Oka et al., 1985). Like Igf2, H19 maps to chromosome 7 in the mouse but is maternally expressed (Bartolomei et al., 1991), both genes are physically linked (Zemel et al., 1992) with a reciprocal allelic expression. Newborn mice that carry the H19 null mutant are 28% larger than normal littermates; however, the size increase can be eliminated by double mutation of H19 and Igf2 simultaneously (Leighton et al., 1995). Two other imprinted genes are involved in fetal growth control, namely the Mas proto-oncogene (Villar and Pedersen, 1994) and insulin 2 (Ins2) (Giddings et al., 1994).

Interestingly, most imprinted genes are expressed and play an important role in the formation of the placenta (www.mgu.har.mrc.ac.uk/imprinting), therefore indirectly contributing to fetal growth by regulating nutrient transfer to the fetus. Two major imprinted genes have so far been implicated in placental development namely, Mash2, a gene that encodes basic-helix-loop-helix transcription factors which, is responsible for the formation of the spongiotrophoblast layer in the placenta (Guillemot et al., 1995; Guillemot et al., 1994), whereas Igf2 transcript is expressed specifically in the labyrinthine trophoblast lineage (Constancia et al., 2000). Further evidence demonstrating the importance of these genes in placental formation comes from androgenetic embryos

which poorly develop spongiotrophoblast tissue due to the loss of Mash2 (Guillemot et al., 1994). It is noteworthy to mention that Mash2 imprinting is independently regulated from DNA methylation (Caspary et al., 1998; Tanaka et al., 1999).

Imprinted genes have been shown to be involved in cell cycle regulation. This was demonstrated by the identification of p57 (currently Cdknlc) (Hatada and Mukai, 1995; Matsuoka et al., 1996; Taniguchi et al., 1997), a maternally expressed gene that encodes a tissue specific cyclin-cdk inhibitor (Chung et al., 1996; Lee et al., 1995; Matsuoka et al., 1995). Biallelic expression of this gene leads to decreased cellular proliferation, such as those observed in cells derived from parthenogenetic tissues. Conversely, androgenetic cells which presumably are null for p57, have an increased rate of cellular division (Matsuoka et al., 1995; O'Keefe et al., 1997). Furthermore, it has been demonstrated that imprinted genes are responsible for postnatal behavior (Isles and Wilkinson, 2000). Females that lack a functional Pegl and Peg3, which are paternally expressed genes, neglect feeding their offspring (Kuroiwa et al., 1996; Lefebvre et al., 1998; Li et al., 1999; Nishita et al., 1996). Both genes are expressed abundantly in the mouse fetus, particularly in mesodermal tissues and the brain. Similarly, behavior abnormalities were observed in mice lacking Grfl and Ube3a genes, which were characterized by defects in contextual learning and lack of long term memory (Brambilla et al., 1997; Jiang et al., 1998).

2.3. Genomic repression of imprinted genes

2.3.1. Epigenetic repression via DNA methylation

It has been known for some time that DNA methylation is inversely correlated with gene activity, meaning that hypermethylation of DNA sequences are associated with gene repression while hypomethylation correlates with gene activation. A large body of evidence suggests that DNA methylation is involved in a variety of biological processes that include cell differentiation, establishment and maintenance of genomic imprinting, X chromosome inactivation, stability of chromatin structure, DNA replication and carcinogenesis (Hall, 1991; Junien, 1992; Leonhardt et al., 1992; Li et al., 1993b; Rastan, 1994; Razin and Kafri, 1994; Surani et al., 1990b; Surani et al., 1988). Genomic DNA is methylated and maintained in vitro with the use of a group of enzymes called DNA methyltransferases which were identified in mice (Bestor et al., 1988; Okano et al., 1998a; Okano et al., 1998b; Yoder and Bestor, 1998; Yoder et al., 1997) and humans (Finnegan and Dennis, 1993; Yen et al., 1992). DNA methyltransferases target the cytosine residues of CpG islands in key regulatory sites, such as promoters or the imprinting control region (ICR) in the case of imprinted genes, and add a methyl group to the 5-carbon position to suppress transcription (Bestor et al., 1988; Bestor and Ingram, 1983; Pfeifer et al., 1983). Moreover, the role of methylation in genomic imprinting is supported by the demonstration that methyltransferase null mice lack proper expression of imprinted genes (Li et al., 1993a; Surani et al., 1990a). In addition to the fact that removal and disruption of primary DMR sequences in mice resulted in deregulation of imprinted gene expression (Thorvaldsen et al., 1998).

2.3.1.1 Dynamics of DNA methylation in the embryo

The genomic DNA undergoes dynamic alterations in the methylation pattern during early embryonic development (Fig. 3). These changes are described in three steps, firstly the so called erasure, which is characterized by an active wave of DNA demethylation that occurs after fertilization till the blastocyst stage, in which the majority of CpG islands in the embryo are void of methylation (Howlett and Reik, 1991; Kafri et al., 1992; Monk, 1987; Oswald et al., 2000). Recently however, it has been shown that demethylation occurs at a much faster rate whereby genomic demethylation is complete by the morula stage (Santos et al., 2002). It should be noted that imprinted alleles are protected from the genome wide demethylation activity during preimplantation development (Brandeis et al., 1993; Olek and Walter, 1997; Shemer et al., 1996; Tremblay et al., 1997). The physiological importance of the wave of global demethylation in early embryogenesis is unknown. It is thought however, that this process is required to prevent transmission of aberrant epigenetic modifications to subsequent generations and also as a prerequisite for the formation of pluripotent embryonic stem (ES) cells at the blastocyst stage. Two types of DNA demethylation (Fig. 4) have been documented to occur in the early embryo namely, active and passive demethylation. While the first does not require DNA replication, it is a must for the latter (Reik and Walter, 2001; Rougier et al., 1998). Secondly the establishment process, which takes place at the time of implantation throughout gastrulation, is characterized by a wave of global de novo methylation resulting in a rapid increase in DNA methylation levels

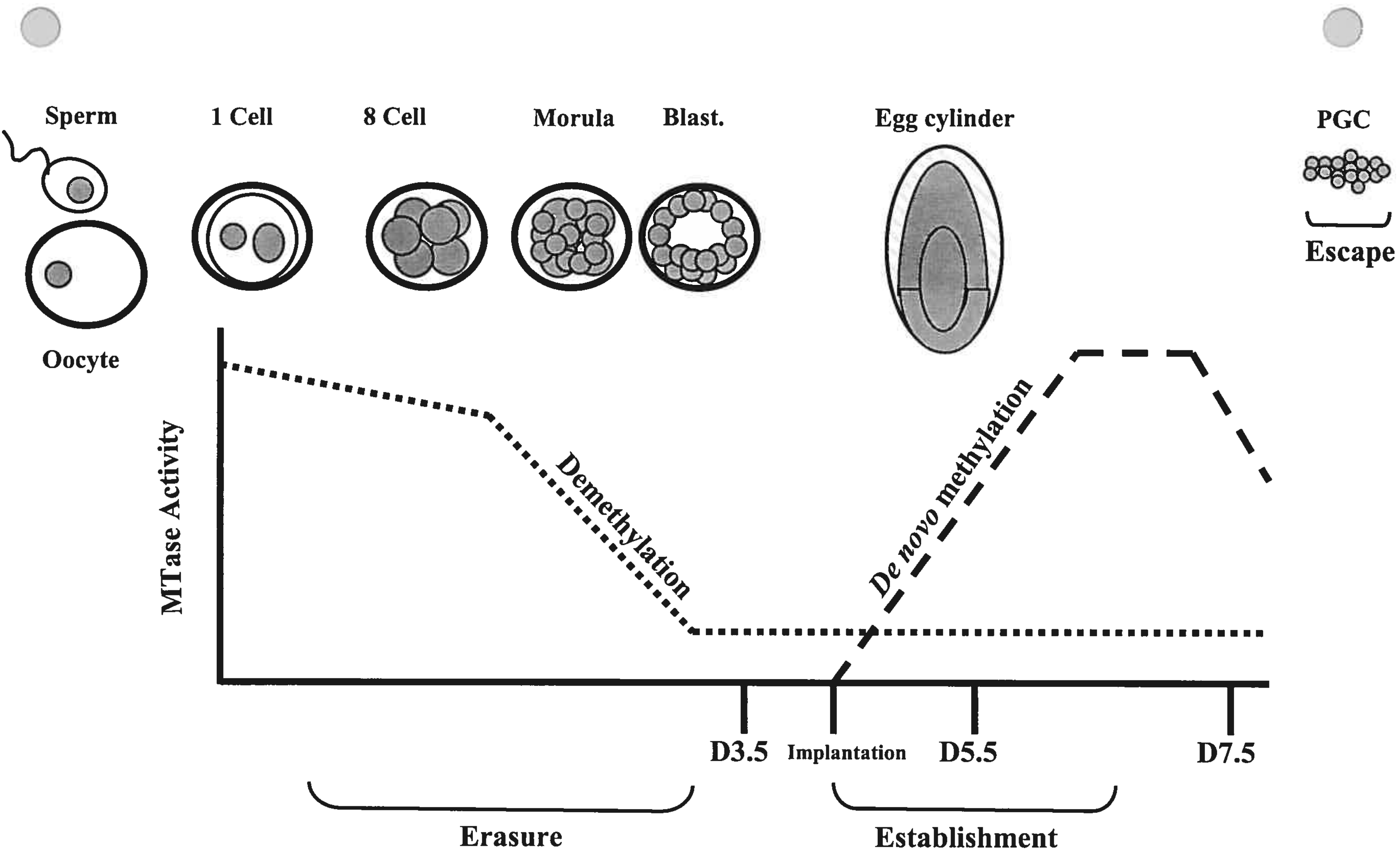


Fig. 3. Dynamic changes in DNA methytransferase activity in the pre and postimplantation mouse embryo. DNA demethylation occurs shortly after fertilization till the blastocyst stage. After implantation a wave of de novo methylation take place throughout gastrulation. Adapted from Li et al 1997, with slight modifications.

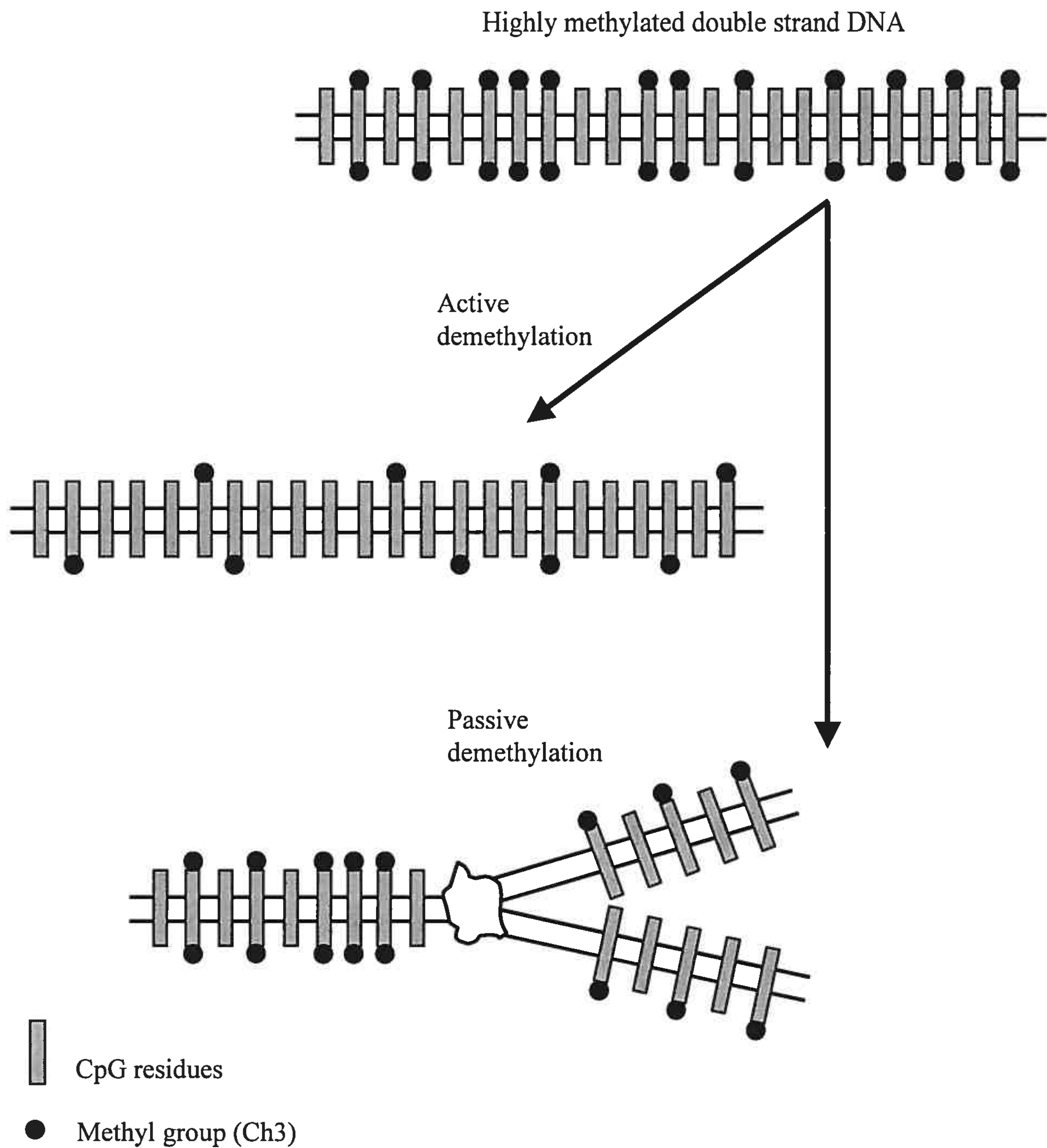


Fig. 4. Dynamics of global DNA demethylation patterns in early embryos. Demethylation occurs in the absence of Dnmt1 with continued rounds of DNA replication (passive demethylation) or actively without DNA replication (active demethylation). Adapted with modifications from Reik and Walter 2001.

during post-implantation development (Chaillet et al., 1991; Jahner et al., 1982; Kafri et al., 1992; Monk, 1988; Razin and Shemer, 1995; Sanford et al., 1987; Stoger et al., 1993; Tremblay et al., 1995). Recent work indicates that the de novo methylation wave take place as early as the blastocyst stage and is restricted to the inner cell mass (ICM) (Santos et al., 2002). Thirdly, the so called gametogenesis escape, whereby the primordial germ cells (PGCs) of both sexes of the developing embryo escape the wave of de novo methylation and remain highly undermethylated (Monk et al., 1987; Sanford et al., 1987) until they begin to differentiate as gametes (sperm and oocyte) (Kafri et al., 1992). It is widely believed that perturbation in methylation dynamics in preimplantation embryos leads to developmental abnormalities and growth disorders.

2.3.1.2 DNA methyltransferases

The importance of DNA methyltransferases in genomic imprinting was demonstrated by the fact that mice lacking maintenance methyltranferase (Dnmt1) gene fail to show allele-specific methylation, thereby disrupting the expression profile of imprinted genes (Li et al., 1993b). Furthermore, embryos deficient in DNA methyltransferase have hypomethylated genomes and die early in embryogenesis due to abnormal expression of imprinted genes (Caspary et al., 1998; Li et al., 1993b; Li et al., 1992). DNA methyltransferases (Fig. 5) play a pivotal role in the establishment and maintenance of imprinting during early pre- and postimplantation stages (Li et al., 1993b; Surani et al., 1990b).

So far few methyltransferases (for reviews see Bestor, 2000) have been identified namely; Dnmt1, the maintenance methyltransferase which can recognize hemi-methylated CpGs, as DNA replicates it adds a methyl group to the daughter DNA strand (Bestor and Ingram, 1983; Gruenbaum et al., 1982; Leonhardt et al., 1992; Pfeifer et al., 1983). Although Dnmt1 is known for maintenance DNA methylase activity, it was demonstrated that it has a significant de novo methylase activity (Yoder et al., 1997). The second DNA methyltransferase was called Dnmt2 (Okano et al., 1998b; Yoder and Bestor, 1998) which was found in prokaryotic and eukaryotic organisms. Dnmt2 knock-out ES cells failed to interrupt either de novo methylation or maintenance of hemimethylated DNA sequences, suggesting a limited role in genomic de novo and global maintenance methylation in ES cells (Okano et al., 1998a). A third methyltransferase (Dnmt3) was discovered in two forms in the mouse, type a and b,

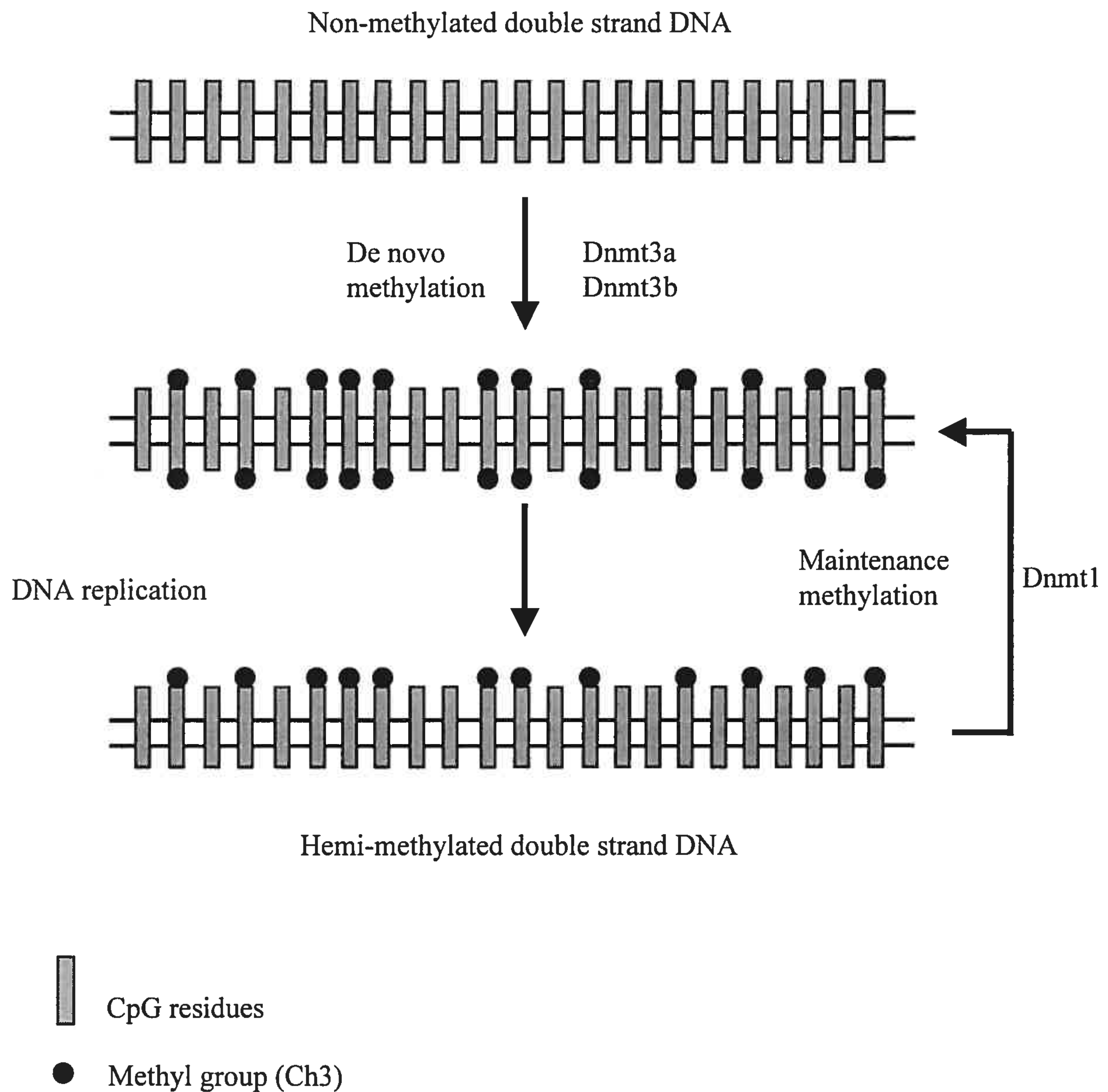


Fig. 5. Dynamics of de novo and maintenance DNA methylation patterns in dividing cells. Methylation is introduced into unmethylated DNA by de novo methylation enzymes Dnmt3a, Dnmt3b and Dnmt1. Following DNA replication, Dnmt1 recognize the hemi-methylated DNA sequence and introduce a methyl group on the opposite strand. Adapted with modifications from Bird 1999.

that are present at low levels in somatic and differentiated ES cells (Okano et al., 1998b). Dnmt3a and Dnmt3b lack the capacity to maintain the methylation of hemimethylated DNA sequences but interestingly, have high affinity for de novo methylating "blank" genomic DNA rendering them as a prime candidate for de novo methylase (Okano et al., 1998a). This was evident by experiments demonstrating that Dnmt3a was responsible for de novo methylation of DNA sequences in Drosophila, which usually lack any methylated regions (Lyko et al., 1999), and also in experiments of bi-knock-out ES cells of both forms which showed absence of newly methylated regions and early death at gastrulation (Okano et al., 1999). While Dnmt3a knockout mice develop to term but die around 3-4 weeks after birth, embryos deficient in Dnmt3b fail to develop beyond 9.5 days after fertilization. Moreover, it has been shown that Dnmt3a and Dnmt3b have an overlapping function in preimplantation embryos since their expression was found localized mainly in the embryonic ectoderm (Okano et al., 1999). Furthermore, it has been shown recently that Dnmt3L, a protein sharing homology with DNA methyltransferases, interacts with Dnmt3a and Dnmt3b to carry out de novo methylation of imprinted genes in germ cells (Bourc'his et al., 2001; Hata et al., 2002). It is interesting to note that together with Dnmt3L, other proteins have been identified lately that interact directly with DNA methyltransferases including PCNA, DMAP1, HDAC1, HDAC2, pRB and RP5b, however, neither their cooperative function nor the mechanism by which they target DNA methyltransferases has been determined yet (Bachman et al., 2001; Fuks et al., 2001; Robertson et al., 2000a). With all the methyltransferases and the interacting proteins known so far, it is possible that the maintenance and establishment of methyl

imprints in the sperm, oocyte and fertilized embryo may still depend on additional novel DNA methyltransferases yet to be identified (Howell et al., 2001; Oswald et al., 2000).

2.3.1.3 Affect of DNA methyltransferase inhibitors (5AzaC)

5-Azacytidine (5AzaC) is a potent chemical agent that is known to induce global DNA demethylation (Creusot et al., 1982; Juttermann et al., 1994; Michalowsky and Jones, 1987). The drug 5AzaC is a nucleoside analog which incorporates into cellular DNA and irreversibly inhibits DNA methyltransferases by forming a covalent binding complex on the DNA methyltransferase enzyme (Bender et al., 1998; Juttermann et al., 1994). It has a short life span and is typically administered to patients with carcinogenic illnesses by continuous subcutaneous injections over several days (Chitambar et al., 1991; Glover and Leyland-Jones, 1987; Goldberg et al., 1993). Treatment of somatic female cell lines with 5AzaC induces partial reactivation of the inactive X chromosome, evident by the expression of X-linked genes, decompaction of the heterochromatin site and advanced replication timing (Jablonka et al., 1985; Schmidt et al., 1985). Moreover, it is worth noting that drugs that inhibit DNA methylation are highly toxic, due to their interference with the methylation machinery (Davidson et al., 1992; Juttermann et al., 1994; Yoshida et al., 1990).

Since imprinted gene expression is dependent on hypermethylation of DMRs, treatment of cells with 5AzaC results in many cases in upregulation of imprinted genes due to loss of imprinting (LOI). While several imprinted genes were overexpressed in response to 5AzaC exposure in preimplantation stage embryos (Baqir and Smith, 2001a),

embryonic (Nishita et al., 1999) and gastric (Kang et al., 2000) carcinoma cells, mouse fibroblast cells (El Kharroubi et al., 2001; Grandjean et al., 2001; Pedone et al., 1999) and human fibroblast cells (Hu et al., 1998), some imprinted genes, such as p57, were not induced in embryonic rhabdomyosarcoma cells (Chung et al., 1996). Moreover, it is interesting to note that 5AzaC can bring about demethylation of highly methylated sequences in silenced endogenous non-imprinted genes (Jones and Taylor, 1980; Jones et al., 1982), retroviruses (Groudine et al., 1981) and genes involved in tumor gastric cells (Kang et al., 2000).

