I. Introduction.

An epigenetic phenomenon is defined as a "mitotically and/or meiotically heritable change in gene function that cannot be explained by changes in DNA sequence" (Wu and Morris, 2001). Epigenetic factors modify, restrict or enhance the potential for genes to be expressed with the consequence that cells may be able to follow a different phenotypic pathway.

Nuclear reprogramming describes a "switch in gene expression of one kind of cell to that of another unrelated cell type mediated by epigenetic factors" (Gurdon and Melton, 2008).

These epigenetic heritable modifications mark genomic regions and act as stable instructions for the specification of chromatin organisation and structure that dictate transcriptional states.

DNA methylation changes and the modification of histones account for the major epigenetic alterations of nuclear reprogramming that may contribute with other events to restore totipotency. DNA methylation is a reversible signal and can change in response to environmental and other signals (Ramchandani *et al*., 1999). The DNA molecule can be modified at the 5' position of cytosine present in CpG dinucleotide sequences by addition of a methyl group (Razin and Riggs, 1980). De novo and maintenance DNA methylation reactions are catalized by DNA methyltransferases (DNMTs).

Patterns of cytosine methylation are distinct for each cell type and confer cell type identity (Szyf, 2005). DNA methylation patterns are closely linked to chromatin structure. Unmethylated DNA is typically associated with an active chromatin configuration while methylated chromatin is associated with inactive chromatin. The traditional view has maintained that cytosine methylation precedes chromatin structure and that DNA methylation attracts methylated cytosine binding proteins, which in turn recruit repressor complexes (Jones *et al*., 1998; Nan *et al*., 1998). The repressor complexes contain histone deacetylases, which further contribute to a repressive chromatin state. From this perspective, cytosine methylation is the primary epigenetic mark responsible for repressive chromatin structure.

Recently, an alternative to this model has been described in which the state of chromatin determinates, or influences, DNA methylation or demethylation (Szyf, 2005). Chromatin controls the genetic information by either presenting an accessible nucleosomal structure or by organising higher order structures that prevent the accessibility of the underlying DNA. This function is carried out by the histones modifications. Histone are major carriers of epigenetic information, and covalent modifications on the histone N-terminal tails function as master on/off switches that determine whether a gene is active or inactive. Histone tails are subjected to a wide range of posttranslational modifications, including acetylation, phosphorylation, and methylation (Strahl and Allis 2000). This modifications can exist in multiple combination and together comprise what is being referred to as the "histone code" (Jenuwein and Allis, 2001). During reprogramming, histone modifications influence associations of proteins and protein complexes that regulate gene transcription or repression by altering the availability of genes to transcription factors. Histone acetylation is typically associated with a transcriptionally permissive state. Histone acelyl transferases (HATs) are the responsible to add acetyl groups to histone tails (Gibbons, 2005). The methylation of lysine residues can have a dual role in transcription (Szyf, 2005). While some modifications are traditionally considered as repressive and are localized preferentially to constitutive or facultative heterochromatin (e.g., methylated H3K9, H3K27, or H4K20) others like methylated H3K4, H3K36, or H3K79 have been shown to be linked with active transcription (Schübeler *et al*., 2004; Miao and Natarajan, 2005). Some results indicate, however, that on the whole genome scale the active methylation marks are underrepresented when compared to the repressive methylation marks indicating that the majority of histone methylation can be associated with transcriptionally inactive chromatin or heterochromatin (Jones *et al*., 1998; Nan *et al*., 1998; McKittrick *et al*., 2004; Thomas *et al*., 2006).

All these changes involve a remodelling, not of the underlying genetic sequences that comprise the genome, but of the epigenetic features that overwrite the gene sequences and find interpretation in new gene expression (Surani, 2001).

I.1. *In vivo* reprogramming.

I.1.1. Reprogramming during the development.

Two phases of large scale epigenetic reprogramming have been described during mammalian development. The first takes place during the germ cell development, where the loss in DNA methylation is associated to the erasure and subsequent resetting of imprinting marks (gametic imprinting) in the developing germ-line cells (Lee *et al.*, 2002; Hajkova *et al.,* 2002). The second takes place throughout the early embryo development. After fusion of the oocyte with the sperm cell, in a pre-zygotic stage, both maternal and paternal genomes undergo passive and active demethylation, respectively, excluding that of imprinted genes and some repetitive DNA sequences (phase I). At the blastocyst stage most methylation marks are removed and the genome, in particular that of the inner cell mass, is de novo methylated during implantation in mice (Reik *et al.*, 2001) (phase II). During this phase (II), despite the general loss of DNA methylation, gametic imprinting is maintained, at least in some species such as mice (Dean *et al.*, 2003).

I.1.1.1. Reprogramming in the germ line.

- Genomic imprinting.

Genomic imprinting is a form of epigenetic gene regulation that results in expression from a single allele in a parent-of-origin-dependent manner. This form of monoallelic expression affects a small but growing number of genes and is essential to normal mammalian development (Ideraabdullah *et al.,* 2008).

Usually, imprinting is achieved through DNA methylation of imprinting control regions (ICRs) and then the appropriate allele is silenced throughout development and the entire life by covalent methylation of CpG dinucleotides by the de novo methyltransferase, Dnmt 3a (Constancia *et al.,* 2004). A typical feature of imprinted genes is that they are found in clusters on the chromosomes and the ICRs exert a regional control of expression of genes from that cluster (Reik *et al.,* 2001).

Deletion of ICRs results in loss of imprinting of adjacent genes (Fitzpatrick *et al.,* 2002; Mancini-Dinardo *et al.,* 2006). Although all imprinting clusters comprise an ICR that controls the allelic expression at that domain, the DNA sequences of individual ICRs show little similarity with that of the others (Kacem and Fiel, 2009)

Failure of these regulatory mechanisms leads to perturbed gene expression and results in developmental abnormalities and cancers (Feinberg and Tycko, 2004)

The germ cell precursors, PGCs, during migration and in the gonads, undergo erasure of methylation, including that of imprinted genes and the inactive X chromosome, most likely by an active mechanism, as well as partial erasure in some repetitive DNA sequences (Hajkova, *et al.,* 2002; Maatouk *et al.,* 2006). Once the genomes of PGCs have been demethylated, the male PGCs enter mitotic arrest and the female PGCs enter meiosis arrest. Although these two processes (demethylation and cell-cycle arrest) may be uncoupled, there are advantages in stopping proliferation to keep chromosome stability in the germ cells (Roelen and Chuva de Sousa Lopes, 2008). For example, demethylated centromeres are decondensed and may be functionally altered (Xu *et al.,* 1999). Demethylation also leads to a higher frequency of structural abnormalities in chromosomes (Chen *et al.,*, 1998).

- Chromatin dynamics during germ cell epigenetic reprogramming.

The chromatin in nascent mouse PGCs (developmental stage: E8.5) experiments several changes as loss of methylation of lysine 9 of histone H3 (H3K9me2), enhancement of trimethylation of lysine 27 of histone H3 (H3K27me3) concomitantly with EZH2 (histone-lysine-N-methyltransferase), a polycomb group enzyme. In addition, there is enrichment of methylation of lysine 4 of histone H3 (H3K4me2 and H3K4me3) and of many histone acetylation marks, especially H3K9ac (Hajkova *et al.,* 2008).

This germ cell chromatin signature is established specifically in PGCs (not detected in the contemporary somatic cells) before their entry into the gonads, and is associated with the expression of pluripotency-specific genes: Sox2, Oct4 (Pou5f1), Nanog and stella (Surani *et al.*, 2004). This chromatin state is also potentially crucial for the derivation of pluripotent embryonic germ cells from PGCs between E8.5 and E11.5.

It is against this background that subsequent events follow when PGCs enter into the developing gonads at E10.5 (Hajkova *et al.,* 2008).

The first sign of chromatin changes in gonadal PGCs at E11.5 is a rapid loss of linker histone H1, accompanied by 'loosening' of the chromatin. Although removal of these repressive histone modifications could make the chromatin more 'permissive' for DNA demethylation, they may underlie more profound changes in nuclear structure. Indeed, modifications associated with the transcriptionally active chromatin, such as H3K9ac, are also lost (Hajkova *et al.,* 2002; Lee, *et al.*, 2002).

Whereas the erasure of differential DNA demethylation of imprinted genes in E11.5 PGCs persists until new imprints are imposed later during gametogenesis (Allegrucci *et al.,* 2005), the chromatin decondensation and restructuring are transient. Concomitantly, the H3K9me3 marks reappear and the proteins associated with pericentromeric heterochromatin relocalize forming a pattern resembling that seen in the surrounding somatic cells. Other chromatin changes also revert to the original state, although with diverse kinetics. For example, there is rapid reappearance of linker histone H1, but that of H3K27me3 is slower. Notable some histone modifications are lost altogether, including H3K9ac and H4/H2AR3me2s. The reprogramming process thus exerts the erasure of epigenetic memory at multiple and distinct levels. Importantly, none of these changes are seen in the surrounding somatic cells (Hajkova *et al.,* 2008).

- Acquisition of imprints.

The de novo methyltransferase DNMT3A is required for the establishment of methylation imprints in both male and female germ cells (Kaneda *et al.,* 2004). A related protein, DNMT3-like (DNMT3L), is also essential for imprint establishment (Bourc'his *et al.,* 2001a; Hata *et al.,* 2002). It is thought that DNMT3L interacts with DNMT3A and stimulates its activity, most likely by recruiting DNMT3A to chromatin (Chedin *et al.,* 2002; Jia *et al.,* 2007). How the DNMT3A/DNMT3L complex becomes recruited to ICR sequences is not known.

Several studies tested the hypothesis that close tandem repeat sequences could provide specificity to this process (Neumann and Barlow, 1996). At the mouse Rasgrf1 locus, a tandem repeat sequence neighbouring the ICR was shown to be

essential for imprint establishment (Yoon *et al.,* 2002). Transgenic experiments on the ICRs of the Igf2r and Kcnq1 domains indicate that tandem repeats, if present in multiple copies, contribute to imprint establishment as well as to somatic maintenance of methylation during early embryogenesis (Reinhart *et al.,* 2006). Deletion of the repeated element at the Kcnq1 ICR was found to not affect imprint acquisition (Mancini-Dinardo *et al.,* 2006), and also, repeats close to the Igf2-H19 ICR are not essential for imprint establishment (Lewis *et al.,* 2004; Reed *et al.,* 2001).

Another indication that chromatin organization in germ cells could potentially play a role in imprint establishment comes from the Prader-Willi Syndrome (PWS) imprinted domain on human chromosome 15q11. Its ICR, at the SNRPN gene, has a maternal methylation imprint (Kacem and Fiel, 2009). In some studies, this ICR was reported to become methylated after fertilization of the oocyte only (El-Maarri *et al.*, 2001; Geuns *et al.,* 2003; Kantor *et al.,* 2004). It indicates that the de novo DNA methylation machinery somehow detects the right parental allele in the early embryo. It has been proposed that this could involve a maternally derived chromatin signature (Kaufman *et al.*, 2009). Support for the idea that specific chromatin features could be inherited from the germline independently of DNA methylation comes from the different transgenic studies on the H19-Igf2 ICR. When inserted as a single copy at specific exogenous positions in the genome (Park *et al,* 2004; Tanimoto *et al.,* 2005), acquisition of paternal DNA methylation at the ICR did not happen during spermatogenesis but only after fertilization, during early development.

Nonhistone proteins contribute to the specificity of imprint establishment as well. A recent conditional targeting study in the mouse showed that the zinc finger protein ZFP57 contributes to imprint establishment at the Snrpn ICR in oocytes (Li *et al.,* 2008).

Transcription could potentially play a role in the acquisition and/or maintenance of maternal imprints. Maternally methylated ICRs comprise promoters, some of which transcribe long non coding RNAs (ncRNAs) involved in chromatin repression. Many of these transcripts overlap in the antisense orientation with other transcripts at the imprinted locus in which they are located (Peters and Robson, 2008).

Therefore, double-stranded RNA molecules (dsRNAs) that might affect gene activity via the formation of small interfering RNAs (siRNAs) could be formed (Kacem and Fiel, 2009).

Although Dicer and RNA interference are not involved in the control of imprinted genes in somatic cells (Fukasawa *et al.,* 2006; Morita *et al.,* 2007; Redrup *et al.,* 2009), RNA interference (RNAi) mechanisms could, however, still be involved in the establishment of imprints in germ cells, because the DNA methylation at retrotransposon elements in mammals is controlled by small RNAs during gametogenesis (Aravin and Bourc'his, 2008). It seems too early to conceptually link the involvement of transcription and the role of chromatin in imprint establishment in female germ cells. One possibility, however, could be that transcription through CpG islands brings about a chromatin devoid of H3K4 methylation, which would facilitate recruitment of the DNMT3A-DNMT3L complex and acquisition of de novo methylation, if there is an appropriate spacing of CpG dinucleotides (Kacem and Fiel, 2009). In human cells and in plants, DNA methylation is found in the bodies of highly expressed genes, indicating that transcription could indeed mediate DNA methylation (Ball *et al.,* 2009; Zilberman *et al.,* 2008). Chromatin- and transcription-mediated mechanisms may thus be linked together in the establishment of imprints in female germ cells (Kacem and Fiel, 2009).

Many putative transcription units overlap with transcripts from the opposite strand and the majority of these are noncoding (Katayama *et al.,* 2005; Brosnan and Voinnet, 2009). Several long ncRNAs at imprinted chromosomal domains have been characterized in great detail and have been shown to play essential roles in the control of imprinted gene expression (Peters and Robson, 2008; Umlauf *et al.,* 2008).

The Igf2r domain on mouse chromosome 17 was the first locus where expression of a long ncRNA was demonstrated to be essential (Sleutels *et al.,* 2002). Igf2r is expressed from the maternal allele and comprises an ICR in its second intron which presents maternally derived DNA methylation. Deletion of this ICR on the paternal chromosome led to disruption of imprinted expression of Igf2r and flanking genes (Zwart *et al.,* 2001). On the paternal chromosome, transcription initiates from the ICR and is in the antisense orientation compared to the Igf2r transcript. This

noncoding antisense RNA is very large, going across the Igf2r promoter, through an intergenic region, and up to the neighboring gene (Lyle *et al.,* 2000). This noncoding RNA was called Airn and is nonspliced and escapes export from the nucleus (Seidl *et al.,* 2006). It is essential for the paternal silencing of Igf2r and that of two close genes, Slc22a2 and Slc22a3, both expressed and imprinted in the placenta (Sleutels *et al.,* 2002). Although Airn transcription clearly mediates differential gene expression from the maternal allele, imprinted expression of Igf2r seems not to be regulated by Airn itself but possibly via a transcriptional interference mechanism (Pauler *et al.,* 2007).

The Kcnq1 domain on distal chromosome 7 is structurally similar to the Igf2r locus. An intronic ICR of the Kcnq1 domain has maternal DNA methylation and produces a long ncRNA (called Kcnq1ot1, or Lit1) from the unmethylated paternal allele. This ICR, called KvDMR1, is essential for silencing genes on the paternal allele of this domain (Fitzpatrick *et al.,* 2002; Mancini-Dinardo *et al.,* 2006; Shin *et al.,* 2008). Imprinting at the domain is most extensive in the placenta, where it concerns more than ten genes (Lewis *et al.,* 2004; Umlauf *et al.,* 2004).

Several other imprinted domains express long ncRNAs, including the Gnas locus on chromosome 2. Transcription of one of its ncRNAs, the Nesp antisense (Nespas) (Williamson *et al.,* 2006) seems important for repression of Nesp on the paternal allele.

I.1.1.2. Reprogramming during early development.

- Epigenetic status of the gametes.

Initially, the paternal genome is highly condensed in the spermatozoa, partly through its binding by protamines. However, methylation patterns of promoters in sperm, embryonic stem (ES) cells and embryonic germ (EG) cells are surprisingly similar, suggesting that while the sperm is a highly specialized cell type, its promoter epigenome is already largely reprogrammed, resembling a pluripotent state (Farthing *et al*, 2008). The majority of promoters that are hypomethylated in ES and EG cells are also hypomethylated in sperm. This suggests that on the one hand, DNA methylation states at promoters may become reprogrammed during gametogenesis. On the other

hand, reprogramming would not be required for those genes that never become methylated during early embryogenesis or gametogenesis (Albert and Peters, 2009). Important exceptions are the promoters of Nanog, Lefty1, Brd1, Slc5a4a, and Slc39a4 that were highly methylated in sperm while being hypomethylated in ES and EG cells. In addition Oct4 and Sox2 are also found to be methylated in regulatory regions in sperm, albeit outside of the immediate promoter region (Farthing *et at*., 2008).

The maternal genome is arrested at metaphase II with its 2C genome packaged with histones. Microarray studies show that Nanog, Sox2, Tead4, Cdx2, Eomes, Elf5, and Gata6 are not expressed in mature oocytes but become de novo transcribed during preimplantation development (Zeng *et al.,* 2004). In contrast, Oct3/4 is both oocytary and zygotically expressed and functional (Zeng *et al.,* 2004; Foygel *et al.,* 2008.

Puschendorf *et al.,* (2008) identified transgenerational transmission of methylated histones as a novel form of maternal contribution. Specifically, the transmission of H3K9me3, established in oocytes, is required for maintaining the constitutive heterochromatic state at major satellite repeats of the maternal genome in early mouse embryos.

Variants of histones present in the oocyte are involved in wide-scale chromatin remodelling events and have acquired specialised functions during development. H2A.X is the most abundant H2A variant in Xenopus eggs, and the ability to remodel the sperm nucleus to form a paternal pronucleus after fertilization is directly associated with its phosphorylation status (Dimitrov *et al.,* 1994). Phosphorylation of the H2A.X variant is implicated in the initiation of the Meiotic Sex Chromosome Inactivation (MSCI) (Fernandez-Capetillo *et al.,* 2003). Macro H2A, another H2A variant (Pehrson and Fried, 1992), is present in the chromatin of developing and mature oocytes and it seems to be also implicated in the MSCI (Costanzi *et al.,* 2000; Hoyer-Fender *et al.,* 2000).

- Epigenetic reprogramming phase I

At fertilization, the paternal genome has a very different developmental history from the resident maternal genome and must acquire an appropriate epigenetic state to participate in development (Arney *et al.,* 2002). The protamines in sperm chromatin are rapidly replaced with histones. The active remodeling might provide a chance of access to sperm genome for diverse cytoplasmic reprogramming machineries.

Histones incorporated into the male pronucleus are highly acetylated (Adenot *et al.,* 1997; Santos *et al.,* 2002). However, immediately upon histone incorporation H3K4me1, H3K9me1, and H3K27me1 are detected at fertilization and, H3K4me3, H3K9me2, and H3K27me3 become detectable only after DNA replication (Erhardt *et al.,* 2003; Santos *et al.,* 2005; Lepikhov *et al.*, 2004). The early appearance of histone methylation marks implies that histone residues are rapidly deacteylated and then monomethylated by the appropriate histone methyltransferases (HMTases) (Morgan *et al.,* 2005). As this replacement occurs prior to S phase, a particular histone variant, H3.3, is selectively incorporated, probably by the histone chaperone Hira, into the paternal genome (van der Heijden *et al.,* 2005; Torres-Padilla *et al.,* 2006). This constitutes the only event of genome wide deposition of H3.3 in the life of an organism (Santenard and Torres-Padilla, 2009). Interestingly, the histone H3.1 is absent from the paternal pronucleus before DNA replication (van der Heijden *et al.,* 2005). This initial epigenetic asymmetry between the parental genomes is further manifested by differences in histone modifications and localization of numerous epigenetic modifiers such as Ezh2 (Erhardt *et al.,* 2003).

Many sequences in the paternal genome, such as Line1 (Long Interspersed Elements) repeats, are actively demethylated in the zygote (Mayer *et al.,* 2000; Oswald *et al.,* 2000; Lane *et al.,* 2003).

Many regions of the genome do not become demethylated at this stage. These include heterochromatin around centromeres (Rougier *et al.,* 1998; Santos, *et al.*, 2002), IAP retrotransposons (Lane *et al.,* 2003) and paternally methylated imprinted genes (Olek and Walter, 1997). The molecular mechanism of this global DNA demethylation is currently unknown, but correct epigenetic configuration of the paternal chromatin is likely to be important given the fact that the maternal genome escapes this process.

The differences in histone modifications between parental pronuclei may explain the protection of the maternal genome from undergoing DNA demethylation. Histone modifications ordinarily associated with an active chromatin state such as acetylated lysine and H3K4me are found in the female pronucleus (Adenot *et al.*, 1997; Arney, *et al.,* 2002; Lepikhov *et al.,* 2004). Heterochromatic modifications such as H3K9me2/3, H3K27me1 and H4K20me3 largely associated with repressive chromatin organization are also in evidence (Cowell *et al.,* 2002: Erhardt, *et al.*, 2003; Reik *et al.*, 2003; Liu *et al.,* 2004). It is suggested an association between H3K9me2 and protection against DNA demethylation in the female pronucleous (Santos *et al.,* 2005). More recently, Stella was shown to be required for preventing DNA demethylation of the maternal genome. In Stella-deficient oocytes, the maternal genome is massively demethylated (Nakamura *et al.,* 2007). However, as Stella is found in both maternal and paternal pronuclei, additional factors must cooperate to protect the maternal genome from DNA demethylation.

- Epigenetic status of the zygote.

The zygote contains a number of key maternally inherited transcription factors, including some that are essential for pluripotency, such as Oct3/4 and Sox2, as well as epigenetic factors for histone modifications including Polycomb group (PcG) proteins such as Ezh2 and Eed, proteins of histone metabolism, and chromatin remodelers such as Brg1 (Schuettengruber *et al.,* 2007). As the key requirement at this stage of development is to convert the quiescent genome into a transcriptionally competent one, this must be accomplished by maternally inherited factors in the oocyte. Among the maternal factors whose function has been well defined is Brg1, a component of the SWI/SNF chromatin-remodeling complex (Bultman *et al.,* 2006). Loss of Brg1 results in reduced transcription and arrest at the two-cell stage. Another example is Npm2, whose presence in the oocyte is essential for histone deacetylation and heterochromatin formation surrounding the nucleoli (Burns *et al.,* 2003). As mentioned before, macroH2A is present in the chromatin of developing and mature oocytes, but it is immediately lost from the maternal chromatin in the zygote following fertilization and reappears only after the 8-cell stage (Chang *et al.,* 2005). Because

macroH2A inhibits chromatin remodeling (Angelov *et al.,* 2003), it is possible that its rapid disappearance following fertilization is necessary to render the zygotic chromatin permissive for remodeling and epigenetic reprogramming (Santenard and Torres-Padilla, 2009; Ziegler-Birling *et al.,* 2009).

In mouse, from the late zygote to the two-cell stage, when the embryonic genome becomes activated (MZT), the epigenetic status of the parental genomes starts to become less distinct, with the exception of DNA methylation. The overall differences in DNA methylation persist for one to two cleavage divisions, followed by a passive and steady decline through preimplantation development (Mayer *et al.,* 2000).

- Epigenetic reprogramming phase II.

A second major reprogramming of DNA methylation patterns occurs after in the early embryo. Sequences in the maternal genome are passively demethylated during the cleavage divisions in the preimplantation mouse embryo (Monk *et al.,* 1987; Howlett and Reik, 1991), presumably due to the exclusion of Dnmt1o. Dnmt1o, protein inherited from the oocytes, is excluded from the nucleus during the first three cleavage divisions (Carlson *et al.,* 1992; Bestor, 2000) accounting for the loss of methylation by a passive mechanism, which results in unequally methylated sister chromatids (Oswald *et al.,* 2000). Many different types of sequences lose methylation at this stage but imprinted genes retain their germline imprints raising questions about the DNMT responsible and the recognition of regions where DNA methylation is to be maintained. Curiously, Dnmt1o only enters the nucleus at the eight cell stage, and it has been argued that this is needed for the maintenance of imprinted methylation (Howell *et al.,* 2001). It is not known whether Dnmt1s (the somatic form of Dnmt1) is required for specific maintenance of imprints during preimplantation. Unique histone modifications may guide DNA methylation maintenance of imprinted regions. There are quantitative differences in passive demethylation between mammalian species that have been analysed. Perhaps, this is related to the differences in timing of blastocyst cavitation and development of progenitors of the embryonic and extraembryonic lineages. To date, extensive changes of DNA methylation during this

period have not been reported outside of mammalian, suggesting that this is a mammalian specialization (Morgan *et al.,* 2005).

To what precise extent histone modifications are reprogrammed during passive DNA demethylation is not yet clear. In the mouse, it seems that H3K4me, H3K9me and H3K27me are not globally altered (Erhardt *et al.,* 2003), but in bovine embryos, both heterochromatic histone methylation and H3K9ac decrease and then increase in advance of major genome activation (Santos *et al.,* 2003). More transient histone marks, such as phosphorylation and arginine methylation, undergo changes during the cell cycle in mouse embryos (Sarmento *et al.,* 2004; Nowak and Corces*,* 2004) that are likely associated with DNA replication rather than reprogramming, but to what extent this differs from cell cycle regulation of these marks in more differentiated cells is not clear at present.

Genome-wide hypomethylation at the morula stage is then followed by lineage specific de novo methylation beginning at the blastocyst stage (Santos *et al.,* 2002), presumably carried out by Dnmt3a and Dnmt3b. It is possible that this de novo methylation leads to epigenetic silencing of key promoters during early development (Okano *et al.,* 1999).

I.1.2. Reprogramming during *in vivo* regeneration.

I.1.2.1. Regeneration ability.

Some invertebrates, such as planarians or annelid worms, can re-grow all missing body parts when cut into small pieces, while some vertebrates, such as amphibians and fishes, can replace lost limbs (Endo *et al.,* 2000), tails (Tseng and Levin, 2008), lens (Malloch *et al.,* 2009), retina (Vergara and Del Rio-Tsonis, 2009), and several internal organs and repair skeletal muscle, peripheral nervous system and CNS (Satoh *et al.*, 2009). Fish can also re-grow amputated hearts (Raya *et al.,* 2003). Mammals (including humans) can repair damage to skeletal muscle and peripheral nervous system and can recover from damage to the liver, but lack the ability of amphibian and fish to re-grow appendages, heart, lens, retina, and CNS (Stoick-Cooper *et al.,* 2007a).

Regeneration is defined as a process that allows an organism to regain the function of an organ or structure damaged by injury or disease. Three types of regeneration in vertebrates can be distinguished (Stoick-Cooper *et al.,* 2007a): (1) "Epimorphic": regeneration via formation of a blastema, a population of progenitor cells that arises through epithelial mesenchymal interactions and contains intrinsic morphogenetic information that is required to re-pattern the regenerating structure (e.g., limb, tail, and fin regeneration); (2) "Compensatory growth": here it is not the damaged part of an organ that is restored, but uninjured parts of the organ compensate for the loss by growth (e.g., after removal of two lobes of the liver, the third lobe grows until the original mass of the liver is restored), and; (3) "Tissue regeneration": repair of local, limited damage to an organ predominantly via restoration of only one cell type (e.g., skeletal muscle). Our interest is focused in the first type

I.1.2.2. Epimorphic regeneration.

Perhaps the most spectacular regenerative events in vertebrates represent epimorphic regeneration. Thomas Hunt Morgan used the term "epimorphosis" to define regenerative processes involving proliferation (Morgan, 1901). A more contemporary definition of epimorphic regeneration reflects regeneration with the presence of a specialized structure called the blastema, and the reconstitution of complex tissue with multiple cell types (Poss *et al.*, 2003)

The blastema is a collection of heterogenous mesenchyma-like cells located between the stump tissues and the wound epidermis with which they establish exchanges of various types. It involves strict growth controls and cell reprogramming occurring in adult tissues followed by sequential steps of cell differentiation and patterning leading to the faithful restoration of only the lost parts (Poss *et al.,* 2003).

I.1.2.3. Origin of the blastema in limbs and fins regeneration.

There are several cell types present at the surface of the amputation cut that are able to participate in the formation of a blastema, including cells of the connective and muscle tissue, cartilage, scleroblasts, pigment cells, the Schwann cells, the endothelial cells forming the capillaries, and the blood cells (Akimenko *et al.,* 2003).

In vitro and *in vivo* evidence demonstrates that muscle tissue dedifferentiates from multinucleated myotubes (in culture) and myofibers (*in vivo*) to form mononucleated cells that proliferate and contribute to the blastema in limb regeneration (Namenwirth, 1974; Lo *et al.,* 1993; Kumar *et al.,* 2000; Echeverri *et al.,* 2001; Brockes and Kumar 2002). Tanaka *et al.,* (1999) have shown that the bloodclotting proteinase thrombin may act as an extracellular signal that induces this process, as it can indirectly induce S-phase re-entry in cultured newt myotubes. It is also known that, intracellularly, phosphorylation of the retinoblastoma (Rb) protein and expression of the homeobox protein Msx1, a transcriptional repressor that is expressed in many regenerating systems, is required for myotube cell cycle re-entry *in vitro* (Tanaka *et al.,* 1997; Kumar *et al.,* 2004).

Cartilage cells also have been shown to dedifferentiate to participate in the blastema formation during limb regeneration (Steen, 1968; Namenwirth 1974; Muneoka *et al.,* 1986), but extensive studies have not been done to uncover the molecular signals involved in this process either.

In fin fish regeneration, cytologic studies show that some cells of the connective tissue have an altered phenotype: from stellate-shaped they become rounded (Becerra *et al.,* 1996). This change may indicate a modification in the differentiation state of these cells and/or a cytologic modification that may favour migration. In addition, many bromodeoxyuridine (BrdU) incorporation analyses (Poleo *et al.,* 2001a; Santos-Ruiz *et al.,* 2002; Nechiporuk and Keating, 2002) suggests that cells of the connective tissue at the level of the stump enter the cell cycle, making them excellent candidates for blastema formation in fin regeneration, supporting the hypothesis that the blastema forms through a process of dedifferentiation of local mature cells similar to the mechanism taking place during regeneration of amphibian limb.

There is no evidence of the scleroblast involve in bone regeneration in the fin regeneration. There is no indication that endothelial cells participate in the formation of the blastema either (Akimenko *et al.,* 2003).

Participation of derivatives of Schwann cells to limb regeneration was reported in the regenerating newt limb (Maden, 1977), but has not yet been shown in fin regeneration.

Alternatively, blastema cells could originate from one or several stem cell populations, quiescent in the normal fin and residing at the level of each mature tissue of the stump (Nechiporuk and Keating 2002). It is possible that either mature fin cells dedifferentiate or that quiescent stem cells are activated to form this pluripotent mass; however there is not direct evidence for either process. To date, only the melanocytes of the zebrafish caudal fin have been proposed to originate from a population of stem cells distributed along the intraray tissue and, after fin amputation, to differentiate and invade the regenerate region through migration (Rawls and Johnson, 2000, 2001). White cells, a specific type of pigment cells of the zebrafish fin, could also originate from nonpigmented stem cells (Murciano *et al.,* 2002). There is no indication that these stem cells give rise to other cell types participating to the formation of the blastema during regeneration. Therefore, in addition to some pigment cells, putative stem cells are not better candidates than local mature cells undergoing cell dedifferentiation.

In urodeles, it has been recently shown that pax7-expressing muscle satellite cells, which are well-described muscle progenitor cells in mammalian muscle formation and regeneration, become mitotically active after limb amputation (Morrison *et al.,* 2006). Moreover, pax7-expressing cells are found in the blastema, suggesting that these cells may participate in blastema formation. This is a surprising and somewhat controversial finding, however, because most research in the field points to dedifferentiation as playing the major role in forming the urodele blastema.

This observation leads to the conclusion that blastema formation could be the result of two cellular mechanisms, not mutually exclusive, depending upon the cell types present in the stump.

- Blastema potency.

As discuss above, experiments have led to wide acceptance that tissues dedifferentiate to form pluripotent cells.

There is evidence of upregulation of some transcription factors related to the pluripotent state, such as Sox2, Klf4 and c-myc during the early stages of newt lens regeneration. However, the absence of Oct4 and nanog expression might indicate why the newt cells do not become pluripotent (Maki *et al.,* 2009). Recently, Kragl *et al.* (2009) found that each tissue, in axolotl, produces progenitor cells with restricted potential. So, the blastema would be a heterogeneous collection of restricted progenitor cells. In axolot limb regeneration, cells keep memory of their tissue origin and the positional identity is a cell-type-specific property of blastema cells, in which cartilage-derived blastema cells harbour positional identity but Schwann-derived cells do not (Kragl et al., 2009).

These recent results show that the complex phenomenon regeneration may be achieved without complete dedifferentiation to a pluripotent state.

I.1.2.4. Common mechanism in molecular regulation of regeneration.

While the scale, the complexity, and the cellular mechanisms of regeneration of different organs and structures are obviously quite different, some common principles in their molecular regulation can be identified (Stoick-Cooper *et al.,* 2007a).

In all vertebrates, signals regulating the immune response are also good candidates for triggers of regenerative processes. Indeed, both in skeletal muscle and liver regeneration, the immune system provides important signals during early phases of the regenerative processes. Similar cytokines, have been shown to be required for progenitor cell proliferation in regenerating liver and muscle. Whether cytokine signalling is also involved in the regulation of heart regeneration has not been tested (Stoick-Cooper *et al.,* 2007a). Likewise, very little is known about the role of cytokine signalling in epimorphic regeneration of appendages. The complement components C3 and C5, which are implicated as triggers of liver regeneration, have been shown to be

expressed in specific domains during newt limb and lens regeneration (Kimura *et al.,* 2003), but their functional involvement has not been tested. Interestingly, the chemokine SDF-1 was proposed to be a negative regulator of zebrafish fin regeneration (Bouzaffour *et al.,* 2009).

Newts appear to have adopted the blood-clotting factor thrombin as an essential signal regulating dedifferentiation and proliferation of cells during muscle and lens regeneration (Imokawa and Brockes 2003). Thrombin induces cell cycle re-entry of cultured newt myotubes and is activated selectively on the dorsal margin of the iris, which after injury of the lens can dedifferentiate and replace the lens (Imokawa *et al.,* 2004). If thrombin activity in the eye is blocked, lens regeneration is impaired.

It is noteworthy that newts and the Japanese freshwater fish Misgumus are the only vertebrate species known to be able to regenerate their lenses, while even axolotls, which are otherwise champion regenerators, are incapable of doing so (Godwin and Brockes 2006). Similarly, muscle dedifferentiation, which thrombin appears to be involved in, might be quite an exceptional feat, only found in urodele amphibians.

A comparison of signals known to regulate cell proliferation and specification in different regenerating systems reveals that FGF signaling is implicated in almost all of them. FGFs have several essential roles during epimorphic regeneration of urodele limbs, anuran tails, and fish fins and in regulating the wound epidermis (WE) formation, blastema formation and proliferation, and positional memory (Godwin and Brockes, 2006). FGF signaling is also required for zebrafish heart (Raya *et al.,* 2003) and liver (Kan *et al.,* 2009) regeneration and is implicated in the regulation of cell proliferation and differentiation in regenerating skeletal muscle and mammalian liver (Ross *et al.,* 2001). Nevertheless, it is remarkable that FGFs appear to be universal regulators of regeneration, despite the different cellular mechanisms driving regeneration of these systems and the different cell types being regenerated.

Likewise, Wnt/β-catenin signaling appears to be involved in many regenerative processes as well (Stoick-Cooper *et al.,* 2007b). It is one of the first signals known to be activated during zebrafish fin and heart regeneration, is required for correct WE

patterning in fins and amphibian limbs, and for blastema formation and proliferation in fins (Lin and Slack, 2008). In addition, Wnt/β-catenin signaling appears to have a role in liver regeneration and can activate progenitor cells during skeletal muscle regeneration (Godwin and Broches, 2006).

Although, there are evidences that point to Msx transcription factors are general tissue-independent markers of the regenerative response in zebrafish (Raya *et al.,* 2003), comparison of gene expression profiles from regenerating zebrafish caudal fin, heart muscle, and neural retina revealed an elevated number of shared genes even though different cellular substrates are required for regeneration of these diverse structures (Quin *et al.*, 2009).

I.2. *In vitro* reprogramming.

I.2.1. Molecular circuitry of pluripotency.

The gene-expression program of pluripotent cells is a product of regulation by specific transcription factors, chromatin-modifying enzymes, regulatory RNA molecules, and signal-transduction pathways. Recent studies have provided new insights into how the key cell regulators work together to produce the pluripotent state (Loh *et al*., 2008; Jaenisch and Young, 2008).

I.2.1.1. Pluripotent cells in culture.

Culture-Induced Reprogramming Pluripotent cells have been derived from embryonic sources such as blastomeres and the inner cell mass (ICM) of the blastocyst (ES cells), the epiblast (EpiSC cells), primordial germ cells (EG cells), and postnatal spermatogonial stem cells (‘‘maGSCs,’’ ‘‘ES-like’’ cells). Donor cells from the germ cell lineage such as PGCs or spermatogonial stem cells are known to be unipotent *in vivo*, but it has been shown that pluripotent ES-like cells (Conrad *et al*., 2008), or maGSCs (Guan *et al*., 2006), can be isolated after prolonged *in vitro* culture. While most of pluripotent cell types were capable of *in vitro* differentiation and teratoma formation, only ES, EG, embryonic carcinoma (EC), and spermatogonial stem cell-derived maGCSs or ES-like cells were pluripotent by more stringent criteria, as they were able to form postnatal chimeras and contribute to the germline. Recently, multipotent adult spermatogonial stem cells (MASCs) were derived from testicular spermatogonial stem cells of adult mice, and these cells had an expression profile different from that of ES cells (Seandel *et al*., 2007) but similar to EpiSC cells, which were derived from the epiblast of postimplantation mouse embryos (Brons *et al*., 2007; Tesar *et al*., 2007). While both MASCs and EpiSCs were able to differentiate *in vitro* and to generate teratomas *in vivo*, they were unable to form chimeras in contrast to ES, EG, EC, and maGSCs cells. MASCs and EpiSCs were similar to human ES cells in many ways: they required FGF but not LIF for growth, they were able to express trophoblast markers *in vitro*, and they displayed expression profiles that were more typical of human than mouse ES cells. These similarities raise the possibility that the embryonic origin of

human ES cells may be the epiblast stage in contrast to that of mouse ES cells, which are derived from the ICM. It may be that the present isolation protocols of human ES cells using FGF and activin selects against ‘‘true’’ ES cells and results in cells that resemble mouse EpiSCs rather than mouse ES cells (Lovell-Badge, 2007). It is possible that the existing human ES cells, the murine EpiSCs and MASCs are multipotent cell types that are endowed with a more restricted developmental potential than pluripotent mouse ES cells.

I.2.1.2. Transcription factors that regulate pluripotency

- Oct3/4

The transcription factor Oct3/4 is strongly involved in the maintenance of self-renewal of pluripotent cells. Genome-wide studies in human and mouse revealed a large panel of target genes with Oct-regulatory elements and many targets have frequently been implicated in ES cell signalling. A large number of these genes possesses regulatory elements for the transcription factors Sox2 and Nanog in close proximity that were found to be co-occupied in genes specifically positively or negatively regulated in ES cells (Boyer *et al*., 2005, Loh *et al*., 2006). A number of putative regulatory factors for Oct3/4 have been identified (Niwa, 2007). These include the enhancers Lrh1 (Gu *et al*., 2005), retinoic acid receptor:retinoid X receptor heterodimers (Sylvester and Scholer, 1994; Ben-Shushan *et al*., 1995) and SF1 (Barnea and Bergman, 2000) and the repressors Cdx2 (Niwa *et al*., 2005), COUP I+II (Sylvester and Scholer, 1994; Ben-Shushan *et al*., 1995) and Gcnf (Fuhrmann *et al*., 2001). Gcnf has been shown to recruit the de-novo methyltransferase Dnmt3 to the Oct3/4 promoter and to promote its methylation (Sato *et al*., 2006).

- Sox2.

The transcription factor Sox2 (SRY-type high mobility group box 2) is part of a large family of 20 proteins that share a similar HMG box DNA-binding motif. So far, it is the only Sox-protein found to have a crucial function in sustenance of ES cell pluripotency. Downregulation of Sox2 in murine ES cells promotes ES cell differentiation (Ivanova *et al*., 2006). Sox2 regulatory elements in gene promoter

regions are often found in close proximity to Oct3/4 and Nanog binding sites (Boyer, 2005). Several genes specific to ES cells are transcriptionally regulated by the combined action of Sox2 and Oct3/4. These include apart from Sox2, Oct3/4 and Nanog genes, as mentioned in the Oct3/4 section, also Fgf4 (Yuan *et al*., 1995), Utf-1 (Nishimoto *et al*., 1999), Fbx15 (Tokuzawa *et al*., 2003) and Zfp206 (Wang *et al*., 2007). It is possible that other ES-cell associated genes are regulated by combination of Sox2 with different transcription factors as in the case of Rex1, which is mainly activated by a combination of Sox2 and Nanog (Shi *et al*., 2006).

- c-Myc.

The helix-loop-helix/leucine zipper transcription factor Myc is associated with a number of cellular functions including cell growth, differentiation and proliferation but also with oncogenic transformation. c-Myc has been proposed as a major downstream target for two pathways that support maintenance of pluripotency: the LIF (leukaemia inhibitory factor)/STAT3 and the Wnt signalling cascades. However, its specific role in self-renewal of ES cells has not been thoroughly characterized yet. The first pathway associated with c-Myc, LIF signalling, is routinely used in murine ES cell culture but it does not appear to be necessary for the culture of human ES cells (Humphrey *et al*., 2004). LIF triggers by binding to a hetero-dimeric LIF-receptor a signalling cascade that results in activation and nuclear translocation of the transcription factor STAT3. STAT3, when overexpressed, is sufficient for the continued self renewal of mouse ES cells even in absence of LIF (Matsuda *et al*., 1999). c- Myc transcriptional activation was found to be one of the downstream targets of STAT3 in ES cells (Cartwright *et al*., 2005).

- Klf4.

The Kruepel-type zinc-finger transcription factor Klf4 is, like c-Myc, a downstream target of activated STAT3 in LIF-induced ES cells. Its overexpression leads to sustained expression of Oct3/4 and inhibition of differentiation in ES cells (Li *et al*., 2005). Similar to Sox2, Klf4 can also act as a co-factor for Oct3/4-mediated regulation of gene transcription. However, this seems to apply only to a very limited number of genes, including Klf4 itself and Lefty1 (Nakatake *et al*., 2006). There is so far no

evidence that Klf4 is required to exert a similar function for other target genes of Oct3/4 or Sox2. Klf4 may be indirectly involved in the upregulation of Nanog protein by repressing p53 (Rowland *et al*., 2005), a negative regulator of Nanog (Lin *et al*., 2005).

I.2.1.2 Autorregulatory circuitry of pluripotency

- Genetic regulation

Oct4, Sox2, and Nanog all bind to their own promoters, as well as the promoters of the genes encoding the two other factors (Boyer *et al*., 2005). This autoregulatory circuitry (see Fig. 1) suggests that the three factors function collaboratively to maintain their own expression. Oct4 and Nanog co-occupy and share a substantial portion of their target genes. In the mouse system, Nanog co-occupies 44.5% (345) of Oct4-bound genes (Loh *et al*., 2006) while 353 genes are co-bound by NANOG, OCT4 and SOX2 in the human ES cells (Boyer *et al*., 2005). These findings strongly suggest that the Oct4, Sox2 and Nanog converge and collaborate to regulate common genomic targets, which include both active and inactive genes (Boyer *et al*., 2005; Loh *et al*., 2006). One strategy that Oct4 and Nanog maintain pluripotency is by promoting the expression of downstream self-renewal genes while simultaneously repressing the activity of differentiation-promoting genes.

Loh *et al*. (2006) perturbed the level of transcription factors of interest, and showed that only a subset of the bound genes is responsive to the downregulation of Oct4 and Nanog levels, indicating that only a fraction of the binding loci are functional. They have short-listed 372 genes as primary Oct4 targets and further identified an Oct4 downstream gene, Tcl1, as a critical regulator of stem cell proliferation (Matoba *et al*., 2006). Ivanova *et al*. (2006) applied RNAi-mediated knockdown in a large-scale screen and identified 8 genes (Nanog, Oct4, Sox2, Tbx3, Esrrb, Tcl1, Dppa4 and Mm.343880) that are essential for sustaining an undifferentiated state of the ES cells. Thus, the mechanisms by which the 8 identified factors maintain pluripotency may include the direct repression of differentiation-promoting genes (Ivanova *et al*., 2006). The corresponding genes of numerous members in the interactome, including Esrrb, Rif1 and Sall4, have also been identified as bound targets of Oct4, Sox2 or Nanog (Boyer *et al*., 2005; Loh *et al*. 2006). Wu *et al*. (2006) showed that Sall4 physically

interacts with Nanog. ChIP analysis has further revealed considerable overlap in the binding sites of Nanog and Sall4, suggesting co-regulation of downstream target genes that include Nanog, Sall4, Oct4 and Sox2 (Loh *et al*., 2008). Together, these findings demonstrated that auto- and co-regulatory feedback loops are common phenomena in the ES cell transcriptional circuitry. Such a tightly inter-regulated pluripotency network that is intricately connected by protein-protein interactions may serve to stabilize gene expression patterns during self-renewal while ensuring a rapid response to differentiation cues (Loh *et al*., 2008).

The recurrence of factors such as Esrrb, Tbx3 and Sall4 identified in the various genome-wide studies (Boyer *et al*., 2005; Ivanova *et al*., 2006; Loh *et al*., 2006; Matoba *et al*. 2006; Wang *et al*., 2006) is an indication of their importance in the pluripotency regulatory network. However, functional and mechanistic studies supporting specific roles of the newly identified pluripotency-associated genes are limited.

- Epigenetic regulation.

The master regulators of pluripotency (see Fig.1 and Fig.2, pag. 28-29) occupy the promoters of active genes encoding transcription factors, signal transduction components, and chromatin-modifying enzymes that promote ES cell self-renewal (Boyer *et al*., 2005; Loh *et al*., 2006). However, these transcriptionally active genes account for only about half of the targets of Oct4, Sox2, and Nanog in ES cells. These master regulators also co-occupy the promoters of a large set of developmental transcription factors that are silent in ES cells, but whose expression is associated with lineage commitment and cellular differentiation (Boyer *et al*., 2005; Loh *et al*., 2006). Silencing of these developmental regulators is almost certainly a key feature of pluripotency, because expression of these developmental factors is associated with commitment to particular lineages. MyoD, for example, is a transcription factor capable of inducing a muscle gene expression program in a variety of cells (Davis *et al*., 1987). Therefore Oct4, Sox2, and Nanog likely help maintain the undifferentiated state of ES cells by contributing to repression of lineage specification factors. HOX gene clusters, in differentiated fibroblast cells but not in the ES cells, are bound by UTX (Lan *et al*., 2007), which corresponds to the demethylation of H3K27me3 and a concomitant

activation of the downstream genes (see Fig1, pag. 28). Lastly, it has been suggested that UTX may modulate the binding of Polycomb Repressive Complexes (PRC), which have been shown to regulate H3K27me3 at developmental genes including the HOX genes in ES ells (Boyer *et al*., 2006; Lee at al., 2006). Polycomb-group (PcG) proteins have been implicated to play an important role in maintaining pluripotency. Genome-wide studies have revealed that PcG proteins bind at many target genes containing H3K27me3 domains at their promoter regions (Boyer *et al*., 2006; Lee at al., 2006). These PcG-bound genes are repressed in ES cells, and are preferentially upregulated during cell differentiation.

Most of the transcriptionally silent developmental regulators targeted by Oct4, Sox2, and Nanog are also occupied by the Polycomb group (PcG) proteins (Bernstein *et al*., 2006; Boyer *et al*., 2006; Lee *et al*., 2006), which are epigenetic regulators that facilitate maintenance of cell state through gene silencing. The PcG proteins form multiple polycomb repressive complexes (PRCs), the components of which are conserved from Drosophila to humans (Schuettengruber *et al*., 2007). PRC2 catalyzes histone H3 lysine-27 (H3K27) methylation, an enzymatic activity required for PRC2-mediated epigenetic gene silencing. H3K27 methylation is thought to provide a binding surface for PRC1, which facilitates oligomerization, condensation of chromatin structure, and inhibition of chromatin remodeling activity in order to maintain silencing. PRC1 also contains a histone ubiquitin ligase, Ring1b, whose activity appears likely to contribute to silencing in ES cells (Stock *et al*., 2007). How the PcG proteins are recruited to genes encoding developmental regulators in ES cells is not yet understood. Some of the most conserved vertebrate sequences are associated with genes encoding developmental regulators, and some of these may be sites for DNA-binding proteins that recruit PcG proteins.

Recent studies revealed that the silent developmental genes that are occupied by Oct4, Sox2, and Nanog and PcG proteins experience an unusual form of transcriptional regulation (Guenther *et al*., 2007). These genes undergo transcription initiation but not productive transcript elongation in ES cells. The transcription initiation apparatus is recruited to the promoters of genes encoding developmental regulators, where histone modifications associated with transcription initiation and the

initial step of elongation (such as H3K4methylation) are found, but RNA polymerase is incapable of fully transcribing these genes, presumably because of repression mediated by the PcG proteins (see Fig.2, pag.29). These observations explain why the silent genes encoding developmental regulators are generally organized in ‘‘bivalent’’ domains that are occupied by nucleosomes with histone H3K4me3, which is associated with gene activity, and by nucleosomes with histone H3K27me3, which is associated with repression (Azuara *et al*., 2006; Bernstein *et al*., 2006; Guenther *et al*., 2007).

The presence of RNA polymerase at the promoters of genes encoding developmental regulators (Guenther *et al*., 2007) may explain why these genes are especially poised for transcription activation during differentiation (Boyer *et al*., 2006; Lee *et al*., 2006). Polycomb complexes and associated proteins may serve to pause RNA polymerase machinery at key regulators of development in pluripotent cells and in lineages where they are not expressed. At genes that are activated in a given cell type, PcG proteins and nucleosomes with H3K27 methylation are lost (Bernstein *et al*., 2006; Boyer *et al*., 2006; Lee *et al*., 2006; Mikkelsen *et al*., 2007), allowing the transcription apparatus to fully transcribe these genes. The mechanisms that lead to selective activation of genes encoding specific developmental regulators are not yet understood, but they almost certainly involve signals brought to the genome by signal transduction pathways and likely involve H3K27 demethylation by enzymes such as the JmjC (JumonjiC histone demethylase) domain- containing UTX and JMJD3 proteins (Lan *et al*., 2007).

The functional significance of the bivalent domains was evaluated recently in three independent studies, in human and mouse ES cells (Mikkelsen *et al*., 2007; Pan *et al*., 2007; Zhao *et al*., 2007). It was found that bivalent domains are present in differentiated cells and thus are not a unique feature of the epigenome of ES cells. Moreover, there is a prevalent occurrence of H3K4me3 modification that accompanies H3K27me3 mark on genes that do not have any roles in developmental processes. This raises the question of whether the bivalent domains are uniquely found at developmental genes. In summary, the role of bivalent domains in ES cell pluripotency remains controversial and awaits further confirmation.

- Linking the genetic and epigenetic systems.

Genome-wide location study revealed that Oct4 binds within Jmjd1a and Jmjd2c genes (Loh *et al*., 2006) (see Fig. 1, pag28). These genes encode for histone demethylases of the JmjC family (JHDM). Oct4 positively regulates the expression of these two HDMases (Loh *et al*., 2007). Importantly, depletion of Jmjd1a and Jmjd2c resulted in cell differentiation, indicating that these HDMases play essential roles in pluripotency. Jmjd1a positively regulates the expression of Tcl1, a gene regulating self renewal (Ivanova *et al*., 2006; Matoba *et al*., 2006) by binding and demethylating H3K9me2 at its promoter regions (Loh *et al*., 2007). The induction of H3K9me2 at Tcl1 after Jmjd1a depletion resulted in reduced Oct4 binding to the Tcl1 promoter. On the other hand, Jmjd2c regulates the expression of Nanog by modulating the demethylation of H3K9me3 at its promoter region (Loh *et al*., 2007). In the absence of Jmjd2c, the chromatin of the Nanog promoter is marked with H3K9me3 which leads to the recruitment of repressor proteins (Loh *et al*., 2007). These findings provide evidence for the regulation of chromatin accessibility in ES cell epigenome through the modulation of repressive H3K9me3 marks. This suggests a novel mechanism whereby Oct4 can upregulate the expression of its target genes indirectly by transcriptional activation of chromatin modifiers that keep target sites in a permissive chromatin state.

Activation of JHDMs by Oct4 may not be the sole mechanism that links genetic network to the epigenetic regulation of gene expression. Oct4 and Nanog have been shown to bind genes encoding chromatin-modifying complexes in both mouse (Loh *et al*., 2006) and human ES cells (Boyer *et al*., 2005). Zhou *et al*. (2007) detected significant site enrichment of Oct4 and its co-regulators in the conserved upstream regions of Phc1-bound genes (a member of PcG proteins) which are normally repressed in ES cells. This raises the possibility that Oct4 and its co-regulators may have a role in recruiting or modulating polycomb function. Other lines of evidence also support the connection between epigenetic system and transcriptional network. The protein interactome mapped for key transcriptional factors in mouse ES cell has uncovered the enrichment of chromatin modifiers in the interaction network (Wang *et al*., 2006). Specifically, histone deacetylase NuRD, polycomb group, SWI/SNF chromatin

remodeling complexes and co-repressor KAP1 are found to interact either directly or indirectly with Oct4 and Nanog (Wang *et al*., 2006). These results suggest that Oct4 and Nanog may govern the chromatin state of pluripotent ES cells by regulating or interacting with chromatin modifiers.

Fig 1. An interplay of transcription and epigenetic factors in the regulation of pluripotency.

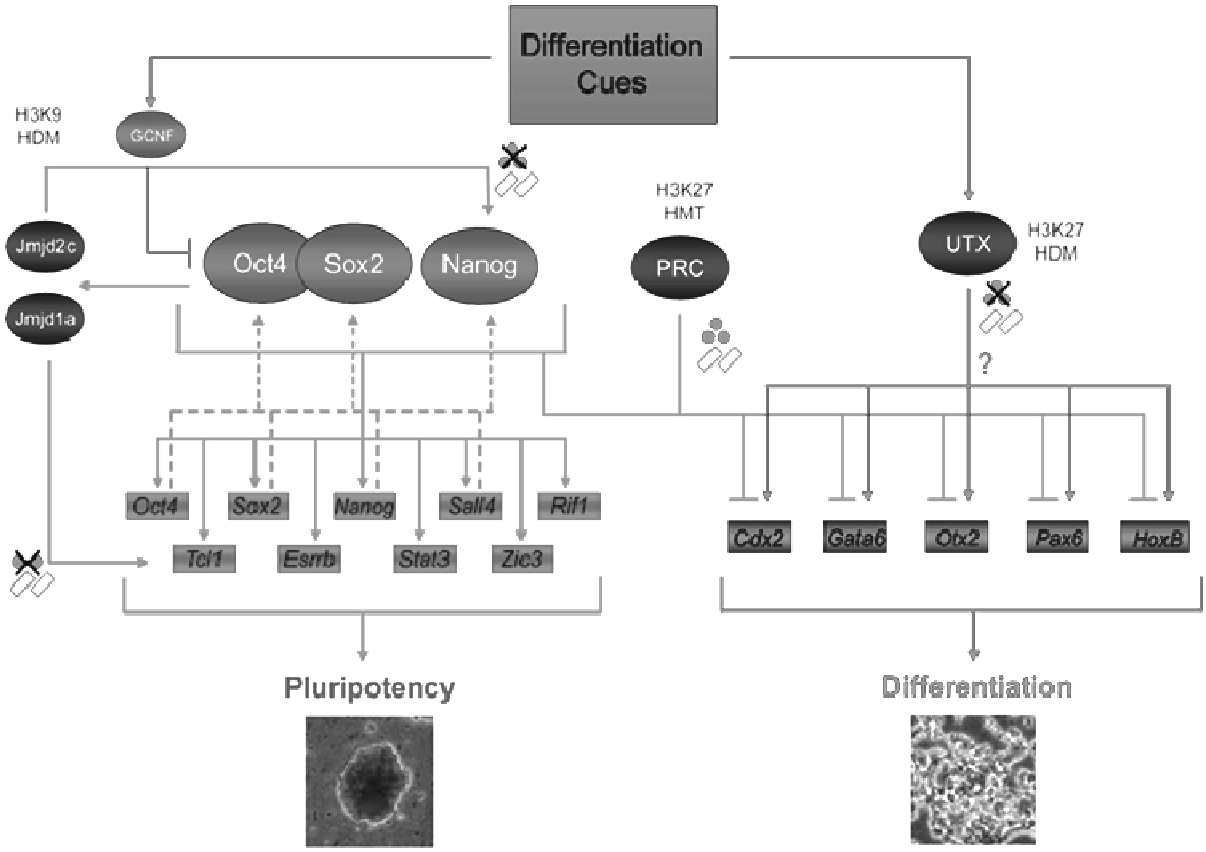
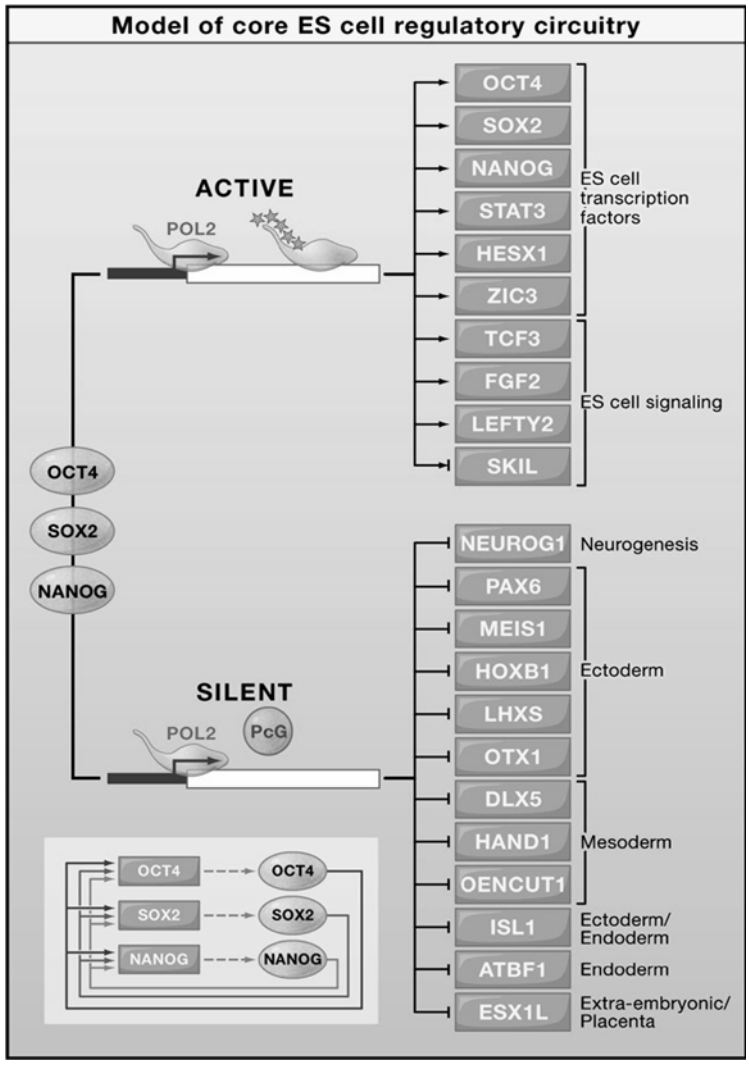


Fig 2. Model of core ES cell regulatory circuitry


Model of core ES cell regulatory circuitry
ACTIVE
POL2
OCT4
SOX2
NANOG
SILENT
POL2
PcG
OCT4
SOX2
NANOG
OCT4
SOX2
NANOG
STAT3
HESX1
ZIC3
ES cell transcription factors
TCF3
FGF2
LEFTY2
SKIL
ES cell signaling
NEUROG1
Neurogenesis
PAX6
MEIS1
HOXB1
LHXS
OTX1
Ectoderm
DLX5
HAND1
OENCUT1
Mesoderm
ISL1
Ectoderm/ Endoderm
ATBF1
Endoderm
ESX1L
Extra-embryonic/ Placenta

I.2.2. Strategies of somatic cell reprogramming.