2.3.2. Epigenetic repression via histone deacetylation

A large body of evidence indicates that epigenetic control of gene expression in mammals is achieved by a combination of DNA methylation and chromatin modification (For reviews see Cheung et al., 2000; Kouzarides, 1999; Spencer and Davie, 1999). It has been known for some time that chromatin structure is associated with the acetylation pattern of the histones at individual lysine residues by regulating the interaction between nucleosomes (Grunstein, 1997). Histone acetylation is often associated with activated transcription and deacetylation correlates with transcriptional repression (Struhl, 1998; Tazi and Bird, 1990). Evident by the fact that euchromatins are usually open and often hyperacetylated thereby enabling transcription factors to access the DNA and form an environment that is active (Grunstein, 1997; Kuo and Allis, 1998). Conversely, closed configured chromatin (heterochromatin), which is hypoacetylated would block transcription factors and render the chromatin in a repressive state (Fig. 6) (Berger, 1999;

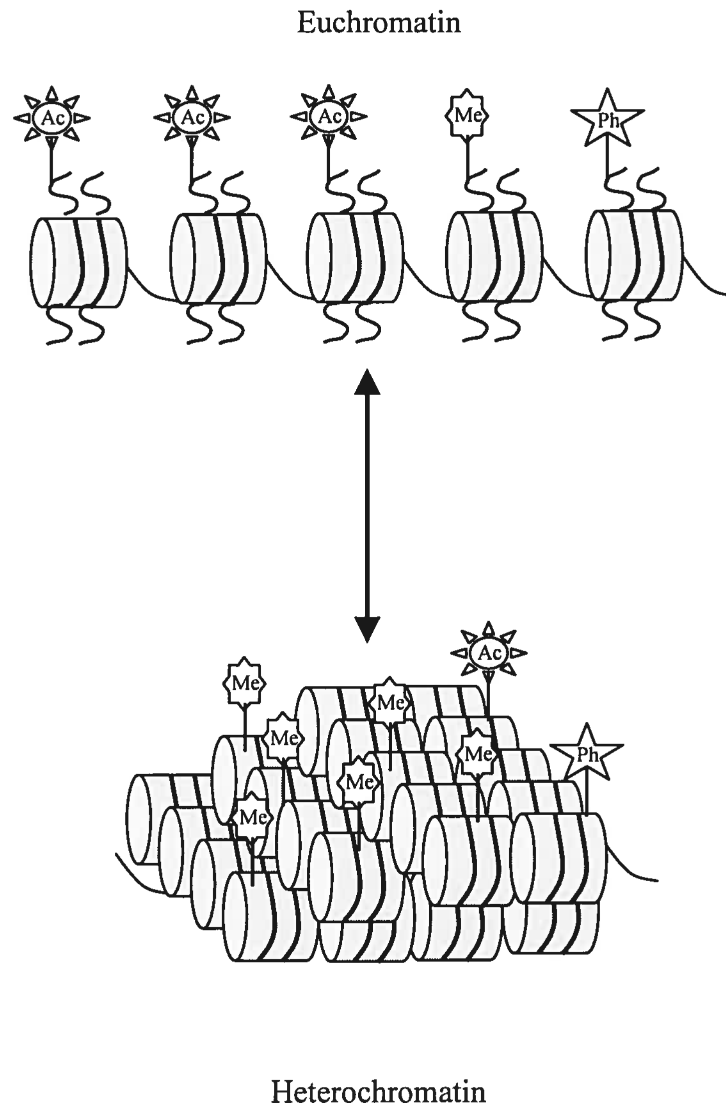


Fig. 6. Models for active chromatin (euchromatin), which is open and often hyperacetylated and repressed chromatin (heterochromatin) which is structurally closed and hypoacetylated thereby blocking transcription factors. Both models shows histone tail modifications such as methylation and acetylation. Adapted with modifications from Jenuwein and Allis 2001

Kuo and Allis, 1998). This model was supported by the fact that, while transcriptionaly suppressed regions including telomeres, centromeres, heterochromatin and methylated sites are found to be acetylation free (Brown et al., 1997; Cortes et al., 1999; Ekwall et al., 1997; Grunstein, 1997; Nan et al., 1998), active genomic sequences are euchromatic and harbor highly acetylated histones with no CpG methylation (Grunstein, 1997). Another piece of evidence comes from the fact that X-linked genes that are expressed have their histone H4 hyperacetylated, while X-linked genes that are silenced are usually hypoacetylated (Gilbert and Sharp, 1999; Jeppesen, 1997; Jeppesen and Turner, 1993). It should be noted however, that in few rare cases, it has been shown that acetylation of specific lysine residues is associated with gene repression (De Rubertis et al., 1996; Pazin and Kadonaga, 1997; Turner, 1991). Repressed chromatin structures usually recruits histone deacetylases (HDACs) to promoter regions or key regulatory sites to repress genomic transcription (De Rubertis et al., 1996).

2.3.2.1. Affect of histone deacetylase inhibitors (HDACs)

Histone deacetylases are the enzymes responsible for the removal of acetyl moieties from specific lysine residues of core histones, thereby creating a positively charged environment. The interaction between the positively charged lysine residues reduces nucleosome mobility on the DNA strand rendering it inaccessible to the transcriptional machinery (Cheung et al., 2000; Wolffe and Hayes, 1999). The inhibitory affect of HDACs on gene expression can be reversed by use of drugs that inhibit HDACs, such as sodium butyrate, trapoxin and trichostatin A (TSA). Moreover, it is worth noting

that drugs that inhibit HDACs are highly toxic due to their interference with the architectural structure of the chromatin (Davidson et al., 1992; Yoshida et al., 1990), and might cause cellular arrest in G1/G2 of the cell cycle (Sugita et al., 1992; Yoshida and Beppu, 1988), apoptosis (Medina et al., 1997) and antiproliferative effects (Sambucetti et al., 1999; Sugita et al., 1992). Although imprinted gene expression is controlled by DNA methylation, recent reports have shown that histone deacetylation is involved in the silencing pathway of genomic imprinting. This was demonstrated by experiments showing that several imprinted genes were overexpressed in response to HDAC inhibitors exposure in various cell types including human fibroblast (Hu et al., 1998), mouse embryonic stem cells (Baqir and Smith, 2000), preimplantation stage embryos (Baqir and Smith, 2001a), embryonic fibroblast (El Kharroubi et al., 2001; Yoshioka et al., 2001) and cells from newborn mice (Pedone et al., 1999). However, it should be noted that some imprinted genes, including Snrpn, U2af1-rs1 and H19, appear to remain unaltered by TSA exposure (El Kharroubi et al., 2001; Gregory et al., 2002; Pedone et al., 1999; Saitoh and Wada, 2000; Yoshioka et al., 2001).

2.3.3. Epigenetic repression of imprinted genes via DNA methylation and histone deacetylation

While it is evident that beside DNA methylation in imprinted genes (Brandeis et al., 1993; Ferguson-Smith and Surani, 2001; Neumann and Barlow, 1996), other mechanisms are involved in genomic repression pathways, such as those caused by HACDs in plants (Pazin and Kadonaga, 1997; Tian and Chen, 2001). The two silencing

processes were brought together by experiments showing that the inactive X chromosome is hypoacetylated (Jeppesen and Turner, 1993; Keohane et al., 1996; Wakefield et al., 1997), in addition to the fact that differential histone H3 and H4 acetylation has been found at the CpG island of DMR1 of SNRPN gene (Saitoh and Wada, 2000). Further evidence comes from experiments that show HDAC inhibitors reactivated the transcription of sequences repressed normally by DNA methylation. For instance, TSA was responsible for the increased expression of a transfected methylated gene in mouse fibroblast cells (Eden et al., 1998) and sodium butyrate in the case of methylated non-imprinted episomal reporter gene (Bender et al., 1998). Similarly, both sodium butyrate/TSA, like 5AzaC were able to restore the expression of a methylated transgene (Pikaart et al., 1998). In addition, it has been reported that TSA can substitute for 5AzadC in restoring the transcriptional silencing from previously methylated plant ribosomal RNA genes (Chen et al., 1997). Moreover, it has also been shown more specifically that the alteration in gene expression was due to genome wide or site-specific DNA demethylation caused by HDACs in various organisms namely, fungus (Selker, 1998), virus (Szyf et al., 1985), transfected plasmid (Cervoni and Szyf, 2001) and in mouse skin fibroblast cells (Hu et al., 2000). Conversely, another form of interplay between DNA methylation and histone deacetylation was demonstrated by recent studies showing that the methyltransferase inhibitor 5Aza-dC induced hyperacetylation of a hypermethylated region (Saitoh and Wada, 2000; Takebayashi et al., 2001). Much of the methylation-acetylation connection was pointed at a recently identified group of methyl-cytosine specific binding proteins (MeCP1 and MeCP2) that are mainly localized in heterochromatin sites and capable of recruiting histone deacetylases (Fig. 7)

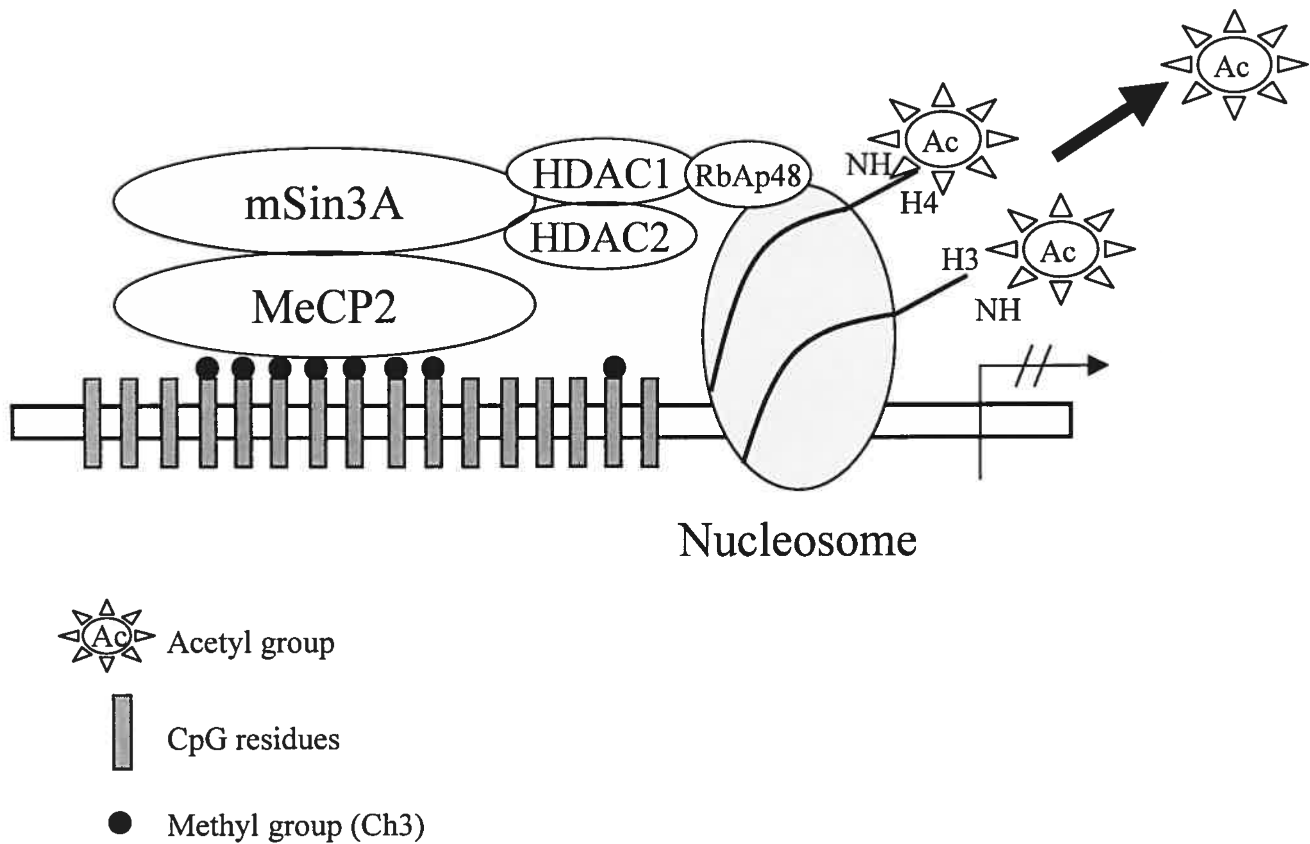


Fig. 7. Diagram of the repression process. The repression complex is formed initially by the interaction of MeCP2 with highly methylated CpG residues which then recruits a co-repressor (mSin3A) to which HDACs are associated. The deacetylases removes acetyl moieties from lysine residues of histone H3 and H4. Adapted with modifications from Razin 1998.

via a transcriptional repression domain (TRD) (Cross et al., 1997; Hendrich and Bird, 1998; Jones et al., 1998; Nan et al., 1997; Nan et al., 1998). Indeed, it has been demonstrated that the repression of H19 caused by the methylation of the imprinting control region (ICR) is associated with MeCP2 and presumably forming a repressive complex with deacetylase activity (Drewell et al., 2002).

Furthermore, it is intriguing to note that the link between DNA methylation and histone acetylation was further strengthened by studies that showed that not only DNA methyltransferases, the enzyme required to methylate genomic sequences, but also Dnmt3L, a protein that interacts with de novo methyltransferases, to be actively associated with HDACs (Aapola et al., 2002; Fuks et al., 2000; Fuks et al., 2001; Robertson et al., 2000a; Rountree et al., 2000; Wade et al., 1999).

2.4. Effect of serum starvation (medium) on imprinted gene expression

During early embryogenesis, extensive genomic modifications such as DNA methylation occur to accommodate the requirement of later developmental stages. Perturbation of this process would normally lead to deregulation of imprinted genes, growth disorders, pregnancy complications and unexpected miscarriages. There is increasing evidence that environmental factors such as in vitro culture and manipulation of preimplantation stage embryos can give rise to phenotypic, genetic and epigenetic abnormalities at postimplantaion and postnatal stages of development (Walker, 1996; Young et al., 1998). Much of the blame was assigned to the use of serum and amino acids in culture medium. For instance mouse embryos cultured in Whitens medium showed

H19 LOI, whereas KSOM AA medium had no affect on H19 expression (Erbach et al., 1994). Similarly, augmentation of amino acids in KSOM AA compared to Whitens medium increased the expression of Igf2 and Igf2r in preimplantation mouse embryos (Ho et al., 1995). More recently, it has been shown that culture of mouse embryos in serum free medium led to H19 overexpression and was associated with undermethylation of the imprinting control region (Doherty et al., 2000). Conversely, the addition of serum to mouse embryo culture resulted in alteration in the expression of two important growth regulatory imprinted genes namely, Igf2 and Grb10 (DeChiara et al., 1990; He et al., 1998), and was associated with reduced developmental potential after embryo transfer (Khosla et al., 2001a). Moreover, culture of preimplantation bovine embryos in serum restrictive medium, resulted in major alterations in Igf2 expression in the liver and skeletal muscle in day 70 fetuses (Blondin et al., 2000). Much of the deregulation of imprinted genes caused by serum deprivation was attributed to aberrant methylation and acetylation profile of these cells (Ferguson-Smith et al., 1993; Jones et al., 2001; Knosp et al., 1991). This is not surprising though, since it has been demonstrated that serum starvation was capable of altering the enzymatic activity of Dnmt1 and Dnmt3a in vitro (Robertson et al., 2000b), and depletion of polyamine in culture was associated with an alteration in methyltransferase activity in teratocarcinoma stem cells (Frostesjo et al., 1997).

Cell cycle synchronization is required to facilitate the nucleo-cytoplasmic interaction between donor nuclei and the recipient oocyte in animal cloning (Szollosi et al., 1988; Wilmut and Campbell, 1998). Serum-depleted culture conditions are commonly used as a prerequisite to synchronize donor cells before nuclear transfer (Wakayama et

al., 1998; Wilmut et al., 1997). The fact that several cloned animals were derived successfully from serum starved donor cells confirm this notion (Wilmut et al., 1997). However, the resulting pregnancies are often associated with increased miscarriages and post natal mortality, in addition to pronounced developmental malformations such as increased body and organ size (Nagy et al., 1993; Wakayama et al., 1999; Wang et al., 1997). One school of thought have suggested that the observed growth abnormalities in cloned animals are triggered by incomplete epigenetic reprogramming of imprinted genes in the donor cell due to culture condition thereby carrying these alteration to the fetus. This was demonstrated by chimeric experiments, whereby alterations in the epigenetic profile of imprinted genes in ES cells were carried to the fetus (Dean et al., 1998). Yet alone, serum starvation induced alterations in the expression pattern of several imprinted genes in mouse (Baqir and Smith, 2000; Eversole-Cire et al., 1993; Eversole-Cire et al., 1995; Hayashida et al., 1997), rat (Ungaro et al., 1997) and rabbit (Han et al., 1996) somatic cells. Not to mention that serum-constrained culture conditions caused an aberrant methylation profile (Eversole-Cire et al., 1995; Ferguson-Smith et al., 1993) and acetylation pattern of core histones in fibroblast cells (Knosp et al., 1991).

2.5. Imprinted gene expression in ES cells

ES cells are derived from the blastocysts ICM, they are pluripotent and give rise to the embryo proper, amnion, yolk sac and the chorioallantoic portion of the placenta (for review see (Rossant, 2001). Unlike embryonic germ cells which are totipotent, ES cells retain their appropriate parental imprints, as assessed by the developmental potential

of chimeras (Allen et al., 1994; Mann et al., 1995). ES cell culture require the presence of exogenous factors such as leukemia inhibitory factor (LIF) (Smith et al., 1988; Williams et al., 1988), they lose their totipotency in the event that LIF is withdrawn from the medium (Stewart et al., 1992). Moreover, ES cell derivation and unlimited capability for division make them more vulnerable to epigenetic changes, and therefore unstable in culture (Dean et al., 1998; Humpherys et al., 2001). Evidently by the fact that allele-specific methylation and expression patterns of imprinted genes in ES cells are unstable with progressive passage in culture (Baqir and Smith, 2000; Nagy et al., 1993; Szabo and Mann, 1994). This was confirmed by the failure of late passage ES cells to produce live offspring by tetraploid embryos (Nagy et al., 1990; Nagy et al., 1993; Wang et al., 1997) and by nuclear transfer (Rideout et al., 2000).

Recently it has been demonstrated that DNA mathylation profiles in ES cells are different than those observed in somatic tissues (Shiota et al., 2002) not to mention that differences in the levels of DNA methyltransferase activity between ES and somatic cells exists. For instance, unlike somatic cells in which Dnmt1 is shown to be the most abundant methyltransferase, ES cells and early stage embryos are known to contain high levels of de novo methylation activity (Jahner et al., 1982; Lei et al., 1996; Li et al., 1992; Palmiter et al., 1982; Robertson et al., 1999; Stewart et al., 1982). Moreover, in contrast to somatic cells, ES cells deficient of Dnmt1 are viable in culture, although they contain minimal levels of genomic methylation (Lei et al., 1996; Nan et al., 1996). Similarly, ES cells lacking MeCP2, a methyl cytosine binding protein whose activity is associated with histone deacetylases, have no effect on the survivability of ES cells (Tate et al., 1996). Moreover, it seems that Dnmt2 has a limited role in genomic methylation in ES cells due

to the fact that it is expressed at low levels and fails to methylate transfected retroviral DNA (Okano et al., 1998b). While Dnmt3a and b methyltransferases were shown to be expressed abundantly in undifferentiated ES cells, their expression levels were found to be minimal in somatic cells (Okano et al., 1998a). Although Dnmt3a and Dnmt3b are expressed at low levels in somatic cells, it is unknown why they exist, since most somatic tissues had already undergone the process of de novo methylation.

Contrary to ES cells, somatic cells are neither totipotent nor do they require the addition of LIF in culture, they are highly methylated and possess all the chromatin modifications characteristic of differentiated cells. The stability of genomic imprinting and methylation profiles in somatic cells was demonstrated by experiments showing that allele-specific repression of the H19 gene can be observed in the liver of 90 year old individual (Ekstrom et al., 1995). In fact it has long been shown that methylation profiles are increasingly protected in somatic cells and tissues (Stein et al., 1982; Wigler et al., 1981). Furthermore, differences in response to 5AzaC and TSA between ES versus somatic cells clearly indicates that somatic cells are more resistant to changes in methyltransferase and histone deacetylase activity and may require higher dose, extended time of exposure and/or multiple rounds of treatment to exert an effect on imprinted gene expression (Baqir and Smith, 2001b; El Kharroubi et al., 2001; Pedone et al., 1999).

Epigenetic reprogramming is poorly characterized in ES cells, however, it has been shown that chimeras with Dnmt$^{-/-}$ ES cells resulted in somatic cells carrying normal levels of genome-wide methylation but failed to establish methylation profiles of imprinted genes (Shemer et al., 1997; Tucker et al., 1996). Similarly, aberrant epigenetic alterations in ES cells failed to be corrected during post-implantation development, and

were associated with irregular imprinted gene expression and phenotypic abnormalities (Dean et al., 1998). Normal monoallelic methylation and expression patterns of imprinted genes were only restored when passed through the germ line (Tucker et al. 1996).
It is noteworthy to mention that Dnmt1 deficient ES cells, although retaining about 30% of normal methylation levels, are highly susceptible to mutations, genomic rearrangements and chromosomal translocations, probably through increased frequency of homologous recombination within demethylated DNA sequences (Chen et al., 1998; Ji et al., 1997; Miniou et al., 1994).

2.6 Epigenetic reprogramming in cloned embryos

Proper embryonic development requires that all genetic and epigenetic events that take place in the newly transferred nuclei be reprogrammed correctly by the oocyte to ensure normal development to term (Inoue et al., 2002; Kikyo and Wolffe, 2000; Solter, 2000). Currently, the success rate of producing cloned animals derived from somatic cells is very low (~1%) (Rideout et al., 2001; Wakayama et al., 1998), and in many cases is associated with growth abnormalities, miscarriages, enlarged placenta, respiratory difficulties and high mortality rate after birth (Lanza et al., 2000; Tanaka et al., 2001; Wakayama et al., 1998; Wakayama et al., 1999). On the other hand, ES nuclear donor cell are used successfully with higher developmental rate (~4%) in cloning experiments (Eggan et al., 2001; Rideout et al., 2001; Rideout et al., 2000; Wakayama et al., 1999; Zhou et al., 2001). Although, micromanipulation techniques were to blame for the inheritance of epigenetic errors to the next generation (Roemer et al., 1997), it is

increasingly evident that the epigenetic content of the nuclear donor plays an important role in the success of embryo cloning (Humpherys et al., 2001; Rideout et al., 2001). This was demonstrated by the fact that somatic cell epigenetic reprogramming was incomplete and often lacked correct methylation patterns in cloned embryos (Kang et al., 2001; Ohgane et al., 2001). Similarly, mice cloned from ES cells also displayed aberrant embryonic methylation profiles (Humpherys et al., 2001). Furthermore, epigenetic errors were carried to a later stage of development and was expressed in the placenta and the skin of cloned animals, which again revealed inappropriate re-establishment of methylation profiles in these tissues and interestingly, the degree of aberrations varied among cloned offspring (Humpherys et al., 2001; Ohgane et al., 2001). With all the epigenetic irregularities reported in cloned mice, few imprinted genes remained to be expressed at normal levels and development to term was achieved under these conditions (Humpherys et al., 2001; Inoue et al., 2002). It is interesting to note however, that improper gene expression in non-imprinted housekeeping genes including G6PD, PGK, Glut1and FGF4 (Daniels et al., 2000; Wrenzycki et al., 2002; Wrenzycki et al., 2001), in addition to developmentally important genes such as Oct4 and heat shock proteins (Hsp) (Boiani et al., 2002; Wrenzycki et al., 2001) was observed in cloned animals, many of whom survived to term.

Recently, a few investigators have attempted to synchronize the methylation pattern of the donor cell by mimicking that of the early preimplantation embryo, which is characterized to have minimal levels of DNA methylation. This was achieved by the use of 5AzaC as a demethylating agent for donor fibroblast bovine cells. However, this treatment neither improved the development to the blastocyst stage nor pregnancy rate

after nuclear cloning (Jones et al., 2001). Others tried to epigenetically remodel donor somatic cells by the use of TSA, a histone deacetylase inhibitor, which has been shown to increase the transcriptional and translational activity in preimplantation stage embryos (Memili and First, 1999). Similar to 5AzaC, TSA failed to improve developmental rates of cloned bovine embryos (Tani et al., 2001).

Although ES donor cells are epigenetically unstable in culture compared to somatic cells, other dissimilarities emerged recently such as the demonstration that DNA methylation patterns in ES cells are different from those observed in somatic tissues (Shiota et al., 2002), nonetheless, they differ in methyltransferase activity levels as well (Okano et al., 1998b). In addition, the mutation rate in ES cells is less frequent than somatic cells due to suppression of the mitotic recombination pathway in ES cells. However, the predominant form of mutation in ES cells was uniparental disomy (UPD) (Cervantes et al., 2002; Shao et al., 1999). Interestingly, UPD is a major cause for disturbances in the expression of imprinted genes (Sotomaru et al., 2002; Sotomaru et al., 2001) and in many cases they are either lethal or lead to a variety of growth disorders in mice and humans (Cattanach and Beechey, 1990; Georgiades et al., 2000; Kotzot, 2001; Preece and Moore, 2000; Robinson, 2000). Furthermore, epimutations, or the occurrence of abnormal mutations in the epigenetic system (Reik and Walter, 2001), for instance, mutations that arise in DNA methyltransferases or histone deacetylases which might be responsible for the failure to reprogram imprinted genes correctly in cloned embryos. In fact it has been reported lately that cloned bovine embryos express low levels of Dnmt1 (Wrenzycki et al., 2001), which indeed may lead to genomic epimutations. Interestingly, it has been shown that ICF (Immunodeficiency, centromeric instability, facial anomalies)

syndrome in human which is characterized by mental retardation and tongue enlargement arise from mutations in Dnmt3b gene (Hansen et al., 1999; Okano et al., 1999; Xu et al., 1999). Similarly, Rett syndrome in humans, which is characterized by mental retardation in females was found in patients carrying mutations in MeCP2 (Amir et al., 1999; Lee et al., 2001; Wan et al., 1999; Wan et al., 2001). Therefore, it might be possible that these epimutations were carried from the nuclear donor cell due to culture conditions, in vitro manipulation or perhaps aging (Dean et al., 1998; Khosla et al., 2001a; Reik et al., 1993) into the enucleated oocyte. This is supported by the fact that Dnmt3b knockout mice develop at a very low rate to term and exhibit some of the ICF symptoms (Okano et al., 1999), similarly, MeCP2 deficient mice poorly develop to term and may exhibit symptoms of Rett syndrome (Tate et al., 1996).

2.7. Aberrant regulation of imprinted genes and Growth disorders

Aberrant imprinted gene expression is widely expected to cause abnormal embryonic growth and development, since virtually all imprinted genes play a critical role in this process. It has long been known that several imprinted genes have been implicated in the Beckwith-Wiedemann syndrome (BWS), these genes include IGF2, H19, P57 and KVLQT (Catchpoole et al., 1997; Joyce et al., 1997; Lee et al., 1997; O'Keefe et al., 1997). BWS is characterized by fetal and post natal growth enhancement, organomegaly such as gigantism of the kidney, liver, adrenal and tongue (Maher and Reik, 2000), in addition to the likelihood that the majority of affected individuals will develop Wilms tumor (WT), which is characterized by LOI of IGF2 (Moulton et al.,

1994; Steenman et al., 1994) or other embryonic neoplasms (Elliott and Maher, 1994; Pettenati et al., 1986; Reik and Maher, 1997; Ward, 1997), that have aberrant methylation and imprinted gene expression profiles (Frevel et al., 1999; Taniguchi et al., 1995). It is likely that BWS is mainly caused by elevated levels of IGF2 expression due LOI and is associated with transcriptional repression and hypermethylation of the maternal H19 allele (Joyce et al., 1997; Okamoto et al., 1997; Reik et al., 1995). Similarly, BWS patients display 50% and 20% LOI for LIT1 and IGF2 respectively (Feinberg, 2000a; Horike et al., 2000). More recently, deregulation of imprinting expression of Igf2 and p57 has been shown to display many of the characteristics of BWS, including placentomegaly, or the gigantism of the placenta and organomegaly of several tissues (Caspary et al., 1999; Grandjean et al., 2000).