Several different strategies such as cellular extracts exposition, cellular fusion, nuclear transplantation and culture induced reprogramming have been employed to induce the conversion of differentiated cells into an embryonic state.

I.2.2.1. Reprogramming by cellular extracts.

Pluripotency is established in different ways in the embryos of amphibians and mammals. In amphibians, unlike mammals, transcription from the zygotic genome is not activated until the mid-blastula stage (MBT), thus the establishment of pluripotency is directed by molecules stored in the egg (Morrison *et al*., 2006). This suggests that the chromatin of somatic cells exposed to amphibian oocytes might be remodelled to a pluripotent status directly, without the intervening cleavage stages that are required to produce pluripotent cells during mammalian development. Byerne *et al*. (2003) demonstrated that mammalian nuclei injected into the germinal vesicle (GV) of Xenopus oocytes can re-express the Oct4, directly, in the absence of DNA replication, accompanied by demethylation of the Oct4 promoter. Also, amphibian extracts have induced germ cell alkaline phosphatase (GCAP) in 293T cells and human primary leukocytes, but did not express surface antigens characteristic of pluripotent cells, indicating that reprogramming was incomplete (Hansis *et al*., 2004). Recently, epigenetic marks in somatic chromatin are remodelled to resemble pluripotent nuclei by amphibian extracts (Bian *et al*., 2009). Molecules present in axolotl oocyte extracts induce the reduction of the overall levels of H3K9me3, HP1, and DNA methylation of somatic cells, and they increase the levels of H3K9ac (Bian *et al*., 2009). Moreover, several factors that are involved in nuclear remodeling or dedifferentiation, such as ISWI (Kikyo *et al*., 2000), FRGY2a/b (Gonda *et al*., 2003), and especially, BRG1 (Hansis *et al*., 2004) and nucleoplasmin (Tamada *et al*., 2006) present in Xenopus extracts are also able to induce reprogramming events in mammalian cells.

Mammalian egg extracts have not commonly used because they are much smaller which makes difficult to prepare a sufficient volume of cell free extracts. Therefore, extracts from porcine germinal vesicle (GV) oocytes were used and induced

partial dedifferentiation after cell culture, as well as activated pluripotent marker genes, especially NANOG, were activated (Miyamoto *et al*., 2009).

Direct exposure of human somatic cell lines to the extracts of embryonic carcinoma cells or ES cells (Tarenger *et al*., 2005; Freberg *et al*., 2007) seems to have met with partial success in the reversion of some aspects of cell differentiation too. In most cases, re-expression of pluripotency marker, especially Oct3/4, were reported. It has been suggested that some studies cannot properly exclude the possibility that the reported re-expression of pluripotency- associated genes is due to material from the pluripotent cells (Hochedlinger and Jaenisch, 2006).

I.2.2.2 Reprogramming by pluripotent cell fusion.

Developmental pluripotency has been demonstrated in cell hybrids made between murine ESCs and somatic cells (Matveeva *et al*., 1996; Matveeva *et al*., 1998; Tada *et al*., 2001), as well as, embryonal carcinoma and even embryonic germ cells (Pralong *et al*., 2006). Hybrids contain reconfigured chromatin (Kimura *et al*., 2004), are pluripotent (Tada *et al*., 2003), and express stem cell markers such as Oct4 and Nanog (Hatano *et al*., 2005). Human fibroblasts have been successfully reprogrammed when they were fused with human ESCs (Cowan *et al*., 2005). In addition, human myeloid precursor cells have been reprogrammed to a pluripotent state by fusion with human ESCs (Yu *et al*., 2006).

However, hybrid cells lack therapeutic potential because of their abnormal ploidy (Pralong *et al*., 2006). Enucleation of ES cells before fusion may not be feasible in circumventing this problem as it has been shown to abolish the ability of the remaining ES cytoplast to re-activate expression of pluripotency markers in hybrids with somatic cells (Do and Scholer, 2004). The reprogramming activity of ESCs has been shown to reside within the nuclear compartment because karyoplasts, but not cytoplasts, derived from ESCs can bring about reprogramming of somatic cells upon fusion (Do and Scholer, 2004). The universal chromosome elimination cassette (CEC) can eliminate of a single embryonic stem cell (ESC)-derived chromosome 11 or 12, and also both copies of chromosome 6, which harbor pluripotency-associated genes including Nanog. Hybrid-cell pluripotency is attributed to the expression of Nanog from

the reprogrammed somatic-cell nuclei. So, the selective removal of chromosomes is possible but may be unfeasible for the complete set (Matsumura *et al*., 2007).

I.2.2.3. Reprogramming by somatic cell nuclear transfer.

It has been shown that nuclear transplantation, by which nuclei from fully differentiated cells are transplanted into oocytes or enucleated eggs, is able to reprogramme a differentiated state into a totipotent state and promote the development of a whole organism (Gurdon and Byrne, 2003). This can involve the erasure of the differentiated gene expression pattern, and also its epigenetic state, in the donor nucleus, followed by the re-establishment of totipotency in nuclear transplant embryos (Ng and Gurdon, 2005a).

- Reprogramming mechanisms after nuclear transfer.

  - Active demethylation.

The exact nature of the active demethylation is not well understood. Active demethylation is operatively defined as loss of methylation in the absence of DNA replication.

Three basic mechanisms have been proposed (Armstrong *et al*., 2006). The first and most simplistic would involve direct removal of methyl groups from the major groove of DNA. Although the mechanism by which this is achieved is uncertain, methyl binding domain protein (MBD2) has been shown to possess demethylase activity (Cedar and Verdine, 1999), with methanol as the stable leaving group. The methylated DNA binding protein 2 (MBD2) suppress methylated promoters by recruiting the chromatin remodeling complex NuRD, which contains HDACs (Ng *et al*., 1999). Independent attempts at verification of this result, by two different groups, failed to find demethylase activity for MBD2 (Hendrich *et al*., 2001). A second possible mechanism envisages the replacement of 5-methylcytosine by cytosine or removal of the CpG dinucleotide by either base or nucleotide excision repair (Klimasauskas *et al*., 1994). For this reason, the uridine deglycosylase enzyme methyl binding domain protein binding 4 (MBD4) was proposed as a potential demethylase because of its role

in DNA repair (Wu *et al*., 2003) although paternal-specific demethylation appeared to occur normally in MBD4 null fertilized oocytes (Santos and Dean, 2004). A third possibility proposes hydrolytic deamination of 5-methylcytosine by cytidine deaminases (AID and Apobec1) resulting in the conversion of 5MeC to thymidine. Genes for both proteins are expressed in oocytes (Morgan *et al*., 2004). However, this process would require considerable energy input and as such is the least likely mechanism (Armstrong *et al*., 2006).

It has been suggested that the activity of demetilases and cytidine deaminases coupled with base excision repair theoretically could result in demethylation without DNA replication (Eilertsen *et al*., 2007).

- Passive demethylation.

In the normal fertilized embryo, DNA replication appears to facilitate chromatin remodelling to create an embryonic genome. The first round of DNA replication is required to allow transcriptional activation of marker genes at the 2-cell stage in the mouse, while the second round of replication is required to silence those genes that are transiently expressed (Latham and Schultz, 2001). It may be that DNA replication also plays a critical role in the process of nuclear reprogramming, so that each round of DNA replication allows a greater array of genes to be reprogrammed. The number of rounds of replication required is not known, but it is likely that the process of reprogramming continues after implantation. The passive demethylation process in SCNT occurs to the same extent as in normal development (Beaujeaun *et al*., 2004) and thus seem unaffected by the putative introduction of different forms of Dnmt with somatic cell (Chung *et al*., 2003).

- Chromatin remodelling.

After transfer, nuclear envelope breakdown has occurred (Czolowska *et al*., 1984; Szollosi *et al*., 1988; Kim *et al*., 2002; Gao *et al*., 2004a), and the chromosomes are condensed in association with diminishing maturation promoting factor (MPF) activity (Szollosi *et al*., 1986, 1988; Kim *et al*., 2002; Gao *et al*., 2004a; Latham, 1999). During this time, somatic H1 linker histone variants are completely removed and

replaced with the oocyte-specific H1FOO variant (Gao *et al*., 2004a; Teranishi *et al*., 2004). H1FOO assembles on to the incoming somatic cell DNA within as little min of nuclear transfer. Short time later, depending of the species, no detectable somatic H1 variants remain associated with the chromatin (Gao *et al*., 2004a). This occurs in 100% of cloned constructs, indicating that this process is highly efficient. Interestingly, the ability of the ooplasm to remove somatic H1 from the chromatin is developmentally regulated, diminishing within 2–4 h of activation, and the timing of this loss is affected by the presence/ absence of the oocyte spindle chromosome complex, indicating that the spindle chromosome complex possesses factors that regulate a variety of process within the embryo in addition to chromosome segregation (Gao *et al*., 2004a).

As development proceeds through the 2-cell stage to the 4-cell stage, the H1FOO is largely eliminated from normal fertilized embryonic nuclei and the somatic H1 variants reappear, at a low level during the 2-cell stage and then at a greatly increased abundance at the 4-cell stage (Gao *et al*., 2004a). Thus there is a period during the 2-cell stage of a relative paucity of H1 linker protein of any type. This may lead to a temporarily diminished ability to regulate gene transcription. The array of genes that may be transcribed in cloned embryos during this transient period of transcriptional promiscuity potentially differs from the array of genes transcribed in fertilized embryos. Evidence that this is the case can be seen in the unusual culture requirements of cloned embryos (Latham, 2005).

It seems to be that nuclear remodeling of histone H3 phosphorylation and acetylation, but not H3 methylation (H3K9), of injected somatic nuclei in cloned pig took place in the oocytes under regulation by the oocyte cytoplasm (Bui *et al*., 2006). The chromosomes of somatic cells showed changes in histone H3 dephosphorylation and reacetylation, similar to oocytes after their activation. In contrast, histone H3 methylation (H3K9) of somatic cell nuclei did not show any significant change after injection and electroactivation of the oocytes.

The polycomb repressive complex 2 (PRC2) component genes (Eed, Ezh2, and Suz12), which are responsible for the generation of H3K27me3, were expressed at lower levels in the mouse cloned embryos. Reduced expression of PRC2 component

genes in cloned embryos results in defective modification of H3K27me3 to the differentiation-related genes in pluripotent ICM cells (Zhang *et al*., 2009).

SNF2-type ATP-dependent chromatin factors (Brahma, Brg1, SNF2H, SNF2L, CHD3, and CHD5) contribute to epigenetic reprogramming and the relative amount of these factors in the donor cell affects developmental potential of the reconstructed embryos. Higher morula/blastocyst rates were correlated with lower amount of SNF2L and CHD3 transcripts (Magnani *et al*., 2008).

It has been suggested that the inefficiency of NT success could reflect the poor ability of oocytes citoplasmic factors to entirely remove the epigenetic features of a somatic cell, a function that is not normally required of the oocytes (Cervera, 2004).

- Consequences of aberrant epigenetic reprogramming.

A. Aberrant demethylation patterns.

The studies of several groups have shown that the somatic genome used in NT does not respond so readily to the demethylation activity of the oocytes (Han *et al*., 2003a; Kang *et al*., 2001,2002), and in most cases the level of methylated DNA remains much higher than in normal embryos, a state more reminiscent of somatic cells.

After fusion with the recipient sheep oocytes the methylation patterns of the adult fibroblast donor nucleus appear to either stay methylated or partially demethylated (Beaujean *et al*., 2004). At 2-cells stage, in sheep SCNT embryos, methylated and partially demethylated nuclear types can still be distinguished; however, highly methylated embryos do not seem to survive the subsequent claveage division beyond the 8-cells stage. This suggests that the initial nuclear demethylation after fusion is crucial for further development, at least in ovine (Beaujean *et al*., 2004).

Alterations in global methylation levels have been observed in abnormal or dead bovine SCNT foetuses or calves, compared with either normal controls or apparently normal clones. Some studies reported hypermethylation (Hiendleder *et al*. 2004) and others, hypomethylation (Cezar *et al*. 2003). Such conflicting data emphasise the importance of comparing the same DNA sites in equivalent tissues and developmental stages between studies and using comparable methodologies.

The significance of these observations is unclear as the effect of altered methylation levels on gene expression is generally unknown. It is not possible to conclude if the altered methylation levels caused the abnormalities and developmental failure or if abnormal development has lead subsequently to improper DNA methylation (Niemman *et al*., 2008).

B. Epigenetic memory.

The term "epigenetic memory" refers to the transmission of a gene expression state through multiple cell generations in the absence of initiation signals and genetic variation (Bird, 2002). It is generally believed that the inheritance of these epigenetic marks, from mother to daughter cells, is crucial for the maintenance of a cell differentiation state. The epigenetic memory could be propagated by various epigenetic mechanisms such as DNA methylation, histone modifications, replacement of histone variants, Polycomb group (PcG) and Trithorax group (TrxG) proteins (Ringrose and Paro, 2004), among others.

Cloned embryos display persistent expression of some molecular markers of somatic nuclear donor cells. Some Xenopus nuclear transplant embryos showed a persistent expression of their donor-specific genes in the wrong cell lineage (Ng and Gurdon, 2005b) even after more than 24 mitotic cell divisions (Ng and Gurdon, 2008). In addition to aberrant gene expression, some cloned embryos have aberrant epigenetic patterns, affecting DNA methylation and histone 3 lysine 9 (H3K9) methylation, so that they resemble the donor epigenetic state (Dean *et al*., 2001; Santos *et al*., 2003).

Bovine cloned embryos display nucleoli with a transcriptionally active morphology, indicating that reprogramming of rRNA genes is slow (King *et al*., 1996; Lavoir *et al*., 1997). They also display heterogeneity in mitochondrial morphology, indicating possible alterations in mitochondrial gene expression (King *et al*., 1996). Centromeric heterochromatine, which is incompletely methylated in normal blastocyst, remained more methylated in cloned bovine embryos. The persistence of high methylation level in centromeric heterochromatine my also be a source of disturbance of early embryonic activity, because heterochromatine has been involved

in gene silencing in mammals and other organisms (Brown *et al*., 1997; Henikoff, 2000).

Ultrastructural analysis of bovine nuclear transfer embryos revealed widespread transcriptional silencing following nuclear transfer and then transcriptional re-activation much like normal embryos, but with an excess production of ribosomes (Kanka *et al*., 1996). One study examining the change in methylation status of specific sequences showed that the CpG sites in the galanin gene that were methylated in the donor cell DNA were demethylated after nuclear transfer and underwent re-methylation at exactly the same positions in a manner that recapitulated the events in normal bovine IVF development (Kang *et al*. 2002).

Bovine clones also expressed elevated levels of insulin-like growth factor 2 (Han *et al*., 2003). In transplants of mouse embryo fibroblasts to bovine oocytes, Hsp 70.1 was not silenced after nuclear transfer (Arat *et al*., 2003). In addition, cloned mouse embryos prepared with myoblast nuclei continue to express the glucose transporter GLUT4, which is normally expressed in muscle but not in early embryos (Gao *et al*., 2003).

Other molecular markers expressed in donor cells are down-regulated after nuclear transfer. The TEC-3 surface antigen appears to be silenced correctly in cloned embryos made with blastomere nuclei, and is then re-expressed at the appropriate time (Van Stekelenburg-Hamers *et al*., 1994; Trounson *et al*., 1998). Phosphofructokinase, eIF1A and MyoD was also downregulated in bovine clones (Bordignon *et al*., 2001)

Along with the altered expression of specific molecular markers, other molecular alterations are evident in effects on basic cellular processes in cloned embryos. For example, expression of Dnmtsom, the somatic form of the DNA methyltransferase, Dnmt1, in cloned embryos occurs in striking contrast with normal embryos (Chung *et al*., 2003). Normal embryos express the mRNA for the somatic form, but not the protein (Ratnam *et al*., 2002). The expression of this protein thus indicates a loss of appropriate post-transcriptional mechanisms that normally prevent this from happening. Nuclear import of the abundant supply of the oocyte form of Dnmt1 (Dnmto) is also inhibited in clones (Chung *et al*., 2003), indicating possible

defects or alterations in the array of proteins that can be transported from cytoplasm to nucleus. Alterations in cytoplasm–nuclear protein transport may be particularly relevant to the overall ability of nuclear reprogramming to proceed, as reprogramming should be dependent upon access of specific transcription and chromatin remodelling factors to the nuclear compartment.

The foregoing observations thus indicate that nuclear reprogramming remains largely incomplete during the pre-implantation period, with some genes being silenced and others not being silenced (Latham, 2005).

Along with persistent activation of somatic cell characteristics, a number of studies have revealed deficiencies in activating embryonically expressed genes. The expression of Oct4 is variable between cells (i.e. mosaic) and reduced in a majority of cloned embryos (Boiani *et al*., 2003), and Oct4-related genes are likewise under-expressed (Bortvin *et al*., 2003). It has found that the transcription requiring complex (TRC), a complex of proteins that provides a transiently expressed marker of embryonic genome activation, is expressed in cloned embryos, but this expression is generally reduced relative to controls (Gao *et al*., 2004b).

Thus, the slow and incomplete pattern of silencing of somatic genes is accompanied by similarly slow or incomplete activation of embryonic genes, revealing at a molecular level the slow, progressive nature of nuclear reprogramming (Latham, 2005).

C. X-chromosome-linked development and gene expression.

- X-chromosome inactivation in mammals.

In general terms, transcription of X-linked genes is restricted to a single, active X chromosome (Xa) and in females inhibited on the other inactive X chromosome (Xi).

The X-inactivation process is initiated early in embryogenesis by transcription of XIST from one of the two X chromosomes that is set to be inactivated (Xi) and subsequent coating of the same X chromosome by the untranslated XIST RNA (Brockdorff, 2002; Heard, 2004; Chang *et al*., 2006). The choice of which X chromosome becomes inactive appears to be under an imprinted control, where random X-inactivation occurs in the inner cell mass derivatives and preferential

inactivation of the paternal X occurs in the trophoblast derivatives (Chang *et al*., 2006). Immediately after XIST RNA coating begins, the Xi undergoes various chromatin modifications such as loss of methylation on H3 lysine 4 (H3K4), methylation of histone H3 lysine 9 (H3K9) and methylation on H3 lysine 27 (H3K27) and these changes lead to transcriptional silencing (Peters *et al*., 2002, Plath *et al*., 2003, Chadwick and Willard, 2004) and late-replication of the Xi (Keohane *et al*., 1999). However, histone modification such as H3K27 methylation is not sufficient for silencing of the X chromosome (Plath *et al*., 2003). Hence, the inactive state is synergistically maintained through other chromatin modifications such as hypoacetylation at histone H4, macroH2A recruitment and DNA methylation (Csankovszki *et al*., 2001). Nonetheless, the functional links between methylated DNA and histones on the X chromosome are extremely stable and are maintained throughout all subsequent cell divisions and life (Avner and Heard, 2001).

- Effects of SCNT on X-chromosome-linked mRNA expression.

One of the epigenetically regulated developmental mechanisms potentially affected by faulty reprogramming is X-inactivation. Indeed, some SCNT embryos and offspring have been shown to exhibit aberrations in the X-chromosome inactivation. Although the inactive X of the donor cells can be successfully reactivated by the recipient cytoplast, there was heterogeneity within SCNT mouse blastocysts for X-inactivation, with cells showing zero, one or two inactive X chromosome(s) (Nolen *et al*., 2005).

The use of a donor cell line containing an X chromosome which causes preferential inactivation of the normal X chromosome resulted in aberrant inactivation patterns in the organs and tissues of stillborn and dead new-born calves which included random inactivation of the normal and abnormal X, and inactivation of both X chromosomes (Niemann *et al*., 2008).

In bovine embryos, XIST RNA, the initiator of X-inactivation, was found in samples taken from pools of male SCNT blastocysts, but not male in vivo blastocysts (Nino-Soto *et al*., 2007). The pattern of X-inactivation in aborted bovine SCNT foetuses and dead newborn calves was found to be altered (Xue *et al*., 2002). Placental samples

exhibited random X-inactivation as opposed to the non-random preferential paternal X-inactivation seen in normal controls and healthy SCNT calves. In addition, nine out of ten X-linked genes examined in various organs and tissues of the dead cloned calves were aberrantly expressed.

D. Telomere length and somatic cloning.

Telomeres are the natural ends of linear chromosomes and play a crucial role in maintaining the integrity of the chromosomal DNA by preventing loss of terminal coding DNA sequences and preventing end-to-end chromosome fusion. Telomerase is critically involved in maintaining normal telomere length and is active in haematopoietic, cancer and germ cells and in early embryos at the blastocyst stage (Blasco *et al*., 1999). Changes in the telomere length are closely related to ageing and cancer (de Lange, 2002). As a general rule, some loss of telomere length occurs with each cell division as a result of the incomplete replication of the lagging strand.

Telomeres of Dolly, the cloned sheep, which was derived from an adult mammary epithelial cell, were shorter when compared with age-matched, naturally bred counterparts and correlated with telomere length of the donor cells (Shiels *et al*., 1999). Subsequently, however, the vast majority of cloning studies reported that telomere length in cloned cattle, pigs, goats and mice are comparable with age-matched, naturally bred controls even when senescent donor cells were used for cloning (Jiang *et al*., 2004, Betts *et al*., 2005, Jeon *et al*. 2005, Schaetzlein and Rudolph, 2005). The regulation of telomere length is to some extent related to the type of donor cells employed for cloning. The telomere length in cattle cloned from fibroblasts or muscle cells was similar to that of age-matched controls, while clones derived from epithelial cells did not have telomeres restored to normal length (Miyashita *et al*., 2002). A check point for the elongation of telomeres to their species determined length has been discovered at the morula to blastocyst transition in bovine and mouse embryos (Schaetzlein *et al*., 2004). Telomeres were at the level of the donor cells in SCNT morulae, whereas at the blastocyst stage telomeres had been restored to normal length. The telomere elongation process at this particular stage of embryogenesis is

telomerase dependent since it was abrogated in telomerase deficient mice (Schaetzlein *et al*., 2004).

I.2.2.4. Reprogramming by ectopic expression of transcription factors: induced Pluripotential Stem Cells (iPS cells).

Takahashi and Yamanaka (2006) found that differentiated cells can be reprogrammed to an embryonic-like state by introducing four factors, Oct3/4, Sox2, c-Myc, and Klf4, under ES cell culture conditions. Unexpectedly, Nanog was dispensable. Initially mouse embryonic fibroblasts (MEFs) and tail-tip fibroblasts (TTFs) of mice homozygous for a knocking of a neomycin-reporter cassette into the Fbx15 (also known as Fbxo15) gene locus were employed. Selection of cells indeed allowed growth of ES-cell-like colonies and cells derived by sub-culturing these ES-like colonies were designated as 'induced pluripotent stem (iPS)' cells. These cells, exhibited the morphology and growth properties of ES cells and express some ES cell marker genes. Subcutaneous transplantation of iPS cells into nude mice resulted in tumours containing a variety of tissues from all three germ layers. Following injection into blastocysts, iPS cells contributed to mouse embryonic development. Fbx15 iPS cells, however, had the different gene expression and DNA methylation patterns compared with ES cells and did not contribute to adult chimaeras. The generation of iPS cells from fibroblasts is a gradual process that takes between 15 and 20 days upon infection of somatic cells with retroviruses expressing Oct4, Sox2, Klf4, and c-Myc, giving rise to iPS cells at a frequency of less than 0.1% (Maherali *et al*., 2007; Takahashi and Yamanaka, 2006; Wernig *et al*., 2007).

- Improving iPS cells obtaining.

As well as selection for Fbx15 (Takahashi and Yamanaka, 2006), iPS cells were also isolated using drug selection for Oct4, or Nanog (Maherali *et al*., 2007; Okita *et al*., 2007; Wernig *et al*., 2007). Curiously, iPS cells produced with Fbx15 selection were less potent than ES cells, while iPS cells produced with either Oct4 or Nanog selection appeared functionally and molecularly indistinguishable from ES cells, suggesting that Fbx15 is a less stringent selection marker than Oct4 and Nanog. Fbx15-selected iPS

cells contributed to diverse tissues in midgestation embryos; however, these embryos succumbed at midgestation. Consistent with this observation, only part of the ES cell transcriptome was expressed in iPS cells, and methylation analyses of the chromatin state of the Oct4 and Nanog promoters demonstrated an epigenetic pattern that was intermediate between that of fibroblasts and ES cells. Nanog is essential for embryonic development and is required for the maintenance of pluripotency by suppressing differentiation into primitive endoderm. Fbx15, in contrast, is not essential for pluripotency or development despite its exclusive expression in ES cells (Chambers *et al*., 2007).

Although Nanog selection allowed the generation of high quality iPS cells, the c-myc retrovirus reactivation, which may result in tumor formation, was observed (Okita *et al*., 2007). Omission of c-Myc from the reprogramming cocktail further reduces the efficiency and delays the process (Nakagawa *et al*., 2008; Wernig *et al*., 2008). Established iPS cells show silencing of retroviral genes and the re-expression of endogenous pluripotency genes such as Oct4 and Nanog (Maherali *et al*., 2007; Okita *et al*., 2007; Wernig *et al*., 2007). Moreover, iPS cells reactivate the silenced X chromosome in female cells, restore telomerase activity, and re-establish a genome-wide histone methylation pattern characteristic of ES cells (Maherali *et al*., 2007; Takahashi and Yamanaka, 2006).

A doxycycline-inducible lentiviral system has been generated to transiently express the four reprogramming factors c-Myc, Klf4, Oct4, and Sox2 in fibroblasts (Werning *et al*., 2008: Welstead *et al*., 2008). iPS cells produced with this system are pluripotent and give rise to chimeras when injected into blastocysts, similar to retrovirally produced iPS cells. It has recently shown that drug selection is not required to obtain iPS cells (Blelloch *et al*., 2007; Maherali *et al*., 2007; Meissner *et al*., 2007). However, omission of drug selection increases the number of false positive colonies, such as transformed cells or cells that failed to receive all four viruses, and necessitates a trained eye to identify ES cell-like colonies. A major advantage of the inducible system over constitutive expression systems is that it allows for the ‘‘self-selection’’ of reprogrammed cells in the absence of drug selection and obviates the need for ES cell expertise. After stably reprogrammed cells have been generated and doxycycline has

been withdrawn, cells that survive go on to reactivate the endogenous pluripotency program, while unstable reprogramming intermediates and transformed colonies disappear, likely through differentiation or apoptosis (Stadtfeld *et al*., 2008).

To achieve more efficiency in the iPS obtaining, the Dox-inducible lentivirus vectors were used to generate a "secondary system" of iPS cells. This was achieved by infecting fibroblasts with Dox-inducible lentiviral vectors carrying the four reprogramming factors (Brambrink *et al*., 2008; Stadtfeld *et al*., 2008). When cultured in the presence of Dox, multiple iPS lines were generated that could be propagated independently of Dox. As a next step, 'primary' iPS lines were injected into blastocysts to generate embryonic or adult mouse chimeras, thus allowing clonal expansion and their re-differentiation into multiple somatic cells types in vivo. Because the injected iPS lines carried a constitutively expressed antibiotic-resistance gene, homogenous iPS-derived somatic cell populations such as embryonic fibroblasts, mesenchymal stem cells, neural precursors and lymphocytes could be isolated that carried identical provirus integration patterns as those in the primary iPS cell line (Hanna *et al*., 2008; Werning *et al*., 2008). Cultivation of these 'secondary' somatic cells in the presence of doxycycline efficiently generated 'secondary' iPS cells, which grew independently of Dox and were shown to be pluripotent by stringent criteria. Two major advantages are offered by this strategy. First, because secondary somatic cells do not require new vector-mediated factor transduction, cells that are difficult to infect can be reprogrammed. Second, the approach avoids the genetic heterogeneity produced by direct viral infection of somatic cells (Hanna *et al*., 2008; Werning *et al*., 2008). More recently, it has also established a secondary system for human cells (Hockemeyer *et al*., 2008).

Despite the use of non-integrated adenoviral vectors, as the lentiviral system (Stadtfeld *et al*., 2008), the genetic transfer of viral into somatic cells is critical. Specifically, because of uncontrolled insertions into the genome, lentiviral and retroviral vectors may potentially activate endogenous oncogenes when iPS cells generated by such methods would be transplanted (Nienhuis *et al*., 2006).

- Which techniques can be regarded as alternatives?

At first, the use of non-viral vectors may be an alternative. Repeated transfections of expression plasmids containing the cDNA of the four pluripotency-associated genes into mouse embryonic fibroblasts resulted in virus vectorfree iPS cells without evidence of plasmid integration (Okita *et al*., 2008). An optimal solution would be to induce reprogramming directly by chemical factors ('small molecules'), which specifically modulate the epigenetic status of the cells. Small molecules offer an alternative to replace virally transduced transcription factors with chemical signalling cues responsible for reprogramming. For example, a specific inhibitor of histone methyltransferases in conjunction with the transduction of only two transcription factors (Oct4 and Klf4) enabled mouse foetal neural progenitor reprogramming into iPS cells (Shi *et al*., 2008). Also, iPS cells have been recently generated from human fibroblasts in absence of exogenous Sox2 using an inhibitor of lysine-specific demethylase (Li *et al*., 2009). Another molecule, the histone deacetylase inhibitor valproic acid (VPA), enabled pluripotency reprogramming of human fibroblasts with two pluripotency-associated transcription factors (Huangfu *et al*., 2008). Recently small molecule screening scaffols to identify compounds that functionally replace the reprogramming factor Klf4.

This demonstrates that there are specific cell types which may be more easily reprogrammed into iPS cells and would allow reprogramming by using only one or two transcription factors. Recent data showed that adult mouse neural stem cells (which endogenously express Sox2) could be reprogrammed by viral transfer of only one transcription factor, Oct-4 (Kim *et al*., 2009).

A novel approach was recently reported to reduce the number of viruses by delivering reprogramming factors in a single virus using 'self-cleaving' peptides, which facilitated efficient polycistronic expression from a single promoter (Carey *et al*., 2009). In this case, the four reprogramming factors, Oct4, Sox2, Klf4 and c-Myc, could be expressed from a single virus to generate iPS cells. The study showed that insertion of the transduced pluripotency genes is not required for in vitro reprogramming. In addition, a virus-free approach was applied with the direct delivery of transcription factor proteins to somatic cells by the use of protein transduction domains or

immunoliposomes (Heng and Richards, 2008). Moreover, genetic strategies that allow removal of the viral vectors after successful transfer of pluripotency associated genes from the iPS cells were reported. Two groups demonstrated successful reprogramming of mouse fibroblasts by using doxycycline-inducible transcription factors delivered by 'piggyBac' transposition (Woltjen *et al*., 2009) and showed that the individual vector insertions needed for introducing the reprogramming factors could be removed from the iPS cells (Kaji *et al*.,2009).

A further step forward to successful reprogramming of adult human cells was the derivation of reprogramming 'factor-free' iPS cells by the use of Cre-recombinase excisable viruses (Soldner *et al*., 2009) and the use of episomal vectors (Yu *et al*., 2009). The data presented evidence that iPS cells after removal of transducing viral vectors or episomes were free of transgene sequences.

Although these elegant techniques avoid the permanent integration of viral vectors, cells may still be genetically or epigenetically altered by the previous vector integration.

Therefore, the recent report on protein-induced reprogramming of mouse embryonic fibroblasts without genetic modification indicated a further breakthrough (Zhou *et al*., 2009). The authors demonstrated the generation of protein-induced pluripotent stem (piPS) cells by repeated transductions of four recombinant cell-penetrating reprogramming proteins (Oct4-11R, Sox2-11R, Klf4-11R and c-Myc-11R) in combination with valproic acid (VPA) treatment. However, further methodical improvements will be necessary to increase the reprogramming efficiency, a requirement for routine application.

- Sequential expression of pluripotent markers.

The mechanism and nature of molecular changes underlying the process of direct reprogramming remain largely mysterious (Jaenisch and Young, 2008)

The immediate response to induction of the reprogramming factors in wild-type MEF cells is observed by de-differentiation state and up-regulation of proliferative genes (Mikkelsen *et al*., 2008). De-differentiation is evident in a significant decrease in expression levels of typical mesenchymal genes expressed in MEFs (for example, Snai1

and Snai2). The proliferative response is evident in up-regulation of genes with functions such as DNA replication (Poli, Rfc4 and Mcm5) and cell cycle progression (Ccnd1 and Ccnd2). This response may be consistent with expression of reprogramming factor c-Myc (Yamanaka, 2007; Adhikary and Eilers, 2005).

The expression of stress-induced and anti-proliferative genes also experiment strong increase. In particular, upregulation of Cdkn1a and Cdkn2a is detected, which encode cyclin-dependent kinase (CDK) inhibitors that are key effectors of multiple differentiation and tumour suppressor pathways (Mikkelsen *et al*., 2008). Cdkn1a is a downstream target of the reprogramming factor Klf4 (Rowland and Peeper, 2005), whereas Cdkn2a is known to be activated by deregulated c-Myc expression (Gregory *et al*., 2005). This response was followed by gradual up-regulation of genes associated with differentiating MEFs on days 12–16.This suggests that induction of the reprogramming factors triggers normal 'fail-safe' mechanisms that act to prevent uncontrolled proliferation, which may prevent the majority of cells from reaching a stably dedifferentiated state.

Strong up-regulation of lineage-specific genes from unrelated lineages is also detected. These include axon guidance factors (Epha7 and Ngef), epidermal proteins (Krt14, Krt16, Ivl and Sprr1a) and glomerular proteins (Podxl). It is suggested that this gene activation reflects responses to the reprogramming factors Sox2 and Klf4, which, independent of their roles in ES cell regulation, function in neural, epidermal and kidney differentiation (Rowland and Peeper, 2005; Yamanaka, 2007).

In embryonic stem cells, virtually all high-CpG promoters (HCPs) are enriched with H3K4me3. Some pluripotency- and germ-line specific genes show loss of both H3K4me3 and H3K27me3 in somatic cells, and this correlates with DNA hypermethylation (Imamura *et al*., 2006).

At all pluripotency- and germ-line-specific genes examined in the iPS cells, the promoters have regained H3K4me3-enrichment and show DNA hypomethylation. At genes encoding lineage-specific transcription factors that are transcriptionally silent in embryonic stem cells, the pattern is typically re-established (Mikkelsen *et al*., 2008).

So, it is suggested that direct reprogramming to a pluripotent state involves re-activation of endogeneous pluripotency-related genes, establishment of an 'open'

chromatin state (as indicated by genome-wide H3K4me3 enrichment and DNA de-methylation), and comprehensive Polycomb-mediated repression of lineage-specifying genes (as indicated by chromatin states involving H3K27me3-enrichment) (Mikkelsen *et al*., 2008).

I.2.2.5. Differences in the mechanisms of reprogramming.

Will the mechanism of reprogramming be the same in nuclear transfer to eggs or iPS experiments? Probably not. It may due to the following considerations. (1) Unlike SCNT, in vitro induced reprogramming by factors seems to depend on active proliferation of the somatic cells. (2) Reprogramming by SCNT likely occurs in a shorter time span than in vitro reprogramming by factors. For example, Oct4 is activated at the 2 to 4 cell stage in cloned embryos (Boiani *et al*., 2002), and major chromatin modifications are detected early after nuclear transfer (Santos *et al*., 2003) suggesting that the epigenetic state of the somatic donor genome is reset within a few cell divisions. In contrast, in vitro reprogramming has been shown to be a protracted process that proceeds over many weeks.

A general hypothesis to explain why to reset the gene expression becomes increasingly difficult as cells become more differentiated is the idea of “fletting access” (Gurdon and Melton, 2008). The combinations of DNA binding or chromosomal proteins become increasingly tightly associated with the regulatory regions of inactive genes. Even though most proteins are thought to dissociate from DNA at frequent intervals of seconds or a few minutes (Catez *et al*., 2004), and in a few instances for longer (Yao *et al*., 2006), a multicomponent complex as a whole may have a very long dwell time on inactive genes. It will be a very rare event for a sufficient number of individual proteins in a complex to dissociate from a chromosome at the same time for a gene region to be accessible to reprogramming factors. In embryonic cells, most genes (and in differentiated cells, the active genes) will be in a decondensed configuration with relatively short dwell times for multicomponent complexes (Gurdon and Melton, 2008).

According to this view, the probability of reprogramming taking place in nuclear transfer, cell fusion and iPS experiments would depend on the statistical access

frequency of gene regulatory regions together with the duration and concentration of transcription or other regulatory factors. The concept of fleeting access will be the same, but the actual reprogramming molecules will be different. It is know that eggs have very high concentrations of certain molecules such as nucleoplasmin and histones B4 and H3.3. The eventual identification of egg-reprogramming molecules may well be able to enhance the efficiency of the iPS (Gurdon and Melton, 2008).

I.3. Cell reprogramming in biomedicine.

I.3.1. Regenerative medicine.

Researchers are taking three primary approaches in an attempt to enhance the regenerative capacity in mammals: (1) transplantation of stem cells, progenitor cells, differentiated cells or, recently iPS cells into the injured organ (2) transplantation of cell-seeded scaffolds (both biodegradable and nonbiodegradable) into the damaged region; and (3) induction of endogenous regeneration through the activation of resident stem cells or by inducing cells in the vicinity of the injury to dedifferentiate and once again acquire the properties of stem cells or progenitor cells

I.3.1.1. Cell therapy.

- Transplantation of human Embryonic Stem cells (hES cells).

Few years ago, there has been growing interest in stem cells potential for clinical exploitation in the repair or replacement of an ever-expanding range of failing or damage organs (Hadjantonakis and Papaioannou, 2001). ESCs might be introduced for treating several pathologies, including diabetes (Soria *et al*., 2000), spinal cord injuries (Hendricks *et al*., 2006) and liver (Duan *et al*., 2007) and heart (Kofidis *et al*., 2005) transplantation. ES cells could be derived from several sources such as from cryopreserved embryos or surplus embryos derived from *in vitro* fertilisation procedures, from embryos resulting from anomalous fertilisation (Cervera, 2004). However, ES cells from previously detailed sources, certainly provoke immune rejection and the need of immunosuppressive treatment for the rest of the patient's life (Clarke and van der Kooy, 2009). It has been suggested that hESCs are in some way immune privileged and do not invoke the same immune response as other allogeneic tissue. It has been shown that MHC class II molecules are not expressed on hESC or their differentiated progeny, and it has even been shown that hESCs do not illicit T-cell activation *in vitro*; however, more recently, it has been shown that they express low levels of MHC class I, which is upregulated on differentiation and further modulated by inflammatory cytokines such as interferon gamma (Hyslop *et al*., 2005). However, hESC

lines in which MHC molecules or other immune effectors have been deleted by genetic manipulation have been obtained (Hyslop *et al*., 2005).

The only way to avoid the immune rejection consists of obtaining ES cells from the patient him/herself by nuclear transfer, process known as therapeutic cloning.

- hES cells from embryos produced by nuclear transfer: Therapeutic cloning.

Therapeutic cloning involves the transfer of the nucleus of a somatic cell from the individual, who requires the replacement or supplementation of diseased or damaged tissue, into an enucleated donor oocyte, which would then be used to derive a blastocyst and subsequently isolate hESC lines that would be genetically and immunologically compatible cells and tissues to the patient (Lanza *et al*., 1999; Yang *et al*., 2007). However, despite the claims of the South Korean Dr Hwang Woo-suk in 2005 that have subsequently been shown to be fraudulent, SCNT has not been successfully performed in human oocytes. It appears that the process is more complicated in humans than in animals where this technology has been used successfully in many species (Mountford, 2008).

Therapeutic cloning has arisen as consequence of two major scientific advances: (1) the success of nuclear transfer (NT) from adult somatic cells in several species (Wilmut *et al*., 1997; Tanaka and Kanagawa, 1997; Wakayama *et al*., 1998), and (2) the derivation of ES cells from human embryos (Thompson *et al*., 1998).

Because therapeutic cloning requires obtaining and cell ablation of blastocyst stage cloned embryos, it raises complex ethical questions (Shapiro, 1999), which can fall into three categories. The first pertains to all cell replacement technologies using stem cells derived of human embryos. A second objection has to do with the fear that therapeutic cloning opens the way to the eventual cloning of a human being through the reproductive uses of these technologies. A third set of objections focuses on perceived threat to women's health and freedom, from the potential market in human eggs that therapeutic cloning may create.

- hES cells from interespecific somatic cell nuclear transfer.

Mechanism regulating early embryonic development may be conserved among mammalian species, in that bovine oocyte cytoplasm supports early development of NT embryos from many mammalian species including cow, sheep, pig, rat (Dominko *et al*., 1999; Sugawara *et al*., 2009), chimpanzee (Wang *et al*., 2009) and human (Lanza *et al*., 1999). It has been shown that human somatic cell nuclei can be reprogrammed using non-human mammalian oocytes (rabbit oocytes) to develop to the blastocyst stage at least (Chen *et al*., 2003).

Nuclear transfer ES cells (NTES) isolated from interespecific NT reconstructed embryos at the blastocyst stage possess many properties of hES cells; they retain a normal kariotype, expression of surface markers, special growth requirements, such dependence in feeders and independence in LIF, capabilities of self-renewal, formation of embryoid bodies and differentiation into cells of all three germ layers (Chen *et al*., 2003).

The main problem in interespecific NT is the mitochondia mosaicism in the reconstructed embryos since the fate of mitochondria from donor cells and from recipient oocytes is unclear (Katayama *et al*., 2006; Zhong *et al*., 2008). Also, the potential spread of animal-derived pathogens to the human patient (surpass the species barrier) is a complication of interespecific derivation of NTES cells, which must be addressed (Cervera, 2004). Recently, Tachibana *et al*. (2009) conducted chromosomal transfer into recipient primate oocytes to yield live offspring or monkey ESCs with no contribution of donor mitochondria. This approach may offer a reproductive option to prevent mtDNA disease transmission in affected families.
Once this strategy is proved to be safe, it could solve oocyte supply limitations to derive hES cell lines from NT embryos.

- Transplantation of adult stem cells (AS cells).

Non embryonic stem cells, also called adult stem cells (AS cells), are lower in the stem cell hierarchy. They are thought to have lost the pluripotent capability that ESCs have. However, throughout the organism's life, AS cells retain a multipotent differentiation potential. Non-ESCs can be derived from several sources including

amniotic fluid (De Coppi *et al*., 2007), umbilical cord tissue (Wang *et al*., 2004), fat tissue (Zuk *et al*., 2001), central nervous system (Gage, 2000), bone marrow (Caplan, 1991), retina (Tropepe *et al*., 2000) and skin (Toma *et al*., 2001).

- Stem cell niche and cellular plasticity.

A niche consists of signalling molecules, intercellular communication and the interaction between stem cells and their neighbouring extracellular matrix (Bajada *et al*., 2008). This three-dimensional microenvironment is thought to influence/ control genes and properties that define 'stemness' of the stem cells, i.e. self-renewal or development to committed cells (Watt and Hogan, 2000). An interesting theory put forward is that stem cells might be terminal differentiation cells with the potential to display diverse cell types, depending on the host niche. Adult stem cells that are implanted into a totally different niche (different germ layer) can potentially differentiate into cell types similar to those found in the new environment. For example, human neuronal stem cells were found to produce muscle cells when they were implanted into skeletal muscle (Galli *et al*., 2000). Bone marrow cells were found to differentiate into neuronal cells when they were transplanted into a neural tissue (Zhao *et al*., 2002; Mezek *et al*., 2003).

In addition, 'transdifferentiation' of liver cells to islet cells was achieved (Alam and Sollinger, 2002). These findings showed the possibility of 'strong' niche influence leading to adult stem cell plasticity (the ability to dedifferentiate into cells from other lineages). Thus, the distinction between ESCs and non-ESCs is blurred further than previously thought. The enthusiasm generated by these findings on stem cell plasticity has been countered by a degree of controversy and scepticism in many research centres. In particular it has been suggested that cell–cell fusion, rather than plasticity, is the cause of these results (Wurmser and Gage, 2002; Wurmser *et al*., 2004). Recent papers have demonstrated *in vitro* cell–cell fusion of neural (Ying *et al*., 2002) or bone marrow cells (Terada *et al*., 2002) with male-derived ESCs. Again, cell fusion of BMSCs with Purkinje neurons, cardiomyocytes and hepatocytes was observed *in vivo* (Alvarez-Dolado *et al*., 2003). Thereafter, these cells showed a dual phenotype, possessing a large nucleus containing numerous nucleoli and a tetraploid number of chromosomes.

Furthermore, the presence of XXXY-positive nuclei provided the strongest argument that cell fusion in animal studies is a possible cause of stem cell plasticity. However, very low levels of fusion were observed. Thus, these cannot completely explain the significant amount of regeneration obtained in animal studies of cell implantation for retinal, hepatic and cardiac conditions (Orlic *et al*., 2002).

- Transplantation of induced pluripotent cells (iPS cells).
    - A critical point of view.

It is generally accepted that *in vitro* reprogramming of somatic cells into induced pluripotent stem cells offers new applications in basic research, diagnosis and cell therapy, and make possible to surround the practical and ethical questions related to hES, hNTES cells and the iSCNT.

It seems that iPS cells promise nearly everything embryonic stem cells do in the cell therapy field, what is true, but the applied research with ES and AS cells was far from the achieving the objectives marked. Ten years after the discovery of human ES cells, scientists are still working on standardizing procedures for coaxing pluripotent cells to become mature tissue. It is a critical problem: pluripotent cells used in therapies could trigger dangerous tumors. And even though scientists can easily prompt pluripotent cells to become beating heart cells in a lab dish, no one has yet perfected a way to get such cells to integrate into the body's tissues to replace or repair their diseased counterparts.

As occurred with the SCNT technique, in wich cells from all tissues, species and ages were used as nuclear donors without taking into account the real use of the works, the last year dozens of papers reported the iPS cells obtaining from many tissues and from patients with a particular disease, and even they were differentiated to the damaged tissue. To induce reprogramming *in vitro* is, without doubt a great advance with many attractive applications in several research fields, which should be treated more cautiously.

- What to do with stem cells research?

It would be a serious mistake to conclude that recent developments in iPS cell research avert the need for ongoing research on hES cells. There are many important reasons why iPS cell research must be conducted hand in hand with hES cell research. There are overwhelming scientific justifications for proceeding with hES cell research, which is precisely why it is important to maintain a realistic perspective on iPS cell research vis-à-vis hES cell research (Hyun *et al*., 2007).

- Embryonic stem cells are considered the gold standard. They have been studied for more than a decade, and their common origin from embryos suggests, to most scientists, that they will be less variable than iPS cells derived from different tissue types. Recently, Jaenisch and collegues (Soldner *et al*., 2009) characterized human iPS cells before and after the extra genes had snipped themselves out. Cells that still contained extra copies of the reprogramming genes expressed 271 genes differently from embryonic stem cells; with the genes gone, that number dropped to 48. There is so much anecdotal evidence saying that iPS cells do not do as well or that they are different from embryonic stem cells but it's just unpublished (Baker, 2009). The cells could be intrinsically unique because they do not come from embryos, or they might differ from embryonic stem cells because current methods for creating iPS cells are inadequate (Baker, 2009).

- Researchers have not yet agreed how to evaluate iPS cells. The most rigorous test of reprogramming involves inserting reprogrammed mouse cells into an embryo, implanting it into a surrogate mother, letting the chimaeric mice grow to adulthood, and waiting to see if the reprogrammed cells go on to make sperm or eggs that produce healthy offspring. The ability to contribute to a brand new embryo shows that the biological settings in the original cells have been reset (Baker, 2009).

Such tests are ethically unacceptable in humans, so the standard assay, borrowed from human embryonic stem cells, involves injecting human cells into an immune-compromised mouse and waiting six to eight weeks to see if the cells form a tumour called a teratoma (Okita *et al*., 2008). Naturally occurring teratomas can grow

into a knot of differentiated tissues, including hair and bone, but for transplanted cells to win the iPS label, researchers just need to see a mass of differentiated cells representing all major classes of tissue. Researchers say that it is not uncommon for cells that seem fully reprogrammed in terms of appearance and surface markers to fail to form teratomas (Baker, 2009).

- hES cells will have to be used as important controls to examine the safety and abilities of human iPS cells. In the case of mouse iPS cells, the selection method can be improved by using ES cells as a control. It must be emphasized that at present ES cells derived from embryos represent the only pluripotent cells that are genetically unmodified. In the course of early human iPS cell research, up-to-date knowledge of hES cells will be essential for informing scientists' understanding and analyses of human iPS cells (Hyun *et al*., 2007).

Thus, research of human stem cell research must proceed together. Science is a practice that works best when it is approached with an open and creative mind. Research into one approach can inspire new ideas in unpredictable and exciting ways. As a case in point, the inspiration for iPS cell research came from an earlier stem cell study in which human body cells were reprogrammed by fusion with hES cells (Cowan *et al*., 2005). From this earlier study it was hypothesized that hES cells have defined factors that induce pluripotency, thus leading to the first iPS cell breakthrough in 2006 (Takahashi and Yamanaka, 2006). In short, the recent advancements in iPS cell research would not be possible if it were not for the many years of dedicated hES cell research that preceded them. The iPS cell research cannot advance without hES cell research (Hyun *et al*., 2007).

- Therapeutic potential of iPS cells.

One of the promises of patient-specific ES cells is the potential for customized therapy of diseases. Previous studies have shown that disease-specific ES cells produced by nuclear cloning in combination with gene correction can be used to correct an immunologic disorder in a proof-of-principle experiment in mice (Rideout *et*

al., 2002). In a similar approach, it has recently demonstrated that iPS cells derived from skin cells of a mouse with sickle cell anemia were able to fully restore normal blood function when transplanted into diseased mice (Hanna *et al*., 2007). Finally, it has shown that iPS cells can be efficiently differentiated into neural precursor cells giving rise to neuronal and glial cell types in culture. Neural precursors derived from iPS cell were able to improve behavior in a rat model of Parkinson's disease upon transplantation into the adult brain demonstrating the therapeutic potential of directly reprogrammed fibroblasts for neuronal cell replacement in an animal model (Wernig *et al*., 2008c).

Recently, somatic cells from Fanconi anaemia patients have been reprogrammed to pluripotency. These cell lines appear indistinguishable from human embryonic stem cells and iPS cells from healthy individuals. These “corrected” Fanconi-anaemia-specific iPS cells can give rise to haematopoietic progenitors of the myeloid and erythroid lineages that are phenotypically normal (Raya *et al*., 2009).

An unresolved question is whether one somatic cell type can be converted into another cell type without prior dedifferentiation to a pluripotent state, by direct trans-differentiation. Recently, the *in vivo* conversion of exocrine pancreas cells to endocrine insulin-producing cells has been achieved by expression of three transcription factors (Zhou *et al*., 2008). It will be a major challenge for future work to utilize our current knowledge of transcriptional networks active in different somatic cell types to achieve the direct reprogramming of somatic cells to cells of a different germ layer in the Petri dish.

- iPS cells: more breakthroughs wanted.

Although impressive progress was made in 2008-2009, several more breakthroughs are needed before cellular reprogramming yields its first cure for disease.

For reprogramming to be safe enough to use in cell therapy, an efficient way to trigger it must be found and, to understand exactly how the process works is needed. Although dozens of labs have used the technique, what is happening inside the

reprogrammed cell remains a mystery, and a combination of chance events seems to determine which rare cells end up being reprogrammed (Gurdon and Melton, 2008).

The original reprogramming recipe relies on viruses to insert the reprogramming genes into the infected cell's genome, altering the DNA permanently. Scientists are wary of that approach for a couple of reasons. First, the inserted DNA could interrupt existing genes, for example, those that guard against cancer, leaving the cells likely to form tumors. And although the inserted genes seem to turn off after reprogramming is finished, allowing the cell's own genes to take over, scientists worry that the inserted genes could be reactivated or could have other subtle effects on the cell. For that reason, labs around the world are working on other ways to trigger reprogramming.

To be useful, reprogramming also needs to become much more efficient. Most experiments have managed to reprogram less than one in 10,000 cells. In what seems to be a lucky break for the field, however, two groups recently showed that called keratinocytes are particularly easy to reprogram (Aasen *et al*., 2008; Amoh *et al*., 2009). Researchers can reprogram roughly 1% of the keratinocytes they treat, and the process takes only 10 days instead of the several weeks that other cells require.

Moreover, another intrinsic problem of the reprogramming technology has to be solved: detailed molecular analysis of data showed that quite often reprogrammed cells keep the genetic/epigenetic ‘memory’ of adult cells even after reprogramming into the pluripotent state.

Methods have to be developed or adapted from existing human ES cell protocols to evaluate the differentiation potential of reprogrammed cells and to scale up the amount of iPS-derived donor cells. Such methods established for human ES-derived cells are lineage-specific directed differentiation and selection techniques to generate pure populations of specialised cells. For example, human ES cells have been used to generate functional neurons essential to cure senso-motoric deficits in an animal model of Parkinson’s disease (Cho *et al*., 2008) or to develop insulin-producing cells that reverted glycaemia in diabetic animals (Kroon *et al*., 2008). At first, these techniques have to be adapted to reprogrammed iPS cells. However, sometimes experimental data showed reduced efficiency of iPS cell differentiation, which may be

due to yet unknown mechanisms related to the genetic/epigenetic 'memory' of reprogrammed cells.

Considering therapeutic applications of human iPS derived cells, the graft has to fulfil disease-specific requirements, including correct cell integration, migration and survival within the surrounding tissue of the recipient. Therefore, one of the most important questions at present is whether human iPS cells will generate functional cells of the desired phenotypes at sufficient amounts (Rolletschek and Wobus, 2009).

Another essential problem that has to be considered is the problem of cell ageing in somatic tissues of adults. Tissue cells with increasing age are known to enrich recessive mutations due to mutagenic effects of stress factors and reactive oxygen species. Such mutations would be present also in reprogrammed iPS cells. Therefore, it has to be clarified whether it will be possible to generate high quality patient-specific iPS cells also from old-age donors (Rolletschek and Wobus, 2009).

It has recently been shown that mobilized peripheral blood (mPB) cells can be reprogrammed to pluripotency (Loh *et al*., 2009). However, adult mPB cells will have the potential disadvantages that they may have accumulated genomic alterations as a result of aging or disease and that the pharmacological treatment used to mobilize the adult hematopoietic stem cell compartment represents a health risk for the donor (Anderlini, 2009). In this way, Cord Blood (CB) cells are considered an alternative to bone marrow (BM) as a source of hematopoietic stem cells for transplantation. CB cells can be collected without any risk for the donor, are young cells expected to carry minimal somatic mutations, and possess the immunological immaturity of newborn cells (Rocha *et al*., 2004). Recently, it has been achieved the reprogramming of CB cells to pluripotency by retroviral transduction of four (Oct4, Sox2, Klf4, c-myc), three (Oct4, Sox2, Klf4,), and as few as two (Oct4, Sox2) transcription factors, without the need for additional chemical compounds (Giorgetti *et al*., 2009).

1.3.1.2. Endogenous regeneration.

An alternative goal is to identify small molecules that will stimulate an organism's own endogenous progenitor cells to promote regeneration.

To date, this strategy is being developed using animal models which have the ability to regenerate parts of the body by themselves.

- Endogenous regeneration studies in zebrafish and other low vertebrates.

While quite a bit about the molecular mechanisms and the signaling pathways regulating regenerative processes is known at present, why some tissues and organs regenerate well, while others do not is not quite understood. Furthermore, why salamanders and fish are able to form blastema and regenerate complex structures, while human cannot, is not only difficult to accept, but is also not well understood. A common belief is that regenerative capacity is elevated in lower animals but has diminished in higher ones. However, a more thorough review of the regenerative potential across the animal kingdom reveals that also some "primitive" forms do not regenerate, while close relatives do. For example, some annelids can regenerate the whole body from small fragments, while similarly complex nematodes do not regenerate at all (Slack, 2007). Likewise, urodele amphibians (salamanders) are the champions of vertebrate regeneration, while anuran amphibians have significantly lower regenerative capability (Stoick-Cooper *et al*., 2007a). It thus appears that organism complexity is a rather poor predictor for regenerative capacity. Regeneration can be considered a "pristine" quality of all tissues and structures and not a specific evolutionary adaptation of a few organisms and organs. However, this quality is easily lost during evolution and thus appears to carry some evolutionary cost. Yet, from this point of view, it appears reasonable to assume that the basic machinery necessary for regeneration still exists in human organs and that it might be the triggers of regeneration that are missing.