Optimal feto-maternal interactions between the placenta and the fetus are required to ensure normal fetal growth and development. Placentomegaly, has been reported in in vitro fertilized and cloned animals (Wakayama et al., 1998; Wakayama et al., 1999). Much of the blame caused by growth anomalies due to placental defects has been pointed at imprinted genes since they are abundantly expressed in the placenta in addition to the fact that recent uniparental disomy experiments for distal chromosome 12, which contains a cluster of imprinted genes (Gtl2 and Dlk1), revealed a variety of placental defects such as abnormal distribution of the placental layers (Georgiades et al., 2001; Georgiades et al., 2000; Schmidt et al., 2000; Takada et al., 2000).

Although Mash2 (Guillemot et al., 1995), Igf2 (Caspary et al., 1999; Sun et al., 1997), p57 (Caspary et al., 1999; Zhang et al., 1998), Igf2r (Eggenschwiler et al., 1997) and Esx1 (Li and Behringer, 1998) play important roles in the development and formation of

the placenta, it seems that the imprinted Ipl (Tssc3) gene play a critical role in this process. Mice knockout of Ipl, which is located on distal chromosome 7 within the same cluster that contain p57-Igf2-H19, whose expression is restricted only in extraembryonic tissues (Qian et al., 1997) displayed abnormal overgrowth in the placenta (Frank et al., 2002).

Interestingly, several phenotypic abnormalities observed in BWS that are caused by aberrant imprinting patterns, such as fetal overgrowth, skeletal abnormalities, placentomegaly and enlargement of internal organs (liver and heart) are similar to those reported in the so called large offspring syndrome (LOS) in cattle and sheep (Farin and Farin, 1995; McEvoy et al., 1998; McEvoy et al., 2000; Schnieke et al., 1997; Sinclair et al., 2000; Thompson et al., 1995; Young and Fairburn, 2000; Young et al., 1998). It is widely anticipated that deregulation of the Igf2-H19, p57-LIT1 and Igf2-p57 locus are prime candidates for LOS (Caspary et al., 1999; Feinberg, 2000b). This is evident in recent reports demonstrating that epigenetic alteration in Igf2r expression is associated with fetal overgrowth in sheep due to culture medium (Loewith et al., 2001). Conversely however, a 20% drop in fetus weight was attributed to down regulation of Igf2 transcripts due addition of serum in the medium of preimplantation mouse embryos and was associated with reduce viability (Khosla et al., 2001a). These findings are in agreement with others (Arny et al., 1987; Caro and Trounson, 1984) who reported comparable effects of serum on the post-implantation viability of mouse embryos.

Moreover, embryo manipulation and nuclear transfer experiments can result in alterations in gene expression profiles (Latham et al., 1994; Reik et al., 1993). Thus leading to growth abnormalities, this was evident in pronuclear transfer experiments which resulted

in a 15% growth deficiency in some of the animals and was epigenetically inherited by the next generation (Roemer et al., 1997). Recent reports on cloned animals have described abnormalities during post-implantation development of reconstructed embryos that are similar to those of LOS (Campbell et al., 1996; Cibelli et al., 1998; Kato et al., 1998; Keefer et al., 1994; Kubota et al., 2000; Schnieke et al., 1997; Wells et al., 1997). Similarly, nuclear cloning experiments in the mouse, produced abnormally large placenta (Wakayama et al., 1998; Wakayama et al., 1999), which were characterized by major disturbances in the architectural ratio of the layers that form the placenta; this was associated with alterations in the expression pattern of several imprinted genes (Tanaka et al., 2001). Furthermore, completely ES cell-derived mice and fetuses obtained from high-passage ES cell lines which presumably harbor an inappropriate epigenetic program, were reported to have also increased weight at birth along with high incidences of postnatal death (Dean et al., 1998; Nagy et al., 1993; Wang et al., 1997).

3. Rational, Hypothesis and Objectives

3.1. Rational

The success rate of embryo cloning is extremely low and the surviving animals suffer from respiratory illnesses, skeletal abnormalities and dysfunctional organs due to organomegaly. Much of the phenotypic defects observed in cloned fetuses and newborn animals point towards improper epigenetic reprogramming of developmentally important genes, including those that are imprinted. While much of the work to improve cloning efficiency has focused on cell cycle synchronization and the origin of the donor cell, little has been done to characterize the epigenetic status of donor nuclei prior to transfer. For instance, while serum depleted cultures has been widely used to synchronize donor cells in G0 of the cell cycle, little is known about the epigenetic modification associated with the use of such protocol. Moreover, while DNA methylation has been attributed to the monoallelic silencing of imprinted genes, no thorough investigation has been carried out on other repression mechanisms, namely histone deacetylation, during preimplantation development and in ES cell cultures.

Finally, while several protocols focus on cell cycle synchronization of donor cells, no information is available on the induction of epigenetic alterations to donor nuclei prior to transfer. Given the fact that the oocyte cytoplasm fails to correct abnormal methylation profiles of somatic donor cells (Kang et al., 2001; Ohgane et al., 2001) further justifies the need for such studies.

3.2. Hypothesis

Imprinted gene expression pattern is susceptible to culture conditions, alterations in DNA methylation and histone acetylation in mouse embryonic stem cells, preimplantation stage embryos and cloned embryos.

3.3. Objectives

1. To characterize the expression pattern of two imprinted genes, Igf2 and H19, in embryonic stem cells induced into quiescence (growth arrest) by means of serum starvation, confluency, and also to investigate whether passages of ES cells at progressive numbers would affects the expression profile of imprinted genes.
2. To characterize the expression pattern of several imprinted, non imprinted and housekeeping genes in mouse embryonic stem cells under a state of hypomethylation, hyperacetylation or a combination of both in mouse ES cells.
3. To monitor the expression profile of imprinted genes in preimplantation mouse embryos and parthenogenetic embryos under a state of hypomethylation and hyperacetylation.
4. To determine whether the cloning procedure is able to reprogram the expression of imprinted genes of donor nuclei (ES or somatic) exposed to drugs that modify histone acetylation and DNA methylation.

4. Articles

4.1. Article one

Growth Restricted *In Vitro* Culture Conditions Alter the Imprinted Gene Expression Patterns of Mouse Embryonic Stem Cells

Growth Restricted *In Vitro* Culture Conditions Alter the Imprinted Gene Expression Patterns of Mouse Embryonic Stem Cells

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ABSTRACT

Embryonic stem (ES) cell-derived clones and chimeras are often associated with growth abnormalities during fetal development, leading to the production of over/under weight offspring that show elevated neonatal mortality and morbidity. Due to the role played by imprinted genes in controlling fetal growth, much of the blame is pointed at improper epigenetic reprogramming of cells used in the procedures. We have analyzed the expression pattern of two growth regulatory imprinted genes, namely insulin like growth factor II (Igf2) and H19, in mouse ES cells cultured under growth restricted conditions and after in vitro aging. Culture of cells with serum-depleted media (starvation) and at high cell density (confluence) increased the expression of both imprinted genes and led to aberrant methylation profiles of DMR2, a key Igf2 regulatory region. These findings confirm that growth constrained cultures of ES cells are associated with alterations to methylation of the regulatory domains and the expression patterns of imprinted genes, suggesting a possible role of epigenetic factors in the loss of developmental potential.

INTRODUCTION

Mammalian embryos and cells used to reconstruct embryos by nuclear transfer are often exposed to *in vitro* culture conditions that differ substantially from the natural milieu in which they grow and differentiate in vivo. Serum is commonly added to chemically defined solutions not only to provide a macromolecule substrate but also to supply growth-enhancing factors required for cell proliferation. Furthermore, because serum-depleted culture medium leads to mitotic cell arrest at G0 of the cell cycle, a stage embedded in the G1-phase in which both total and specific protein synthesis is decreased (Darzynkiewicz et al., 1996; Larsson and Zetterberg, 1986), serum-depleted culture conditions are commonly used to synchronize donor cells before nuclear transfer to clone mammals (Wakayama et al., 1998; Wilmut et al., 1997). Cell cycle synchronization before DNA replication is required to facilitate the interaction of donor nuclei with metaphase-stage host cytoplasm (Szollosi et al., 1988) and to maintain the correct ploidy (Campbell et al., 1996). Moreover, G0 arrest by serum starvation is believed to render donor chromatin more amenable to epigenetic reprogramming in the differentiation pathway of cells. However, G0 cell cycle synchronization can also be obtained by contact inhibition in confluent monolayers and the production of viable clones confirms that at least some aspects of epigenetic reprogramming does occur successfully in the absence of serum starvation (Cibelli et al., 1998; Shiga et al., 1999). Nonetheless, pregnancy loss at different stages of gestation and fetal abnormalities are often observed with reconstructed and *in vitro* derived embryos exposed or not to serum (Holm et al., 1996; Keefer et al., 1994; Lane and Gardner, 1996). Therefore, further studies are necessary to determine the

molecular mechanisms by which cell cycle arrest, both in the presence and absence of serum, may affect the developmental program of donor cells used to reconstruct embryos.

Genomic imprinting is a mechanism that allows some genes to be expressed from only one of the parental alleles, often in a tissue or developmental stage specific manner (Barlow, 1995). Insulin-like growth factor II (Igf2) and H19 are located on the distal region of chromosome 7 in the mouse and are expressed from either the paternal or maternal alleles (Bartolomei et al., 1991; DeChiara et al., 1991). Moreover, both genes seem to have a common regulatory mechanism which falls under the control of several differentially methylated regions (DMRs) (Brandeis et al., 1993; Feil et al., 1994; Hark et al., 2000; Moore et al., 1997). Aberrant methylation profile of these regions, particularly the DMR2 region, is often correlated with deregulation of both genes. The role of Igf2 in regulating fetal growth and development has been demonstrated by its null mutation mutant mice, which produced smaller size animals (DeChiara et al., 1990). Moreover, excessive expression of Igf2 by loss of imprinting in Beckwith-Wiedemann syndrome is associated with increased size at birth and other fetal abnormalities in humans (Morison et al., 1996; Reik and Maher, 1997). Together, these results suggest that the aberrant expression patterns of Igf2 and H19 are implicated in the abnormal fetal growth observed after exposure of normal and reconstructed embryos to *in vitro* environments.

Embryonic stem (ES) cells, which are derived from the inner cell mass of the blastocyst, can be used to produce entire animals both through microinjection into tetraploid blastocyst (Nagy et al., 1990) and by nuclear transfer (Wakayama et al., 1999). However, the resulting pregnancies are often associated with elevated pre- and postnatal mortality and pronounced developmental malformations such as increased size and

swollen edematous skin (Nagy et al., 1993; Wakayama et al., 1999; Wang et al., 1997). Improper epigenetic reprogramming of imprinted genes including both Igf2 and H19 has been suggested to be implicated in fetal growth defects (Dean et al., 1998). Previous studies with mouse embryonic fibroblast cell lines have indicated that imprinted genes are up-regulated upon confluence and serum starvation (Eversole-Cire et al., 1993; Eversole-Cire et al., 1995; Hayashida et al., 1997). Moreover, upregulation of the expression patterns of imprinted genes were also observed in ES cell, induced to differentiate *in vitro* into embryoid bodies, suggesting that *in vitro* handling effects are similar among somatic and embryonic cells undergoing differentiation (Allen et al., 1994). Therefore, to further understand the role imprinted genes play in developmental abnormalities caused by cell handling procedures, the expression patterns of Igf2/H19 and the methylation profile of DMR2 were examined in ES cells cultured under both growth restrictive conditions and after prolonged culture *in vitro*. The expression profiles of non-imprinted genes Gapdh, Oct4 and Gas6 were also examined to determine the effect of growth restrictive conditions on housekeeping, differentiation and cell arrest/stress, respectively.

MATERIALS AND METHODS

Embryonic stem cell culture

The mouse ES cell line H106 was established in our laboratory from F1 females (C3H X C57Bl6, Charles River Canada, St-Constance, QC) and used previously to produce germline chimeras (unpublished data). Undifferentiated ES cells were cultured

on a feeder layer of inactivated murine mitomycin C-treated fibroblasts cells in DMEM medium supplemented with 20% v/v heat inactivated fetal bovine serum, 10^3 U ml^{-1} of leukemia inhibitory factor (LIF, ESGRO), 1% v/v of nucleosides, L-glutamine, non-essential amino acids (all from GibcoBRL; Burlington, ON) and 0.1 mM β-mercaptoethanol (Sigma, St. Louis, MO). At each passage, cells were counted and plated at different densities according to each experimental protocol. Alkaline phosphatase (AP) activity, a marker for undifferentiated ES cells, was measured using standard techniques (Buehr and McLaren, 1993). Briefly, cells were washed with PBS prior to fixation with 4% paraformaldehyde for 30 min at room temperature, AP staining was carried out for 45 min at room temperature in dark conditions.

RNA Preparation and RT-PCR analysis

After removal of the culture medium from the ES cells, total RNA was isolated with TRIzol (GibcoBRL) as described in the manufacturers protocol. Three micrograms of total RNA was reverse transcribed in a total volume of 20μl reaction mix containing 1X RT buffer (50 mM KCL, 10 mM Tris-HCL, pH 8.3, GibcoBRL), 0.5 μg oligo (dT), 3 mM $MgCl_2$, 1 mM of each of the four dNTPs, 10 mM DTT, 1 U/μl RNase inhibitor (Rnasin; Promega, Madison, WI) and 10 U of AMV reverse transcriptase (Promega). Samples were incubated at 42°C for 60 min, 50°C for 5 min and 72°C for 10 min. After cDNA synthesis, 1 μl of RNase H (GibcoBRL) was added to the mix and incubated at 37°C for 15 min followed by 5 min at 90°C and the cDNA was diluted in 200 μl Tris-EDTA (pH 8.3). PCR reactions were carried out in a final volume of 50 μl in a DNA thermal cycler (Hybaid, OmniGene). The amplification mixture contained 10 μl of the

cDNA solution, 0.4 μM primers, 1 U of Taq polymerase, 200 μM of each dNTP, 10 mM Tris-HCl (pH 8.3), 50 mM KCl and 3 mM $MgCl_2$. Samples were held for an initial denaturation cycle at 80°C for 4 min, followed by several cycles (26 to 32) consisting of denaturation at 95°C for 45 sec, annealing at 56°C for 45 sec and extension at 72°C for 1 min. The number of amplification cycles was optimized in preliminary experiments to yield PCR products in the exponential phase of the amplification. PCR primers for Igf2 amplified a 197 bp product (Eversole-Cire et al., 1993), 564 bp for H19 (Eversole-Cire et al., 1995), 507 bp for Gas6 (Fleming et al., 1997), 586 bp for Gapdh (Eversole-Cire et al., 1995), 257 bp for Globin (Davis et al., 1996) and 550 bp for Oct4 (forward: 5'-CGAGGAGTCCCAGGAC ATGAAA-3' and reverse: 5'-TGGGGGCAGAGGAAAGGATACA -3'). As a negative control for possible contamination, cDNA was replaced with autoclaved-DEPC treated water. Band intensities were quantified by densitometric scanning (FotoDyne software) and corrected with band intensities of a control housekeeping gene (Gapdh; Glyceraldehyde-3-phosphate dehydrogenase) or globin (exogenous standard; GibcoBRL) in the case of passage by using an image densitometry analysis system (Collage 3.0 software system).

DNA bisulfite modification and melting peak analysis

Genomic DNA was extracted with TRIzol (GibcoBRL) as described in the manufacturers protocol. Purified DNA was digested with *Bam*HI and *Eco*NI, which do not cut within the desired region of methylation analysis (Weber et al., 2001). Prior to bisulfite treatment, digested DNA was purified with MinElute cleanup kit (Qiagen, Mississauga, ON) according to manufacturers protocol. Sodium bisulfite modification

was performed using the CpGenome DNA modification kit (Intergen, Hornby, ON), purified with the Geneclean II kit (Bio101, Carlsbad, CA), eluted in 25μl sterile H_2O and stored at -20°C in dark until PCR analysis was conducted. Primers and melting maps were created using the MELT94 software (http://web.mit.edu/osp/www/me lt.html) and according to previous reports (Sheffield et al., 1989; Worm et al., 2001). The primers were designed to amplify a 208 bp product in a region containing a 14 CpG island. This region is located on DMR2 of the Igf2 gene (U71085), which is known to be methylated on the paternal allele (Brandeis et al., 1993; Feil et al., 1994; Weber et al., 2001). As a positive control for hypermethylation of the paternal allele, liver DNA extracts of d15 fetus were used. Primers were designed to contain GC clamps as follows: [5′-(CGGGCGGGGG)CCAATATAACACCTAA AAACAATC-3′] and [5′-(CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG)TTTGATTTATTG ATGGTTGTTGGA-3′] and melting curves were obtained on a lightCycler™ apparatus (Roche Diagnostics, Laval, QC). Prior to PCR amplification 3-5μl of bisulfite treated DNA was mixed with 0.5μl TaqStart antibody (Clontech, Palo Alto, CA) for 5 min and added to the PCR master mix containing: 5μl SYBR green I mix (Roche Diagnostics), 0.5μM primers (Gibco BRL) and 3mM $MgCl_2$ in a total volume of 20μl. The amplification program consisted of an initial denaturation step of 10 min at 95°C, followed by 38 cycles of denaturation for 0 sec at 95°C, annealing for 5 sec at 56°C and extension for 20 sec at 72°C. Fluorescence readings for melting curve analysis were measured between 62°C and 98°C at a rate of 0.05 °C/sec. Melting curves were generated with lightCycler™ software, version 5.28 (Roche Diagnostics).

Normalization and statistics

Normalization of gene expression analysis was carried out by dividing the value of each gene with that of the correspondent endogenous standard (Gapdh) or exogenous standard (Globin) in the case of ES cell passage number analysis. Values were corrected and expressed relative to a non-treated control. All experiments were repeated three times. Data were analyzed by ANOVA and mean values were statistically compared using the Tukey-Kramer test with differences at $P \leq 0.05$ being considered significant.

RESULTS

Effects of serum starvation

In order to examine the effects of serum starvation on the ES cell replication, differentiation and the control of expression of imprinted genes, ES cells (passage 11) were seeded at 1×10^5 in 35 mm dishes, cultured for 2 days with serum and then cultured for another 2 and 4 days in serum-depleted medium (0.5% v/v). Although cell count analysis indicated growth arrest during the first 2 days of serum starvation, an increase in cell numbers was observed at day-4 of culture in serum-depleted medium, indicating a re-initiation of mitotic activity regardless of low serum concentration (Fig. 1A). Staining of ES colonies with alkaline phosphatase (AP) after 2 days in serum-depleted medium yielded a majority of colonies with moderate staining (Fig. 1B, center) compared to the strong staining in controls (Fig. 1B, left), whereas cultures for 4 days yielded many colonies with weak or no staining. Although many colonies remained undifferentiated at day-2, these results indicate the initiation of a differentiation process by day-4 of culture

in serum depleted medium. Using melting peak analysis, we next investigated the epigenetic status of the Igf2 gene by monitoring methylation patterns of DMR2, a 14 CpG island located within a 208 bp fragment of a differentially methylated region (Fig. 1C). In contrast to somatic DNA from fetal liver (positive control), which showed high levels of methylation (Fig. 1C, line 5), non-starved ES controls and 2-day starved ES cells displayed lower methylation patterns (Fig. 1C, lines 2 and 3). Moreover, ES cells exposed to serum-depleted medium for 4 days (Fig. 1C, line 4) showed a shift towards higher methylation patterns similar to those observed in somatic cells, indicating an increase in the number of methylated CpG islands within the DMR2 of Igf2 following extended serum starvation.

Expression levels of Igf2, H19, Oct4, Gas6 and Gapdh genes in serum-starved ES cells are shown in Figure 2. The overall expression of both imprinted transcripts (Igf2 and H19) was upregulated upon serum starvation with increasing time in culture (day 2 and 4). The observed fold increase for Igf2 at day-2 and 4 of culture was 5.1 and 10 fold (Fig. 2A), respectively. However, H19 expression was affected to a lesser degree and was only evident after 4 days in serum-depleted culture conditions (1.4 fold; Fig. 2B). In addition, monitoring the expression of Gas6, a member of a family of genes associated with growth arrest caused by serum deprivation or contact inhibition (Ciccarelli et al., 1990; Fleming et al., 1998; Schneider et al., 1988), showed an elevated expression pattern of 5.1, 7.9 fold in 2 and 4-day starved ES cells, respectively (Fig. 2C). Nonetheless, preliminary experiments with flow cytometry revealed that serum deprivation for 2 days caused an increase in cells arrested at G0/G1 of the cell cycle compared to non-starved controls (59% vs 24%), indicating that cells were actively dividing. Oct4 expression, a

transcription factor which is expressed exclusively in totipotent ES cells, decreased to basal levels following 4 days of culture in serum-depleted medium (Fig. 2D), indicating that ES cells were undergoing differentiation and confirming our findings of weaker AP staining (Fig. 1B). Nonetheless, Oct4 expression remained present regardless of serum starvation, indicating that at least some ES cells did not differentiate, which is consistent with the weak AP staining of some colonies (Fig. 1B, right). To examine the reversibility of the effects observed on expression patterns, ES cells cultured for 4 days in serum-depleted medium were washed and cultured for another 2 days in medium containing 20% serum. Igf2 and H19 expression was downregulated (4.7 and 1 fold, respectively), indicating partial reestablishment of the expression levels observed before serum starvation. Similarly, Gas6 expression was downregulated, indicating that the cells reinitiated mitotic replication (6.5 fold down from 8 fold). In general, these observations suggest that imprinted and non-imprinted gene expression in ES cells is affected by growth inhibitory conditions induced by serum-depletion. Moreover, with the exception of Oct4, the addition of serum to serum-starved ES cells seems to reverse the expression of imprinted and non-imprinted genes to levels observed prior to serum starvation.

Effects of cell density

We next sought to examine the expression of Igf2 and H19 in ES cells at different densities during a 5-day culture period at confluence. To induce growth arrest by contact inhibition, ES cells were seeded at high density (30×10^5 in a 35 mm petri dish). Cell counts (Fig. 3A) show no increase in cell numbers after seeding followed by an increase from day 2 to 4, indicating that even at high densities ES cell number doubled at an

exponential rate. Nonetheless, a plateau was reached at day-4 and 5 of culture when up to 90% of the surface was covered by multilayered ES colonies, indicating growth arrest due to confluent culture conditions. Staining with AP showed mostly positively stained colonies at day-3 of culture, indicating the presence of some undifferentiated cells in the centre of most colonies. Nonetheless, many colonies showed no AP staining after 5 days of continuous culture, indicating a high degree of differentiation under high density conditions (Fig. 3B right). Melting peak analysis of the methylation profile of DMR2 of Igf2 showed an increase in methylation at day 4 (Fig. 3C, line 3) compared to that of non-confluent cultures at day 2 (Fig. 3C, line 2). However, the shift in methylation patterns observed in confluent cultures did not match the fully methylated pattern found in the liver (Fig. 3, line 4).

Expression levels of Igf2, H19, Oct4, Gas6 and Gapdh were monitored at 24 h intervals for 5 days during ES cell cultures (Fig. 4). The overall expression of Igf2 was indeed upregulated in a gradual manner (1.9, 2.5, 4, 4.5 fold). Similarly, H19 was also overexpressed, however, not to the same level as Igf2 (1, 1.1, 1.3, 1.4 fold), whereas the non-imprinted control Gapdh did not vary during culture. Concurrent with the differentiation of the outer border and loss of AP staining of the ES colonies at 3 days of culture, Oct4 expression showed a 0.4, 0.3 fold decrease at day 4 and 5, respectively. Since Oct4 expression was never completely abolished, these results indicate the continuous presence of some undifferentiated cells, consistent with the positive AP staining (Fig. 3C, left). These findings confirm previous reports showing the presence of undifferentiated cells among highly dense ES cultures and when LIF was withdrawn from the medium (Rathjen et al., 1990; Sasaki et al., 1992). In support of the continuous

presence of undifferentiated ES cells at day-5, trypsinization followed by seeding at low density led to the formation of morphologically normal ES colonies, i.e. high nuclear : cytoplasmic ratio, uniform tightly packed round colonies and positive AP activity (data not shown).

Combined effects of serum starvation and cell density

Since serum starvation and confluence had similar effects on both imprinted genes, we wished to ascertain whether imprinted gene overexpression patterns were similar at high (30 x 10^5) and low (1 x 10^5) ES cell seeding densities during 2 days in the presence and absence of serum-depleted medium (Fig. 5). Although only minor non-significant alterations were observed with H19 expression, Igf2 transcription levels were significantly increased in both high and low seeding densities. However, Igf2 up-regulation in serum-depleted medium was comparable among low (3.5 fold) and in high (2.7 fold) seeding densities, indicating that confluence conditions do not substantially affect the response of ES cells to serum starvation. Moreover, Igf2 overexpression response to different seeding densities did not vary between starved (1.9 fold) and non-starved (2.5 fold) ES cells, indicating that the serum depletion does not alter substantially the response of ES cells to confluence conditions.

Effects of passage number

ES cells were recovered at early (n=8), intermediate (n=22) and late (n=36) passages to examine the effects of ES cell aging on the expression levels of Igf2 and H19 during continuous passages *in vitro*. ES cells were trypsinized and seeded at 30 x 10^5

using 35 mm dishes at 3 to 4 day intervals. Since the expression of Gapdh fluctuated between passages (data not shown), exogenous globin mRNA was added during the RNA extraction procedure and used as an external standard. Although Igf2 expression remained stable throughout early and intermediate passages, a dramatic decrease was observed during late passage (0.5 fold; Fig. 6A). Similarly, H19 (Fig. 6B) expression remained constant during early and intermediate passages and was downregulated in late passage (0.7 fold; Fig. 6B). These results suggest that the epigenetic imprint of ES cells is unstable after long *in vitro* culture periods. It is worth noting that the upregulation of Igf2 and H19 was observed at all three passages following serum starvation treatment (data not shown), indicating that even aged ES cells will respond to growth arrest stimulatory conditions.