In support of this view, it has been shown that terminally differentiated mouse muscle cells in culture can be stimulated to dedifferentiate, which salamander muscle cells do during limb regeneration, when treated with extracts from salamander regenerating limbs (McGann *et al*. 2001). Similarly, the diminished regenerative potential of skeletal muscle in old mice can be reversed by joining their circulatory systems with those of young mice (Conboy *et al*. 2005). Such heterochronic parabiosis restores the activation of Notch signaling in regenerating muscle and the proliferation

of satellite cells. Thus, extracellular factors exist that can trigger regeneration in systems that normally do not regenerate. Triggering spectacular regenerative events, like limb regeneration, in humans is a distant goal of regenerative medicine, but manipulation of several factors has been found to improve certain aspects of regeneration.

Most differentiated vertebrate cells withdraw from the cell cycle, which requires the tumor suppressor protein Rb that inhibits expression of genes needed for cell cycle entry. While normal-cycling cells can inactivate Rb by phosphorylation and thus enter the cell cycle, differentiated cells such as mammalian muscle do not respond to growth stimuli (like serum in culture), since the kinases that normally phosphorylate Rb appear to be inhibited (Stoick-Cooper *et al*., 2007a).

In salamanders, however, skeletal muscle cells and cardiomyocyte cells re-enter the cell cycle in response to serum in culture, and they do so by phosphorylating and thereby inhibiting the Rb protein (Tanaka *et al*. 1997; Bettencourt-Dias *et al*. 2003). Thus, the retained ability to regulate Rb phosphorylation might be one explanation for the plasticity and regenerative capacity of differentiated salamander cells. Indeed, Rb-deficient mouse muscle cells can be stimulated to re-enter the cell cycle in response to serum (Schneider *et al*. 1994), and mouse differentiated post-mitotic auditory hair cells likewise re-enter the cell cycle by induced deletion of Rb (Sage *et al*., 2005).

The oncogenes ErbB2 and ErbB3, two members of the EGFR family, are essential for mounting a successful regeneration response in vertebrates. ErbB, PI3K and NRG1 are components of a permissive switch for migration and proliferation continuously acting across the amputated fin from early stages of vertebrate regeneration onwards that regulate the expression of the transcription factors lef1 and msxB (Rojas-Muñoz *et al*., 2009).

Expression of Msx homeobox proteins is a hallmark of the blastemas in regenerating amphibian and fish appendages and tails and is also induced in regenerating zebrafish heart. It is thus conceivable that mammals lack the ability to regenerate these structures due to a failure to activate Msx expression. In support of this hypothesis, forced overexpression of Msx1 in cultured mammalian muscle cells has been shown to cause loss of differentiation markers and fragmentation into

mononucleated cells (Odelberg *et al*., 2000). These dedifferentiated cells could be induced to differentiate along chondrogenic, adipogenic, myogenic, and osteogenic lineages.

While the importance for dedifferentiation of differentiated cells during regenerative processes is disputed, these results point to the possibility of improving mammalian regeneration by adding factors that regulate regeneration in lower vertebrates.

- Overexpression of signaling molecules can improve regeneration.

The identification of signaling factors and pathways that positively regulate regeneration has prompted efforts to augment regeneration by overactivating these signalling pathways. Intriguingly, in many cases, regeneration can be enhanced by such manipulations or, more rarely, even triggered in systems that normally do not regenerate (Stoick-Cooper *et al*., 2007a).

As described above, FGF signaling plays essential roles in blastema-mediated regeneration of amphibian and fish appendages. Intriguingly, implantation of beads soaked with FGF10 has been reported to be sufficient to reactivate regeneration in Xenopus limbs at later stages of development where limbs have lost their regenerative capacity (Yokoyama *et al*., 2001). More dramatically, FGF2-soaked beads can stimulate embryonic chick limb buds, which do not regenerate, to regenerate digit-like structures (Taylor *et al*., 1994). Thus, activation of FGF signaling holds the promise of improving the regenerative capacity of nonregenerating appendages.

Similarly, targeted overexpression of IGF in skeletal muscle improves muscle regeneration in response to injury, betters the phenotype of a muscle dystrophy mouse model, and enhances the regenerative capacity of old mice (Musaro *et al*., 2001; Barton *et al*., 2002; Rabinovsky *et al*., 2003). Systemic IGF overexpression even triggers heart muscle regeneration in response to heart failure in mice.

Wnt/β-catenin signaling is required for zebrafish and salamander fin and limb regeneration (Kawakami *et al*., 2006; Stoick-Cooper *et al*., 2007b). The transient activation of Wnt/β-catenin signaling by overexpression of Wnt8 was sufficient to increase proliferation in the regenerating zebrafish fin, but did not augment overall

regeneration. In contrast, prolonged increase of Wnt/β-catenin signalling in fish heterozygous for a loss-of-function mutation of axin1, a negative regulator of Wnt/β-catenin signaling, resulted in accelerated regeneration (Stoick-Cooper *et al*., 2007b). Similarly, Kawakami *et al*. (2006) found that overexpression of constitutively active Wnt/β-catenin can rescue fin regeneration in a fish line that is mutant in an as-yet unidentified gene causing variable fin regeneration defects. Furthermore, constitutively active Wnt/β-catenin could induce, albeit at low frequency, partial regeneration in developing Xenopus hindlimbs at a stage at which untreated limbs did not regenerate (Kawakami *et al*., 2006). It is noteworthy that Wnt/β-catenin signaling is strongly up-regulated during zebrafish heart regeneration (Stoick- Cooper *et al*., 2007b); thus, manipulation of Wnt signalling has the potential to be beneficial for regenerative therapies.

It is obvious that attempts to augment regeneration by overactivation of potent signaling molecules, which regulate proliferation and specification of many cell types and some of which, like Wnt/β-catenin signaling, are involved in tumor formation, will have to deal with the issue of unwanted side effects (e.g., cancer). In this regard, it is encouraging that overactivation of Wnt/β-catenin signaling in zebrafish regeneration does not appear to induce mispatterning or cancerous overgrowth (Kawakami *et al*., 2006; Stoick-Cooper *et al*., 2007b). However, it is possible that a system with high regenerative capacity, like a fish fin, is adapted to limit such side effects, but attempts to induce regeneration in a normally nonregenerating organ might be more prone to such problems.

- Interference with signaling molecules can augment regeneration.

Regenerative processes need to be tightly regulated to avoid overgrowth, mispatterning, and tumor formation. A few signals that negatively regulate regeneration have been identified. From a therapeutic standpoint, these are very interesting, since it is typically easier to interfere with the function of a gene than to enhance it.

Myostatin is a highly specific inhibitor of muscle growth (see above), and mice lacking myostatin display improved skeletal muscle regeneration (McCroskery *et al*.

2005). Furthermore, administration of Myostatin neutralizing antibodies increases muscle mass and muscle strength in Mdx mice, which serve as a model for Duchenne muscular dystrophy (Bogdanovich *et al*., 2002). Thus, myostatin is an excellent candidate for therapeutic intervention in degenerative muscle diseases and muscle wasting syndromes.

Wnt5, likely activating a Wnt/β-catenin independent signaling pathway, acts as an inhibitor of zebrafish fin regeneration (Stoick-Cooper *et al*., 2007b). Thus, fish mutant for one of the two wnt5 paralogs, wnt5b, exhibit faster fin regeneration. Wnt5 has been shown to act as an inhibitor of Wnt/β-catenin signalling in several systems, including the fish fin (Stoick-Cooper *et al*., 2007), indicating that it might interfere with fin regeneration by inhibiting β-catenin signaling. It will be interesting to test whether β-catenin independent Wnt signaling is active in other regenerative systems and whether its activity correlates with regenerative capacity.

I.3.2. Cancer research.

Cancer has been defined as a disease driven by genetic alterations, including mutations in tumor-suppressor genes and oncogenes, as well as chromosomal abnormalities (Feitsma and Cuppen, 2008). However, the study of normal human development has identified that in addition to classical genetics, regulation of gene expression is also modified by 'epigenetic' alterations (Sharma and Jones, 2009. Epigenetic mechanisms are essential for normal development and maintenance of tissue specific gene expression patterns. Global changes in the epigenetic landscape are a hallmark of cancer. Recent advancements in the rapidly evolving field of cancer epigenetics have shown extensive reprogramming of every component of the epigenetic machinery in cancer including DNA methylation, histone modifications, nucleosome positioning and non-coding RNAs, specifically microRNA expression (Sharma and Jones, 2009).

The zebrafish is now emerging as an effective system for the study of the fundamental aspects of tumorigenesis. In keeping with the striking anatomical and physiological similarity between fish and mammals, zebrafish develop a wide spectrum of cancers resembling human malignancies (Kent *et al*., 2002). The most common

target tissues for spontaneous neoplasia are the testis, gut, thyroid, liver, peripheral nerve, connective tissue, and ultimobranchial gland. Less common target tissues include blood vessels, brain, gill, nasal epithelium, and the lymphomyeloid system (Kent *et al*., 2002).

The potential for zebrafish as a cancer model derives from its strengths as an experimental system for developmental biology. Despite 450 million years of evolutionary distance, the pathways that govern vertebrate development including signaling, proliferation, cell movements, differentiation, and apoptosis-indeed, the same pathways that are often misregulated in tumorigenesis-are highly conserved between humans and zebrafish (Amatruda and Patton, 2008). In addition, zebrafish have been found to develop almost any tumor type known from human, with similar morphology and, according to gene expression array studies, comparable signaling pathways. However, tumor incidences are relatively low, albeit highly comparable between different mutants, and tumors develop late in life. In addition, tumor spectra are sometimes different when compared with mice and humans. Nevertheless, the zebrafish model has created its own niche in cancer research, complementing existing models with its specific experimental advantages and characteristics (Sharma and Jones, 2009).

Strategies used in the cancer study in zebrafish include carcinogenic treatments, forward genetic screens for proliferation and genomic instability, reverse genetic target-selected mutagenesis to inactivate known tumor suppressor genes, the generation of transgenics to express human oncogenes and transplantation of mammalian cancer cells.

I.3.2.1. Treatment with mutagens.

Historically, researchers appreciated the relative ease of treating fish with carcinogens because the chemicals can be dissolved or suspended in water and the animals can be exposed for longer time periods. When exposing zebrafish to different compounds [e.g., 7,12-dimethylbenz(a)anthracene, N-nitrosodimethylamine, and N-nitrosodiethylamine], liver and intestinal tumors (Khudoley, 1984; Stanton, 1965) pancreas carcinomas (Mizgireuv and Revskoy, 2006), epithelial tumors in thyroid, and

testis; mesenchymal tumors in cartilage, blood vessels, muscles, and connective and lymphoid tissues and neural tumors (Spitsbergen *et al*., 2000) were observed.

I.3.2.2. Mutants from forward genetic screens.

The largest impetus for zebrafish to become an important animal model was its suitability for forward genetic screens. Since the first mutagenesis experiments in the Nusslein-Volhard lab (Mullins *et al*., 1994), screens have been carried out for almost any type of phenotype, including phenotypes related to cancer (Murphey *et al*., 2006). A screen for proliferation defects takes the advantage of the fact that many oncogenes and tumor suppressors are actually essential for development (Bessa *et al*, 2008). Homozygous mutant embryos are therefore lethal, and heterozygotes, due to haploinsufficiency or loss of heterozygosity, causes genome instability and increased cancer susceptibility (Shepard, 2005, 2007).

I.3.2.3. Reverse Genetics: Target-Selected Inactivation of Tumor Suppressor Genes.

Since knockout technology in zebrafish became available by means of target-selected mutagenesis (Wienholds and Plasterk, 2004), several mutants for known tumor suppressor genes have been generated (Yuan *et al*., 2009).

Targeted knockout strategies such as those used for making mouse knockouts are not available in zebrafish. The current strategy makes use of random N-ethyl-N-nitrosourea mutagenesis combined with targeted selection of mutations in the gene of interest, which means that the researcher is dependent on the random point mutations that are induced. However, the positive aspect of this is that the generated point mutations can be more similar to the type of spontaneous mutations that occur in human cancer patients than the large gene deletions or insertions in mouse knockouts (Feitsma and Cuppen, 2008).

For tp53, the most frequently mutated gene in human cancers, two zebrafish mutants were isolated: one with a missense mutation in the DNA-binding domain and one with a missense mutation that affects protein structure in a heat-sensitive manner (Marchler-Baver *et al*., 2009).

The second most frequently mutated tumor suppressor in human cancers, pten, has undergone a gene duplication in zebrafish. Faucherre *et al*. (2008) isolated nonsense mutants for both ptena and ptenb.

The first DNA repair genes that have been mutated in zebrafish are the mismatch repair genes msh1, msh2, and msh6 (Feitsma *et al*., 2007), which are involved in the repair of small replication errors such as base mismatches and insertion/deletion loops. Homozygous mutants were genomic unstable, as shown by the occurrence of variation in lengths of microsatellite sequences in their DNA (Feitsma and Cuppen, 2008).

One of the disadvantages of the use of random mutagenesis in the knockout procedure is that each fish that is retrieved will contain, besides the mutation of interest, several background mutations. Those additional mutations are heterozygous and will probably not have a large effect on developmental phenotypes, but they may of course be of influence on the process of mutation accumulation that is necessary for tumor development (Feitsma and Cuppen, 2008).

I.3.2.4. Transgenic Zebrafish Expressing Mammalian Oncogenes.

The largest number of studies on cancer development in zebrafish thus far comes from transgenic zebrafish expressing mammalian oncogenes. This approach makes use of another advantage of zebrafish as a laboratory animal: the convenience of introducing foreign DNA into zebrafish cells and getting it expressed by injection into one-cell embryos. Many of the models concern lymphomas and leukemias, cancers that rarely occur spontaneously in zebrafish but for which the transgenic model may be of great aid in searching for new treatments (Zhurauleva *et al*., 2008).

Enormous efforts have put into generating optimal zebrafish models for leukemia. Zebrafish expressing mouse c-myc under the zebrafish rag2 promotor to restrict the expression to lymphoid cells have developed (Langenau *et al*., 2003). Also, a transgenic line overexpressing the zebrafish B-cell leukemia 2 (bcl2) gene under the rag2 promotor, in which lymphoid apoptosis is blocked (Langenau *et al*., 2005). Zebrafish expressing constitutively active human NOTCH under the rag2 promotor developed T-cell leukemia in the 40 % of the cases (Chen *et al*., 2007).

Several transgenic models for solid tumors have also been generated. Langenau *et al*. (2007) observed that the zebrafish rag2 promotor that they frequently used showed ectopic expression in undifferentiated cells of the muscle, making it possible to express human activated RAS, a common mutation in human oncogenesis, in these cells, resulting in the development of rhabdomyosarcoma in 47% of the cases. A conditional version of the same activated human RAS under a zebrafish β-actin promoter with a floxed EGFP gene inserted in between was combined with the heat shock–inducible CRE (Le *et al*., 2007). To test the role of activated BRAF in melanoma development, transgenic zebrafish expressing activated human BRAF-V600E under the zebrafish melanocyte mitfa promotor were generated (Patton *et al*., 2005). Finally, human MYCN under the zebrafish myod promotor is expressed in neural tissue and pancreas and, in combination with a human core enhancer, also in muscles (Yang *et al*., 2004)

I.3.2.5. Transplantation of Mammalian Cancer Cells into Zebrafish.

Different groups have been experimenting with transplantation of mammalian cancer cells into zebrafish embryos. This creates an *in vivo* system in which the advantages of cultured human cancer cells are combined with those of transparent zebrafish embryos in which development can be followed. Lee *et al*. (2005) transplanted fluorescently labelled human metastatic melanoma cells into zebrafish blastula-stage embryos and showed that these cells survive, migrate, and divide, and are still present in adults but do not cause cancer or metastases. Another study showed that aggressive human melanoma cells are able to induce a secondary axis or an abnormal head when transplanted into 3-hour-old zebrafish embryos, which was shown to be due to Nodal signalling from the tumor cells (Topczewska *et al*., 2006; Lee *et al*., 2006). In contrast to the above studies where no cancer development was observed, similar human melanoma cells as well as a colorectal and a pancreatic cancer cell line were found to induce tumor-like cell masses when transplanted into 2-day-old zebrafish embryos (Haldi *et al*., 2006).

Transplantation studies can be specifically effective in the study of vasculature remodeling, cancer invasion, and metastasis. Currently, no good *in vivo* model is

available in which such dynamic process can be followed in real time. When transplanted into 2-day-old zebrafish embryos, human and murine tumor cell lines expressing fibroblast growth factor or vascular endothelial growth factor induced rapid neovascularization inside the tumor graft, and this could be inhibited by treatment with antiangiogenic chemicals (Nicoli *et al*., 2007). In another elegant study, researchers used injection of fluorescently labeled human breast cancer cells in 1-month-old zebrafish in combination with three-dimensional modeling to show how the human cells interact with vessels and invade in tissues. They showed that expression of vascular endothelial growth factor induces openings in vessel walls that can be used for invasion, which in turn is stimulated by RhoC expression (Stoletov *et al*., 2007).

I.3.3. Human disease studies: triggering, evolution and pharmaceutical research.

The mechanisms by which one disease develops can be studied both *in vivo* and *in vitro*.

Human ES cells obtaining and their *in vitro* differentiation to a lot of type of tissues opened the possibility of studying *in vitro* the evolution of specific diseases. hESC can be genetically manipulated using conventional techniques (Strulovici *et al*., 2007), in this way, disease-specific mutations can be mimicked and the effect of the genetic alterations on cellular proliferation and differentiation along the affected lineage can be studied in detail.

The ES cells obtaining from the patients involves the therapeutic cloning so, the study *in vitro* (iPS cells) the development mechanism of one disease allows to jump the human nuclear transplant. Pathomechanisms of diseases, including Parkinson's disease and Huntington's disease, juvenile-onset type 1 diabetes mellitus or amyotrophic lateral sclerosis (Park *et al*., 2008) are being studied using iPS cells. Recently, skin fibroblasts from a patient with spinal muscular atrophy were reprogrammed into proliferative iPS cells that maintained the disease genotype and were able to differentiate into motor neurons. The iPS cells showed selective deficits typical for the disease as well as drug responsiveness (Ebert *et al*., 2009). This example demonstrates that iPS cells may be a promising tool to study the mechanisms of diseases and the effects of drugs thus enabling the development of new therapies. For instance, iPS

cells differentiating *in vitro* may be used as an alternative system for screening of embryotoxic and/or teratogenic substances (Caspi *et al*., 2008). However, as treated before, more knowledge and advances need to be for using the iPS.

An extension of basic research towards the clinic is the proposed use of hESC in *in vitro* testing assays, such as chemical genetic screen. Chemical genetic screening can be described as a discovery approach in which chemicals are assayed for their effects on a defined biological system (Kaufman *et al*., 2009). For example, it has been proposed that toxicity testing on human tissue would be a valuable addition or alternative to animal testing that is currently undertaken. However, the reality is that so far it has been impossible to get large quantities of normal human tissue and what is available has not been successfully maintained nor expanded *in vitro* to useful amounts (Oh and Choo, 2006). Now, hESCs offer the potential to generate very large numbers of cells with the properties of normal human tissues. The liver is the organ most affected by drug toxicity and is the site of xenobiotic converting enzymes such as the cytochromes that metabolize drugs and can alter their bioactive properties. Hence, pharmaceutical companies have undertaken the development of scale-up protocols to generate vast numbers of hepatocytes from hESC to screen new and existing drugs for toxicity and metabolism in a human system (Ahuja *et al*., 2007). Similarly, cultures of cardiomyocytes and neural cells would be valuable in such studies (Mountford, 2008).

These cultures of normal human tissue could be used as control material in the investigation of on-target drug actions (Mountford, 2008). In order to be clinically useful, a drug should selectively affect diseased cells and not normal cells. This necessity for drug selectivity will become increasingly important in future in cancer therapeutics. A number of cancers have now been found to have a stem cell component that is responsible for fuelling the development and persistence of tumours. To date, cancer stem cell (CSC) populations have been isolated from acute and chronic leukaemias, breast cancer, prostate cancer and neurological tumours among others (Al-Hajj, 2007). In order to target these disease sustaining CSCs without harming the normal stem cells that are essential for tissue survival, it will be necessary to undertake very rigorous testing for drug selectivity.

Human ESCs offer an ideal resource for the generation of tissue-specific stem cell populations that cannot be obtained from any other source. Finally, as mentioned above, it is possible to introduce genetic mutations or modifications to hESC (Strulovici *et al*., 2007) that would allow drug screening against individual gene product targets within the context of a human system. It is difficult to obtain viable diseased human tissue from many organs and thus hESC would offer the option to recapitulate disease in a human system instead of using genetically manipulated animal models.

However, the only way to study *in vivo* the causes that trigger a disease and its evolution is by the use of animal models. *In vitro* cellular behaviour is different than *in vivo*, where cells undergo movements and interactions, somatic and germ cells undergo reprogramming and acquire new imprints, etc.

Many factors suggest that the zebrafish is a powerful tool for the study of human diseases: characterization of an exceptionally large number of genes involved in vertebrate pathways, patterning, pathfinding and connectivity in the central nervous system (CNS) have all been deciphered and correlate with the human CNS (Driever *et al*., 1996; Alestrom *et al*., 2006; transparency of embryonic zebrafish facilitates analysis of single neuron activity during the execution of normal and pathological behaviour. Touch and behavioural responses such as movement patterns can be monitored (Guo, 2004). Larvae and adults can display pathological and behavioural phenotypes that are quantifiable and related to those seen in man.

Other characteristic that point to the zebrafish as a complete model for disease studies is the achievement of ES cells (Fan *et al*., 2004a), which allow realizing *in vitro* complementary studies. The reverse genetic techniques of gain-of-function by mRNA injection and gene knockdown by morpholino injection, as was before mentioned, have become standard for the zebrafish system. Micro-injection of antisense morpholino oligonucleotides into zebrafish embryos at the single cell stage can be used to suppress the translation of a particular gene during early development (Heasman, 2002). The morpholino strategy has been successfully used to study the function of particular genes in normal development and the consequences of altered gene function in disease, including the neurodegenerative disease spinal muscular

atrophy (Nasevicius and Ekker, 2000). The main disadvantage of the morpholino technique, however, is that they lose their efficacy after day 5 of the development.

One important technical challenge to address in the future of zebrafish reverse genetics is the achievement of conditional knockdowns (or knockouts) of specific genes. Efforts to achieve homologous recombination in zebrafish ES cells that can then reconstitute the germ line have not yet been fully rewarded; although it should be remembered that to date the mouse remains the only organism where targeted knockouts can be achieved, with even the closely related rat proving intractable (Skromne and Prince, 2008).

The *in vivo* drug discovery studies represent an essential step with important limitations in mammals (Kari *et al*., 2007).

Zebrafish characteristics allow for flexible, rapid and scalable chemical screen design (Barros *et al*., 2008). Besides the advantages above mentioned, only milligrams of compound are needed for screening in 96-well plates and the larvae can live in as little as 50 µL of fluid. In addition, chemical screening is facilitated by the fact that zebrafish are reasonably tolerant to dimethylsulphoxide concentrations generally used in such technologies and small molecule compounds dissolved in the swimming medium can reach target tissues via passage through the skin of the larvae (Rombough, 2002). Given these advantages, it is not surprising that screening platforms using zebrafish are now emerging as they provide the high content of an *in vivo* assay that can be easily and inexpensively applied throughout the crucial hit to lead and lead optimization stages of the drug discovery process (Goldsmith, 2004; Parng, 2005; Zon and Peterson, 2005; Rubinstein, 2006, Parng *et al*., 2007).

Some approach using zebrafish as model in drug discovery are the following. Small molecules have been identified that suppress mutation-caused cardiovascular defect (Peterson *et al*., 2004) or cell cycle arrest (Stern *et al*., 2005), and those that modulate the embryonic heart rate in wild type (Burns *et al*., 2005). In addition, many drugs with known effects in humans have been shown to cause similar effects in zebrafish (Langheinrich *et al*., 2003; Milan *et al*., 2003). Moreover, a recent study has

identified a potent small molecule, prostaglandin E2, through zebrafish-based small molecule screening for chemical regulators of haematopoietic stem cell (HSC) homeostasis. Remarkably, this small molecule also performs similar action in mammalian HSCs, thus validating that zebrafish-based drug discovery can potentially lead to therapeutic compounds for human conditions (North *et al*., 2007).

I.4. Nuclear reprogramming in zebrafish. Technical aspects.

Zebrafish nuclear transplant and somatic and germ-line chimaerism are two main techniques to raise many biomedicine studies in zebrafish.

1.4.1. Nuclear transplant.

I.4.1.1. Donor nucleus somatic cells.

- Donor cell types.

Donor cell types frequently used in nuclear transfer in teleost fishes are blastomeres (Wakamatsu, 2001). Besides embryonic cells, either larvae somatic cultured cells (Lee *et al*., 2002) or adult somatic cultured cells (Bubenshchikova *et al*., 2007; Luo *et al*., 2009; Siripattarapravat *et al*., 2009b) are recently used too. The transplant has been performed using cells from primary cultures (Lee *et al*., 2002; Bubenshchikova *et al*., 2007; Siripattarapravat *et al*., 2009b) as well as from subcultures (Lee *et al*., 2002). This is an important aspect taking into account that chromosomal abnormalities are often in long cultures (Kaftanovska *et al*., 2007).

- Somatic cell culture.
    - Cell obtaining.

Adult fishes can be anesthetized with an eugenol (Grush *et al*., 2004) or with tricaine solution (Lee *et al*., 2002). Caudal fins must be dissected and disinfected. Huang *et al*., (2003) uses 0.04% sodium hypochlorite solution for 3 min. for disinfecting the larval tissue in zebrafish and Ju *et al*., (2003) uses a concentration of 0.4% sodium hypochlorite for 30 s. for caudal fins. The embryonic cells are not usually disinfected because of the embryo is originally isolated into the chorion.

    - Cell culture.

The fish somatic cell cultures are usually not C02 dependents and the incubation temperature, at atmospheric pressure, rounds 28.5 ºC (Westerfield, 2007). The Leibovitzs' L-15 basic medium, supplemented with 15-20% of fetal bovine serum (FBS) and antibiotics (penicillin and streptomicine), is the most used both in medaka

and zebrafish, as culture medium for primary cultures (Bubenshchikova *et al*., 2007; Westerfield, 2007). To establish both primary and long-term primary cultures in zebrafish, DMEM (Dulbecco's modification of Eagle's medium) has used (Huang *et al*., 2003; Siripattarapravat *et al*., 2009b).

When fish blastula cells are used as donors, the blastoderm is usually isolated from the yolk using a fine glass needle and a hair loop (Hu *et al*., 2002, Wakamatsu *et al*., 2001). Then, donor blastomeres are obtained by blastoderm disaggregation in a Ca++ and Mg++ free Hanks' solution (Cardona-Costa and García-Ximénez 2007). In medaka, blastoderms are dissociated into individualized cells by being pippeted in Ca2+ - and Mg2+ -free PBS (Phosphate Buffered Saline; Wakamatsu *et al*., 2001).

I.4.1.2. Recipient.

- Unfertilized oocyte obtaining.

In zebrafish, mature oocytes are usually directly obtained from the ovarian cavity by a gentle extrusion (Westerfield, 2007). For that, the female has to be anesthetized with an eugenol (Grush *et al*., 2004) or tricaine (Lee *et al*., 2002) solution. Instead, in medaka, eggs are often extracted sacrificing the specimen and dissecting the cavity (Kaftanovskaya *et al*., 2007).

- Characteristics of non activated oocytes.

Selman *et al*. (1993) established the zebrafish oocyte development into five stages. The earliest stage I oocyte contains no yolk and appears as a transparent ball of cytoplasm surrounding a central germinal vesicle (GV). By stages II and III, has increased the in size due to yolk uptake, while the VG remains in the center of the oocyte. At stage IV the oocyte undergoes maturation, marked by both the migration of the GV to the future animal pole of the embryo and the break down of the nuclear envelope. This migration is the first morphological sign of polarity in the oocyte and the future embryo. The oocytes increase slightly in size, become translucent, and their yolk becomes non-crystalline. After maturation, oocytes are ovulated and become stage V eggs when are capable of being fertilized.

Teleost eggs, display a remarkably variety of adaptations. Morphologically, mature zebrafish eggs differ significantly from those of medaka. While the latter posses a central yolk mass, separated from the peripheral cytoplasm by a continuous yolk membrane (Gilkey at al., 1978), zebrafish eggs posses, many individual membrane-bound yolk globules that are homogenously intermingled with ooplasm throughout the mature oocyte (Beams *et al*., 1985). However, although not structurally separated by a continuous membrane, these eggs possess a functional ooplasm cortex some 15-20 µm thick where yolk globules are excluded. When mature zebrafish eggs are discharged from the ovarian stroma and come in contact with the spawning medium, they activate (Hart and Yu, 1980; Hart and Fluck, 1995; Sakai *et al*., 1997). These partenogenetically activated eggs then proceed to expand their chorions and undergo normal ooplasmic segregation. After several abortive cleavages, however, they fail to develop further. If sperm are present in the spawning medium, an identical series of steps are observed but cell division is normal and development proceeds (Lee *et al*. 1999).

- Oocyte activation.

Activation of the recipient oocytes is of major importance to the outcome of NT experiments, because artificial activation causes the reduction in MPF and MAPK levels that both provoke reconstructed MII arrested oocytes to activate and to continue further "normal" development, and affects the time interval for donor nucleus exposure to reprogramming ooplasmic factors.

Oocyte activation comprises a sequence of cellular changes, all of which must be faithfully completed to assure development to term. These events include the display of intracellular calcium oscillations, which triggers all other activation events, including cortical granule exocytosis to prevent polyspermy, completion of meiosis with extrusion of the second polar body, and formation of male and female pronuclei, DNA complement replication, and first mitotic cleavage (Horner and Wolfner, 2008).

The complete (not abortive) artificial egg activation in zebrafish is very difficult compared with mammals because, at present, no electric and/or chemical treatment has to be efficient. The osmolarity requirement for the eggs maintenance in a non

activate state must be around 320 mOsm since this is the osmolarity inside the ovarian cavity (Westerfield, 2007). The natural activation is triggered reducing the medium osmolarity down to 35 mOsm (Westerfield, 2007). To reach a good fecundation rate, the main guides (Nüsslein-Volard and Dahm, 2002: Westerfield, 2007) recommend that the time passed from the egg obtaining to the artificial fecundation must be minimum, but longer delays are possible (90 min.) using rainbow trout or coho salmon ovarian fluid (Allende and Weinberg, 1994) or Hank's saline supplemented with 0.5% BSA (Hatta *et al*., 1994). However, Cardona-Costa *et al*. (2009a), in a modified egg medium advise to not extend this time above 60 min. Recently, it has shown that zebrafish eggs keep their fertilizability cultured in Chinook salmon ovarian fluid (CSOF) for 5h (Siripattarapravat *et al*., 2009a). This time is still short compared with mammals in which the oocytes can be kept up to several hours, in adequate conditions, before the artificial fecundation (Stricker, 1999).

- Dechorionation.

NT in zebrafish has realized using activated eggs (Lee *et al*., 2002, Lou *et al*., 2009) because the treatment usually employed for dechorionation, protease treatment, activates the oocytes. To dechorionate mechanically a non activated zebrafish egg is nearly imposible. However, in medaka, due to the mycropile is easily detected, NT can be carried out into chorionated eggs (Bubenshchikova *et al*., 2007). Recently, in zebrafish, non dechorionated eggs were also used as receptor, despite the difficulty to localize the micropyle (Siripattarapravat *et al*., 2009b).

- Enucleation.

In mammals, enucleation is usually realized by metaphasic plate staining and its extirpation as caryoplast by the enucleation-transplant pipette. Although it is the normal process, a large number of variants exist, such us blind enucleation.

In fishes, enucleation is still a critical step for nuclear transfer due to find the exact localization of the metaphasic plate. In most fish species, like zebrafish and medaka, the maternal pronucleus is located under the micropyle, the unique chorion structure by where the spermatozoa can entry into the egg in the normal fertilization.

In medaka, the micropyle is easily visible in non activated oocytes and it is possible to aspirate the metaphase plate to enucleate them. However, in zebrafish, to find the microplyle is more difficult (Huang *et al*., 2003), what involves that the unique feasible possibility to remove the female pronucleus, after activating the oocyte, is to be able to visualize the PB1 at the animal pole. So, using the polar body as reference, the pronucleus can be removed by sucking out a very small amount of cytoplasm just below the polar body (Lee *et al*., 2002).

In various fish species it is also possible to functionally enucleate the oocyte through ionic irradiation or UV irradiation (Wakamatsu *et al*., 2001; Ungar *et al*., 1998). Also, Siripattarapravat *et al*. (2009b) used laser-ablated metaphase II eggs as recipients. Moreover, due to teleost fishes can be viable triploids, even in several cases even tetraploids, the nuclear transfer can be performed without removing the female pronuclei (Wakamatsu *et al*., 2001, Li *et al*., 2003).

I.4.1.3. Nuclear transfer technology.

- Micromanipulation media.

The micromanipulation media depends on the oocytes state. If oocytes are dechorionated, thus also activated, they are placed in Hank's saline solution (Lee *et al*., 2002; Luo *et al*., 2009). Metaphase II eggs have been transplanted in the Chinook salmon ovarian fluid (CSOF) to keep them in a non activated stage (Siripattarapravat *et al*., 2009b).

- Nuclear microinjection

In fishes, the NT technique exclusively employed is the microinjection (Wakamatsu *et al*., 2001; Lee *et al*., 2002; Bubenshchikova *et al*., 2007), which is also used in mice (Wakayama *et al*., 1998). The donor cell must be deposited under de oocyte membrane situated under the micropyle, where the nucleus was located (Lee *et al*., 2002) (see Fig.3, pag.82).

Two very important exigencies must be accomplished for a successful nuclear transfer in fishes. On the one hand, we have to ensure the donor cell lysis and the full

contact between the perinuclear cytoplasm of the transplanted cell and the ooplasmic region of the animal pole.

In medaka, the injection is usually through the micropyle (Wakamatsu *et al*., 2001; Bubenshchikova *et al*., 2007) both into activated and non activated eggs. However in zebrafish, only one work reported the microinjection througt the micropyle (Siripattarapravat *et al*. 2009b)

I.4.1.4. Embryo culture.

The development of cloned embryos remains poor, with respect to the number of embryos that develop to term (Wimut *et al*., 1997; Wakayama *et al*., 1998; Ono *et al*., 2001). Much recent discussion about the basis for the limitations in cloned embryo development has centered on the question of whether the donor somatic cell nucleus is reprogrammed efficiently. Comparatively little attention has been devoted to the possible effect of culture environment on clone development.

The *in vitro* culture environment exerts significant effects on embryo development in mammals. Complex culture media are used during the development to blastocyst state and affects epigenetic information in the embryonic nucleus. Serum in culture media can alter epigenetic information related to genomic imprinting (Khosla *et al*., 2001), and a transient relaxation of imprinting at the 8-cells stage has been reported for the H19 gene in mouse embryos, which is dependent upon the culture medium employed (Doherty *et al*., 2000). Thus, early effects of culture environment have the potential to exert long-term effects on development in mammals.

In fresh water fishes the embryo culture medium is very simple, dechlorated and decalcificated water is enough for the whole development because of it is its natural environment. So, in principle, the media no contain additional components to alter the epigenetic pattern. The requirements of temperature for embryos and cell cultures are similar, 28ºC, but not osmolarity requirements, which are 35 mOsm for dechorionated embryos and 280-310 mOsm for somatic cells (Westerfield, 2007).

1.4.2. Germ-line chimaerism.

Germ-line chimaerism is a technique that has proved to be useful to produce viable gametes when transplanted embryonic cells or embryo stem cells colonize the germinal ridges in recipient embryos and, in a final step, to obtain offspring from such transplanted cells (Ma *et al*., 2001; Fan *et al*., 2004bc).

1.4.2.1. Donor cells.

Blastomeres (Fan *et al*., 2004b; Francisco-Simao *et al*., 2009), ES cells (Fan *et al*., 2004c) and PGCs (Ciruna *et al*., 2002) have been used as donors in zebrafish chimaerism.

Donor blastomeres are obtained by blastoderm disaggregation in a Ca++ and Mg++ free medium (Cardona-Costa and García-Ximénez 2007).

1.4.2.2. Recipient embryo.

- Characteristics of the recipient embryo.

Chimaerism have to be performed at mid blastula (MBT) stage since that is the state in which cells remains pluripotent (Lin *et al*., 1992). Although cells obtained from more advanced stages have also used as donors reaching similar chimaerism rates (Nakagawa and Uneo, 2003)

Dechorionated embryos are usually employed since chorionated embryos turn into the chorion, what make very difficult to fix them for transplanting. As in the NT technique, dechorionation is also achieved by protease treatments.

- Recipient embryo germ-line penalizing.

The success rates are still quite limited. Strategies usually employed to accomplish this aim have focused on minimizing and even cancelling the cell participation of recipient embryos in development and, particularly, in gamete production to increase the contribution of transplanted cells in establishing the germ line (Carsience *et al*., 1993).

This end can be achieved by chemical treatments such as, in mammals, culture atmosphere, media toxicity by the addition of substances such as ethanol (Brown *et al*.

1979), by physical treatment (Aige-Gil and Simkiss 1991; Strahle and Jesuthasan, 1993; Ikegami *et al*., 1997; Li *et al*., 2001) or by ploidy manipulation (Luo and Li, 2003). Recently, an UV radiation has proposed to be used as penalizing agent for zebrafish embryos (Francisco-Simao *et al*., 2009). However, the more effective technique to block the recipient germ line development is using the recent advances of reverse genetic (morpholinos technique; Ciruna *et al*., 2002)

1.4.2.3. Chimaerism technology.

- Osmolarity media.

Manipulation is usually carried out in a low osmolarity environment (Lin *et al*., 1992; Ma *et al*., 2001). However, it was proved that MBT embryos could support high osmolarities (300 mOSm) for 1h without further damage in the development (Pérez-Camps and García-Ximénez, 2008). In addition, Francisco-Simao and Cardona-Costa *et al*. (2009) reported that the low osmolarity commonly used in chimaerism experiments does not affect the viability of the transplanted cells, but that it affects embryonic survival. This results advise to carry out the chimaerism in a high osmolarity media (300 mOsm).

- Cell injection.

Unlike mammals, where chimaerism technology can carry out by aggregation among embryos or among embryo and cells (Neganova *et al*., 1998; Krausslich and Brem, 1985), the cell microinjection is the technique usually employed in teleost fish. The number of injected cells per recipient embryo ranged from 50 to 100 cells and they are deposited into the animal pole as described by Lin *et al*. (1992), specifically, they must be placed into the lower part of the blastoderm (Nakagawa and Ueno, 2003).

Cells can be picked using a microinjection pipette of 50 µm inner diameter and injected into the embryos held with a 260 µm outer diameter holding pipette (Francisco-Simao *et al*., 2009) (see Fig.4, pag.82). Alternatively, there are automatic techniques of transplantation were embryos are immobilized in methyl cellulose (Nüsslein-Volard and Dahm, 2002).

1.4.2.4. Embryo culture.

Embryos must culture in dechlorinated and decalcified water at 28.5ºC (Westerfield, 2007). There are no exigencies related to supply the medium, as occurs in mammals.

Fig.3. Nuclear microinjection.

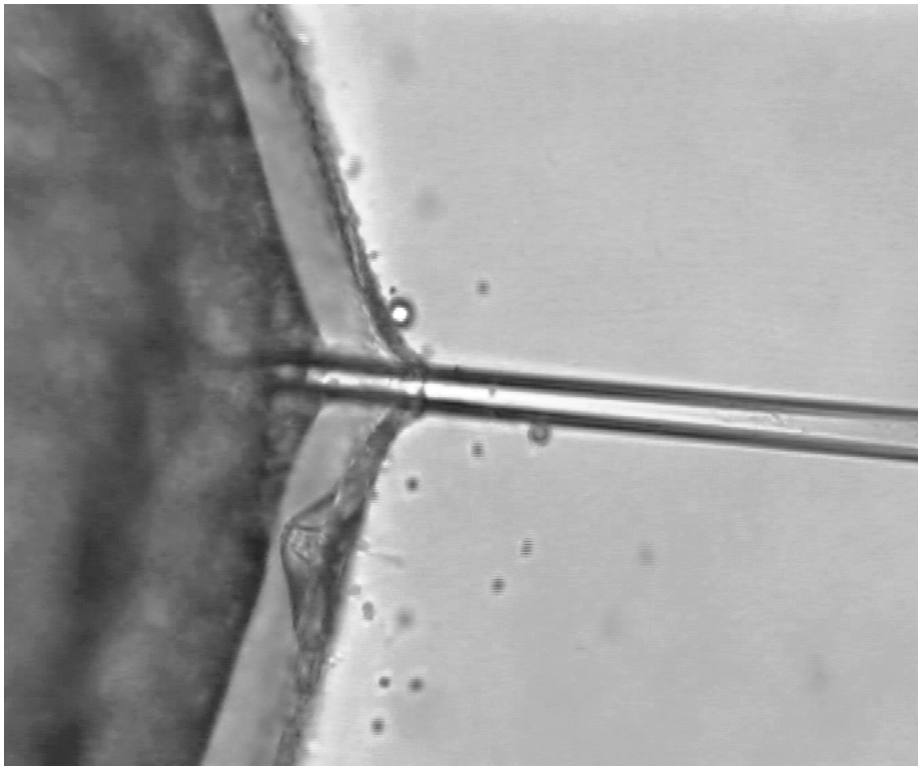
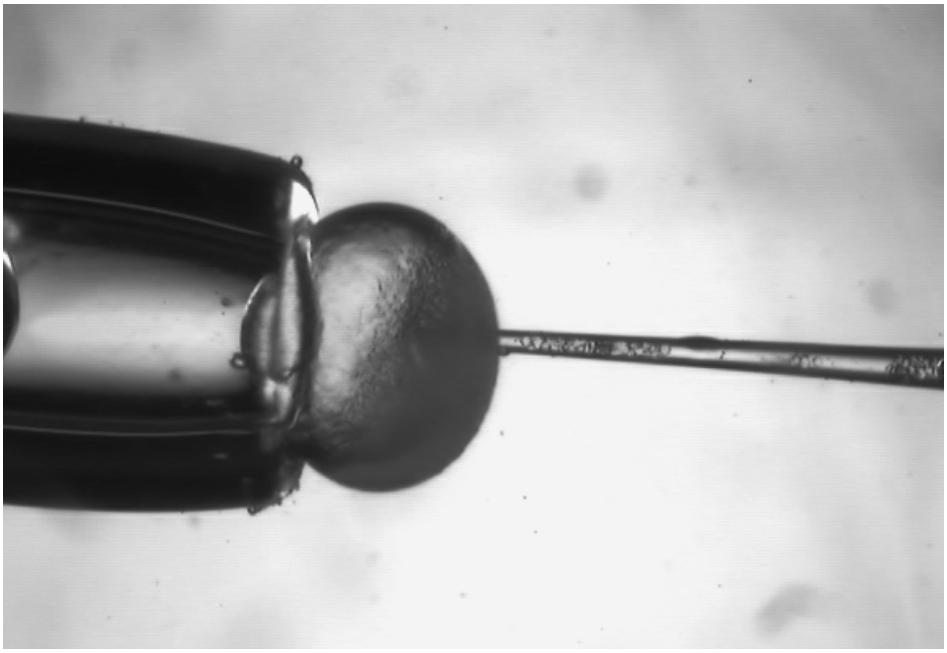


Fig.4. Cell injection.



The aim of the present work was:

1. To evaluate, using the chimaerism technique, the reprogramming degree reached by the blastema cells generated after the caudal fin amputation.

2. To analyze the integration degree of nuclei from adult cultured fibroblasts depending on the place of the nucleus insertion into the recipient egg, the egg activation stimuli and the reconstructed egg ploidy.

3. To test the possible existence of gametic imprinting through the reconstruction of diploid gynogenetic embryos by transplant of somatic haploid nuclei derived from haploid gynogenetic larvae.

The first objective to reach in the present work was to have a chimaerism technique capable to adapt to diverse cellular types. So, in the experiment I entitled **"Ultraviolet radiation and handling medium osmolarity affect chimaerism success in zebrafish"**, the consequences on the germ line chimaersm derived from two main factors were studied: the osmolar compatibility between transplanted cell medium and intraembryonic environment, and the embryo penalization, especially PGCs precursor cells, by ultraviolet radiation.

In parallel to the experiment I, the conventional chimaerism technique (without penalizing the embryo and without taking into consideration the osmolar compatibility) was employed to test the blastema cells totipotency in the experiment II presented under the title of **"Evaluation of presumptive caudal fin blastema cells as candidate donors in intraspecies zebrafish (*Danio rerio*) chimaeras"**

Regarding the adult fibroblast reprogramming by nuclear transplant, four experimental works were realized.

In the first of them, experiment III, published as **"Definition of three somatic adult cell nuclear transplant methods in zebrafish (*Danio rerio*): before, during and after egg activation by sperm fertilization",** the effects generated on the integration degree, and therefore the reprogramming, by the fact that the NT were performed using metaphase II eggs or zygotes, were studied. This involved different locations of the transplanted nucleus, into the central region or into the animal pole respectively. Furthermore, the permissible favourable effect derived from an initial tetraploid condition induced by the transplanted eggs fertilization was evaluated.

Results from the experiment III, in which adult specimens were obtained by the transplant of adult somatic nuclei into the central region of metaphase II eggs activated by sperm fertilization, led to carry out the following work. In the experiment IV, the reprogramming degree of the somatic nuclei transplanted into the egg central region, when there was no nuclear helper role derived from the fertilization, was

studied. In addition, this work aimed to test whether a limited egg aging improved the transplanted nuclei integration. Results were presented in the paper entitled **"Transplant of adult fibroblast into the central region of metaphase II eggs resulted in mid blastula transition (MBT) embryos".**

One of the main problems in the NT technique success, both in mammal and in fish, concerns the deficit of the activating stimulus when it is not triggered by the sperm fertilization. In mammals, the oocytary activation in NT experiments is complemented by the application of electrical or chemical-electrical stimuli, which notably improve the clone development. With this aim, a sequence of electrical pulses to activate non manipulated eggs beyond that was induced by the water contact was established in the experiment V. The obtained results were presented the paper **"Electroactivation of zebrafish (*Danio rerio*) eggs"**

In the last study of those dedicated to the nuclear reprogramming, experiment VI, was entitled **"Comparison of different activating stimuli efficiency in zebrafish nuclear transplant"** in which different activating stimuli, even the electrical, were compared when they were integrated together with the NT technique.

Finally, the last experimental part, experiment VII, was dedicated to the preliminary study of the genomic imprinting. Few decades ago raised the interest of the imprinting in mammals. However, this phenomenon has barely been contemplated in fish, although the parthenogenetic developmental ability is higher than in mammals. Based on this fact, the zebrafish can be an excellent model system to studies of this nature. As a starting point for these studies, in the work entitled **"Reconstruction of heteroparental gynogenetic diploid condition by nuclear transplant in zebrafish"**, gynogenetic haploid larvae were obtained by parthenogenetic egg activation using radiated sperm, which tissues were *in vitro* cultured. These haploid somatic cells were transplanted into metaphase II eggs or into recently activated oocytes.

All these experiments are presented as follows.

ESTUDY I. Ultraviolet radiation and handling medium osmolarity affect chimaerism success in zebrafish*.

ABSTRACT

The effects of a predefined ultraviolet radiation dose (0.529 mW/cm^2 for 30s) together with two different micromanipulation media osmolarity (30 mOsm/kg vs 300 mOsm/kg) were tested on embryo survival at different developmental stages and on the somatic (skin) and germ-line chimaerism rates.

Somatic (13 %, 6/47 adults) and germ-line chimaerism (50 % pigmented F1 larvae) were detected only in the UV treated recipient embryos micromanipulated in a 300 mOsm/kg medium. From the results obtained, we concluded that the conditions cited above were the most suitable to improve somatic and germ-line chimaerism rates in zebrafish.

KEY WORDS: Germ-line chimaerism, Embryo, Osmolarity, Zebrafish.

* This paper has been submitted to the journal "Zygote" with the following reference: Francisco-Simao M, Cardona-Costa J, Pérez-Camps M and García-Ximénez F. (2009). Ultraviolet radiation and handling medium osmolarity affect chimaerism success in zebrafish Zygote, (submitted).

INTRODUCTION

The chimaerism technique has proven useful to obtain offspring in which a part of the gametes comes from embryonic cells or embryo stem cells (Ma *et al*., 2001; Fan *et al.,* 2004).

The colonisation of transplanted cells in the presumptive chimaera, both at somatic and germ-line levels, depends on the preponderance of transplanted cells over the recipient cells. To facilitate colonisation, different treatments can be applied with the aim of penalising the recipient embryo or some specific structures, as occurs with PGCs precursors (Carscience *et al.*, 1993). To this end, chemical products (Swartz, 1980) or ionising radiations (Joly *et al*., 1999; Li *et al*., 2002) have commonly been used. The use of ultraviolet (UV) radiation is of interest because it is cheaper, easier and less dangerous than other kinds of radiation, and no special installations are required for its use. In our lab, a UV radiation dose to penalise recipient embryos was defined specifically for *wild* and *gold* zebrafish strains (Francisco-Simão *et al*., 2009). On the other hand, in another work, Cardona-Costa and Francisco-Simão *et al*., (2009) observed that the micromanipulation medium osmolarity (30 vs 300 mOsm/kg) could affect recipient embryo survival, possibly due to the rupture of the osmolarity barrier when the microinjection pipette punctured the outer embryonic layer.

In this context, the aim of the present work was to test the effect of the previously defined radiation dose (0.529 mW/cm^2 for 30s) together with the micromanipulation medium osmolarity (300 or 30 mOsm/kg) on the germ-line chimaerism efficiency in zebrafish.

MATERIAL AND METHODS

Embryos at the early blastula stage from two different strains (*wild*: donors; *gold*: recipients) were used. All chemical products and culture media were from Sigma-Aldrich (Madrid, Spain)

UV irradiation of gold (recipient) embryos

According to previous results obtained in our lab (Francisco-Simão *et al.*, 2009), *gold*-type embryos were treated with UV radiation to improve the colonisation of

transplanted cells during chimaeric embryo development. Briefly, embryo irradiation was carried out almost to mid blastula transition (MBT) stage without dechorionation. They were held in 35 mm-Petri dishes (corning) as containers with system water. A vortex (MS1-IKA) at 200 rpm was used with the aim of homogenising the radiation area during UV exposure. A UV germicide lamp (General Electric, 30W) was used. Irradiation was carried out at 62 cm of focus-object distance. The radiation dose applied was 0.529 mW/cm^2 and was measured by a USB 4000 (Miniature Fiber Optic Spectrometer; Ocean Optics Inc. First In Photonics, USA). After irradiation, embryos were kept at room temperature for 30 min and then dechorionated.

Chimaerism technique

Donor MBT blastomeres (non radiated cells) from *wild* specimens were obtained by blastoderm disaggregation, in modified Hanks' buffered salt solution (HBSS) medium free of Ca^{++} and Mg^{++} (Cardona-Costa and García-Ximénez, 2007).

The chimaerism was performed using a Nikon inverted microscope (Nikon Europe B.V, Badhoevedorp, Netherlands) equipped with two Leitz micromanipulators (Leica, Wetzlar, Germany). Two separated media drops were placed in a Petri-dish (90 mm) and covered by mineral oil. One of them composed of HBSS (300 mOsm/kg) medium free of Ca^{++} and Mg^{++} contained the isolated blastomeres and the other one was the handling medium in which the chimaerism was performed, composed of HBSS (300 mOsm/kg) or HBSS-10% (30 mOsm/kg) medium (Pérez-Camps and García-Ximénez, 2008) depending on the experimental group carried out. During the manipulation process, the cells were picked with a microinjection pipette of 50 µm inner diameter and injected into the embryos held with a 260 µm outer diameter holding pipette. The number of injected cells per recipient embryo ranged from 50 to 100 cells and they were deposited into the animal pole as described by Lin *et al.* (1992); specifically, into the lower part of the blastoderm (Nakagawa and Ueno, 2003). Manipulated embryos were placed in 35 mm cell culture dishes at 28.5ºC for 5 days in HBSS-10% (30 mOsm/kg).

Surviving embryos at 30-60 minutes were considered as the initial number. The further survival rates were assessed at 24h, 48h, 72h and 5 days post-chimaerism.

Then, surviving embryos were raised to adulthood where skin pigmentation from adult chimaera and their F1 progeny was registered.

Experimental design

Four experimental groups were established by combining embryo recipient UV radiation (30s UV vs non radiated) and the micromanipulation medium osmolarity (30 vs 300 mOsm/kg). Differences among groups in survival rates of different stages were tested. Somatic and germ-line chimaerism were evaluated in adults.

Overall germ-line chimaerism rate estimation

In this work, the parameter used to compare the osmolarity media and UV effect on germ-line chimaerism rates assumed that all the adult specimens obtained (male and female) in each experimental group provided a single "hermaphrodite and simultaneous" gonad. In this way, depending on the treatment applied, the relative frequency of gametes produced (whether sperm or eggs) from donor cells (*wild*) or from recipient (*gold*) could be estimated by melanocyte presence in the larval skin, because the marker from *wild* specimens (pigmentation) is dominant over *gold* specimens, so only offspring from *gold-gold* gametes pairing will be non pigmented. To this end, embryos from the four experimental groups were collected for 8 weeks and their skin pigmentation (*wild or gold*) was evaluated at 48 h developmental stage (Lin *et al*., 1992).

At least three replicates were done in all experimental groups. Results were analysed using the Chi-square test. When a single degree of freedom was involved, Yates' correction for continuity was performed.

RESULT AND DISCUSSION

Results from experiment I are shown in Table I. In the irradiated groups, significant differences were observed in the survival rate at 24h between the two handling media osmolarity (300 mOsm/kg: 50% and 30 mOsm/kg: 36%; $p<0.05$). However, this difference gradually disappeared from the 48h to 5 days stage, and even

in the global survival (cumulative survival). Moreover, in the non radiated groups, micromanipulation media osmolarity did not affect embryo survival rates at any developmental stage. These results could indicate that the osmotic shock produced when chimaerism is performed in 30mOsm/kg micromanipulation medium osmolarity does not apparently affect long term survival in a relevant manner (Cardona-Costa and Francisco-Simão *et al*., 2009). As was expected, global survival (cumulative survival) rates in the irradiated groups were significantly lower than in non-irradiated (Francisco-Simão *et al*., 2009).

It may be noted that the number of males was higher than females in all experimental groups. No interpretation of this observation could be made because the system and factors of phenotypic sex determination are unknown in zebrafish (Saito *et al.*, 2007).

Only 6 (4 males and 2 females) from the 47 total adults showed *wild* skin pigmentation and all of them belonged to the 300mOsm-30s UV experimental group (Table 2). Moreover, it should be emphasised that high rates of *wild* offspring (50%) were also only observed in the 300mOsm-30sUV group (Table 3). This fact confirms that the presence of pigmentation acts as an excellent sign of germ-line chimaerism in zebrafish (Lin *et al*., 1992). In medaka fish, the gamma irradiation of recipient embryos also favoured the appearance of large pigmentation signals from donor cells and, in parallel, a significant increase in germ-line chimaerism (Joly *et al*., 1999).

According to the results obtained, the penalisation of recipient embryo with a radiation dose of 0.529 mW/cm^2 for 30s together with the manipulation in 300mOsm/kg handling medium osmolarity was the combination that obtained the best somatic and germ-line chimaerism rates.

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Table 1: Survival rates of transplanted embryos. Embryos were irradiated or not (controls) and manipulated in different osmolarity media (30 and 300 mOsm/kg).

	30s of UV		**Non radiated**	
	300 mOsm/kg	**30 mOsm/kg**	**300 mOsm/kg**	**30 mOsm/kg**

Initial number of embryos	188	232	54	108
Normal embryos at 24h	93/188 (50%)[a]	82/232 (36%)[b]	37/54 (69%)[c]	59/108 (55%)[ac]
(abnormal)	(30)	(38)	(6)	(16)
Normal embryos at 48h	74/93 (80%)[a]	69/82 (84%)[ab]	35/37 (95%)[ab]	55/59 (93%)[b]
(abnormal)	(21)	(31)	(4)	(9)
Normal embryos at 72h	64/74 (87%)	62/69 (90%)	28/35 (80%)	53/55 (96%)
(abnormal)	(22)	(24)	(3)	(5)
Normal embryos at 5 days	59/64 (92%)	54/62 (87%)	26/28 (93%)	48/53 (91%)
(abnormal)	(12)	(23)	(5)	(3)
Global survival at 5 days	(31%)[a] 59/188	(23%)[a] 54/232	(48%)[b] 26/54	(44%)[b] 48/108

Columns with different superscripts are statistically different (p<0.05)

Table 2: Sex distribution and pigmented marks in adult presumptive chimaeras.

Experimental groups	Nº of final adults fishes			Nº of adults with pigmented marks		
	Total	Male	Female	Total	Male	Female
30 mOsm - Non UV	13	13	0	0	0	0
30 mOsm - 30s UV	11	7	4	0	0	0
300 mOsm - Non UV	9	7	2	0	0	0
300 mOsm - 30s UV	14	8	6	6	4	2
Total	47	74% 35/47	26% 12/47	13% 6/47	67% 4/6	33% 2/6

Table 3: *Wild* skin pigmentation rates in F1 larvae assessed at 48 h.