DISCUSSION

We have performed a detailed investigation on the overall expression pattern of Igf2 and H19 in mouse ES cells under growth inhibitory conditions, i.e. serum starvation and confluence. Our results show that Igf2 is upregulated upon serum starvation in ES cells, which is consistent with earlier observations in somatic cells in mouse (Eversole-Cire et al., 1993; Eversole-Cire et al., 1995; Hayashida et al., 1997), rat (Ungaro et al., 1997) and rabbit (Han et al., 1996), suggesting a common regulatory mechanism controlling Igf2 transcription in embryonic and differentiated somatic cells. Interestingly, it is worth noting that H19 was less affected in serum-deprived cultures than Igf2 (including this study), suggesting that the epigenetic control of H19 is more stable than

Igf2 and better able to resist environmental changes in ES and somatic cultures. However, although the upregulatory pattern of both imprinted genes in somatic cells was somewhat similar to embryonic cells (this study), the magnitude was much lower in the latter, suggesting differences in the epigenetic regulation between somatic and embryonic cells.

Although little is known about the molecular events associated with the response of cells to growth inhibitory conditions, DNA methylation has been correlated with the expression level of imprinted genes (Brandeis et al., 1993), and growth constrained conditions in somatic cells (Eversole-Cire et al., 1995; Ferguson-Smith et al., 1993). These findings are consistent with our observation of Igf2 overexpression and its association with an increase in methylation profiles of a 14 CpG island located in DMR2 and with previous studies showing that growth arrest by confluency increased methylation by 3 fold (Pieper et al., 1999). Nonetheless, both these findings conflict with a recent report showing that methyltransferases are down-regulated upon confluency (Robertson et al., 2000). Since methyltransferases are responsible for maintaining or *de novo* methylating DNA sequences, one would expect a decrease rather than an increase in methylation at increased cell densities. Several scenarios may explain the above discrepancy. First, previous studies were all performed using somatic cells that contain lower levels of methyltransferase activity when compared to ES cells (Lei et al., 1996; Stewart et al., 1982). Second, since ES cells exposed to growth inhibitory conditions do not fully arrest in G0, as indicated by flow cytometry and late logarithmic phase in the growth curve analysis, it is likely that methylation continues to occur throughout treatment, regardless of starvation or confluency. In fact, the same study showed no

major alterations in methylation patterns between logarithmically growing and late log phase cells, suggesting that a decrease in methylation occurs only when cells are arrested for long periods in G0 (Robertson et al., 2000). Third, it cannot be ruled out entirely that alterations of the methylation status reported in this specific site of DMR2 could occur differently in other important imprinted control regions elsewhere in the genome. Finally, since others have also shown no change in the methylation patterns between serum fed versus serum deprived cultures (Kang et al., 2001), it is likely that variability is due to differences in the ES cell line used in each experiment. One might speculate that these alterations in methylation patterns depend on the starting epigenetic "package" of each cell type prior to treatment.

The observation that there were no shift in the methylation profile of the DMR2 from ES cells starved for 2 days regardless of a significant increase in Igf2 expression (5 fold), supports the notion that epigenetic mechanisms other than DNA methylation are involved in controlling the expression pathway of imprinted genes. It has been observed that inhibitors of histone deacetylases (sodium butyrate and TSA), which render the chromatin in a hyperacetylated state, can lead to an overexpressed pattern of imprinted genes (Hu et al., 1998; Pedone et al., 1999). At the same time, TSA has been shown to inhibit cellular growth and, therefore, it is possible that growth inhibitory conditions increases the acetylation level of histones leading to the overexpression of imprinted genes. This hypothesis is strengthened by earlier findings showing that serum starvation is capable of altering the acetylation status of core histones in fibroblast cells (Knosp et al., 1991). Therefore, it is tempting to speculate that DNA methylation and histone

acetylation act jointly in regulating the transcriptional activity of imprinted genes during growth arrest.

Since Oct4 expression and AP staining decreased concurrently with growth arrest treatments, it is unclear whether imprinted gene overexpression was attributed exclusively to decreased cellular proliferation or also to differentiation. Nonetheless, the presence of Oct4 expression and AP staining throughout this study and other previous experiments in which ES cell growth arrest was induced (Rathjen et al., 1990; Smith et al., 1992), suggest that cellular differentiation is unlikely to be a prominent player in the activation of imprinted gene expression. Moreover, Oct4 expression after replacing serum to starved ES cells did not reverse to values observed before starvation, suggesting that Igf2 downregulation was independent of differentiation.

The expression of Igf2 and H19 during various passages was examined, our data demonstrate that the overall expression of imprinted genes was not perturbed between early (n=8) and intermediate (n=22) passages whereas at late (n=36) passages there was a 50% drop in the expression of both transcripts. These data suggest that the progressive loss of gene expression with ES cell aging is associated with alteration in the methylation profile of imprinted genes. Therefore, it is conceivable that the reduced dosage of imprinted gene transcripts in late passage ES cells accounts for the developmental abnormalities and organ defects observed in chimeras derived from late passage ES cells (Nagy et al., 1990; Nagy et al., 1993; Wang et al., 1997) and the failure to produce mice through cloning by nuclear transfer (Rideout et al., 2000) or tetraploid-derived chimeras (Wang et al., 1997). Furthermore, recent advances in embryo cloning technology indicates an advantage in using serum starvation to synchronize donor cells in a quiescent

state (Campbell et al., 1996; Hill et al., 2000; Wilmut et al., 1997; Zakhartchenko et al., 1999), suggesting that G0 arrested cells harbor a modified chromatin which is capable of supporting development to term. Although the exact nature of these alterations in chromatin configuration are yet to be identified, a large body of evidence is accumulating on the role of genomic imprinting as a key player in regulating embryonic growth. The role of serum starvation on ES cells used as nuclear donors to produce cloned mice has been down played recently by the absence of growth abnormalities during gestation (Wakayama et al., 1999). This contrasts with our findings showing increased expression of Igf2 after serum starvation, which would presumably cause fetal overgrowth defects. Previous reports on Igf2 perturbation, such as paternal duplication of chromosome 7 (Ferguson-Smith et al., 1991), pronuclear microinjection with Igf2 transgene (Ward et al., 1994) and the use of ES cells with abnormal Igf2 expression (Dean et al., 1998) and carrying an Igf2 transgene to produce chimeras (Sun et al., 1997), have all displayed prenatal overgrowth, organ enlargement and skeletal defects. It is worth noting that the examples above lead to permanent alterations in Igf2 expression whereas serum starvation and confluence are reversible epigenetic modifications, as indicated by the re-introduction of serum to starved ES cells (Fig. 2A), that may be corrected by nuclear-cytoplasmic interactions during pre- and post-implantation development.

In summary, this study demonstrates that growth inhibition by serum starvation or confluence leads to an overexpressed pattern of Igf2, and to a lesser extent to H19, in mouse ES cells. Moreover, overexpression patterns were associated with perturbation of a the key methylated domain of the Igf2 gene. Further molecular analysis is required to characterize the mechanisms which mediate the expression of imprinted genes and the

epigenetic factors involved (methylation profiles and acetylation status of histones). This information is particularly relevant to the use of cell quiescence prior to nuclear transfer procedures to determine whether serum starvation is an advantageous procedure for preparing donor cells for cloning.

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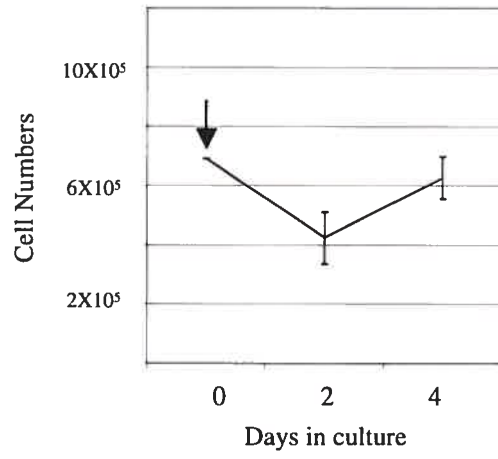
FIG. 1. Effects of serum starvation on mouse ES cell cultures. (A) Cell counts at different days after starvation. Arrow indicates the start of serum deprivation regime. (B) Staining of ES colonies with alkaline phosphatase (AP) before serum starvation (left), or at 2 days (center) and 4 days (right) after serum starvation. Arrowhead indicates strong to weak AP staining. (C) Melting peak analysis showing: line 1, negative control (H_2O); line 2, non-starved control at 2 days after seeding; line 3, serum starved for 2 days; line 4, serum starved for 4 days; line 5, fetal fibroblast cells (high methylayion), used as positive control.

A



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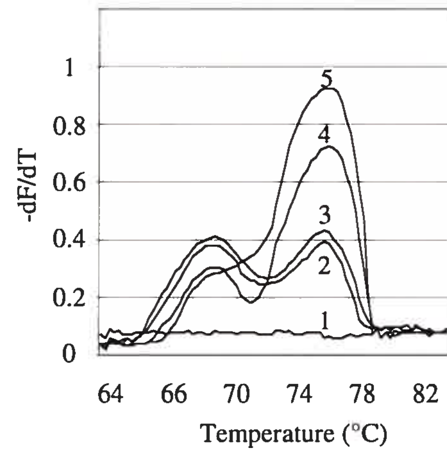

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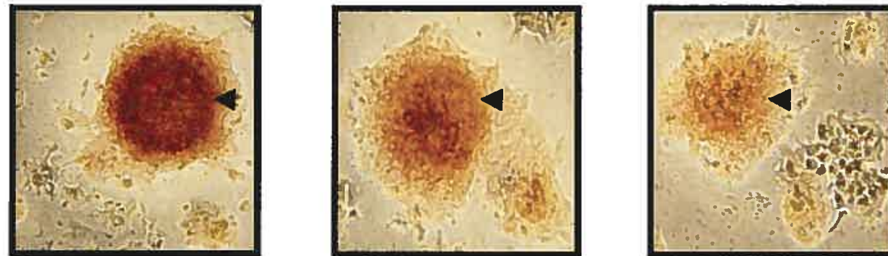
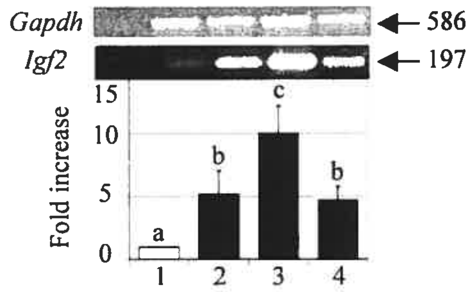


FIG. 2. Effects of ES cell serum starvation on gene expression Igf2 (A), H19 (B), Gas6 (C) and Oct4 (D). ES cells were cultured for 2 days in the presence of 20% serum (group 1; controls, open bar), or of 0.5% serum for 2 (group 2) or 4 days (group 3). After 4 days of serum starvation, some cells were cultured for another 2 days with 20% serum (group 4). First blank lane on the gel is the negative control for possible PCR contamination (no cDNA). Data is presented as mean (SEM) fold increases relative to controls of triplicate experiments. Different superscripts denote significant differences among groups ($p < 0.05$).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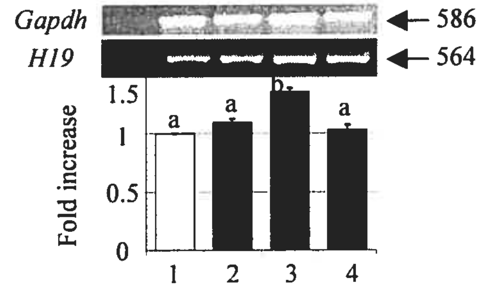
Gapdh
Igf2
586
197
Fold increase
15
10
5
0
a
b
c
b
1
2
3
4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Gapdh
H19
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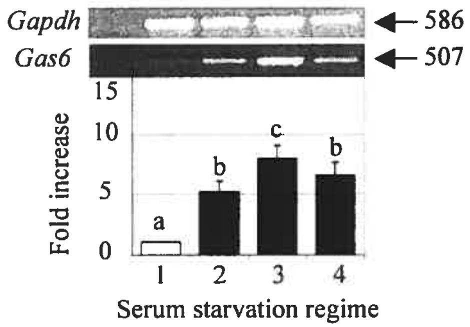
564
Fold increase
1.5
1
0.5
0
a
a
b
a
1
2
3
4


C
Gapdh
Gas6
586
507
Fold increase
15
10
5
0
a
b
c
b
1
2
3
4
Serum starvation regime

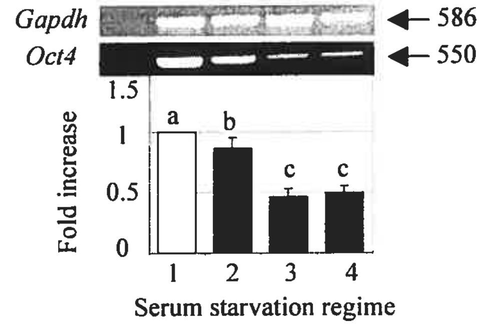
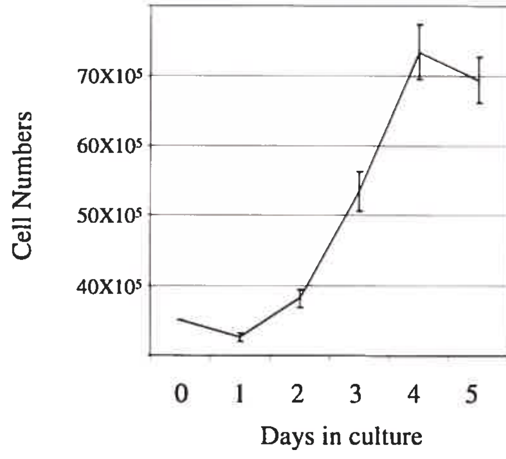
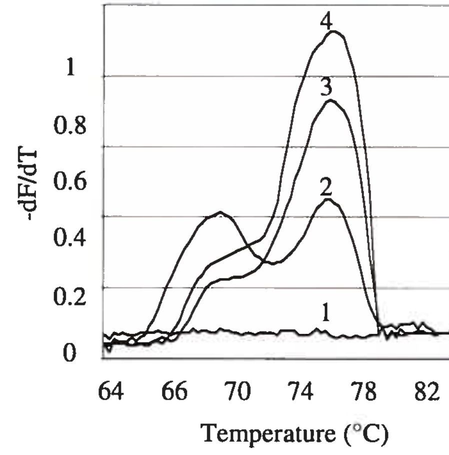

D
Gapdh
Oct4
586
550
Fold increase
1.5
1
0.5
0
a
b
c
c
1
2
3
4
Serum starvation regime

FIG. 3. Effects of cell confluence on mouse ES cultures. (A) Cell number at progressive days of culture after seeding. (B) Staining with alkaline phosphatase (AP) of day-3 semi-confluent (left) or day-5 fully confluent ES culture (right). Arrowhead indicates AP-positive colony. (C) Melting peak analysis showing negative control (line 1), control non-confluent ES cells at 2 days after seeding (line 2), fully confluent culture at 4 days post seeding (line 3), and highly methylated DNA from fetal liver (line 4, positive control).

A



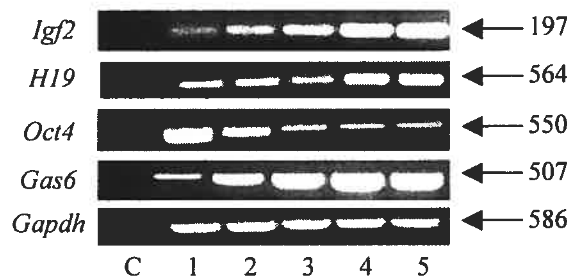
C



B

FIG. 4. Effects of ES cell confluence on the gene expression. (A) PCR products showing Igf2, H19, Oct4, Gas6 and Gapdh transcripts at progressive days in culture. First blank lane on the gel is the negative control (no cDNA). (B) Densitometric analysis of gene expression at different days of culture. The data is presented as means (± SEM) of triplicate experiments. Expression was significantly altered for all genes at increased cell densities during progressive days in culture ($p < 0.001$).

A



B

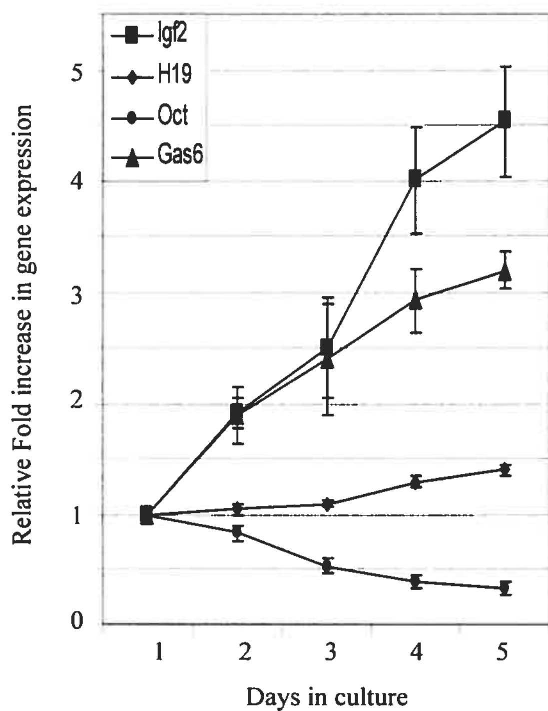
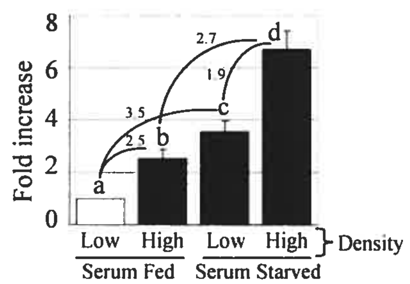


FIG. 5. Combined effects of ES cell density and serum starvation on Igf2 (A) and H19 (B) expression. Densitometric values showing Igf2 and H19 expression levels standardized with the corresponding value of Gapdh and expressed as arbitrary units. The data is presented as means of triplicate experiments. Connecting lines and corresponding values indicate comparative fold differences among groups.

A



B

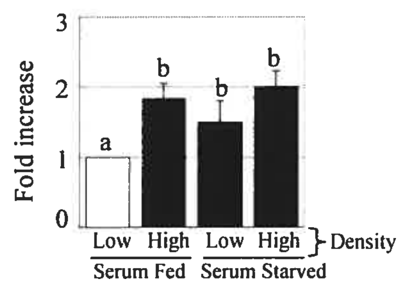
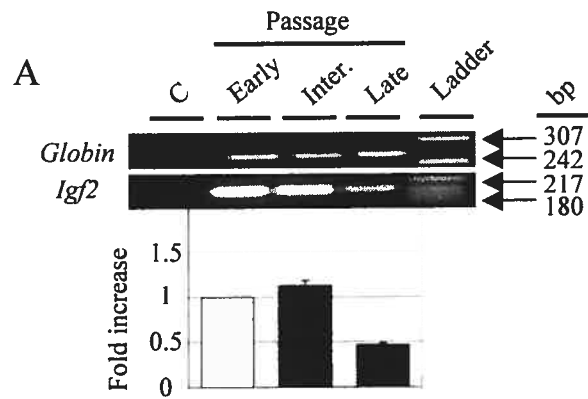
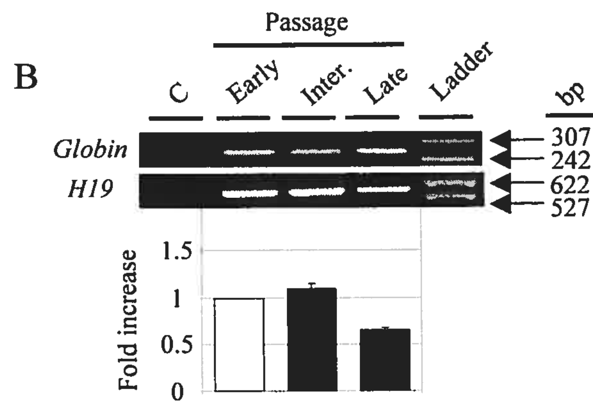


FIG. 6. Effect of ES passage number (in vitro aging) on gene expression. ES cells were harvested at passage 8 (early), passage 22 (intermediate) and passage 36 (late) on the expression of Igf2 (A) and H19 (B). First blank lane on the gel is the negative control (C, no cDNA). Each passage was standardized with an exogenous RNA control (globin) and normalized relative to the expression of early passage (open bar, 1 fold). The data is presented as means (SEM) of triplicate experiments. Different superscripts denote significant gene expression differences among the groups ($p < 0.05$).


A
Passage
C
Early
Inter.
Late
Ladder
bp
Globin
Igf2
307
242
217
180
Fold increase
1.5
1
0.5
0


B
Passage
C
Early
Inter.
Late
Ladder
bp
Globin
H19
307
242
622
527
Fold increase
1.5
1
0.5
0

4. Articles

4.2. Article two

Acetylation and Methylation Alter Imprinted Gene Regulation in Embryonic Stem Cells

Acetylation and Methylation Alter Imprinted Gene Regulation in Embryonic Stem Cells

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Key words: gene regulation, ES cells, imprinting, histone acetylation, DNA methylation

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ABSTRACT

Histone acetylation and DNA methylation are important in regulating the expression of imprinted genes in fetal and adult somatic tissues. To better understand the role of these epigenetic mechanisms in controlling the expression of imprinted genes during embryogenesis, embryonic stem cells were exposed to inhibitors of DNA methyltransferases (5AzaC) and/or histone deacetylases (TSA) and analyzed for gene expression. Most imprinted genes were overexpressed following exposure to 5AzaC or TSA alone and responded in either an additive or synergistic manner when exposed to both drugs together. Interestingly, Igf2 expression was not affected by 5AzaC but increased after exposure to TSA alone and in conjunction with 5AzaC. Moreover, the expression of several imprinted genes remained high or, in some cases, increased only several hours after drug removal. Together, our results suggest that DNA methylation and histone acetylation play jointly an important epigenetic role in governing imprinted gene expression in embryonic cells.

INTRODUCTION

Embryonic stem (ES) cells are currently under close scrutiny due to their potential use as a source of tissue in regenerative medicine, including the controversial application in therapeutic cloning. However, the in vitro culture environment used to establish and maintain ES cell lines can cause alterations to the epigenetic profile of these cells, leading to the deregulation of developmentally important genes, particularly imprinted genes. Genomic imprinting is a unique epigenetic phenomenon whereby the expression of a certain number of genes is expressed exclusively either from the paternal or maternal allele. Although the epigenetic program that ensures proper expression of imprinted genes is not known, accumulated evidence suggests that the monoallelic expression of imprinted genes falls under the control of DNA methylation of CpG sequences found in key regulatory sites, called differentially methylated regions (DMRs) (Bird and Wolffe, 1999; Feil et al., 1994; Li et al., 1993; Plass et al., 1996; Stoger et al., 1993). Aberrant methylation profile of these regions is often correlated with deregulation of imprinted gene expression leading to fetal abnormalities and growth disorders (Dean et al., 1998). Recent studies have suggested a mechanistic link between DNA methylation and histone deacetylation that might contribute to genomic silencing (Fuks et al., 2000; Robertson et al., 2000; Wade et al., 1999). Moreover, we and others have shown recently that inhibitors of DNA methylation (5AzaC) and histone deacetylases (TSA) can modulate the expression of imprinted genes in preimplantation stage mouse embryos (Baqir and Smith, 2001), and in somatic cells of mice (El Kharroubi et al., 2001; Grandjean et al., 2001; Pedone et al., 1999) and humans (Hu et al., 1998). Due to their origin from the

inner-cell-mass of the blastocyst, ES cells differ from somatic cells in DNA methylation levels and the enzymatic activity of methyltransferases (Lei et al., 1996; Stewart et al., 1982). Moreover, unlike what is seen in somatic cells, MeCP2, a methyl cytosine binding protein associated with histone deacetylases, has no effect on the survivability of ES cells (Tate et al., 1996), thus further suggesting that ES cells have a unique epigenetic profile and enzymatic machinery that differs from fetal or adult somatic cells.

Because so little is known about the effects of DNA methylation and histone acetylation in embryonic cells, we undertook studies to analyze the expression pattern of several imprinted genes in mouse ES cells following treatment with either 5-Aza-Cytidine (5AzaC; DNA demethylation agent) and/or Trichostatin A (TSA; inhibitor of histone deacetylase). Moreover, since it has been reported that inhibitors of histone deacetylase cause DNA demethylation in several organisms (Cervoni and Szyf, 2001; Hu et al., 2000; Selker, 1998; Szyf et al., 1985), we have also investigated the methylation status of a crucial differentially methylated region (DMR2) of the Igf2 gene in ES cells treated with TSA and 5AzaC.

METHODS

Embryonic stem cell cultures and treatments

Undifferentiated ES cells (line H106 with a C57Bl/6 x C3H genetic background) at passage 17 were cultured in DMEM medium supplemented with 20% v/v heat inactivated fetal bovine serum, 10^3 U/ml of leukemia inhibitory factor (LIF ESGRO), 1%

v/v of nucleosides, L-glutamine, non-essential amino acids (all from GibcoBRL; Burlington, ON) and 0.1 mM β-mercaptoethanol (Sigma, St. Louis, MO). Exponentially growing cells were cultured in ES medium supplemented with either 0.05 μM 5 Aza-Cytidine (5AzaC, Sigma), a specific inhibitor of DNA methylation, or 100 nM of trichostatin A (TSA, Sigma), an inhibitor of histone deacetylases, for 24 h. Dosages were established in pilot experiments, which indicated that higher concentrations resulted in the immediate death of ES cells. Similar doses have been used previously with ES cells (Juttermann et al., 1994) and preimplantation stage embryos (Baqir and Smith, 2001). Alkaline phosphatase (AP) activity, a marker for undifferentiated ES cells, was measured using standard techniques (Buehr and McLaren, 1993). Briefly, cells were washed with PBS prior to fixation with 4% paraformaldehyde for 30 min at room temperature, AP staining was carried out for 45 min at room temperature in dark conditions

RNA Preparation and RT-PCR analysis

Total RNA was isolated with TRIzol reagent (GibcoBRL) as described in the manufacturers protocol. Three micrograms of total RNA was reverse transcribed in a total volume of 20 μl reaction mix containing 1X RT buffer, 0.5 μg oligo (dT), 3 mM $MgCl_2$, 1 mM dNTPs, 10 mM DTT, 1U/μl RNase inhibitor (Rnasin; Promega, Madison, WI) and 10 U reverse transcriptase. Samples were incubated at 42°C for 60 min, 50°C for 5 min and 72°C for 10 min. The PCR amplification mixture contained 10 μl of cDNA, 0.4 μM primers, 1 U of Taq polymerase, 200 μM of each dNTP, 10 mM Tris-HCl (pH 8.3), 50 mM KCl and 3 mM $MgCl_2$. Amplification cycles (26 to 32) consisted of

denaturation at 95°C for 45 sec, annealing at 56°C for 45 sec and extension at 72°C for 1 min. Specific primers were obtained for Igf2 (Eversole-Cire et al., 1993), H19 (Eversole-Cire et al., 1995), Igf2r (Morgan et al., 1987), Peg1 (Beechey, 2000), p57 (Hatada and Mukai, 1995), Gas6 (Fleming et al., 1997), Gapdh (Davis et al., 1996), Globin (Bird, 1986) and Oct4 (forward: 5'-CGAGGAGTCCCAGG ACATGAAA-3'and reverse: 5'-TGGGGGCAGAGGAAAGGATACA -3'). Band intensities were quantified by densitometric scanning (FotoDyne) and corrected with an exogenous standard (rabbit globin RNA, Lifetechnologies) by using Collage 3.0 software system.