Experimental groups	Total embryos	*Gold* pigmented embryos	*Wild* pigmented embryos
30 mOsm – Non UV(*)	105	105	0(0%)[a]
30 mOsm – 30s UV	25	24	1(4%)[a]
300 mOsm – Non UV	36	36	0(0%)[a]
300 mOsm – 30s UV	494	254	240(50%)[b]

Data in rows with different superscripts are statistically different (P< 0.05)

(*) As all were males, *gold* females were introduced to make the germ-line chimaerism assessment feasible.

STUDY II. Evaluation of presumptive caudal fin blastema cells as candidate donors in intraspecies zebrafish (*Danio rerio*) chimaeras *.

ABSTRACT

The blastema is a regenerative tissue with remarkable pluripotency. The aim of this work done on zebrafish (*Danio rerio*) was to define technical procedures required for obtaining and integrating blastema cells into embryos at the mid blastula transition stage (MBT) and the effect on survival, as well as the capacity to produce pigmented chimaeras. *Wild* type blastema cells were injected into *gold* type MBT embryos (E). *Wild* MBT blastomere cells were also injected into *gold* type MBT embryos as a control (C1). A second control group, C2, was not subjected to any manipulation. Survival was evaluated at 24, 48 and 72 h after performing the chimaerism, and the rate of adult chimaeras evaluated. The results showed significant differences in embryo survival between the E and C1 groups in embryo survival at 24 and 48 h postchimaerism (24 h: E-83.49% vs C1-54.8%, $p < 0.05$; 48 h: E-98.83% vs C1-85.13%, $p < 0.05$). There was no significant difference, at any time, between E and C2. The results at 72 h for E and the controls (E-89.41%; C1-84.12% and C2-92.55%) indicate that insertion of blastema cells does not have a negative effect on embryo development. The results in adults (E: 0 chimaeras from 7 specimens; C1: 5 chimaeras from 17 specimens) suggest that the dedifferentiation grade of the blastema cells may not be enough to generate germ-line chimaeras, but their condition of potentially dedifferentiating cells may be an advantage when using them as donor nuclei in somatic cloning by nuclear transplant.

KEY WORDS: Biodiversity, Chimaerism, Embryo, Zebrafish.

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INTRODUCTION

The cell type used to carry out a biodiversity preservation strategy is important. Until now, current cryopreservation techniques have not enabled the preservation of embryos from species with meroblastic development, such as fish. However, it is possible to store embryonic cells (Cardona-Costa and García-Ximénez, 2007) which can give rise to germ-line chimaeras (Lin *et al*., 1992), although this involves destroying the donor embryo of the lineage to be preserved.

During caudal fin regeneration in fish, a regenerative tissue appears called blastema that grows atop each fin ray, between the wound epidermis and the amputation surface of the stump, which can regenerate the fin structure (Poss *et al*., 2003). It is not clear whether blastema formation involves cellular dedifferentiation, the activation of quiescent cells, or both processes, but its pluripotency is remarkable (Akimenko *et al*., 2003; Poss *et al*., 2003).

If blastema cells are pluripotent embryo stem cells (ESCs), they would be ideal candidates for use as donor cells in germ-line chimaerism with the aim of preserving biodiversity of these animal species, as these undifferentiated cells can be obtained from adult specimens without causing any permanent damages to them. For this reason, it would be interesting to determine the potency of blastema cells and to investigate if it is possible to integrate them in the germ-line of chimera specimens, which has not been reported to date.

The aim of this work on zebrafish (*Danio rerio*), was to test the capacity of blastema cells to colonize and integrate into chimaeras, defining the technical procedures required and the effect on embryo and larval survival, as well as the capacity to produce pigmented chimaeras.

MATERIAL AND METHODS

Adult zebrafish kept in a 6:3 proportion (females: males), and fed granular food supplemented with recently defrosted hen egg yolk and shrimp meat (Simao *et al*., 2007), where used to obtain chorionated embryos at the mid-blastula transition (MBT) stage (1,000 cells), the optimal stage to carry out chimaerism (Lin *et al*., 1992). All chemicals and the culture media were from Sigma-Aldrich.

After washing with tap water on nylon mesh, embryos were selected under a microscope and washed again with tap water. Embryos in a suitable development stage and perfectly clean were kept in dechlorinated and decalcified tap water (Westerfield, 2007). No bleach treatment was applied, but sterilized media and materials (pipettes) and aseptic conditions were used.

Dechorionation was carried out by pronase treatment (1.5 mg mL-1 in H10), being H10 Hanks buffered salt solution (HBSS) diluted 10% in distilled water, v/v) followed by immersion twice in H10 with an osmolarity of 35 mOsm. Damaged embryos were discarded and only intact embryos were used in the experiments.

To remove the yolk, sterile hypodermic needles were used to puncture the yolk sac, and the embryo was then placed on a liquid surface where the yolk was removed immediately from the embryo by surface tension. The intact blastoderms were placed in HBSS without Ca2+ or Mg2+. The blastoderm was disaggregated into blastomeres using a stretch and fire polished Pasteur pipette with an approximately inner diameter of 1/5 the size of a zebrafish blastoderm.

The blastema cells were from adult *wild* type zebrafish. Animals were anaesthetized with a clove oil solution (100 µL in conventional tap water; Grush *et al*., 2004) and the caudal fins were amputated. After 4 days at 28.5ºC these fish were again anaesthetized and the distal region, where blastema was growing, was cut and kept in H10. The tissue was cleaned with a 0.2% bleach solution for 2 min, then washed twice in H10 and finally incubated for 30 min in HBSS without Ca2+ or Mg2+. After this, the blastema region was isolated from the rest of the tissue using a scalpel, under a microscope. To dissociate blastema cells, three 100 µL drops of different incubation media were placed on a Petri dish, one of Trypsin-EDTA and two of L-15 media with 20% bovine foetal serum. The three drops were covered with mineral oil. The whole blastema was first incubated in the Trypsin-EDTA drop for 5 min and the cells were dissociated using a pulled Pasteur pipette. Treatment was stopped adding 10 µL of serum to the drop, next cells were washed twice in L-15 medium by transferring them to the other two drops, and finally cell viability was checked using 0.4% trypan blue.

Around 50-100 presumptive blastema cells were injected per recipient embryo into the marginal zone, where primordial germ cells (PGCs) are localized in MBT embryos The micromanipulation was carried out in H10 medium. Both blastema and MBT cells, were kept in HBSS without Ca2+ or Mg2+. The outer diameter of the injection micropipette was 30 µm for blastema cells and 50 µm for MBT cells. Micromanipulation was carried out using a Leitz micromanipulator inverted microscope (Nikon ECLIPSE TE200). After performing the chimaerism, survival of remaining cells was tested using the trypan blue test.

One experimental and two control groups were established. In the experimental group (E) *wild* type blastema cells were injected into *gold* type MBT embryos. To evaluate the effect of the cell type on embryo and larval survival, *wild* type embryonic cells at the MBT stage were also injected into *gold* type MBT embryos (first control group, C1). The aim was to evaluate the technical efficiency and the effect of chimaerism on survival, the second control group (C2) was not subjected to any manipulation and embryos were kept in H10 for the same period of time as those that were micromanipulated. At least 100 embryos were manipulated, both in the experimental group (E) and in the control groups in the different sessions.

Survival was evaluated at 24, 48 and 72 h after performing the chimaerism, and the rate of adult chimaeras was evaluated after about three months.

Results were analysed using the Chi-square test (Statgraphics Plus 4.0). When a single degree of freedom was involved, the Yates correction for continuity was performed.

RESULTS AND DISCUSSION

During growth and elongation of the blastema, this transitory structure generates a cell proliferating-differentiation balance (Nechiporuk and Keating, 2002). This characteristic is important when selecting donor cells for transplanting because the regenerative outgrowth has different kinetic, morphological and molecular qualities in the days after amputation. In this work, the blastema used in our chimaerism experiments was recovered at 4 d post-amputation. At this stage, the transition between blastema formation and blastema outgrowth begins and the

blastema precursor cells have just undergone the dedifferentiation process and have a high proliferation rate (Poleo *et al*., 2001), so most cells retrieved will be undifferentiated.

In our results, cells from the blastema differed in their morphology to cells from a non- amputated fin with regard to size and lobopodia signs (Harvey, 1983), typical movements of undifferentiating cells. The blastema cells tolerated both manipulation and treatments (bleach, trypsin, etc.) to isolate them, as shown by the viability tests after each session of chimaerism.

With regard to embryo viability, survival of embryos that supported the manipulation process, were evaluated at 24, 48 and 72 h after performing the chimaerism (103 from 134 in E; 135 from 202 in the control group 1, C1). The technical efficiency of embryos that survived manipulation was 76.86% in blastema cells and 66.83% in blastomeres at the MBT stage. Embryo survival is shown in Table 1. Statistical analysis of the results showed significant differences ($p < 0.05$) between the E and C1 groups in embryo survival at 24 and 48 h postchimaerism. This means that insertion of blastema cells involves less immediate damage on embryo survival than insertion of blastomeres at the MBT stage. The cause of the increase in deaths at 24 h, in the control group C1 may have been due to pipette size (30 µm for blastema cells vs 50 µm for MBT cells) because mechanical damage caused by the injection process are higher as micropipette size increases. There were also significant differences ($p < 0.05$) between the two control groups, C1 and C2, at 24 and 48 h. However, at no time was there a significant difference between group E and group C2. Another factor to take into account in embryo viability is the number of cells injected. Although blastema cells are larger than somatic cells (fibroblasts), they are much smaller than blastomere cells (Figure 1). This means that mechanical damage caused by injection of MBT blastomeres is greater than the damage caused by injection of the same number of blastema cells. The survival at 72 h, in E and the controls (C1 and C2) (Table 1) supports the previous conclusion that mechanical damage in the manipulation process causes the differences observed. Thus, although blastema cells have molecular qualities and expression patterns that differ from the blastomeres at the MBT stage, embryo survival is higher in the former. It is worth noting that zebrafish embryos generates a

favourable microenvironment for keeping somatic cells in an undifferentiated state and human metastatic cells can survive, divide, and even migrate and integrate into zebrafish embryos (Lee *et al*., 2005; Piliszek *et al*., 2007).

The results of this work suggest that insertion of blastema cells does not have a negative effect on embryo development for at least three days post fertilization.
Although the process of cell dedifferentiation explains the formation of the blastema after a limb lesion in aquatic urodele amphibians (Straube *et al*., 2004; Brockes and Kumar, 2005; Straube and Tanaka, 2006), in teleost fishes there are no molecular markers available to prove the cellular reprogramming before forming the blastema. However, several cytological (Becerra *et al*., 1996) and cell signalling studies (Santamaría *et al*., 1996; Poleo *et al*., 2001a; Nechiporuk and Keating, 2002; Santos-Ruiz *et al*., 2002) suggest that dedifferentiation of mesenchymal cells would be a possible mechanism of blastema formation. If this were true, their integration into a chimera specimen would be possible, even in the germ line.

Somatic chimaerism begins to show at 48 h postfecundation, because this is the stage at which the first melanocytes differentiate (Rawls *et al*., 2001). As skin pigmentation chimaerism (Figure 2) is a good marker for detecting germ-line contributions from transplanted cells in zebrafish (Lin *et al*., 1992), the results from adults in this work (E: 0 chimaeras from 7 specimens; C1: 5 chimaeras from 17 specimens; data not shown) suggest that blastema cells are not totipotent, which precludes their use as donor cells in strategies of biodiversity preservation via chimaerism. Although the dedifferentiation grade of the blastema cells may not be enough to generate germ-line chimaeras, their condition of potentially dedifferentiating cells may be an advantage when using them as donor nuclei in somatic cloning by nuclear transplant, increasing technical efficiency in fish.

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Table 1. Embryo survival at different stages

	Experimental group (E) [1]	Control group (C_1) [2]	Control group (C_2) [2]
Initial number	103	132	104
24h post-chimaerism	86/103 (83.4%)[a]	74/135 (54.8%) [b]	96/104 (92.3%) [a]
48h post-chimaerism	85/86 (98.8%) [a]	63/74 (85.1%) [b]	94/96 (97.9%) [a]
72h post-chimaerism	76/85 (89.4%)	53/65 (84.1%)	87/94 (92.6%)
Final	76/103 (73.8%) [a]	53/135 (39.3%) [b]	87/104 (83.7%) [a]

Columns with different superscripts are statistically different (Statgraphics 4.0). 1 Experimental group (E): *wild* type blastema cells injected into *gold* type MBT embryos. 2 Control group 1 (C1): *wild* type MBT cells injected into *gold* type MBT embryos. 3 Control group 2 (C2): embryos not subjected to manipulation.

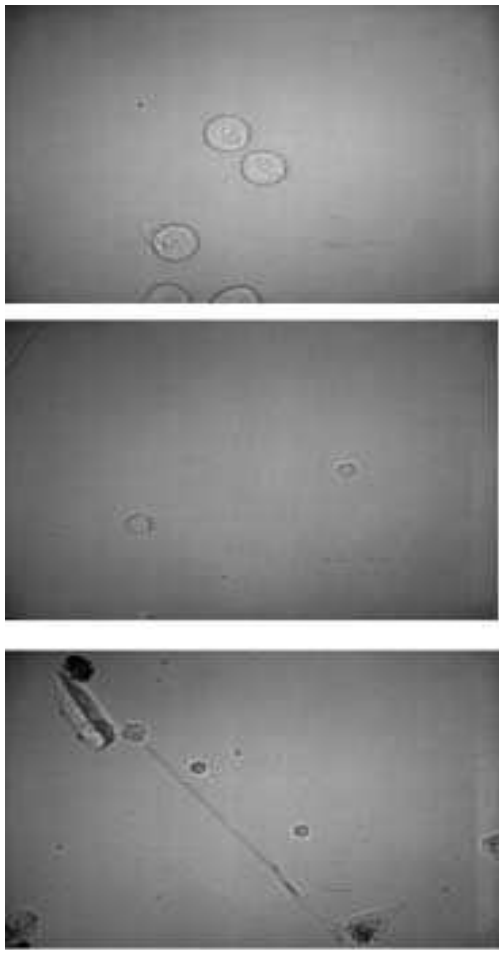

Figure 1. a). Embryonic cells (200x). Blastomeres obtained from embryos at MBT stage (1000-cells). b). Blastema cells (200x). Cells obtained 4 d after caudal fin amputation. c). Somatic cells (200x). Fibroblasts obtained from primary caudal fin cultures**.**

Figure 2. Somatic chimaerism in zebrafish.

STUDY III. Definition of three somatic adult cell nuclear transplant methods in zebrafish (*Danio rerio*): before, during and after egg activation by sperm fertilization*.

Abstract

Zebrafish somatic nuclear transplant has only been attempted using pre-activated eggs. In this work three methods to carry out the nuclear transplant using adult cells before, during and after the egg activation/fertilization were developed in zebrafish with the aim to be used in reprogramming studies. The donor nucleus from somatic adult cells was inserted: (method A) in the central region of the egg and subsequently fertilized; (method B) in the incipient animal pole at the same time that the egg was fertilized and; (method C) in the completely defined animal pole after the fertilization. Larval and adult specimens were obtained using the three methods. Technical aspects related to temperature conditions, media required, egg activation/fertilization, postovulatory time of the transplant, egg aging, place of the donor nucleus injection in each methodology are presented. In conclusion, the technical approach developed in this work can be used in reprogramming studies

KEY WORDS: Nuclear Transplant, Microinjection, Egg activation, Fertilization, Zebrafish.

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INTRODUCTION.

After 1952, when Briggs and King obtained normal hatched tadpoles by blastomere nuclear transfer, nuclear transplant (NT) technology began to be developed for reprogramming studies (Kikyo *et al.*, 2000, Wade and Kikyo, 2002; Li, 2002, Giraldo *et al.*, 2008). Mammalian cloning by nuclear transfer has been successfully achieved in several species (Wilmut *et al*., 1997, Wakayama *et al.*, 1998, Byrne *et al.*, 2007, French *et al.*, 2008) with varied somatic cell types as donors (Campbell *et al.*, 1996; Wilmut *et al.*, 1997, Wakayama *et al.*, 1998, Shiga *et al.*, 1999).

Although fish cloning is less developed, several recent works in medaka have been reported, in which both blastomeres (Bubenshchikova *et al.*, 2005) and somatic larval and adult cells (Bubenshchikova *et al.*, 2005, Bubenshchikova *et al*., 2007, Kaftanovskaya *et al.*, 2007) were used as donors, and non enucleated and activated eggs were used as recipients in all cases. In this species, embryonic nuclear transplants using functionally enucleated and non activated eggs have been achieved only with blastomeres as nuclei donors (Wakamatsu *et al*., 2001).

On the contrary, in zebrafish, the first successful embryonic (10-15 somites) somatic cloning by nuclear transplant with mechanically enucleated and previously activated eggs was described by Huang in 2003. Since this work and to date, to our knowledge, no additional improvement in nuclear transplant techniques in zebrafish has been published by these or any other authors.

To date, due to technical (Nüsslein-Volhard and Dahm, 2002) or biological (Westerfield, 2003) limitations, fish somatic nuclear transplant in these two laboratory species with somatic embryonic (10-15 somites; Huang et al., 2003) or adult cells (Bubenshchikova *et al.*, 2007) has only been attempted using pre-activated eggs as recipients. These limitations have hindered the study of the reprogramming effects of cytoplasmic factors characteristic of the metaphase II status in the oocyte, the effects of the synchrony degree between activation and nuclear transplant, egg aging, or the previous donor cell reprogramming treatments. This is not the case in mammals, in which these reprogramming factors have been studied because somatic adult nuclear transplant has been more easily carried out before, at the same time and after oocyte activation (Cambell *et al.*, 1996; Wilmut *et al*., 1997).

Taking into account that zebrafish is a powerful genetic and developmental model system whose genome has already been sequenced, the aim of this work was to develop three methods to enable nuclear transplant to be carried out using adult cells prior, simultaneously or posterior to the egg activation/fertilization in zebrafish to be used in reprogramming studies.

MATERIAL AND METHODS

Care and maintenance of zebrafish colony.

Two zebrafish (*Danio rerio*) colonies (*wild* and *gold* strains) were established in our laboratory from specimens purchased in a specialized establishment and kept in closed reproduction for five years. Adult zebrafish were kept in 20 L tanks in a 2:1 ratio (females/males) and fed on granular food supplemented with recently defrosted hen egg yolk and shrimp meat (Simao *et al*., 2007). The light cycle was regulated at 14h light/10h dark.

Non activated eggs and sperm collection.

Eggs were collected after evaluating the sexual behavior of both *gold* strain males and females at dawn. Only females that manifested this behavior were anesthetized in an oil clove solution (100 µL in 1L of dechlorinated and decalcified water: system water) for a few minutes and the eggs were obtained by gentle extrusion of the ovary. It is important to prevent eggs coming into contact with fresh water, because they activate immediately. Only good eggs (yellow and translucent color) were kept in Hanks' buffered salt solution supplemented with 1.5% (v/v) of BSA and 0.1 g of NaCl/100 cc of Hanks' medium (egg medium; ph: 7.4 ;osmolarity: 310-320 mOsm) at 8ºC of temperature until their use (1h and 30 minutes as maximum time).

The *gold* zebrafish males that showed reproductive behavior were also anesthetized as described before. The abdominal region was gently pressed while the sperm was being recovered from the genital pore in individually glass microcapillaries (1×90 mm Narishige Scientific Instrument Lab.). A pool from 2-3 different males (0.5-2 µL/male) was diluted in 200 µL of egg medium, which can also keep the sperm in a non activated status and then, the dilution was stored at 8ºC until use.

In vitro fertilization.

In zebrafish, the eggs quickly lose their postovulatory ability to be fertilized (90 min). Moreover, the time between the complete egg activation and in vivo fertilization is extremely short (seconds) in zebrafish (Nüsslein-Volhard and Dahm, 2002). Non activated eggs and sperm were mixed in egg medium and stored at 8ºC until fertilization, whatever the nuclear transplant method (see experimental design). To activate both gametes, 1 mL of system water at room temperature was added to the egg-sperm mixture. After 2-3 min, time required for the fertilization in zebrafish, the 35-mm Petri-dish was full filled with water system for achieving well developing embryos. Further culture was done at 28 ºC.

Primary culture and somatic cell collection.

Somatic cells used as nuclei donor came from *wild* zebrafish caudal fin primary cultures. The tissue was obtained by caudal fin amputation of adult specimens after being anesthetized in oil clove solution. The tissue was cleaned with a 0.2% bleach solution for 2 min, then washed twice in 10% Hanks´ buffered salt solution (H10) and then each tissue fragments were plated individually in a 35 mm Petri-dish (corning). Next, the tissue was incubated in Leibovitz medium supplemented with 20% of FBS and 0.036 g/L of glutamine (L15-FBS) at 28.5ºC (Westerfield, 2003).

Before use, donor cells were incubated in Hanks´ buffered salt solution without Ca^{2+} and Mg^{2+} at room temperature for 30 minutes before performing the nuclear transplant. No additional detachment treatment was realized. Once the cells had come off the substrate, L15-FBS was added and the cell dish was preserved at 5ºC throughout the daily experimental session.

Somatic cell nuclear transplant equipment.

The nuclear transplant was performed using a Nikon inverted microscope equipped with two Leitz micromanipulators. During the manipulation process, the non-dechorionated eggs were held with a 260 µm outer diameter holding pipette and the cells were picked, lysed and injected into the eggs by means of a 10-12 µm inner

diameter microinjection pipette. The microinjection pipette was fire polished, beveled and sharpened.

To perform the nuclear transplant, two separated drops were deposited in a Petri-dish (90 mm) and covered by mineral oil. One of them contained the donor somatic cells and was composed by L-15-FBS medium (300 mOsm). The other drop was the handling drop, which means the place where the nuclear transplant was performed, so the medium was the correspondent depending on the nuclear transplant method tested in each case (see below).

The donor cell was picked up and lysed by aspiration with the injection micropipette before injection. The exact place where the cellular content is to be deposited was dependent on the nuclear transplant method tested in each case (see below).

Nuclear transplant was performed at different temperatures depending on the nuclear transplant method (see below).

Nuclear transplant methods.

In order to carry out the NT whatever the status of the egg activation, three NT methods were developed in which the somatic nuclear transplant was performed prior, simultaneous or posterior to the egg activation by the spermatozoa. Since the aim of this work was to establish these methods technically and they were independently performed, no comparison of their technical efficiencies was made.

Method A: nuclear transplant prior to the egg activation/fertilization.

The somatic cell nucleus was inserted in the central region of the egg. To prevent egg activation, the transplant was performed in a handling drop composed of egg medium and the micromanipulation area was cooled down to 8 ºC. This temperature around the handling zone was reached by air cooled with N_2l. Then, transplanted eggs were individually in vitro fertilized and cultured at 28.5ºC in system water (Westerfield, 2003).

Method B: nuclear transplant simultaneously to the egg activation/fertilization.

In this case, previously mixed non activated eggs and sperm were kept at 8ºC and individually deposited in the handling drop containing system water so that

gametes were activated and fertilized. The micromanipulation area was not cooled (room temperature). The donor nucleus was injected in the incipient animal pole, just where zygote nucleus was being constituted (Wolenski and Hart, 1987). The reconstructed embryos were incubated under the same described conditions.

Method C: nuclear transplant posterior to the egg activation/fertilization

In order to carry out the NT after fertilization, eggs and sperm were previously mixed and activated, then fertilized at room temperature as described in the second technique but, in this case, the NT was realized a few minutes after fertilization, just the time required for visualizing the completely defined animal pole. After injecting the donor nucleus in the animal pole, reconstructed embryos were incubated at the same above described conditions.

Experimental design.

The three techniques tested (A, B and C) were not carried out simultaneously, but were developed and assessed independently. In the three cases, in order to evaluate how post-ovulatory aging affects reconstructed embryo survival, two batches consisting of 3-5 eggs each were transplanted sequentially and compared in each session (A1, A2; B1, B2; C1, C2). Overall, the length of each experimental session did not exceed up 90 min in any case, the maximum time for efficient egg fertilization (Nüsslein-Volhard and Dahm, 2002). In this way, A1, B1 and C1 were manipulated during the first 45 min and A2, B2 and C2 during the last 45 min. A non-manipulated control group was fertilized at the end of each experimental session, at 90 min (CA, CB and CC) to test the egg ability to be fertilized at this time.

Given that the aim of the present work was mainly technical, only the embryo and larval survival rates of reconstructed embryos from the three techniques (A, B and C) were evaluated at different developmental stages: at mid blastula transition (MBT) stage (2h after NT), at 50% epiboly stage (7h after NT), at 24h post-NT, at 48h post-NT and at larval stage (5 days after NT) (Westerfield, 2003). Moreover, at 24h, 48h and at 5 days post-NT, normal and abnormal development was registered. In the non-manipulated control group, only the fertilization rate was evaluated by the survival rate at MBT stage.

At least three replicates were done in all experimental groups. Results were analyzed using the Chi-square test. When a single degree of freedom was involved, Yates' correction for continuity was performed.

RESULTS AND DISCUSSION

Three methodologies for zebrafish somatic nuclear transplant prior, simultaneously and posterior to the egg activation/fertilization in their technical aspects have been established in our laboratory. All three were developed and evaluated using non irradiated eggs activated/fertilized by non irradiated spermatozoa. In this way, the effects on survival and further embryo and larval development can be attributed exclusively to the transplant methodology employed, because the background noise due to the exigency of the spermatozoa for egg activation in zebrafish (Lee *et al.,* 1999) and the developmental limitations caused by a haploid condition (Nüsslein-Volhard and Dahm, 2002) would not exist in this case. On the subject, it has to taken into account that in these fish species, the tri-or tetraploid condition, which could occasionally be derived from the addition of a somatic nucleus to the resident zygote pronuclei, does not affect the embryonic, and even larval, development in a relevant way (Diter et al., 1988; Peruzzi and Chatain, 2003).

As a first general comment regarding the efficiency of the in vivo artificial collection of ovarian oocytes, the sexual behavior synchrony showed by the separate fish colonies must be pointed out, in such a way that a large number of eggs could be collected in some sessions whereas no eggs might be obtained in others. Another relevant consideration concerns the fact that MBT nuclear transplant embryos were obtained in the great majority of the daily experimental sessions, whatever the transplant method used.

A technical advantage common to the three methodologies developed in our laboratory was the avoidance of previous de-chorionation. In fish nuclear transplant, when the oocyte is activated but not enucleated, the donor nucleus is usually inserted in the perinuclear region of the oocyte, the closest as possible to the female nucleus, which is located in the cytoplasm subjacent to the plasma membrane under the micropyle (Amance and Iyengar, 1990). In various teleost species, such as medaka,

catfish or tilapia, the animal pole position can easily be detected in pre-activated oocytes because the micropyle can be visualized at this stage, although, unfortunately, this is not the case in zebrafish (Poleo *et al.*, 2001b).

One possible way to obviate such biological difficulty in zebrafish was attempted in method A, in which the somatic cell nucleus was inserted in the central region of the egg. This point of transplant was chosen because, in terms of probability, the central region will be closer to the female nucleus, whatever its real localization. So, the donor nucleus will be more likely to migrate to the microvillae cluster in animal pole, the place where the fertilization occurs (Wolenski and Hart, 1987), through the cytoplasmic flows together with the pronuclei at the time of the activation.

The earliest manipulated group (A1) reached significant higher survival rates compared with the most aged group (A2) both at epiboly and 24h stage (Table 1). However, in more advanced stages (48h and larval stage), the observed differences did not reach significant levels, probably due to the low number of embryos that developed to these stages. The egg control group (CA) fertilized at the end of each experimental session showed MBT rates equal or higher than the two experimental timing groups, indicating the maintenance of the egg fertilization ability until the end of the transplant session. Anyway, the larval survival rate was 7% (A1:6 larvae from 82 manipulated) in the first group and 5% (6 larvae from 132 manipulated) taking into account the overall two groups.

It must be underlined that to cool down to 8ºC the temperature of the micromanipulation area was decisive to maintain the egg in a non activated state during transplant.

This initial strategy permits the impregnation of the donor nucleus in the reprogramming factors present in the egg at metaphase stage, and in further experiments the effect of different times of donor nucleus impregnation before the activation will be tested.

In method B, the nuclear transplant and fertilization were performed at the same time, which meant transplanting the donor nucleus while the egg was activating. Egg activation and fertilization are both marked by elevation of the chorion and a dramatic reorganization of the yolk cytoplasm. In this way, the animal pole is

segregated through the place where the female nucleus will be located (Wolenski and Hart, 1987). So, this enabled detection of the incipient animal pole to deposit the somatic nucleus in the female perinuclear region.

The survival rate differences between the first (B1) and second group (B2) did not reach significant levels in any assessment. However, it must be pointed out that these differences decreased over time and the survival rates were finally similar at 48 h stage (B1: 19% vs B2: 20%). In this case, the fertilization rate of the final control group (CB) was lower than the Total B, even than the B2, which involved an obvious effect of the egg aging and a very slightly negative effect of the assayed nuclear transplant technique.