DNA bisulfite modification and melting peaks analysis

Genomic DNA was extracted with TRIzol reagent as described in the manufacturers protocol and digested with restriction enzymes (*BamH* I and *EcoN* I) that cuts outside the desired region (Weber et al., 2001). Sodium bisulfite modification was performed using the CpGenome DNA modification kit (Intergen, Hornby, ON). A final step of DNA recovery and purification was carried out with Geneclean II kit (Bio101, Carlsbad, CA) then eluted in 25 µl sterile H_2O. Primers and melting maps were created using the MELT94 software (http://web.mit.edu/osp/www/melt.html) and according to previous reports (Sheffield et al., 1989; Worm et al., 2001). The primers were designed to amplify a 208 bp product in a region containing 14 CpG island. This region is located on DMR2 of Igf2 (U71085), which is known to be methylated on the paternal allele (Brandeis et al., 1993; Feil et al., 1994; Weber et al., 2001). As a positive control for high methylation of the paternal allele, liver DNA extracts of d15 fetuses were used. The

primers were attached to a GC clamps as indicated: [5′-(CGGGCGGGGG)CCAATATAACACCTAAAAACAATC-3′] and [5′-(CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG)TTTGATTTATTGATGGTTGTTGGA-3′]. Melting profiles were generated on a LightCycler™ apparatus (Roche diagnostics, Laval, QC). Prior to PCR amplification, 3-5 µl of bisulfite treated DNA was mixed with 0.5 µl TaqStart antibody (Clontech, Palo Alto, CA) for 5 min and added to the PCR master mix containing: 5 µl SYBR green I mix (Roche diagnostics), 0.5 µM primers (Gibco BRL) and 3 mM $MgCl_2$ in a total 20 µl volume. The amplification program consisted of an denaturation step of 10 min at 95°C, followed by 38 cycles of denaturation for 0 sec at 95°C, annealing for 5 sec at 56°C and extension for 20 sec at 72°C. Fluorescence readings were measured between 62°C and 98°C at a rate of 0.05 °C/S. The experiment was repeated for three times, all of which gave similar results. Melting curves were obtained using the LightCycler™ software, version 5.28.

Normalization and statistics

Normalization of gene expression was carried out by dividing the densitometry value of specific genes with that of the correspondent exogenous standard. All values were corrected relative to the control, which was set as 1 fold. Experiments were performed three times. Data were analyzed by ANOVA and mean gene expression values were compared using the Tukey-Kramer test in which differences at $P \leq 0.05$ were considered significant.

RESULTS

Effect of 5AzaC and TSA on cellular differentiation

The general morphology and alkaline phosphatase (AP) staining of ES cell cultures were examined to determine whether 5AzaC and TSA treatments caused cellular differentiation. Although 5AzaC and TSA caused partial differentiation of ES cells and the appearance of some morphologically irregular colonies, this did not fully eliminate the presence of some undifferentiated cells within treated ES colonies. Indeed, compared to control non-treated cells (Fig. 1 b), a substantial percentage of colonies (~60%) exhibited AP activity following 24 h treatment with 5AzaC and TSA (Fig. 1 c and d, respectively), indicating that exposure to both drugs caused only partial differentiation of ES cells. However, exposure to either drug for 72 h caused the complete loss of AP staining (Fig. 1 e), indicating the disappearance of undifferentiated cells from the ES colonies.

Effect of 5AzaC on gene expression

To examine the effect of DNA demethylation on gene expression, ES cells were cultured for 24 h in the presence or absence (controls) of 5AzaC and analyzed either immediately or, to test the reversibility of the treatment, followed by culture in the drug-free medium for another 24 h or passaged and then cultured for an additional 48 h (Fig. 2). Regardless of the parental origin of the imprint, the expression patterns following

5AzaC treatment varied considerably among different genes. In contrast to controls, Peg1 was overexpressed in response to 5AzaC treatment (1.8 fold) and neither drug removal nor passaging followed by culture altered significantly the profile of treated cells, indicating that the 5AzaC-induced changes to this paternally expressed gene were not easily reversed. On the other hand, Igf2, another paternally expressed gene, was not affected by 5AzaC exposure, indicating that it is not affected by changes in global DNA methylation patterns. As with Peg1, the maternally expressed gene p57 was significantly upregulated (1.7 fold) immediately after exposure to 5AzaC and after 24 h in its absence (1.8 fold). However, in contrast to Peg1, p57 expression was further increased (2.2 fold) following passaging and 48 h culture without 5AzaC, indicating a long-lasting and continuous effect on overexpression. As with Igf2, no differences in H19 expression was detected between 5AzaC-treated and non-treated ES cells. Interestingly, 5AzaC-induced alterations to Igf2r expression were different to those observed with other imprinted genes. Although no effect was observed immediately after exposure to 5AzaC, significant increases in expression were observed both after withdrawal for 24 h (1.6 fold) and after passaging and 48 h in the absence of the drug (1.5 fold), indicating a delayed effect on Igf2r expression in response to 5AzaC. Gapdh, a housekeeping gene known to be completely non-methylated, remained relatively constant in all groups following 5AzaC treatment. Moreover, the expression of Gas6, a member of the family of genes in which expression is associated with growth arrest (Ciccarelli et al., 1990; Fleming et al., 1998; Schneider et al., 1988) showed increased expression immediately (1.5 fold) and after 24 h withdrawal. However, passaging followed by 48 h withdrawal caused a downregulation of Gas6 expression, indicating that the demethylation treatment was reversible. Oct4, a

transcription factor associated with pluripotent and undifferentiated cells, was downregulated following treatment (0.5 fold), indicating that 5AzaC-treated ES cells undergo partial differentiation. These results are supported by ES cell morphology and AP staining, which showed partial loss of undifferentiated cells after 24 h exposure to 5AzaC (Fig. 1 c).

Effect of TSA on gene expression

Following the previous experimental design, ES cells were exposed to TSA to examine the effects of histone acetylation on gene expression of imprinted and non-imprinted genes (Fig. 3). Our data show that Peg1 expression increased (1.8 fold) in response to TSA treatment and remained stable after drug withdrawal and passaging, indicating a non-reversible effect of the drug even after continuous culture in the absence of TSA. Unlike 5AzaC, which had no effect on Igf2 expression, TSA treatment induced a significant upregulation of Igf2 transcripts (1.9 fold). Although Igf2 expression remained high after removal of the drug for 24 h culture, passaging followed by 48 h in TSA-free medium led to complete downregulation of Igf2, indicating a reversible overexpression effect of TSA. We next examined the effect of TSA treatment on the expression profile of maternally expressed genes. p57 expression was upregulated (1.8 fold) following TSA treatment and remained overexpressed for 24 h after drug withdrawal (1.7 fold). Furthermore, passaging of treated cells followed by another 48 h in drug-free medium caused a further increase in p57 transcripts (2.3 fold), indicating a long lasting upregulation effect of TSA. Moreover, although no alterations were observed in H19

expression immediately or after 24 h of drug withdrawal, passage and culture of TSA–treated cells for another 48 h led to a significant increase in H19 transcripts, indicating that perturbation of histone acetylation levels can lead to retarded effects on expression of some imprinted genes. Igf2r expression profiles resembled most previously analyzed imprinted genes whereby TSA treatment led to immediate overexpression, followed by a slight downregulation after 24 h of withdrawal. However, a renewed overexpressed Igf2r profile was observed after passaging and 48 h of withdrawal, indicating again a retarded effect of TSA treatment similar to p57 and H19. Unlike 5AzaC, which showed no effect on Gapdh expression, TSA treatment led to a slight increase in Gapdh transcripts immediately after treatment. However, Gapdh overexpression was completely reversed following drug withdrawal and passaging of TSA-treated ES cells. Similarly, Gas6 expression was upregulated immediately after TSA treatment but, in this case, drug withdrawal led to a gradual decrease at 24 h followed by reversal to basal levels after 48 h in culture, indicating that the epigenetic stress caused by TSA treatment was completely reversible. Although Oct4 was downregulated by TSA treatment (0.5 fold), expression patterns were partially reversed after passaging and culture for 48 h (0.8 fold). Taken together, these results suggest that with the exception of H19, histone deacetylase inhibitors cause a prominent immediate upregulation effect in the expression profile of imprinted genes. However, a retarded effect is also observed with some imprinted genes, i.e. H19, p57 and possibly Igf2r, which is characterized by a renewed increase in expression after several hours in the absence of TSA.

Combined effect of 5AzaC and TSA on the expression of imprinted genes

Because the previous experiments showed that 5AzaC and TSA treatments affect several imprinted genes, we wished to combine treatments with both drugs to determine whether DNA methylation and histone acetylation acted interactively in altering the expression levels of imprinted genes (Fig. 4). Our results indicate that, compared to exposure to a single drug, most imprinted genes responded by upregulation expression in an additive manner, i.e. fold increases of Peg1 (3.2 fold), P57 (3.1 fold) and Igf2r (2.5 fold) were equal to the sum of the individual effects of each drug alone. Similarly, the non-imprinted gene Gapdh (1.3 fold) was slightly upregulated and Oct4 was downregulated (0.2 fold) in an additive manner, indicating that non-imprinted genes can be indirectly controlled through epigenetic alterations. Moreover, Igf2 (3.1 fold) and H19 (1.5 fold) were affected in a synergistic manner, indicating an interrelationship between these two epigenetic processes. Similarly, the non imprinted gene Gas6 was upregulated (4.6 fold) in a synergistic manner when compared to each drug alone. With the exception of Gapdh, reciprocal exposure to each drug in a consecutive manner led to similar patterns of expression in all genes analyzed after simultaneous exposure to both drugs. These results indicate that, with the exception of housekeeping gene (Gapdh), imprinted and non-imprinted genes involved in differentiation (Oct4) and stress (Gas6) respond equally to reciprocal alterations to DNA methylation and histone deacetylation.

Melting peak analysis of DNA methylation

Having shown that inhibiting histone deacetylases with TSA induced the overexpression of several imprinted genes, including Igf2, we determined whether the effect was associated with alterations in the methylation profile of one of a series of several key regulatory sites of this gene. We chose to investigate a 14 CpG island located within a 208 bp fragment of DMR2 of the Igf2 gene using melting peak analysis (Fig. 5). Our data shows that, in contrast to the non-treated ES control group, 5AzaC resulted in a decrease in DNA methylation levels, which was manifested by a shift to a lower melting-temperature peak. Interestingly, no changes occurred in the methylation profile of ES cells treated with TSA, indicating that histone deacetylase inhibitors do not alter methylation patterns in DMR2. As expected for a differentiated somatic tissue, liver DNA (positive control) displayed high levels of methylation. Together, these results indicate that the methylation status of the DMR2 region examined herein is unrelated to the increase in Igf2 expression observed after exposure of ES cells to TSA.

DISCUSSION

Accumulated evidence suggests that the parental-specific silencing of expression depends in part on DNA methylation of CpG sequences found in control regions of imprinted genes (Bird and Wolffe, 1999; Li et al., 1993). However, it is evident that beside DNA methylation other mechanisms are involved in genomic repression pathways, such as those caused by histone deacetylases (HDs) in plants (Pazin and Kadonaga, 1997; Tian and Chen, 2001). In the present study, we demonstrate that artificial induction of DNA hypomethylation and histone hyperacetylation in ES cells is

associated with perturbation of the epigenetic program that controls imprinted gene expression. These results confirm previous findings in somatic cells showing that HDs are indeed involved in the regulatory pathway that governs the expression profile of imprinted genes (El Kharroubi et al., 2001; Hu et al., 1997; Pedone et al., 1999).

The current study shows that the expression pattern of imprinted genes in response to 5AzaC and TSA exposure is similar to our previous work with mouse preimplantation embryos (Baqir and Smith, 2001). This is not surprising since ES cells are derived from the pluripotent inner-cell-mass of the blastocyst, and presumably maintain the same epigenetic "marking" throughout the in vitro transition period required to establish an ES cell line. Our findings with Peg1, which was upregulated with 5AzaC and TSA treatment, contrasts with a recent report with mouse fetal fibroblast cells derived from a day-13 fetus (El Kharroubi et al., 2001). Although our results with 5AzaC agree with previous work with embryonic carcinoma cells (Nishita et al., 1999), the discrepancy between ES and somatic cells could be due to differences in the epigenetic programs of each cell type. Secondly, since Peg1/Mest gene expression is restricted to the mesodermal derivatives (Kaneko-Ishino et al., 1995; Sado, 1993), it is conceivable that the partial differentiation of ES cells in response to 5AzaC and TSA administration led to the appearance of mesodermal derivatives, thereby upregulating the expression of Peg1. The latter is supported by our results with Oct4 expression and AP staining which indicated that treatment with both drugs led to the partial differentiation of ES cell colonies. Furthermore, compared with previous reports with fetal fibroblast cells (El Kharroubi et al., 2001; Pedone et al., 1999), lower dosages and shorter exposure periods to 5AzaC and TSA, indicating that somatic cells are more resistant to changes in

methyltransferase and histone deacetylase activity. Moreover, somatic cells may require a longer exposure and/or multiple rounds of treatment to exert an effect on imprinted gene expression, as indicated from the stability of methylation profiles (Stein et al., 1982; Wigler et al., 1981).

Unlike the expression of Igf2 and p57 in fibroblast cells, which were reversibly downregulated after TSA withdrawal (El Kharroubi et al., 2001), ES cells failed to rapidly reverse the TSA-induced overexpression after culture in drug-free medium, suggesting that somatic cells have a limited *de novo* methylation capacity due to low Dnmt3a and Dnmt3b expression (Okano et al., 1998). While our results with H19, which failed to respond to TSA treatment, are in agreement with previous reports (El Kharroubi et al., 2001; Pedone et al., 1999; Yoshioka et al., 2001), others have shown that H19 is indeed downregulated (Grandjean et al., 2001) or conversely, overexpressed by TSA (Svensson et al., 1998). The fact that, unlike other imprinted genes, H19 was induced only by both drugs is consistent with earlier studies (Pedone et al., 1999), suggesting that the interplay between DNA methylation and histone deacetylation is more complex than expected and might involve multiple epigenetic mechanisms to ensure a correct profile of genomic repression. Moreover, this complexity is also manifested by the unexpected upregulation of H19 only after passaging and culturing treated cells for 48 h, despite a lack of response immediately and 24 h after TSA exposure. This delayed alteration to H19 expression raises new doubts on the normalcy of ES since heritable errors of the epigenetic program can be undetected in the short term and show-up only after several cell cycles.

Methylation-acetylation interactions have proven to be extremely complex. The identification of a group of specific methyl binding proteins capable of recruiting histone deacetylases via a transcriptional repression domain (TRD) clearly illustrates this fact (Cross et al., 1997; Hendrich and Bird, 1998; Jones et al., 1998; Nan et al., 1998). Indeed, it has been shown recently that the repression of H19 caused by the methylation of the imprinting control region (ICR) is associated with MeCP2 by forming a repression complex with deacetylase activity (Drewell et al., 2002). Moreover, DNA methyltransferase itself, the enzyme required to methylate genomic sequences, is actively associated with histone deacetylases (Fuks et al., 2000; Robertson et al., 2000; Rountree et al., 2000). Furthermore, adding to the complexity, recent studies have shown that the methyltransferase inhibitor 5Aza-dC induces hyperacetylation of hypermethylated regions (Saitoh and Wada, 2000; Takebayashi et al., 2001). Together, these reports suggest that many aspects of the epigenetic mechanisms involved in controlling imprinted gene expression remain largely unknown.

In contrast to previous work showing that DNA hypomethylation (Cervoni and Szyf, 2001; Selker, 1998; Szyf et al., 1985) and hypermethylation (Boffa et al., 1994) can be attributed to the use of histone deacetylase inhibitors, TSA treatment in our study did not cause any visible change to the methylation status of a segment within DMR2 of the Igf2 gene. Similarly to our findings, others have demonstrated that TSA treatment causes no alterations in DNA methylation of key regulatory regions in imprinted genes, such as Snrpn, U2af1-rs1 (Gregory et al., 2002) and p57 (El Kharroubi et al., 2001), as well as non-imprinted genes silenced by methylation in cancerous cells (Cameron et al., 1999). Nonetheless, we cannot rule out the possibility that other CpG sites within this region or

perhaps elsewhere, such as DMR1 or the ICR, might be responsible for the overexpressed pattern of Igf2 in TSA-treated ES cells. Indeed, it has been shown that although TSA caused demethylation of some specific genomic sites, other regions remain resistant to such epigenetic modifications (Selker, 1998). Alternatively, while the importance of differentially methylated regions on genomic repression are well known (Bird and Wolffe, 1999; Feil et al., 1994; Li et al., 1993; Stoger et al., 1993), the methylation density and configuration of the repressive domain is shown to play a pivotal role in methylation silencing pathways (Boyes and Bird, 1992; Hsieh, 1994). In fact, it has been demonstrated that histone deacetylase-mediated repression is highly dependent on the density levels of methylated sequences (Lorincz et al., 2000), suggesting that dissimilarities in the concentration of methylated CpG islands could account for differences in how imprinted genes react to 5AzaC and TSA treatments. Moreover, our observation and that of others (Pedone et al., 1999) have shown that some imprinted genes, such as H19, are non-responsive to TSA and require 5AzaC treatment as a prerequisite for expression induction. It is noteworthy to mention that this observation was found in some hypermethylated endogenous genes (Cameron et al., 1999) which in turn suggests that DNA methylation and histone deacetylation can act synergistically to silence gene expression. This suggestion is in agreement with our data which shows that many imprinted genes were further up-regulated by simultaneous addition of 5AzaC and TSA.

Taken together, it is clear that modifications in histone acetylation are involved in regulating the epigenetic control of many imprinted genes in ES cells, possibly acting jointly with DNA methylation. Moreover, these results also show that minor alterations in

the epigenetic process can translate into immediate and/or delayed modifications in the expression patterns of imprinted genes. This highlights a need for caution when deriving or handling ES cells in vitro if they are to be used as nuclear donors for cloning or for therapeutic purposes.

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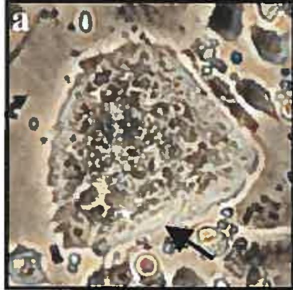
FIG. 1. Morphological and differentiation assessment by alkaline phosphatase (AP) staining. (a) Non-stained undifferentiated ES cell colony bordered by a smooth edge (arrow) and (b) non-differentiated ES colony following AP staining. Partially differentiated ES colony following AP staining after 24 h treatment with either 5AzaC (c) or TSA (d). Fully differentiated ES colony showing absence of AP staining after 72 h treatment with 5AzaC (e) and TSA (f). Arrowheads indicate positive AP staining. Note irregular borders of partially differentiated ES colonies (c, d), and differences in AP staining intensity between the control (b), 5AzaC (c) and TSA (d) treated cells.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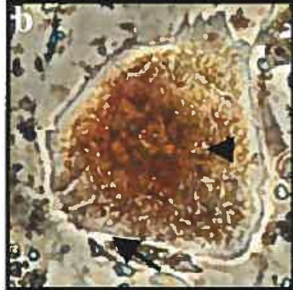
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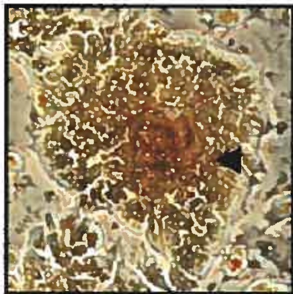
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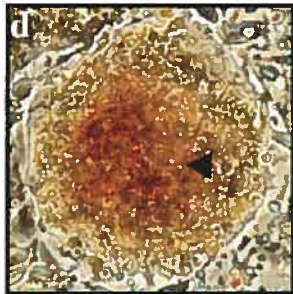
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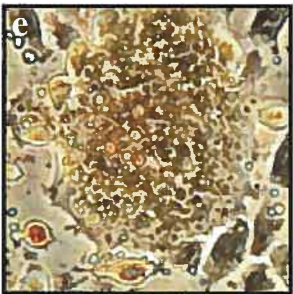
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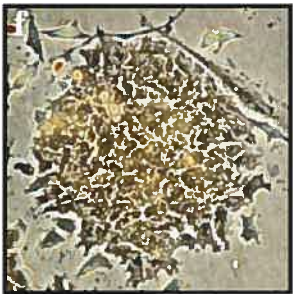
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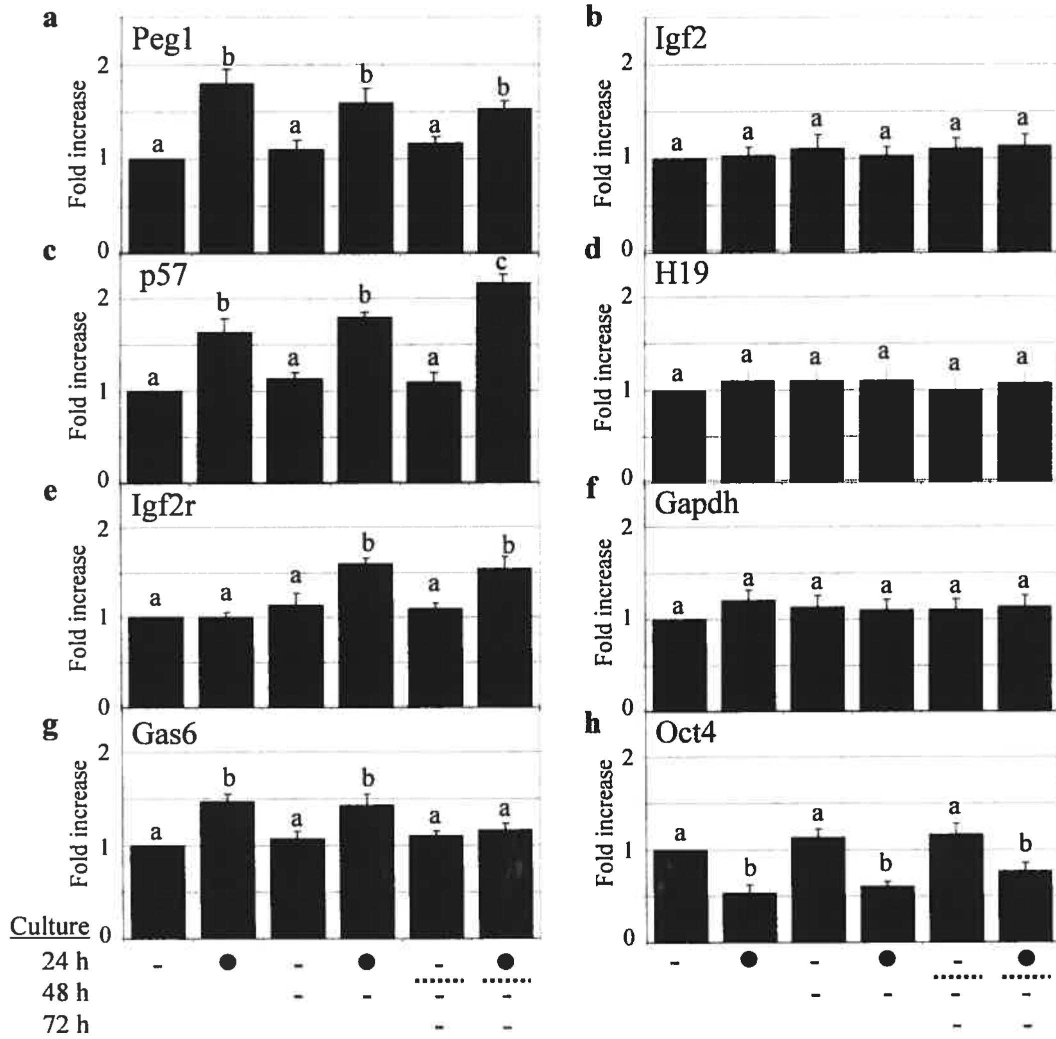
FIG. 2. Effect of 5AzaC on the expression profile of imprinted (a-e) and non-imprinted (f-h) genes in ES cells. ES cells were assessed after 24 h culture in the absence (minus) or presence (circle) of 5AzaC, followed by another 24 h in drug-free medium. A third treatment included passaging treated and non-treated ES cells at 24 h (dots) followed by 48 h of culture without 5AzaC. Expression levels are standardized with an exogenous RNA (globin) and normalized to non-treated controls. Different superscripts denote significant gene expression differences among groups ($p < 0.05$).



a
Peg1
b
Igf2
c
p57
d
H19
e
Igf2r
f
Gapdh
g
Gas6
h
Oct4
Fold increase
Culture
24 h
48 h
72 h

FIG. 3. Effect of TSA on the expression profile of imprinted (a-e) and non-imprinted (f-h) genes in ES cells. ES cells were assessed after 24 h culture in the absence (minus) or presence (circle) of TSA, followed by another 24 h in drug-free medium (gray bars). A third treatment included passaging treated and non-treated ES cells at 24 h (dots) followed by 48 h of culture without TSA. Expression levels are standardized with an exogenous RNA (globin) and normalized to non-treated controls. Different superscripts denote significant gene expression differences among groups ($p < 0.05$).

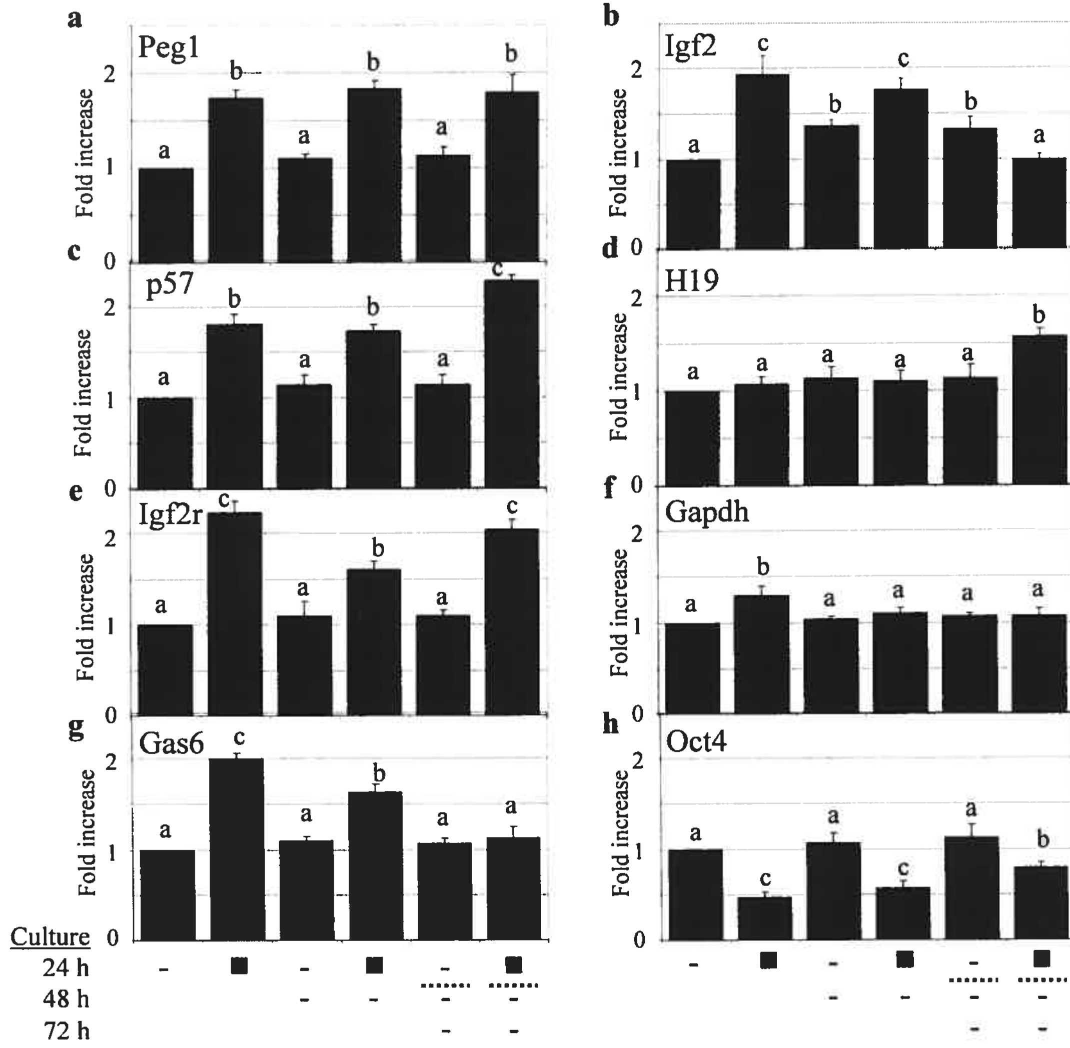
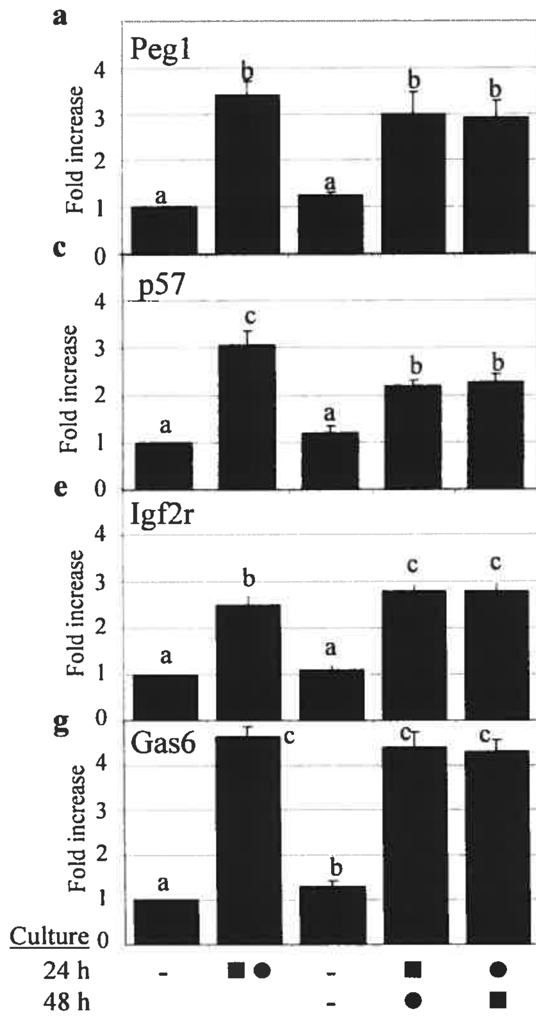

a
Peg1
b
Igf2
c
p57
d
H19
e
Igf2r
f
Gapdh
g
Gas6
h
Oct4
Fold increase
Culture
24 h
48 h
72 h

FIG. 4. Effect of combined and reciprocal addition of TSA (square) and 5AzaC (circle) or control medium (minus) on the expression pattern of imprinted (a-e) and non-imprinted genes (f-h) in ES cells. Expression levels of each gene were standardized with the corresponding value of an exogenous RNA (globin) and normalized to the non-treated control group. Different superscripts denote significant gene expression differences among groups ($p < 0.05$).


a
Peg1
c
p57
e
Igf2r
g
Gas6
Fold increase
Culture
24 h
48 h

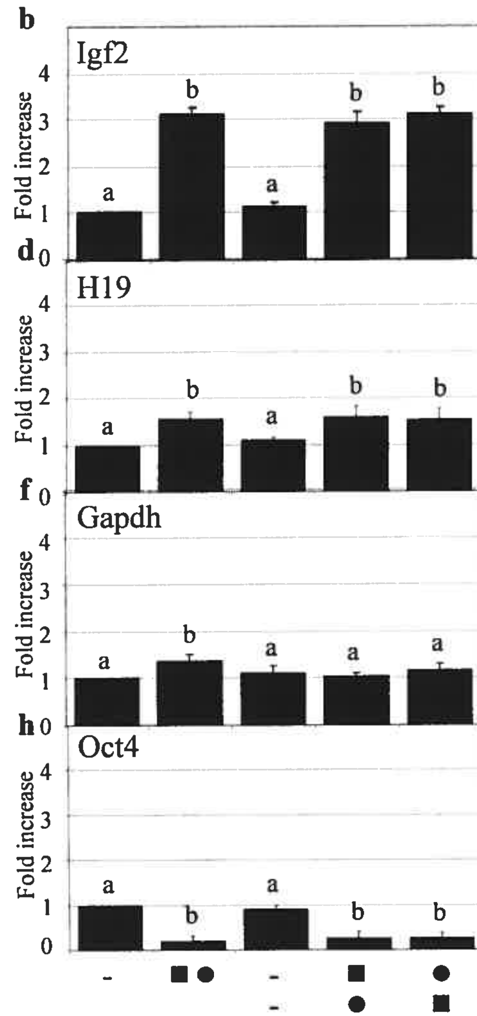
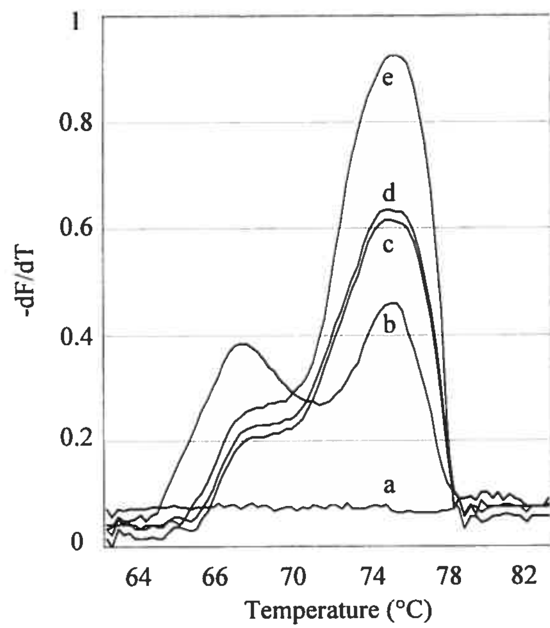

b
Igf2
d
H19
f
Gapdh
h
Oct4
Fold increase

FIG. 5. Melting peak analysis of the DMR2 of the Igf2 gene. Negative control (H_2O; line a), 5AzaC treated (line b), non-treated (line c), TSA-treated (line d) ES cells, and fetal liver cells (highly methylated control; line e). Peaks are presented as negative derivatives of the fluorescence with respect to temperature (-dF/dT).



1
0.8
0.6
0.4
0.2
0
-dF/dT
64
66
70
74
78
82
Temperature (°C)
a
b
c
d
e

4. Articles

4.3. Article three

Alteration of imprinted gene expression in preimplantation embryos exposed to inhibitors of histone deacetylases and DNA methyltransferases

Alteration of imprinted gene expression in preimplantation embryos exposed to inhibitors of histone deacetylases and DNA methyltransferases

Key words: Gene regulation, Early development, Imprinting, Acetylation, Methylation

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Abstract

Genomic imprinting is an epigenetic process responsible for silencing of expression of either the paternal or maternal allele whereby hypermethylation of densely populated CpG regions of genes has been shown to repress expression. Conversely, histone hyperacetylation is often associated with increased gene activity, as indicated by the use of inhibitors of histone deacetylases that are capable of altering the expression pattern of imprinted genes in cultured cells. Imprinted gene expression plays a major role in the initial differentiation in morula-stage embryos. Our objective was to characterize the relative expression patterns of several imprinted and non imprinted genes during preimplantation development after exposure to 5AzaC, an inhibitor of DNA methylation, and TSA, a histone deacetylase inhibitor. By the use of quantitative real time PCR analysis we demonstrate that the expression pattern of the imprinted genes Igf2, Peg1, $p57^{KIP2}$ and Igf2r was upregulated following TSA and 5AzaC treatment. Moreover, although Gapdh expression remained stable, Gas6 and Oct4 expression was altered by both treatments, suggesting exposure to stress and premature differentiation. Finally, although treatment with both inhibitors caused degeneration of several embryos, TSA treatment led to accelerated cell division and early differentiation of some embryos. These results indicate that histone acetylation is indeed involved in regulating the expression pattern of imprinted genes in preimplantation stage embryos and that it complements DNA methylation.

Introduction

Genomic imprinting is a unique epigenetic phenomenon whereby the expression of a gene is solely dependent on its parental origin (reviewed at WWW.mgu.har.mrc.ac.uk /imprinting/). Accumulated evidence suggests that the parental-specific silencing of expression depends in part on DNA methylation of CpG sequences found in control regions of imprinted genes, called differentially methylated regions (DMRs)(Bird and Wolffe, 1999; Li et al., 1993). However, the exclusive DMR control on the expression of imprinted genes has been challenged recently due to the fact that some DMRs are methylated on the expressed allele (Constancia et al., 1998), suggesting that factors other than methylation are involved in regulating imprinted genes. In contrast to the inhibitory effect of DNA methylation on transcription, high levels of histone acetylation are correlated with gene activation (Struhl, 1998), indicating that these epigenetic processes may be antagonistic. Recent reports in plants and fungi demonstrate that the replacement of 5AzaC, a demethylation agent (Creusot et al., 1982), with trichostatin A (TSA), a specific inhibitor of histone deacetylases (Chen et al., 1997; Yoshida et al., 1995), restores the expression of methylated genes (Chen et al., 1997; Selker, 1998), suggesting that histone deacetylases (HDACs) and methylation act together to silence transcription. Moreover, TSA treatment led to elevated expression of a methylated transgene in transfected cells (Eden et al., 1998; Pikaart et al., 1998) and, in cancerous cells, was able to reactivate methylation-repressed genes only after partial demethylation with 5AzaC (Cameron et al., 1999). On the other hand, 5AzaC treatment has been shown to increase the acetylation levels in the silent allele of SNRPN gene (Saitoh and Wada, 2000).

Additional evidence for the orchestration of methylation and histone deacetylation to inhibit transcription comes from studies showing that the heavily methylated inactive X chromosome and heterochromatic regions are depleted of the acetyl moiety on histone H3 and H4 (Jeppesen and Turner, 1993). Moreover, the interdependence between histone acetylation and DNA methylation was strengthened recently by the identification of specific methyl binding proteins (MeCP1 and MeCP2) that recruit histone deacetylases, providing a highly repressed complex (Jones et al., 1998; Nan et al., 1997). Further, it has been shown that DNA methyltransferase 1 is associated with HDAC activity (Fuks et al., 2000; Robertson et al., 2000; Rountree et al., 2000). Finally, it has recently been reported that imprinted gene expression can be modified by alterations in acetylation levels in several cell types, including human fibroblasts (Hu et al., 1998), mouse embryonic stem cells (Baqir and Smith, 2001), fetal and newborn fibroblasts cells (El Kharroubi et al., 2001; Pedone et al., 1999).

As both DNA methylation and MeCP2 are essential to ensure proper embryonic growth and development (Li et al., 1992; Tate et al., 1996), we decided to investigate the effect of TSA during preimplantation in mouse fertilized embryos and parthenogenetic embryos, which lack paternally expressed transcripts. The overall expression level of several imprinted genes (Igf2, H19, Peg1, Igf2r and $P57^{KIP2}$), known to be implicated in early embryonic growth was examined, along with other non-imprinted genes such as Gapdh, Oct4 and Gas6. Our results indicate that imprinted gene expression is altered by inhibiting HDACs during the preimplantation stages of embryogenesis, which suggests that DNA methylation and histone deacetylation are required to coordinate the silencing pathway to guarantee an optimum profile of imprinted gene expression.

Materials and Methods

Embryo recovery and culture medium

Unfertilized eggs and embryos were obtained from virgin C57BL/6 females (4-6 week) which had been mated to C3H/He males (Jackson Laboratories, Bar Harbor, ME). Superovulation was induced by two intraperitoneal injections 46 h apart of 5 IU pregnant mare's serum gonadotropin (PMSG) and 5 IU human chorionic gonadotropin (hCG). *In vivo* derived compact morula-stage embryos were recovered from the uteri of females at 72 h post hCG and used immediately for gene expression analysis.
Fertilized embryos for the *in vitro* control were recovered 20 h post hCG, cumulus cells were removed by a brief exposure to 300 IU/ml of hyaluronidase (Sigma, St. Louis, MO) and cultured for another 52 h to the early morula stage in CZB medium supplemented with 5mg/ml BSA (Sigma) in microdrops under mineral oil at 37°C in an atmosphere of 5% CO_2 in air. Parthenogenetic embryos were produced from unfertilized metaphase II stage oocytes recovered at 18-20 h after hCG administration, denuded from cumulus cells with hyaluronidase. Denuded oocytes were cultured for 3 h in CZB (Ca^{2+} free) (Chatot et al., 1989) containing 10 mM Sr^{2+} to induce activation and 5 μg/ml cytochalasin B for diplodization and cultured for another 52 h in CZB. Cell counting was conducted by mounting embryos on slides and stained with Hoechst 33342 (Pursel et al., 1985) using a fluorescent microscope (Zeiss) at 40X magnification.

5 Aza-Cytidine (5AzaC) and Trichostatin A (TSA) treatment

Embryos were exposed to CZB medium alone (*in vitro* control) or supplemented with 0.1 μM 5AzaC (Sigma) or 100 ng/ml TSA (Sigma) for 12 h. Dosages were established from previous experiments with ES cells (Baqir and Smith, 2001; Juttermann et al., 1994) and preliminarily tested with preimplantation stage embryos to determine the optimum concentration of TSA and 5AzaC. These experiments showed that treatments at concentrations higher than those described above resulted in either immediate death or the formation of blastocysts with an irregular phenotype after 36 h in culture.

RNA Preparation and RT-PCR analysis

Samples were obtained after washing the embryos 3 times with PBS, followed by the addition of 3 pg of rabbit globin RNA. Total RNA was extracted from a pool of 12 morula stage embryos (RNeasy, Qiagen, Mississauga, ON) as described in the manufacturer's protocol. The RNA was eluted from the membrane with 18 μl nuclease free distilled water and reverse transcribed in a total volume of 21 μl (Omniscript, Qiagen). The reaction mix was incubated at 42°C for 60 min, then terminated by 2 steps heating at 50°C for 5 min and 72°C for 10 min and the mixture was then rapidly placed on ice. Reverse transcription was performed in a thermal cycler (Hybaid, OmniGene) to ensure stable temperature. Samples were stored at -20°C until PCR analysis was conducted. Gene transcripts were quantified by real time quantitative PCR analysis on a lightCycler™ apparatus (Roche diagnostics, Laval, QC). Each PCR reaction was carried

out in a total volume of 20 µl containing: SYBR green I mix (Roche diagnostics), 0.5µM primers (Gibco BRL, Burlington, ON), 3 mM $MgCl_2$ and equivalent of one embryo cDNA template, all were added into glass capillaries, sealed, centrifuged briefly (1000 RPM for 10 sec) and placed on the rotor. The PCR primers for Igf2 amplified a 197 bp product (Eversole-Cire et al., 1993), 564 bp for H19 (Eversole-Cire et al., 1995), 186 bp for Igf2r (Morgan et al., 1987), 339 bp for Peg1 (Beechey, 2000), 222 bp for $p57^{KIP2}$ (Hatada and Mukai, 1995), 507 bp for Gas6 (Fleming et al., 1997), 586 bp for Gapdh (Eversole-Cire et al., 1995), 257 bp for Globin (Davis et al., 1996) and 550 bp for Oct4 (5'-CGAGGAGTCCCAGGAC ATGAAA-3') and (5'-TGGGGGCAGAGGAAAGGATACA -3'). The amplification program consisted of an initial denaturation step of 5 min at 95°C, followed by 35-40 cycles of denaturation for 0 sec at 95°C, annealing for 5 sec at 55-59°C (depending on the primer) and extension for 20 sec at 72°C. Amplification was followed by melting curve analysis which consisted of 0 sec at 95°C, 10 sec at 65°C then for 0 sec at 95°C with acquisition on the step mode. As a negative control for possible contamination, cDNA samples were replaced with autoclaved 0.1% DEPC (Sigma) treated water. No increase in fluorescence signal was observed in the absence of template. In addition to melting curve readings, products identity was confirmed by running on 3% ethidium bromide stained agarose gels (not shown), all amplification products were found to match the expected size.

Normalization and statistics

Quantification values were analyzed with lightCycler^{TM} software, version 5.28 (Roche Diagnostics). Normalization of each gene was carried out by dividing the value obtained with that of the corresponding globin exogenous standard (in the case of parthenogenetic embryos) and expressed relative to the value obtained from *in vivo* produced embryos. All experiments were performed for three times and mean gene expression values were compared with the Tukey-Kramer test. For developmental stage analysis and cell counts, differences among groups were evaluated by χ^2 test. Differences at $P \leq 0.05$ were considered significant.

Results

Gapdh, a housekeeping gene known to be non methylated in all stages of the developing embryo (Bird, 1986; Stein et al., 1983), and was used as a control for non imprinted genes. Gapdh expression remained relatively constant in all groups and no significant effect was detected following treatments that modify the level of histone acetylation (TSA) and DNA methylation (5AzaC) (Fig. 1; A). Although no differences were detected between *in vivo* vs. *in vitro* cultured embryos, growth arrest specific 6 (Gas6) expression (Fig. 1; B) was significantly elevated after drug administration (5.2 and 2.9 fold for TSA and 5AzaC, respectively), indicating that treatments had either a negative effect on growth or caused significant stress on embryos. Oct4 expression was downregulated (Fig.

1; C) by both treatments (0.5 fold for both TSA and 5AzaC), indicating that treated embryos may undergo premature differentiation compared to untreated embryos.

We next asked how these treatments would affect the expression profile of maternally expressed genes (Fig. 2). $p57^{KIP2}$ expression was upregulated following both treatments (2 and 1.9 fold), and slightly upregulated in *in vitro* controls (1.4 fold) (Fig. 2; A). Although no major changes occurred after TSA treatment when compared to both *in vitro* and *in vivo* control embryos, H19 expression increased significantly following 5AzaC administration (1.6 fold) (Fig. 2; B). Moreover, Igf2r expression was upregulated in the group of embryos cultured in the presence of TSA (1.5 fold) and 5AzaC (1.4 fold).

We then examined the expression pattern of paternally expressed genes (Igf2 and Peg1) under similar conditions (Fig. 3). While Igf2 was significantly overexpressed in the TSA group (2.8 fold), no difference was found in embryos treated with 5AzaC (1.2 fold) (Fig. 3; A). TSA and 5AzaC-treated embryos displayed significantly higher levels of Peg1 (5.5 and 4.5 fold, respectively) compared to *in vivo* or *in vitro* generated embryos (1 and 1.2 fold respectively).

Parthenogenetic embryos were used as a model to study the effect of inhibiting HDACs and DNA methylation on the expression of paternally repressed genes (Fig. 4). Igf2 expression was increased by TSA treatment while 5AzaC failed to alter significantly Igf2 expression (Fig. 4; A). Moreover, a positive Igf2 signal was detected from the control group of *in vitro* produced parthenogenetic embryos, indicating a biallelic pattern of expression at this stage of development. Furthermore, Peg1, a paternally expressed gene, was significantly elevated after TSA as well as 5AzaC treatment while virtually no

expression was observed in the *in vitro* produced parthenogenetic control embryos (Fig. 4; B).

During the first set of experiments designed to monitor gene expression we observed that a small percentage of TSA treated embryos developed beyond the late morula stage. Therefore, a final experiment was conducted to determine the developmental capacity of 8-cell compact embryos cultured in the presence of TSA and 5AzaC. As expected, most controls and treated embryos developed to the late morula stage after 12 h culture at which stage they had at least doubled the cell number (Table 1). Interestingly, after culture in TSA, close to one tenth of treated embryos developed to the blastocyst stage compared to very few of control and none of 5AzaC-treated embryos within the same window of time (12 h), indicating an accelerated differentiation. Moreover, nuclear counting of TSA-treated group showed an average of 30 cells per embryo compared to 20 in controls and 5-AzaC treated embryos. The apparent increase in the mean cell numbers of the TSA group and the presence of some embryos at the blastocyst stage confirms that TSA treatment accelerated embryonic cleavage rate as well as inducing differentiation. No such effect was observed in embryos cultured in 5AzaC, indicating that these agents acted differently on development. However, neither TSA nor 5AzaC improved the overall developmental competence since treatments were often associated with increasing number of degenerate embryos (50% and 42%, respectively), suggesting that both drugs were toxic to a certain proportion of embryos.

Discussion

Real time PCR analysis provides a powerful tool for accurately monitoring modifications in gene expression. We applied this technology to characterize the expression of several imprinted and non-imprinted genes in preimplantation stage embryos as well as parthenogenetic embryos exposed either to TSA, a specific inhibitor of HDACs (Yoshida et al., 1995), which causes global hyperacetylation of histones or to 5AzaC, an agent known to induce global demethylation (Creusot et al., 1982). In the present study, we demonstrate that artificial induction of hypomethylation and hyperacetylation during early embryogenesis is associated with perturbation of the epigenetic program that controls imprinted gene expression. These results confirm our previous findings (Baqir and Smith, 2001) and of others (Eversole-Cire et al., 1993; Hu et al., 1997; Nishita et al., 1999; Pedone et al., 1999) which show that methylation is indeed involved in the regulatory pathway that governs the expression profile of imprinted genes in embryonic and somatic cells. Moreover, the upregulation of gene expression reported here is consistent with previous observations that TSA leads to overexpression of silenced imprinted genes in numerous cell types, including human fibroblasts (Hu et al., 1998), mouse embryonic stem cells (Baqir and Smith, 2001), fetal and newborn fibroblast cells (El Kharroubi et al., 2001; Pedone et al., 1999). These results contrast with other reports in which Igf2 expression was not altered by inhibitors of histone deacetylases (Eversole-Cire et al., 1993; Grandjean et al., 2001). This discrepancy may be explained by inadequate dosage or the exposure period, which may have been insufficient to potentiate the induction of Igf2 expression (Grandjean et al., 2001). Moreover, sodium butyrate was

used to induce DNA hyperacetylation in the latter study (Eversole-Cire et al., 1993), which is known to not only inhibit HDACs (Kruh, 1982) but also affect other biological activities such as phosphorylation and nuclear proteins (Boffa et al., 1981; Boffa et al., 1994; Yoshida et al., 1995).

Interestingly, a certain percentage of embryos exposed for 12 h to TSA showed accelerated cleavage rate, as indicated by reaching the blastocyst stage earlier than non-treated controls, an effect also reported with bovine embryos cultured in the presence of TSA (Memili and First, 1999). Although it has been shown that cellular proliferation is reduced in parthenogenetic compared to fertilized mouse embryos (Uranga and Arechaga, 1997), it appears that parthenotes exposed to TSA develop at a rate similar to untreated fertilized embryos, suggesting that the activation of paternally repressed genes is related to developmental pace of parthenogenetic embryos. The presence of many degenerated embryos following culture in TSA and 5AzaC can be explained by the known toxic effects of these agents (Gregory et al., 2002; Juttermann et al., 1994). Although previous studies have used higher dosages and longer exposure periods (El Kharroubi et al., 2001; Takebayashi et al., 2001), it is worth noting that treatments with higher dosages and longer exposure than those used resulted in complete embryonic death, whereas lower dosages with shorter window treatments failed to exert substantial alterations in gene expression. However, it remains unknown why some embryos develop to the blastocyst stage more rapidly than expected at this time in development. One might speculate that developmental acceleration is caused, in part, by the deregulation of imprinted genes responsible for the cell cycle control, since Igf2 is a known mitogen (DeChiara et al., 1991; Efstratiadis, 1998) and $p57^{KIP2}$ and H19 have been shown to be

negative regulators of cellular proliferation (Hao et al., 1993; Matsuoka et al., 1995). Following this line of thought, our results with TSA treated embryos indicate that excessive Igf2 expression overrides the effect of H19, which showed less dramatic pattern of overexpression following TSA treatment. Recent studies have also shown that H19 expression remains stable following TSA treatment (Pedone et al., 1999; Yoshioka et al., 2001). Thus, it is conceivable that a profile of imprinted genes biased towards Igf2 might contribute to an increased pace of proliferation in the developing embryo. This suggestion is supported by recent studies showing the importance of balanced Igf2 and $p57^{KIP2}$ expression ratios in some tissues (Caspary et al., 1999; Grandjean et al., 2000). The extensive upregulation of Gas6 expression was surprising since the Gas family of genes has long been correlated with growth arrest in mammalian cells (Ciccarelli et al., 1990; Schneider et al., 1988). It could be argued that the overexpression of Gas6 occurred in response to environmental stress due to the cytotoxic effect of administered drugs, rather than cellular growth arrest. Alternatively, it is also plausible that Gas genes are more specifically induced in cells cultured in nutrient-depleted media, such as serum deprivation, leading to cellular quiescence (Ciccarelli et al., 1990; Schneider et al., 1988). These observations are strengthened by the fact that Gas6 was expressed in actively dividing *in vivo* as well as *in vitro* cultured embryos (Fleming et al., 1997). Moreover, previous reports have shown that Gas6 is involved positively in controlling cellular proliferation (Dormady et al., 2000; Li et al., 1996). The elevated pattern of Gas6 reported in this study was associated with down regulation of Oct4, which agrees with previous experiments showing that Gas6 was induced in differentiated cells (Fleming et al., 1998; Shugart et al., 1995). Furthermore, the decrease in Oct4 expression following

TSA and 5AzaC treatment indicates the presence of differentiated cells within the late morula stage embryo, which is not surprising since several studies have demonstrated that 5AzaC (Michalowsky and Jones, 1989; Santi et al., 1983) as well as TSA (Kouzarides, 1999; Yoshida et al., 1995; Yoshida et al., 1990) play a crucial role in the differentiation process of cells. The lack of difference in the expression of imprinted genes among *in vivo* and *in vitro* embryos cultured in CZB suggests that this medium is capable of maintaining a normal level of expression of imprinted and non-imprinted genes, and extends the work of previous experiments demonstrating that KSOM medium supported optimal levels of expression of imprinted (Ho et al., 1995) and non-imprinted genes (Gas6) (Fleming et al., 1997). The presence of Igf2 transcripts in parthenogenetic embryos is unlikely to be due to the effect of CZB medium, since the bi-allelic expression of Igf2 during preimplantation has been reported elsewhere (Rappolee et al., 1992; Szabo and Mann, 1995).

In summary, we have shown that with the exception of H19, it seems that there is a strong tendency for imprinted genes to be overexpressed by inhibiting HDACs. Additionally, the level of upregulation was dissimilar among the imprinted genes examined here, suggesting that the response of imprinted genes to TSA varies from one gene to another. Whether this is due to differences in their capacity to accumulate acetyl groups onto histones, or to differences in the interaction between HDACs and the methyl content of each gene at that particular stage remains to be investigated.

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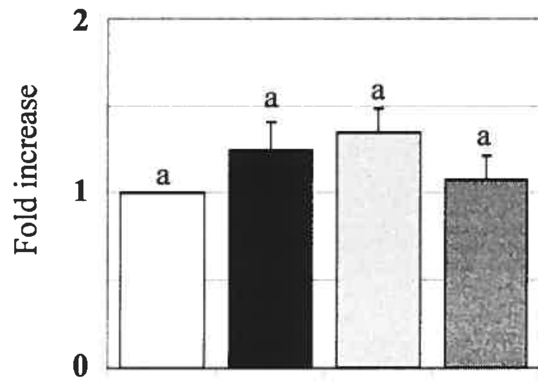
Figure 1. Expression profile of a group of non-imprinted genes, Gapdh (A), Gas6 (B) and Oct4 (C). *In vivo* produced embryos (open bars), *In vitro* cultured embryos (black bars), TSA treated embryos (light grey bars) and 5AzaC treated embryos (dark grey bars). Data are expressed as the mean ± SEM of triplicate experiments (n = 3). Different superscripts denote significant gene expression differences among treatments ($p < 0.05$).

A



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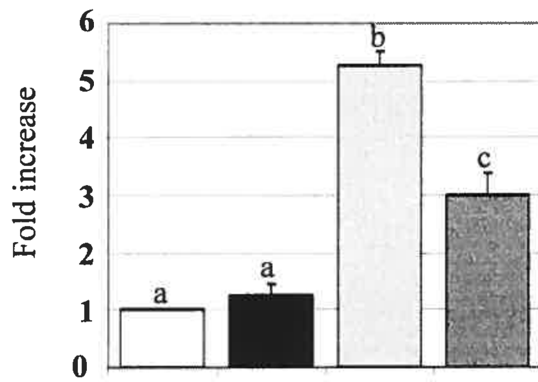

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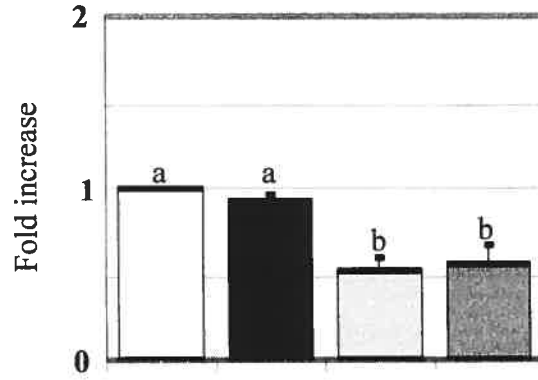
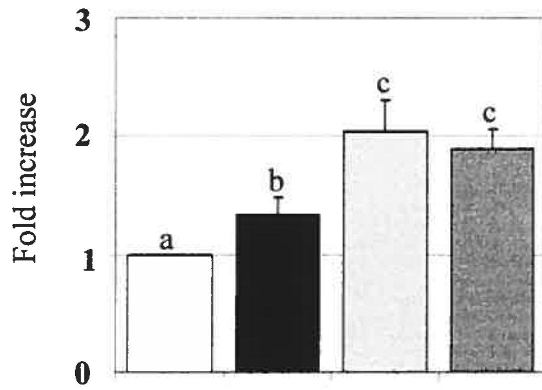


Figure 2. Changes in the expression of maternally expressed imprinted genes $p57^{KIP2}$ (A), H19 (B) and Igf2r (C) expression in preimplantation stage embryos. *In vivo* produced embryos (open bars), *in vitro* cultured embryos (black bars), TSA treated embryos (light grey bars) and 5AzaC treated embryos (dark grey bars). Data are expressed as the mean ± SEM of triplicate experiments (n = 3). Different superscripts denote significant gene expression differences among treatments ($p < 0.05$).

A



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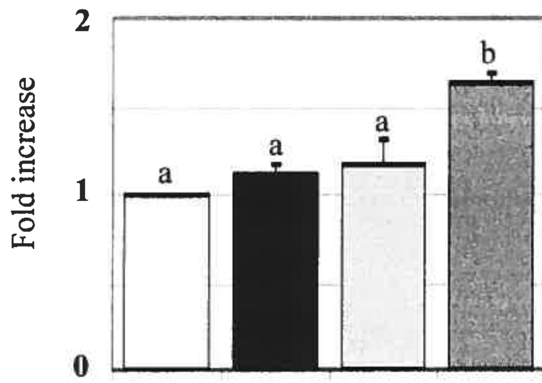

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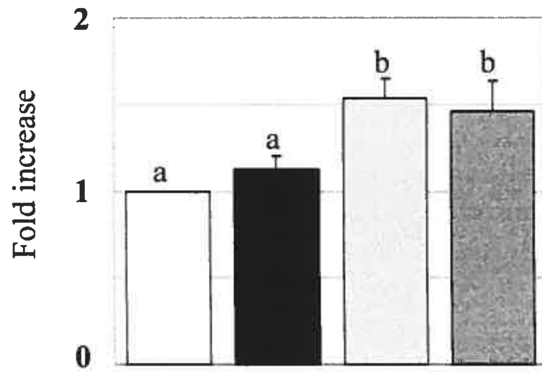
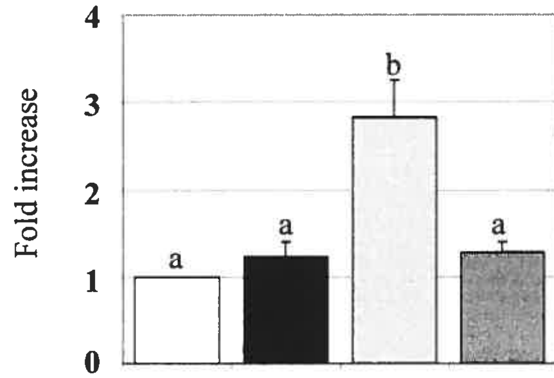


Figure 3. Expression profiles of the paternally expressed imprinted genes Igf2 (A), Peg1 (B). *In vivo* produced embryos (open bars), *in vitro* cultured embryos (black bars), TSA treated embryos (light grey bars) and 5AzaC treated embryos (dark grey bars). Data are expressed as the mean ± SEM of triplicate experiments (n = 3). Different superscripts denote significant gene expression differences among treatments ($p < 0.05$).

A



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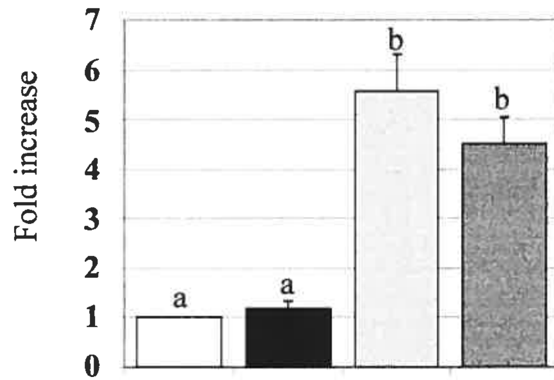
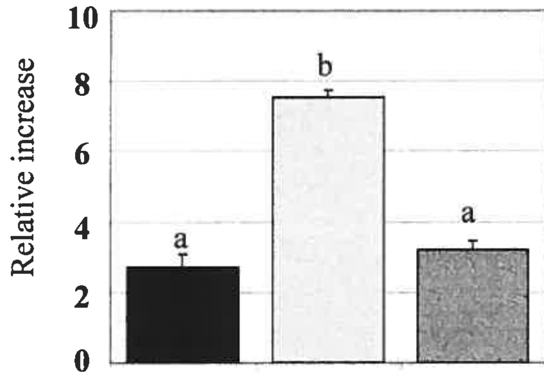


Figure 4. Expression pattern of Igf2 (A) and Peg1 (B) in parthenogenetic derived embryos. *In vitro* cultured embryos (black bars), TSA treated embryos (light grey bars) and 5AzaC treated embryos (dark grey bars). Data are expressed as the mean ± SEM of triplicate experiments (n = 3). Different superscripts denote significant gene expression differences among treatments ($p < 0.05$).

A



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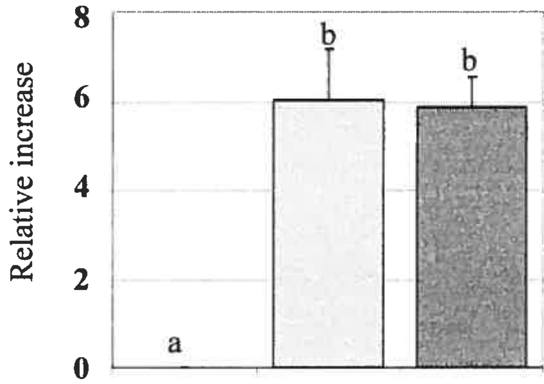

Table 1. Developmental stages and mean cell numbers of early morula stage embryos exposed for 12 h to TSA and 5AzaC.

		Stage of development			Number of cells		
Group	n	Degenerated (%)	Morula (%)	Blastocyst (%)	Morula	Blastocyst	Total
1- *In Vitro*	36	2 (6)[a]	33 (92)[a]	1 (2.8)	19.4 ± 3.4	38.0	21.0 ± 5.9[ab]
2- TSA	52	26 (50)[b]	21 (40)[b]	5 (9.6)	21.0 ±3.4	52.6 ± 13.4	30.0 ± 16.6[a]
3- 5AzaC	31	13 (42)[b]	18 (58)[b]	none	19.6 ±2.2	none	19.6 ±2.2[b]

Different superscripts denote significant differences among treatments ($p < 0.05$)

4. Articles

4.4. Article Four (In preparation)

Partial reprogramming of imprinted gene expression in cloned embryos reconstructed with embryonic stem and fetal fibroblast cells conditioned with 5AzaC and TSA

Partial reprogramming of imprinted gene expression in cloned embryos reconstructed with embryonic stem and fetal fibroblast cells conditioned with 5AzaC and TSA

Key words: embryo cloning, genomic imprinting, epigenetic reprogramming.

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Abstract

Embryo cloning is often associated with high mortality rate and growth abnormalities. Many of the phenotypic irregularities point towards improper epigenetic reprogramming of developmentally important genes, including those that are imprinted. Epigenetic deregulation of imprinted genes arises mainly from aberrant resetting of DNA methylation and/or histone acetylation profiles. Our objective was to investigate imprinted gene expression patterns in embryonic stem (ES) and fetal fibroblast (FF) donor cells preconditioned with 5AzaC, an inhibitor of DNA methylation, and TSA, an inhibitor of histone deacetylases. Subsequently, the ability of the host cytoplasm to reprogram the epigenetic alterations caused by both drugs was monitored by analyzing the developmental potential and gene expression of cloned embryos. Our data show that although pretreatment of both ES and FF donor cells was detrimental to development, some cloned embryos derived from TSA and 5AzaC treated donor cells reached the blastocyst stage earlier than controls, indicating that the epigenetic alterations had accelerated their developmental pace. Moreover, most imprinted genes from cloned embryos derived from treated donor cells showed abnormal patterns of expression, particularly in the advanced blastocyst group. Together, these results indicate that cloned embryos derived from embryonic and somatic cells show slight alterations in gene expression. However, epigenetic modifications to donor cells caused by TSA and 5AzaC before nuclear transfer are not reset during early development, suggesting that histone acetylation and DNA methylation are inadequately reprogrammed after nuclear transfer.

Introduction

Embryo cloning is often associated with miscarriages and growth abnormalities caused by placental defects, respiratory difficulties, fetal overgrowth and disproportionate enlargement of various internal organs (organomegaly) (Eggan et al., 2001; Tanaka et al., 2001; Wakayama et al., 1999; Young et al., 1998). While much of the work to improve cloning outcome has focused on rather empirical aspects of the nuclear donor cell, i.e. tissue origin, cell cycle and differentiation state, little has been done to epigenetically synchronize donor nuclei to the host cytoplasm to which it is transferred. Given the fact that perturbations to the epigenetic program of developmentally important genes, especially those that are imprinted, contribute to the defects observed in cloned animals (Dean et al., 1998; Humpherys et al., 2001; Kang et al., 2001), further justifies the need to investigate experimental procedures that induce epigenetic changes to nuclear donor cells. Epigenetic perturbations in cloned animals have been characterized mainly by deregulation in the expression of imprinted genes, aberrant methylation profiles and improper reprogramming of the imprint (Dean et al., 2001; Kang et al., 2001; Ohgane et al., 2001; Rideout et al., 2001). Although epigenetic errors are often exerted at late stages of development (Dean et al., 1998; Humpherys et al., 2001), failure to properly demethylate somatic nuclei during preimplantation stages (Dean et al., 2001; Kang et al., 2001) supports the notion that better development could be achieved by resetting the epigenetic program in the donor cell prior to nuclear transfer. Moreover, a complete erasure of the epigenetic memory of donor cells would facilitate the reprogramming process in the recipient oocyte by eliminating the need to repair preexisting epigenetic

errors brought in from abnormal culture conditions and improper handling of the donor cell.

Taken together, these observations prompted us to investigate the developmental capacity and imprinted gene expression pattern in preimplantation stage cloned embryos derived from donor cells preconditioned with inhibitors of DNA methylation and histone deacetylation. Moreover, because the epigenetic marking varies considerably between embryonic and somatic cells, we compared the developmental potential and expression patterns of cloned embryos derived from embryonic stem cells and fetal fibroblast cells.

Materials and methods

Donor cell culture, treatment and synchronization for nuclear transfer

Embryonic stem (ES) cells (R1 line) were cultured on a feeder layer of inactivated murine fibroblasts cells (mitomycin C-treated) in standard ES medium. Prior to nuclear transfer, ES cells were plated on gelatin-coated dishes with medium supplemented with 2000 U/ml leukemia inhibitory factor (LIF, ESGRO; Gibco). Fetal fibroblasts (FF) were obtained from mouse fetuses at day 13.5 of gestation and cultured in DMEM (Gibco). Treatment of donor cells consisted of exposure to 0.05 μM 5 Aza-Cytidine (5AzaC, Sigma), a specific inhibitor of DNA methylation or 100 nM trichostatin A (TSA, Sigma), an inhibitor of histone deacetylases (HDs), for 10-12 h. Metaphase synchronization of donor cells was carried out with 3 h incubation with 0.05 μg/ml demecolcin (Sigma).

Nuclear transfer, oocyte activation and embryo culture

Oocytes were collected from oviducts of eight-week old (C57Bl/6 x CBA/J) F1 females at 13h post hCG injection. Cumulus cells were removed with hyaluronidase and oocytes were cultured in M16 medium at 37.5°C under 5 % CO2 in air. Manipulations (Nikon-Narishige, MO-188) were performed under differential interference contrast (DIC equipped with Nikon Diaphot). Oocytes were placed in HEPES-buffered M16 containing 5µg/ml cytochalasin B for enucleation then returned to M16 medium until use. Donor nuclei were obtained from ES cells by gently aspirating in and out of the injection pipette and injected into the cytoplasts of enucleated oocytes as described previously (Zhou et al., 2001). After nuclear injection, reconstructed oocytes were activated in Ca^{2+} free M16 medium containing 10mM Sr^2 for 6 h and CB (5µg/ml). Embryos at 6 h post activation with visible nuclei were considered activated and transferred to fresh M16 medium and incubated in 37.5°C under 5% CO2 in air.

Reconstructed embryos that arrested development at 2- to 3-cell stage (28 h post activation) were fixed and stained with 15µg/ml Hoechst 33342 (Sigma) for 15 min at room temperature. Nuclear abnormalities were visualized with fluorescent microscope (Nikon) at 40X magnification.

RNA Preparation and RT-PCR analysis

Total RNA was extracted from a pool of either 6 or 12 cloned embryos (depending on the availability) with RNeasy kit (Gibco), followed by addition of 3 pg of rabbit globin (exogenous standard) and reverse transcribed in a total volume of 21 µl (Omniscript,

Qiagen, Canada). Gene transcripts were quantified by real time quantitative PCR analysis on a lightCycler apparatus (Roche Diagnostics, Canada). Each PCR reaction was carried out in a total volume of 20 µl containing SYBR green I mix, 0.5µM primers, 3 mM MgCl2 and equivalent of one embryo cDNA templatein sealed glass capillaries. PCR primers used for Igf2 amplified a 197 bp product (Eversole-Cire et al., 1993), 564 bp for H19 (Eversole-Cire et al., 1995), 186 bp for Igf2r (Morgan et al., 1987), 339 bp for Peg1(Beechey, 2000), 222 bp for p57KIP2 (Hatada and Mukai, 1995), 586 bp for Gapdh (Eversole-Cire et al., 1995), 507 bp for Gas6 (Fleming et al., 1997), 257 bp for Globin (Davis et al., 1996) and 550 bp for Oct4 (forward: 5'-CGAGGAGTCCCAGGAC ATGAAA-3' and reverse: 5'-TGGGGGCAGAGGAAAGGATACA -3'). The amplification program consisted of an initial denaturation step of 5 min at 95°C, followed by 35-40 cycles of denaturation for 0 sec at 95°C, annealing for 5 sec at 55-59°C (depending on the primer) and extension for 20 sec at 72°C. Amplification was followed by melting curve analysis which consisted of 0 sec at 95°C, 10 sec at 65°C then for 0 sec at 95°C with acquisition on the step mode.

Normalization and statistics

Quantification values were analyzed with lightCycler™ software, version 5.28 (Roche Diagnostics). Normalization of each gene was carried out by dividing the value obtained with that of the corresponding exogenous globin standard and expressed relative to the value obtained from *in vivo* produced embryos. Data were analyzed by ANOVA

and mean gene expression values were compared using the Tukey-Kramer test in which differences at $P < 0.05$ were considered significant.

Results

Developmental potential of cloned embryos derived from donor cells treated with TSA and 5AzaC

The developmental capacity of ES and FF cloned embryos derived from control and treated (TSA and 5AzaC) donor cells were examined by in vitro culture (Table 1). Our data show that no significant difference in the percentage of cleavage-stage (2-cell) embryos was observed between non-treated ES- and FF- derived cloned embryos at day-1 after nuclear transfer. However, morula and blastocyst formation was much higher in cloned embryos derived from ES cells compared to FF cells (85% and 32% respectively). Moreover, while significantly fewer cloned embryos derived from TSA and 5AzaC pretreated ES donor cells developed to the morula and blastocyst stage at day-3 of culture, no effect was observed in the developmental potential of cloned embryos from TSA and 5AzaC treated FF donor cells. These results indicate that, although nuclei derived from ES cells are better able to reset the developmental program required to support early embryogenesis after nuclear transfer, FF cells are less susceptible than ES cells to the epigenetic changes caused by TSA and 5Aza. Interestingly, while none of the control group embryos were at the blastocyst stage on the third day after nuclear transfer, a few of the cloned embryos derived from TSA and 5AzaC-treated donor cells had

reached the blastocyst stage by day-3, as confirmed by morphological assessment and total cell counts with an average of 56 cell/embryo, indicating that perturbation of the epigenetic program in donor cells by TSA and 5AzaC treatment leads to accelerated developmental pace of a small percentage of cloned embryos.

Because blastomere multinucleation and micronucleation has often been associated with poor developmental potential during preimplantation stages and reduced implantation rates (Alikani et al., 2000; Hardarson et al., 2001; Pelinck et al., 1998), we performed nuclear morphological examination of cloned embryos that were arrested at the 2- to 3-cell stage at day-2 after nuclear transfer (Table 2). The arrested 2-3 cell cloned embryos derived from ES donor cells revealed that the majority of these embryos contained normal nuclei (mononuclei) in the blastomeres (Fig. 1A). However, an increase in abnormal nuclei (multinuclei) (Fig. 1B & C) was observed in the blastomeres of cloned embryos derived from ES cells treated with TSA and 5AzaC (54% and 42% respectively). It is worth noting that multinuclei blastomeres included those of micronuclei morphology (Fig. 1D). These results indicate that cloned embryos derived from ES donor cells are less likely to form morphologically abnormal nuclei. However, treatment with drugs that alter DNA methylation and histone acetylation profiles, negatively effects nuclei configuration.

Although, arrested cloned embryos from FF donor cells displayed mononuclei morphology, high incidence of multinucleation was observed in the blastomeres of cloned embryos generated from FF donor cells treated with TSA (63%). Similarly, treatment of FF donor cells with 5AzaC increased the rate of multinucleation (68%).

In general, compared to cloned embryos derived from ES cells, blastomeres from 2-3 arrested FF cloned embryos displayed similar rate of multinucleation following treatment with TSA and 5AzaC. Together, these results suggest that ES and FF donor cells are susceptible to changes in mehtylation and acetylation profiles, which is manifested by the increased formation of morphologically abnormal nuclei.

Gene expression in donor nuclei

To examine the effect of DNA demethylation and histone acetylation on gene expression in ES and FF donor cells used in nuclear transfer, cells were analyzed after culture for 12 h in the presence of 5AzaC and TSA, respectively (Fig. 2). While Igf2, p57, Igf2r, Gapdh and Gas6 were significantly overexpressed (2.7, 2.6, 1.8, 1.2 and 2 fold, respectively) in ES cells treated with TSA, 5AzaC treatment led to the overexpression of p57, H19 and Gas6 (1.9, 1.7 and 1.6 fold, respectively). These results indicate that whereas some genes respond equally to both drugs (Oct4), others respond but at different levels to both epigenetic alterations (p57 and Gas6), and others will respond only to either TSA (Igf2 and Igf2r) or 5AzaC (H19).

Unlike ES cells, the expression of fewer genes was altered in FF cells after treatment with TSA and 5AzaC. TSA treatment led to the overexpression of p57, Igf2r and Gas6 (2.3, 1.4 and 1.6 fold, respectively) but was unable to alter the expression of Igf2. Similarly, but to a slightly lower extent than ES cells, 5AzaC-treated FF cells caused the overexpression of p57, H19 and Gas6 (1.6, 1.5 and 1.3 fold, respectively). Although both cell types responded with an over-regulatory pattern to TSA and 5AzaC

treatment, the differences in the level of overexpression indicates that ES cells are more prone to alterations in genomic methylation and acetylation levels than FF cells. Moreover, it appears that these epigenetic alterations differ not only in the intensity of their effects but also on the genes targeted by each drug.

Gene expression in cloned embryos

To assess the epigenetic reprogramming capabilities of embryonic and somatic nuclei in cloned embryos, the expression profiles of several imprinted and non-imprinted genes was examined in morula and blastocyst stage embryos derived either in vivo, in vitro or after nuclear transfer using non-treated (control) or TSA- and 5AzaC-treated donor ES and FF cells (Fig. 3). Compared to in vivo-derived controls, the imprinted gene expression profile of in vitro-derived day-3 morula-stage embryos was either equal or only slightly altered, indicating that our culture conditions had little effect on the epigenetic elements controlling their expression. Surprisingly, comparisons between in vitro-derived and cloned morula-stage embryos derived from both ES and FF untreated donor cells showed only minor differences in expression profiles, and in only a small sample of the genes analyzed. For instance, Igf2, p57, H19, Gapdh and Gas6 were all slightly up-regulated in both ES- and FF-derived clones. However, Peg1, Igf2r and Oct4 were down-regulated in FF-derived clones and remained rather unchanged in ES-derived clones, indicating a difference between the epigenetic reprogramming in cloned embryos derived from somatic and embryonic cells.

Gene expression reprogramming in preimplantation stage cloned embryos

The final experiment was conducted to monitor the reprogrammability of imprinted genes in cloned embryos of ES and FF cells that had previously been treated with TSA and 5AzaC (Fig. 3.). Our data show that although Igf2 expression was over expressed in ES cells in response to TSA treatment, cloned embryos derived from TSA-treated ES cells expressed Igf2 in day-3 morula and day-4 blastocysts at similar levels to those of in vitro produced embryos, suggesting that Igf2 expression was corrected during the few cell divisions after nuclear transfer. Interestingly, the advanced blastocysts showed increased Igf2 expression (3 fold), indicating that some cloned embryos are unable to reset Igf2 expression. Moreover, although 5AzaC had no effect on Igf2 expression in ES cells, advanced cloned blastocyst derived from 5AzaC-treated ES donor cells overexpressed Igf2 (2 fold), suggesting that undetected alterations in imprinted gene expression in donor cells may only become evident after nuclear transfer at a later developmental stage. Similarly, Peg1 was overexpressed in the advanced blastocyst group; although no changes in gene expression was detected in the donor ES cells in response to TSA and 5AzaC treatment. Carryover of p57 overexpression from the TSA treated ES cells to the morula stage cloned embryos was detected (2 fold). However, p57 expression was reprogrammed at the blastocyst stage (2.1 fold) compared to cloned blastocysts (1.9 fold). Moreover, the advanced blastocyst at day three exhibited very low levels of p57 expression (0.8 fold), suggesting that aberrant p57 expression is restricted to abnormally cleaving cloned embryos. H19 expression remained to be highly expressed in cloned embryos derived from ES cells treated with 5AzaC at the morula (1.8 fold) and blastocyst

stage (1.8 fold). Similar to p57, Igf2r overexpression in TSA treated ES cells were carried into cloned morula. However, Igf2r expression was corrected in the blastocyst stage (1.6 fold) compared to control cloned embryos (1.5 fold).

We then examined the expression pattern of non-imprinted genes (Gapdh, Gas6 and Oct4) under similar conditions. Although Gapdh was slightly overexpressed in TSA treated ES cells, cloned embryos displayed a carryover of Gapdh upregulation at the morula stage. However, Gapdh expression was corrected at the blastocyst stage (1.4 fold) compared to control cloned derived from non-treated ES cells (1.3 fold). Gas6 was overexpressed in morula and the blastocyst stage embryos cloned from ES cells treated with TSA and 5AzaC, indicating that both drugs had caused significant growth stress on embryos. Oct4 expression was corrected in cloned embryos derived from 5AzaC treated ES nuclear donor cells at the morula (0.8 fold) and the blastocyst (0.6 fold) stage. Interestingly, the advanced cloned blastocysts derived from ES treatment of both TSA and 5AzaC expressed Oct4 at a much reduced levels, confirming their status as blastocyst embryos and suggesting that perturbation of Oct4 expression is limited to a proportion of cloned embryos.

Similar to the previous experiment with ES donor cells, we analyzed the reprogrammability of imprinted genes in cloned embryos derived from FF donor cells. Our data show that although Igf2 was not affected in FF cells following TSA and 5AzaC treatment, cloned embryos aberrantly overexpressed Igf2 at the morula stage (1.7 fold) and the advanced blastocyst at day three (3 fold). No reprogramming of Igf2 occurred in cloned blastocyst from FF treated with TSA (2.9 fold). Moreover, while no difference was found in Peg1 expression between cloned FF embryos treated with TSA and control

cloned embryos, Peg1 was up-regulated in the advanced blastocyst. However, the expression level of Peg1 in the advanced blastocyst was similar to in vitro and in vivo produced embryos. The overexpressed pattern of p57 in TSA treated FF cells was corrected in the morula of cloned embryos (1.2 fold), however the advanced blastocyst under-expressed p57 (0.6 fold). Similarly, although 5AzaC up-regulated p57 in FF cells, cloned morula expressed p57 (1.3 fold) at similar levels to cloned control (1.2 fold). Carryover of H19 overexpression from the 5AzaC treated FF cells to the morula stage cloned embryos was detected (1.5 fold). Advanced cloned blastocyst at day three and normal cloned blastocyst at day four failed to reprogram H19 expression (1.8 and 1.9 fold respectively). Despite an increase in Igf2r expression in TSA treated FF cells, Igf2r was successfully reprogrammed in cloned morula and blastocyst embryos (1.1 and 1 fold respectively). However, advanced cloned blastocyst at day three failed to correct Igf2r expression (1.4 fold). Housekeeping gene Gapdh was not affected in treated FF cells and remained stable in cloned morula and blastocyst embryos. Although Gas6 was overexpressed in TSA and 5AzaC treated FF cells, cloned morula and blastocyst embryos successfully reprogrammed Gas6 expression, indicating that cloned embryos are under no developmental stress. Conversely however, advanced blastocyst at day three remained to express high levels of Gas6. While Oct4 was not expressed in FF cells, cloned morula derived from TSA treated FF cells had higher Oct4 expression than control non-treated FF cloned embryos (0.7 and 0.4 fold respectively). However, the increase in Oct4 expression in cloned embryos derived from TSA treated FF cell is less than in vitro produced embryos (0.7 and 1 fold respectively).

Discussion

Proper embryonic development requires that all genetic and epigenetic events that take place in the newly transferred nuclei be reprogrammed correctly by the oocyte to ensure normal development to term (Inoue et al., 2002; Kikyo and Wolffe, 2000; Solter, 2000). Reprogramming studies in cloned embryos and animals revealed correct X chromosome inactivation (Eggan et al., 2000), restoration of telomerase activity and telomere length (Betts et al., 2001; Lanza et al., 2000; Wakayama et al., 2000) and normal transcription levels of several developmentally important genes (Daniels et al., 2000). Nonetheless, other studies showed that cloned embryos aberrantly express imprinted genes and in many cases are associated with improper methylation profiles (Humpherys et al., 2001; Ohgane et al., 2001). Conversely, our data show that several imprinted and non-imprinted genes were correctly expressed in preimplantation cloned embryos derived from ES and FF donor cells. These results confirm recent findings showing that cloned embryos derived from other donor cells such cumulus and sertoli cells faithfully expressed imprinted genes (Inoue et al., 2002). This discrepancy might be explained by the fact that others investigated imprinted gene expression at postimplantation stages in various tissues (Humpherys et al., 2001) enabling perturbation of imprinted genes at postimplantation stages and perhaps coinciding with the wave of de novo methylation that takes place around implantation. We failed to detect aberrant expression in some imprinted genes following drug treatment of the donor cell prior to transfer. Nonetheless, a pattern of overexpression of these genes was manifested in preimplantation cloned embryos. The aberrant gene expression at later stages remains unknown, and may have

implications on the cloning technique. In fact this might be the reason that perturbation in the epigenetic program may accumulate during embryonic and fetal development to be visible only after climaxing at very late postimplantation stages.

Recent studies have demonstrated that high levels of DNA methylation were brought to morula and blastocyst cloned embryos from the somatic donor nuclei (Dean et al., 2001; Kang et al., 2001), these findings indicated that cloned embryos deficiently demethylated the chromatin at preimplantation stages of development. Therefore, it is conceivable that the reduction of methylation profiles in donor cells prior to transfer might be beneficial for the reprogramming of the imprints. Interestingly, our data show that, while imprinted genes (Igf2, peg1 and Igf2r) were not altered in donor cells treated with 5AzaC remained to be expressed faithfully at the morula and blastocyst stage, whereas the up-regulation of p57 and H19 expression in response to 5AzaC administration remained to be expressed at high levels at the morula and only be reset at the blastocyst stage. In fact it has been shown lately that the DNA in cloned embryos undergo premature de novo methylation as early as the 8 cell stage instead of around the time of implantation (Dean et al., 2001). It is unknown however, whether this early wave of de novo methylation facilitated the reprogramming of imprinted genes at preimplantation stages.

Compared to ES cell-cloned embryos, the low developmental rate to the blastocyst stage in embryos cloned from FF cells indicated that somatic cell nuclei lack appropriate epigenetic repair mechanisms. The further decrease in development in cloned embryos derived from FF donor cells treated with TSA and 5AzaC confirms this notion.

Moreover, although there was a reduction in the development of cloned embryos from ES donor cells treated with both drugs, this reduction in development was higher than those

observed in cloned embryos from FF cells. This indicates that cloned embryos from ES cells endure such treatments and may be capable of partially resetting the epigenetic program. In fact, this might explain the higher survival rate to term and adulthood of embryos cloned from ES cells compared to somatic cloned counterparts (Eggan et al., 2001; Rideout et al., 2000; Wakayama et al., 1999). Additionally, this notion is supported by the fact that many cloned embryos from ES cells survived to term although carrying improper imprinted gene expression (Humpherys et al., 2001). The better survivability of ES cell cloned embryos than those from somatic clones might be due to differences in the epigenetic programming in each cell type and evident by studies showing differences in the methylation profile (Frank et al., 1991; Shiota et al., 2002) and in the levels of DNA methyltransferase activity (Okano et al., 1998) between ES vs. somatic cells.

Our data with Oct4 expression are consistent with recent report showing that cloned embryos express inadequate levels of Oct4 transcripts (Boiani et al., 2002; Bortvin et al., 2003). However, we extend these findings by showing that TSA treatment of FF donor cell improved Oct4 expression at the morula stage. It remains to be investigated however, if the increase in Oct4 expression in cloned embryos from TSA treated FF donor cells will improve their postimplantation survivability.

Strikingly, our drug treatment of donor nuclei has led to the production of rapidly cleaving embryos, although they appear morphologically normal, imprinted gene expression in this group of cloned embryos were completely aberrant. It is more likely that these embryos will lose their developmental potential at postimplantation stages.

Results from the nuclear morphology assessment of cloned embryos showed that treatments of donor cells with TSA and 5AzaC led to the production of a significantly

higher number of multinuclei blastomeres in cloned embryos. It is more likely that dividing embryos may carry similar nuclear abnormalities, therefore, resulting in poor implantation rates. In fact, it has been shown that the presence of multinucleation and micronucleation has a negative impact on implantation rates and postimplantation viability of embryos (Hardarson et al., 2001; Jackson et al., 1998; Kligman et al., 1996; Pelinck et al., 1998). If this was the case, it might explain the high mortality rate around or shortly after implantation of cloned embryos.

The fact that even though clones are derived from the same donor cell population and transferred to the same pool of recipient oocytes are born with or without growth abnormalities, in addition to the existence of significant variation in the degree of methylation among individual cloned blastocysts (Humpherys et al., 2001; Kang et al., 2001; Ohgane et al., 2001), suggests that restoring the epigenetic program is an extremely complex procedure and might involve multiple regulatory mechanisms. However, what triggers the faulty epigenetic reprogramming in specific individual cloned embryos is unknown.

Based on our findings, we propose that ES cell cloned embryos are more efficient in resetting imprinted gene expression pattern at preimplantation stages than somatic cell clones counterparts. Although the reprogramming of imprinted genes is incomplete in cloned embryos derived from TSA and 5AzaC treated donor ES, it remains to be investigated whether such embryos can endure such insufficient reprogramming of the imprints and develop to term.

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Fig. 1. Nuclear transfer embryos arrested at the 2-3 cell stage, stained with Hoechst 33342. (A) Mononuclei 2 cell NT embryo, (B) Multinuclei 2 cell NT embryo, (C) Multinuclei 3 cell NT embryo, (D) Micronuclei 2 cell NT embryo. Polar body (arrow head). Micronuclei (arrow). Scale bar 100 μm.

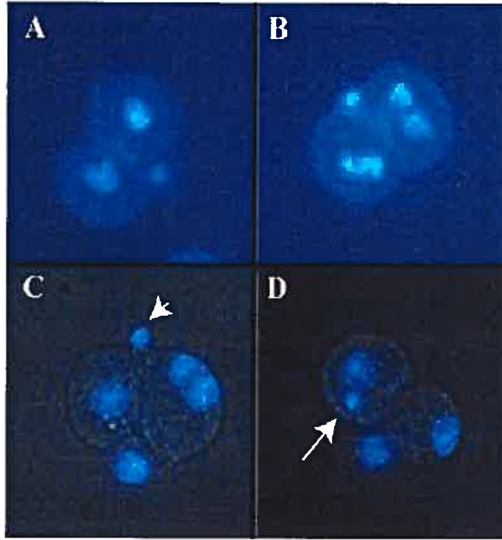
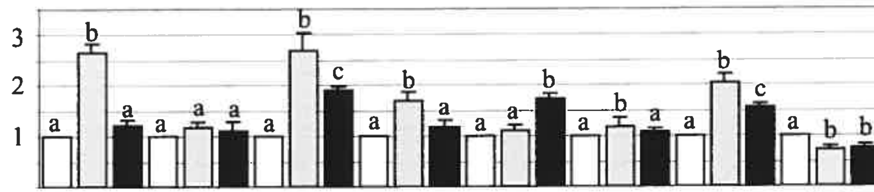

A
B
C
D

Fig. 2. Expression levels of several imprinted and non-imprinted genes in ES and somatic fibroblast donor cells. Controls non-treated cells are in white bars, TSA and 5AzaC treated cells are shown in gray and black bars respectively. Expression levels of each gene were standardized to the corresponding value of an exogenous standard and normalized to the non-treated control group. The data are presented as means of triplicate experiments. Different superscripts denote significant gene expression difference among the groups ($p < 0.05$).

ES Cells



Fibroblast cells

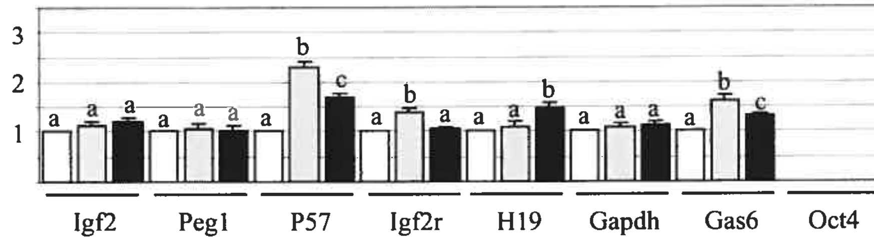


Fig. 3. Expression levels of several imprinted and non-imprinted genes in nuclear transfer embryos derived from ES cells and FF cells pretreated with TSA and 5AzaC. Controls non-treated cells are in white bars, first and second bars are in vivo and in vitro cultured embryos respectively, third and eighth bar are controls of nuclear transfer embryos at the morula and blastocyst stage respectively. Nuclear transfer embryos derived from pretreated donor cells with TSA and 5AzaC are shown in gray and black bars respectively. M=morula, B=blastocyst, B+=advanced blastocyst. Expression levels of each gene were standardized with the corresponding value of an exogenous standard and normalized to the non-treated control group (in vivo). The data of cloned embryos from ES nuclear donor cells is presented as means of triplicate experiments, whereas the data of cloned embryos from FF nuclear donor cells is presented as means of duplicate/triplicate experiments. Different superscripts denote significant gene expression difference among the groups ($p < 0.05$).

Cloned ES embryos

Day 3 Day 4

Igf2

Peg1

p57

H19

Igf2r

Gapdh

Gas6

Oct4

M M M M B+ M B+ B B B

Control TSA 5AzaC Control TSA 5AzaC

Cloned FF embryos

Day 3 Day 4

Igf2

Peg1

p57

H19

Igf2r

Gapdh

Gas6

Oct4

M M M M B+ M B+ B B B

Control TSA 5AzaC Control TSA 5AzaC

Table 1. Developmental potential of cloned embryos generated from ES and FF donor cells pretreated with TSA and 5AzaC. Percentages of embryonic development are of activated oocytes, advanced blast. are those embryos that had a higher developmental pace and was found on day 3 after activation, Blast.=blastocyst.

		No. of oocytes		No. of embryos		
Source of donor cell	Donor cell treatment	Injected	Activated	2-cell (%)	Morula & Blast. (%)	Advanced Blast. (%)
ES	Control	204	195	186 (95)[a]	166 (85)[a]	-
ES	TSA	265	250	231 (92)[a]	146 (58)[b]	9 (4)
ES	5AzaC	95	90	89 (99)[a]	54 (60)[b]	12 (13)
FF	Control	70	66	58 (89)[a]	21 (32)[a]	-
FF	TSA	57	51	45 (88)[a]	13 (26)[a]	1 (2)
FF	5AzaC	132	124	104 (84)[a]	26 (21)[a]	4 (3)

Different superscripts denote significant differences among treatments ($p < 0.05$)

Table 2. Nuclear abnormalities of arrested cloned embryos. Percentages are of total arrested cloned embryos. Multinuclei includes embryos with a micronuclei

Source of donor cell	Donor cell treatment	Arrested embryos at 2-3cell		
		Total	Mononuclei (%)	Multinuclei (%)
ES	Control	14	13 (93)[a]	1 (7)[a]
ES	TSA	13	6 (46)[b]	7 (54)[b]
ES	5AzaC	12	7 (58)[b]	5 (42)[b]
FF	Control	12	11 (92)[a]	1 (9)[a]
FF	TSA	24	9 (38)[b]	15 (63)[b]
FF	5AzaC	32	19 (59)[b]	13 (68)[b]

Different superscripts denote significant differences among treatments ($p < 0.05$)

5. General discussion

The present study provides novel data regarding the reprogramming of imprinted gene expression patterns in ES cells, fertilized and cloned embryos. Genomic imprinting is a unique phenomenon; it is characterized by the uniparental expression of a gene. Unlike housekeeping genes, imprinted genes are hypermethylated on specific CpG islands on the repressed allele, these regions are designated differentially methylated regions (DMRs) (Jones, 1999; Surani et al., 1990a). Perturbation of the epigenetic program in early embryogenesis is often associated with deregulation of imprinted genes leading to aberrant development and abnormal phenotypes.

Embryo cloning is inefficient due to the low developmental rate to the blastocyst stage and to term. Several protocols have been used to improve this technique, including cell cycle synchronization. Although the use of serum starvation to synchronize donor cells prior to nuclear transfer was beneficial for the cloning procedure, many of the produced fetuses and delivered newborns displayed growth abnormalities such as skeletal deformations, placentomegaly and disproportionate enlargement of internal organs (Kruip, 1997; McEvoy et al., 1998; Schnieke et al., 1997; Wells et al., 1997). A large body of evidence suggests that the growth abnormalities observed in cloned animals arise either by transmitting preexisting epigenetic errors from the donor nuclei to the recipient oocyte or due to improper reprogramming by the oocyte environment. The first study in my project demonstrates, for the first time, that imprinted gene expression is upregulated in ES cells cultured under growth inhibitory conditions, namely serum starvation and confluency. The marked increase in Igf2 expression at day 2 of serum starvation with the

absence of any changes in DMR2 of Igf2, indicates that regulatory mechanisms other than DNA methylation may be involved in regulating the expression pathway of imprinted genes. One might suggest that a closely related physiological event which is associated with repressed chromatin, such as histone deacetylation, is responsible for the changes in imprinted gene expression. This notion is supported by earlier findings showing that serum starvation induced alterations in the acetylation status of core histones in fibroblast cells (Knosp et al., 1991). Taken together, the first set of experiments suggests that serum starved nuclear donor ES cells harbor an epigenetically modified chromatin and extra caution is required should ES cells be used as a nuclear donor in embryo cloning or in transplantation medicine for replacement therapy. This suggestion is based on the fact that ES cells carrying aberrant imprinting profile do not limit their capacity to generate live animals from cloned or chimeric embryos, nor hinder their ability to differentiate to various cell types (Dean et al., 1998; Humpherys et al., 2001; Nagy et al., 1990).

The idea of having a secondary epigenetic mechanism beside DNA methylation to control allelic repression in imprinted genes is intriguing. Similar to DNA methylation, histone hypoacetylation is also correlated with transcriptional silencing (Struhl, 1998; Tazi and Bird, 1990). This silencing process has been shown to occur with the assistance of histone deacetylase enzymes that are responsible for ridding the chromatin of active acetyl groups in plants (Pazin and Kadonaga, 1997; Tian and Chen, 2001). In this study, we have shown that TSA, an inhibitor of histone deacetylases was capable of inducing major changes in imprinted gene expression in mouse ES cells. This finding is consistent with other reports in human fibroblast (Hu et al., 1998), mouse embryonic fibroblast (El

Kharroubi et al., 2001; Yoshioka et al., 2001) and cells from new born mice (Pedone et al., 1999).

Although the direct interaction between DNA methylation and histone deacetylation is unknown, it seems likely that the two silencing processes can be brought together by recruiting specific methyl binding proteins (MeCP1 and MeCP2) that are associated with histone deacetylases, thereby forming a repressive complex (Jones et al., 1998; Nan et al., 1998). The identification and characterization of novel members of the MeCP family will greatly enhance our understanding of the biological interactions between DNA methylation and histone deacetylation. Alternatively, histone deacetylases might directly target DNA methyltransferases, the enzymes responsible for de novo and maintenance of correct methylation profiles in imprinted genes. This is supported by recent findings showing that DNA methyltransferases are actively associated with histone deacetylases (Fuks et al., 2000; Robertson et al., 2000a; Rountree et al., 2000). Furthermore, the fact that unlike other imprinted genes, H19 was induced only by dual treatment (TSA and 5AzaC) and consistence with earlier studies (Pedone et al., 1999) suggests that the interplay between DNA methylation and histone deacetylation is more complex than expected and might involve multiple epigenetic mechanisms to ensure a correct profile of genomic repression. Together, these findings raise tremendous concerns regarding the use of ES cells in embryo cloning and transplantation medicine.

Similar to ES cells, we have shown that the expression of several imprinted genes was altered in response to TSA treatment in fertilized and parthenogenetic mouse embryos. The aberrant expression profile of imprinted genes was associated with accelerated cell division and early differentiation of some embryos. The accelerated

cleavage rate to the blastocyst stage is in agreement with earlier reports with bovine embryos cultured in the presence of TSA (Dominko et al., 1999). It is more likely that the developmental acceleration is caused, in part, by deregulation of imprinted genes responsible for the cell cycle control, such as Igf2 and p57, and possibly other non-imprinted genes. Although much of the work has focused on CpG methylation patterns in preimplantation stage embryos which led to the discovery of the wave of DNA demethylation that occurs from fertilization to the blastocyst stage, in which the majority of CpG islands are methylation free (Howlett and Reik, 1991; Kafri et al., 1992; Monk et al., 1987; Oswald et al., 2000), little has been done to characterize the acetylation profile, enzymatic activity of histone deacetylases and the role they play in preimplantation stages.

The epigenetic reprogramming machinery necessary for successful embryo cloning remains largely obscure. However, deleterious alterations of the epigenetic system may create serious aberrations in the expression profile of imprinted genes leading to growth abnormalities and genetic disorders. Moreover, our data show that while complete reprogramming of some imprinted genes occurred in cloned embryos derived from ES and FF donor cells, other imprinted genes remained aberrantly expressed, suggesting, for the first time, that an epigenetic reprogramming apparatus exists in preimplantation stages of embryonic development. Moreover, our findings with imprinted gene expression are supported by a recent studies showing that donor nuclei of cloned embryos undergo incomplete methylation reprogramming (Dean et al., 2001; Ohgane et al., 2001). The existence of such correction machinery implies that additional epigenetic manipulation of the donor nuclei might enhance the overall efficiency of

embryo cloning. Furthermore, it is interesting to note, that with all the epigenetic irregularities and aberrant expression of imprinted genes that exist in cloned embryos, several studies showed that such embryos can develop to term (Dean et al., 1998; Eggan et al., 2001). Thus it is widely anticipated that embryonic development is tolerant to improper reprogramming of imprinted gene expression (Humpherys et al., 2001; Inoue et al., 2002). Further, experiments are required to study the reprogrammability of imprinted genes in postimplantation cloned embryos and their placentas. Our data show that Oct4 is aberrantly expressed in cloned embryos is consistent with earlier report (Boiani et al., 2002). However, we show that other imprinted (p57 and Igf2r) and non-imprinted genes (Gas6) are deregulated in preimplantation cloned embryos. Interestingly, these findings can be used as markers to identify competent cloned embryos at preimplantation stages.

Collectively, the results described here provide information on the cumulative effects of culture conditions, ES cell aging, alterations in acetylation levels, epigenetic reprogramming in cloned embryos on the expression pattern of imprinted genes. Growth constraint conditions and aging of ES cells are critical factors that modify imprinted gene expression in vitro. Moreover, although it seems methylation is the prime epigenetic component that regulates imprinted genes, it is increasingly apparent that other epigenetic events, such as histone acetylation and phosphorilation are involved. Thus, the key to the imprinting mechanism should come from understanding how the imprints are established in the gametes? How DNA demethylation occur during preimplantation stages? What are the DNA demethylation enzymes if any? How can imprinted genes be protected from the wave of DNA demethylation? Is there any additional de novo or maintenance methyltransferases? How can DNA methylation be manipulated artificially in vitro?

What is the exact role of imprinted genes on development? Answering these questions would be advantageous both for our understanding of the disorders of genomic imprinting, produce healthy cloned animals and generate therapeutic cells free of epigenetic errors for transplantation medicine.

6. General conclusion

1. Culture of ES cells under growth constrained conditions is associated with alterations in imprinted gene expression and aberrant methylation profile of key regulatory domain. Additionally, late passage ES cells express imprinted genes at a very low level. Together, the changes in imprinted gene expression might be implicated in the large offspring syndrome and the failure to derive chimeras from late passage ES cells.

2. We conclude that beside DNA methylation, histone deacetylation plays a pivotal role in regulating imprinted gene expression patterns in ES cells. The overexpression effect of TSA or 5AzaC on imprinted genes appears to be non-reversible for at least 24h after drug removal, suggesting that imprinted genes are highly susceptible to changes in methylation and acetylation levels in mouse ES cells.

3. Like DNA methylation, histone acetylation plays an important role in the expression pathway of imprinted genes in preimplantation stage embryos. Additionally, perturbation of the expression pattern of imprinted genes is associated with aberrant pace of development.

4. The cloning procedure is associated with deregulation of some imprinted and non-imprinted genes. Moreover, epigenetic alterations in imprinted genes in donor cells induced by TSA and 5AzaC are partially reprogrammed during preimplantation stages in cloned embryos by unknown factors. Failure to partially reset imprinted gene profile of cloned embryos leads to accelerated cleavage.

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