When the NT was performed posterior to the egg fertilization, method C, the variability in the time required for showing the perivitelline space as an activation signal should be pointed out. This represented a critical point due to the technical difficulty in fixing the egg with the holding pipette because the egg rotated inside the chorion while this space was increasing. With the activation, the micropyle can be more easier detected but, a few seconds after the fertilization, the chorion hardened and the micropyle sealed (Poleo *et al.*, 2001b). This made it very difficult to insert the donor nucleus through this point even if the microinjection pipette was fire polished, beveled and sharpened, as in our case. A possibility to obviate this difficulty could be to de-chorionate the egg after fertilization but, this technique is time-consuming and the first cleavage is very early (minutes) in zebrafish. Moreover, the reduction of the temperature to arrest development before the MBT stage involves embryo lethality (Simao *et al.*, 2007).

Regarding the survival differences between both handling groups (C1 and C2), it should be emphasized that, as in the technique B, the differences observed did not reach significant levels in any case. However, these differences were relevant, ranged from a differential of 10% at MBT stage to up 15 points at the larval stage. The explanation for not reaching significance level could be the low number of surviving embryos that developed to this stage owing to the aforementioned technical difficulty. The survival rate of the control group (CC), compared with the C1 and C2 groups,

showed again a considerable egg aging effect, which means that the time for performing the NT in zebrafish must be shorter in order to avoid (or minimize) such a pronounced negative effect.

Adult specimens showed *gold* phenotype. This fact does not discard a possible mosaicism or that the reconstructed embryos with the donor nucleus incorporated more efficiently did not reach adult stages. However, it has to be into account that the nuclear fate of the transplanted nuclei was not analyzed because, as mentioned previously, the main aim of this work was to establish three nuclear transplant protocols in zebrafish by a technical approach. By this way, after the establishment of these three techniques presented, the nuclear fate will be studied, as well as its integration degree and form in the specimens.

So, the reasonable technical efficiencies achieved in the present work make the use of these three methods interesting for future reprogramming studies by nuclear transplant in this species.

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Table 1. Method A: survival rates of nuclear transplant prior to the egg activation/fertilization.

		A1	A2	Total A	CA
Nº of transplanted eggs		82	50	132	301
Nº of embryos at MBT stage		23 (28%) [a]	6 (12%) [a]	29 (22%)	90 (30%)
Nº of embryos at epiboly stage		18 (22%) [a]	3(6%) [b]	21 (16%)	-
Nº of embryos at 24h post-transplant	normal	12 (15%) [a]	1 (2%) [b]	13 (10%)	-
	abnormal	2	1	3	-
	total	14	2	16	-
Nº of embryos at 48h post-transplant	normal	8 (10%) [a]	1 (2%) [a]	9 (7%)	-
	abnormal	3	1	4	-
	total	11	2	13	-
Nº of survival larvae		6 (7%) [a]	0 (0%) [a]	6 (5%)	-

Columns with different superscripts are statistically different

Table 2. Method B: survival rates of nuclear transplant simultaneously to the egg activation/fertilization.

		B1	B2	Total B	CB
Nº of transplanted eggs		59	54	113	53
Nº of embryos at MBT stage		32 (54%) [a]	19 (35%) [a]	51 (45%)	15 (28%)
Nº of embryos at epiboly stage		22 (37%) [a]	15 (28%) [a]	37 (33%)	-
Nº of embryos at 24h post-transplant	normal	14 (24%) [a]	11 (20%) [a]	25 (22%)	-
	abnormal	6	14	10	-
	total	20	15	35	-
Nº of embryos at 48h post-transplant	normal	12 (20%) [a]	11 (22%) [a]	23 (20%)	-
	abnormal	5	1	6	-
	total	17	12	29	-
Nº of survival larvae		11 (19%) [a]	11 (20%) [a]	22 (19%)	-

Columns with different superscripts are statistically different

Table 3. Method C: survival rates of nuclear transplant posterior to the egg activation/fertilization.

		C1	C2	Total C	CC
Nº of transplanted eggs		49	22	71	374
Nº of embryos at MBT stage		26 (53%) [a]	9 (41%) [a]	35 (49%)	147(39%)
Nº of embryos at epiboly stage		21 (43%) [a]	7 (32%) [a]	28 (39%)	-
Nº of embryos at 24h post-transplant	normal	16 (33%) [a]	4 (18%) [a]	20 (28%)	-
	abnormal	5	1	6	-
	total	21	5	26	-
Nº of embryos at 48h post-transplant	normal	14 (29%) [a]	3 (14%) [a]	17 (24%)	-
	abnormal	3	1	4	-
	total	17	4	21	-
Nº of survival larvae		14 (29%) [a]	3 (14%) [a]	17 (24%)	-

Columns with different superscripts are statistically different

STUDY IV: Transplant of adult fibroblast nuclei into the central region of metaphase II eggs resulted in mid blastula transition (MBT) embryos*.

ABSTRACT

Recently, a novel technical method to perform somatic nuclear transplant in zebrafish using non activated eggs as recipients without the need to detect the micropyle was developed in our lab. However, the use of spermatozoa as activating agent prevented to know if the inserted nucleus conditioned embryonic and early larval developmental ability. The aim of the present work was to test the developmental ability of the embryos reconstructed by transplanting adult fibroblast nuclei into the central region of the metaphase II egg but subsequently activated by only water. In addition, since oocyte ageing facilitates the activation in mammalian oocytes and also leads to metaphasic plate disorganization, this work also pursued to test whether limited aged eggs used as recipients improve the activation and involve some functional enucleation in zebrafish. The adult somatic nucleus located in the central region of the non activated egg resulted in the 12% of mid blastula transition embryos *vs* the 20% when the transplant is in the animal pole ($p \geq 0.05$). This fact suggests that the central region of the non activated metaphase II eggs can be a suitable place for nucleus deposition in nuclear transplant in zebrafish. These results reinforce the possibility to use non activated metaphase II eggs in subsequent reprogramming studies by adult somatic nuclear transplant in zebrafish. Unfortunately, in contrast to mammals, a limited egg ageing (2h) penalized did not improve the activation process in zebrafish nuclear transplant, moreover the hypothesis about a possible functional enucleation was also ruled out.

KEY WORDS: Nuclear Transplant, Activation, Oocyte, Reprogramming, Zebrafish.

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INTRODUCTION

In the few nuclear transplant (NT) studies reported to date in zebrafish (Li *et al*., 2000; Hu *et al*., 2002; Lee *et al*., 2002; Luo *et al*., 2009), the location of the donor nucleus injection place, the animal pole, requires previous oocyte activation because of the micropyle cannot be easily detected in this species (Poleo *et al*., 2001b).

Recently, a novel method developed in our lab made it technically possible to perform the somatic nuclear transplant in zebrafish using mature non activated eggs without the need to dechorionate or localize the micropyle (Perez-Camps *et al*., 2009). In this study, non enucleated oocytes were activated by sperm fertilization after the nuclear transplant. Such strategy was based on the fact that diploidized eggs have been shown as suitable recipients for nuclear transplant in medaka due to the more stable ploidy status, among other aspects (Bubenshchikova *et al.*, 2007). But, the activation by sperm fertilization prevented to know if the donor nucleus conditioned embryonic and early larval developmental ability.

In fish somatic cloning, as in mammals, it is assumed that the nuclear transplant have to do using enucleated oocytes. However, in zebrafish, the resident pronucleus in non enucleated and non fertilized egg receptors seems to degenerate after the nuclear transplant during the first cleavages (Li *et al*., 2000; Li *et al*., 2003). This particularity in zebrafish, not showed in other species as medaka, loach or amphibious (Hu *et al*., 2002), could avoid the need to use whatever enucleation method (mechanical, ionizing and ultraviolet radiations, etc.) including laser.

Since oocyte ageing facilitates the activation in mammalian oocytes, but also leads to metaphasic plate disorganization in this species (Kim *et al*., 1996), it can be hypothesized that a limited ageing in zebrafish eggs could improve the activation response and, perhaps, induce some type of “aging” functional enucleation.

Our lab is focused on developing in zebrafish an efficient somatic cell nuclear transplant technique to be used for reprogramming studies using non activated metaphase II eggs as recipients. The aim of the present work was to test the developmental ability of the embryos reconstructed by transplanting the adult fibroblast into the central region of the metaphase II egg subsequently activated by

only water. In addition, this work pursued to test whether limited aged eggs used as recipients improve the activation and involve some functional enucleation in zebrafish.

MATERIAL AND METHODS

Non activated eggs and sperm collection.

Eggs and sperm were collected after evaluating the sexual behaviour of both *gold* strain males and females at dawn. Eggs were obtained by gentle extrusion of the ovary and sperm was recovered from the genital pore in individual glass microcapillaries (Pérez-Camps *et al.* 2009). Then, non activated gametes were kept in egg medium (Hanks' buffered salt solution supplemented with 1.5% (v/v) of BSA and 0.1 g of NaCl/100 cc of Hanks' medium; ph: 7.4 ; osmolarity: 310-320 mOsm) at 8ºC (Cardona-Costa *et al.*, 2009).

In vitro fertilization.

Non activated eggs and sperm were mixed in a 35-mm Petri-dish and both gametes activated by the addition of a 1 mL of system water. After 2-3 min, the dish was filled with system water to achieve well developing embryos. Further culture was done at 28.5 ºC (Cardona-Costa *et al.*, 2009).

Donor cells culture.

Adult somatic cells used as nuclei donors came from *wild* zebrafish caudal fin primary cultures. Tissue was obtained and treated as Pérez-Camps *et al.* (2009) described and cultured in Leibovitz medium supplemented with 20% of FBS and 0.036 g/L of glutamine (L15-FBS) at 28.5ºC (Cardona-Costa *et al.*, 2009).

Somatic cell nuclear transplant.

Somatic cell nuclear transplant technique, methods and equipment employed was described by Pérez-Camps *et al.* (2009). Briefly:

Method A-Nuclear transplant prior to egg activation/fertilization.

To prevent egg activation, eggs were kept in egg medium at 8ºC until NT, which was performed in a handling drop of egg medium in a petri plate under mineral oil and

the micromanipulation area was cooled to 8 ºC. The somatic cell nucleus was inserted in the central region of the MII egg. Then, transplanted eggs were individually either activated by only water or fertilized by sperm and cultured at 28.5ºC in water (Pérez-Camps *et al.*, 2009).

Method B-Nuclear transplant simultaneous with egg activation/fertilization

When performing NT during the activation only by water, eggs were also kept in egg medium at 8ºC until NT. They were individually deposited in the handling drop containing the water so that oocytes were activated at the moment of transplant. When performing NT simultaneously with the fertilization, non activated eggs and sperm were previously mixed and kept at 8ºC and, as before, individually deposited and transplanted in the handling drop. Donor nuclei were injected in the incipient animal pole in both cases, just where either the female pronucleus or the zygote (male and female) pronuclei were being constituted (Wolenski and Hart, 1987).

Experimental design.

With the aim of testing the developmental ability of embryos reconstructed by transplanting the somatic nucleus into the central region of the non activated egg, the NT were carried out before (method A) and simultaneously (method B) to the egg activation both by sperm and only water as activating stimulus. All nuclear transplants were realized in different sessions immediately after the ovarian egg extraction (0h). In the 0h NT groups, although the literature indicates that eggs can be fertilized until 90 min after their ovarian extraction (Westerfield, 2007; Nüsslein-Volhard and Dahm, 2002), our results in a previous work indicated that, in our hands, development ability drops when NT is performed after the first 45 min following extraction (Pérez-Camps *et al*., 2009). So, we established that the 0h experimental sessions did not exceed 45 min in any case. The nuclear transplants with 2h aged eggs were performed in the same daily session with eggs from the same laying. In this way, to study the egg aging effect on the activation response, results from the 2h NT groups activated only by water were compared with the 0h NT groups activated by water too.

An initial fertilizability control group was done immediately when eggs were extracted to test the egg quality, whatever the time and method of transplant.

Nuclear transplant sessions were considered only when the initial fertility rates were higher than 70%. Activations with water were carried out in parallel to check a possible parthenogenetic development beyond some abortive divisions. Moreover, in all experimental sessions, some oocytes were punctured without nuclear transplant to evaluate the activation efficiency of the micropippete puncture (control groups of activation).

Reconstructed embryos were considered as the developing embryo only when the number of embryonic cells was more than 4 (Lee *et al.*, 1999). In all experiments, survival rates from early embryonic cleavages (>4cell) to larvae (5 days) were evaluated.

At least three replicates were done in all experimental groups. Results were analysed using the Chi-square test. When a single degree of freedom was involved, Yates' correction for continuity was performed.

RESULTS

Central nuclear injection in non activated eggs resulted in embryonic development.

Embryo survival rate differences between the two nuclear transplant methods (transplant before or simultaneously to egg activation) activated by sperm fertilization or by water as the only stimulus were compared (Table 1). Results showed that there were no significant differences in survival rates between the two nuclear transplant methods when the oocytes were activated by sperm. Differences in the embryo survival rates between the two nuclear transplant methods when oocytes were activated by only the water stimulus were not found either.

On the other hand, as was expected, embryonic survival rates differences between reconstructed embryos activated by sperm fertilization and by only water were significant, wherever the nucleus deposition took place (Table 1). Moreover, reconstructed embryos activated only by water reached, at maximum, the MBT stage.

It has to be noted that eggs activated but non transplanted did not show embryonic development. In fact, only very few eggs remained, at most, at the two cell

stage. Moreover, no development signs were observed in punctured non transplanted metaphase II eggs.

Partial egg ageing did not improve the activation.

Because the NT in aged eggs were carried out in the same daily session with eggs from the same female than in the groups previously described at 0h, data corresponding to the freshly eggs were the same presented in the table 1. Differences in the survival rates at early cleavages (> 4 cells) were relevant, although they did not reach significance levels, when young and aged eggs were compared, whatever the NT method, in favour of young eggs (12% vs 3% in NT before activation; 23% vs 7% in NT during activation; $p>0.05$). Differences were also observed at MBT stage but, in this case, significant levels were reached (12% vs 0% in NT before activation; 20% vs 0% in NT during activation). It must be emphasized that none of reconstructed embryos reached the MBT stage when aged eggs (2h) were used. These results suggest that, in contrast to mammals, egg ageing does not involve an improvement in the parthenogenetic development progression or in nuclear transplant efficiency in zebrafish.

The results obtained in this experiment also allowed us to test the possible functional enucleation by ageing. The null survival rates obtained in the 2h transplants ruled out considering a functional enucleation in 2h-aged eggs.

DISCUSSION

In a previous work, we demonstrated that the nuclear transplant in zebrafish before activation of metaphase II eggs was technically possible (Pérez-Camps *et al.,* 2009) but, in such work, the use of spermatozoa as activating agent made it difficult the study, the possible integration of the donor nucleus in the reconstructed embryo. In zebrafish eggs, the mere contact with water causes chorion expansion and cytoplasm segregation, but they either remain at the one cell stage or, in some cases, eggs may develop to some abortive cleavages. In this way, a fertilized embryo is considered as developing when it passes from 2 to 4 well organized cells (Lee *et al.*, 1999). In the present work, we obtained MBT reconstructed embryos by the transplant of adult somatic nuclei in the centre of the non activated eggs, subsequently

activated with water as the only activating stimulus. Moreover, there was no disadvantage at all for the central injection of the nucleus with respect to the nucleus injection in the incipient animal pole.

The obtained results could due to a possible activating action derived from the puncture of the egg with the micropipette during the transplant. In medaka, unfertilized eggs are activated by pricking with a fine glass needle, but most eggs cannot develop further and remain at the one cell stage (Yamamoto, 1944). No data on this phenomenon were found in the reviewed literature in zebrafish. In fact, the only way in which gynogenetic zebrafish embryos have been obtained was by using genetically inactivated sperm by radiation as activating agent (Westerfield, 2007). Moreover, the results here obtained from the puncture of non activated eggs plus water activation (control group of activation) indicate that the only mechanical action of injecting the nucleus does not stimulate by themselves the activation process. So, it can be proposed that, in our case, when no sperm was used as activating stimulus, the nucleus transplanted exerted an activating effect on the nuclear transplant embryo, participating in the development, at least, until MBT stage.

As we hypothesized in method A (central nucleus transplant before the activation), the obtained results suggest that the nucleus transplanted in the centre of the metaphase II egg migrates to the animal pole, may be, through the cytoplasmic flows at the time of activation (Wolenski and Hart, 1987). This result opens the possibility of exposing the donor nucleus to an metaphase II ooplasmic environment for longer and controlled periods of time before activation, a requirement that has proven effective for reprogramming improvement in mammals (Wilmut *et al.*, 1997). Unfortunatelly, the egg culture medium used in the present work is only able to preserve the non activated eggs in good conditions for 1 hour (Cardona-Costa *et al.,* 2009a). A recent study has detected that the Chinook salmon ovarian fluid (CSOF) can preserve non activated eggs for longer periods of time (Siripattarapravat *et al.*, 2009a), but they commercial availability is, at this time, practically null.

Although karyotyping analysis have not realized due to the difficulty in this specie (Hu *et al.*, 2002), the fact that in zebrafish, when nuclear transplant is performed into non enucleated eggs involves the degeneration of the female

pronucleus (Li *et al.* 2000; Li *et al.* 2003), would lead to generate diploid reconstructed embryos, and not triploid, as occurs in other species (Hu *et al*., 2002). Such phenomenon of ploidy correction is not unnusual. In this sense, in medaka, there also seem to be mechanisms that operate in the rearrangement of the chromosome status after the NT (Kaftanovskaya *et al.*, 2007; Bubenshchikova *et al.*, 2007). Mechanisms of this type working in different ways have also been described in mammals (human: Tesarik and Mendoza, 2003; pig: Somfai *et al.*, 2008).

To date, in zebrafish, nuclei from embryonic cells (Hu *et al.*, 2002), from 5-somite embryos (Lee *et al.*, 2002) and from kidney cells of adult male (Luo *et al.*, 2009) have been transplanted into enucleated pre-activated eggs. In our knowledge, in the present work, fibroblasts from primary cultures are used for the first time as donor nuclei in zebrafish nuclear transplant without activation by sperm fertilization. The percentages of reconstructed embryos achieved were similar to those obtained by Lou *et al.* (2009), being even higher when water activation was previous to the NT (20%). However, as Lou *et al.* (2009) the survival rates dropped drastically after reaching the blastula stage.

P.S: During the elaboration of the present manuscript, a new zebrafish nuclear transplant study was published by Cibelli and colleagues (Siripattarapravat *et al*., 2009b), in which adult specimens were obtained from larval cells but not from adult cells.

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Table 1. Survival rates of somatic cell nuclear transplants into non enucleated freshly eggs activated by sperm or by water.

	Method A Transplant before the activation		Method B Transplant during the activation	
	Activated by sperm fertilization	Activated by only water	Activated by sperm fertilization	Activated by only water
Eggs transplanted	20	34	20	30
MBT embryos	9 (45%) [a]	4 (12%) [b]	8 (40%) [a]	6 (20%) [ab]
1day embryos	7 (35%) [a]	0 (0%) [b]	4 (20%) [a]	0 (0%) [b]
5 days larvae	4 (20%) [a]	0 (0%) [b]	3 (15%) [ab]	0 (0%) [b]

Data in columns with different superscripts are statistically different (p<0.05)

Table 2. Survival rates of somatic cell nuclear transplants into non enucleated fresh and 2h-aged eggs activated by water. Data of freshly egg are the same that in table 1.

	Freshly eggs		2h-aged eggs	
	Method A	Method B	Method A	Method B
Eggs transplanted	34	30	34	30
Embryos with more than 4 well orientated cells	4 (12%) [ab]	7 (23%) [a]	1 (3%) [b]	2 (7%) [ab]
MBT embryos	4 (12%) [abc]	6 (20%) [a]	0 (0%) [c]	0 (0%) [bc]

Data in columns with different superscripts are statistically different (p<0.05)

STUDY V: Electroactivation of Zebrafish (*Danio rerio*) Eggs*

ABSTRACT

In zebrafish, initial egg activation by water after being discharged from the ovarian stroma is followed by normal cleavages only in inseminated eggs. When sperm (inactivated or not) is not used as activating agent, reproductive techniques (as either NT or ICSI) are inefficient. In this work, three experiments were performed: In the first, 6 treatments were compared (Voltage x Pulses: L1=2.76x1; L2=2.76x2; L3=2.76x3 and D1=5.40x1; D2=5.40x2; D3=5.40x3). The group D3 showed the best results (32% activated). In the second experiment, al electrical treatment of 20 min duration was carried out. It consisted in a sequence of three equal electrical stimuli every 10 min (of 1 or 3 consecutive DC square pulses for 20 µs each and applied at two voltage levels, 2.76 V or 5.4 V). Only the 20 min treatment with the combination of 3 consecutive pulses at 2.76 V showed significant differences in the activation response with their respective control group (Electroavtivated eggs: 43% vs Control: 18% , $p<0.05$). In the third experiment, negative effects of egg ageing were observed in the activation rates. The best treatment was an electrical pulse sequence of 20 min, where 1 pulse of 20 µs of 5.4 V was applied at 0 min post-activation in system water, at 10 min and finally at 20 min (3 pulses in total). This treatment was able to activate 66 % of fresh and aged (for 1 h) eggs, but without accomplishing normal parthenogenetic embryo development.

KEY WORDS: Activation, Egg, Partenogenetic, Zebrafish.

INTRODUCTION

In zebrafish, egg activation takes place after they are discharged from the ovarian stroma and come into contact with the spawning medium (Sakai, 1997), inducing an intracellular calcium wave discharge (Lee *et al*., 1999). In consequence, activated eggs (oogenesis stage V and thus capable of being fertilized; Howley and Ho, 2000) undergo a programmed series of biological events briefly described by Lee *et al* (1999): contraction of the egg surface, initial small separation of the chorion from the plasma membrane, cortical granule reaction, chorion elevation and ooplasmic segregation. These steps are followed by normal cleavages when a spermatozoon, either genetically inactivated or not, has fertilized the egg (Lee *et al*., 1999; Nüsslein-Volhard and Dahm, 2002. If sperm is not present in the spawning medium, an incomplete parthenogenetic activation takes place, undergoing some abortive cleavages in the best of cases, but without reaching further development (Cardona-Costa *et al*., 2009a), or even the configuration of four well organized cells (Lee *et al*., 1999).

In mammals, oocyte activation is routinely induced or reinforced by electrical pulses (and/or chemicals) after performing somatic cell nuclear transplant (SCNT, Okahara-Narita *et al*., 2007), intracytoplasmic sperm injection (ICSI, Mansour *et al*., 2007; Zhang *et al*., 1997) or in the obtaining of parthenogenetic haploid/diploid embryos (Elsheikh *et al*., 1995; Escribá and García-Ximénez, 2000). Despite this, little attention has been paid to what benefit could be provided by an artificial activation procedure by electric pulses in zebrafish. In this sense, zebrafish NT is still inefficient (Huang *et al*., 2003; Pérez-Camps *et al*., 2009; Siripattarapravat *et al*., 2009b) as also occurs with other techniques such as ICSI (Poleo *et al*., 2001). So, the efficiency in the development of these and other reproductive techniques in this species (i.e. round spermatid injection, ROSI; round spermatid nuclear injection, ROSNI), also depends on efficient procedures for artificial egg activation (Huang *et al*., 2003; Bubenshchikova *et al*., 2007; Pérez-Camps *et al*., 2009). To date, the only activation protocols in zebrafish used water as activating agent (Pérez-Camps *et al*., 2009; Siripattarapravat *et al*., 2009b). So, the electroactivation as an alternative egg activation method in zebrafish

would also permit the experimental evaluation and study of egg activation abilities throughout their egg ageing process, even exceeding the limits of egg fertilizability.

In fact, to our knowledge this methodology has only been attempted in fish for egg activation in NT experiments on medaka fish (Bubenshchikova *et al*., 2007; Wakamatsu, 2008). In this context, the aim of the present work was to establish electrical parameters for the activation of zebrafish intact (non micromanipulated) eggs as well as their activation response throughout their ageing process, but without involving the sperm-mediated stimulus.

MATERIAL AND METHODS

Animal care

Zebrafish (*Danio rerio*) specimens from the *gold* strain were maintained in 20 L aquariums under standard conditions (Westerfield, 2007). Granular food was supplemented with recently defrosted chicken egg yolk and shrimp meat as recommended for egg production in zebrafish in substitution of live food as reported by Simão *et al*. (2007).

Obtaining of gametes and fertilization

The procedure carried out was described in detail by Cardona-Costa *et al*. (2009a). Briefly, gametes were extracted at the same artificial "dawn" time from those aquariums where fish displayed reproductive behaviour. Both males and females were simultaneously anaesthetized with clove oil solution of 100 µl/l (Grush *et al*., 2004) in dechlorinated and decalcified tap water (system water; Westerfield, 2007). All aseptic procedures and sterile instruments were used in subsequent steps. Sperm and eggs were in vivo extracted and maintained inactivated until use at 8 ºC in a modified Hanks 'solution designed as CH (100 ml of Hanks´ supplemented with 1.5 g BSA and 0.1 g ClNa; 320 mOsm, pH 7.4). The motility of selected sperm was 80-100% and eggs were of good quality (translucent, granular and yellowish appearance; Westerfield, 2007).

Control groups of fertilized eggs were formed at the beginning of experiments. To this end, those sessions where the fertilization control was lower than 70 % were discarded. The fertilization procedure was done in a 35 mm Petri dish (Corning) after

carefully mixing 15-20 eggs and sperm solution. Then, 3 to 5 system water drops were added to fertilize eggs (sperm concentration in fertilization was around 2.8 x 10^8 spz/ml, measured with a Thoma counting chamber). Finally, the plate was filled with system water after 5 min.

Control groups activated only by water stimulus were transferred by the same procedure to a Petri dish (Corning) with system water where they were activated only by the water stimulus.

All chemical products and culture media were from Sigma-Aldrich.

Electroactivation

The electroactivation equipment consisted of an Electro Cell Manipulator (ECM 2001; BTX) complemented with a digital oscilloscope from Tektronix. The pulsing chamber used was model 453 from ECM.

The first step of this procedure consisted of the careful selection of 5 to 20 inactivated eggs (per batch) contained in CH medium. With the aid of a Pasteur pipette, previously stretched and fire polished, a part of these eggs (per experimental group) were held in the pulsing chamber, containing system water as electroactivation medium. Immediately, the predefined direct current (DC) square pulse was applied. This pulse was systematically checked through the oscilloscope. Finally, treated eggs were transferred to Petri dishes (Corning) with system water and incubated at 28.5 ºC.

Control groups (see experimental design) came from the rest of the same batch of eggs.

Assessments related to damage/lysis, activation and developmental signs took place immediately after each pulse and at 1 h and 2 h post-activation (after the first contact with system water).

Eggs were considered “activated eggs” when they showed at least one mitotic cleavage apart from the normal activation signs (already described in the introduction; Lee *et al*., 1999).

Experimental design

Preliminary assays

As an initial approach, the electrical conditions reported by Wakamatsu (2008) for medaka egg activation (a double electrical DC pulse of 8.0 V/cm for 20 μs, voltage applied with our employed fusion chamber: 2.76 V) were tested. In addition, different voltages were also tested (2.76 V, 5.4 V, 8.3 V, 11.4 V and 13.8 V; corresponding to 2.76 (x1), 2.76 (x2), 2.76 (x3), 2.76 (x4) and 2.76 (x5)).

Experiment A

In line with results obtained in the preliminary assays, the following electrical parameters were finally tested: 1, 2 or 3 consecutive DC pulses for 20 μs each at room temperature, in system water as electroactivation (ionic) medium. Electric field intensity was established at two levels (E_1 = 8 V/cm vs E_2 = 16 V/cm; voltages with the fusion chamber employed were 2.76 V and 5.40 V respectively). In this way, a total of 6 experimental groups were established in this first experiment (Voltage x Pulses: L1=2.76x1; L2=2.76x2; L3=2.76x3 and D1=5.40x1; D2=5.40x2; D3=5.40x3). Eggs were manipulated during the first 30 min after ovarian extraction. A general control group of activated eggs in system water, but without pulsing, was established in parallel with the experimental groups.

Experiment B

Siripattarapravat and colleagues (2009a) established an elapsed time of 30 min (at 28 ºC culture) between egg activation and the first mitotic cleavage in zebrafish. Experiment B pursued the maintaining of the reduced MPF levels, due to the activation stimulus, but which rise after 10 min of such activation stimulus when this activation process is only promoted by the water stimulus (incomplete parthenogenetic activation). In this way, an electric treatment extended to 20 min was tested, where 1 or 3 consecutive DC pulses for 20 μs each were applied at 0 min, 10 min and after 20 min of their initial activation in water (pulsing medium). The 2.76 V (L1, L3) and 5.4 V (D1, D3) voltage levels were used. Four groups were established (B1= L1 x3; B2= L3 x3 and B3= D1 x3; B4= D3 x3).

Eggs were manipulated during the first 30 min after their ovarian extraction. A general control group of activated eggs in system water, but without pulsing, was established in parallel with the experimental groups.

Experiment C

Because zebrafish egg ageing is incompatible with fecundation but still tolerates (for longer) the activation stimulus, this third experiment pursued the assessment of the better/worse response of egg activation in aged eggs previously stored at 8ºC in CH medium (non activating medium[5]) for different times.

The better combinations from among all experimental groups tested were selected for their best and similar egg activation rates by electric pulses, and used in this experiment. So, the experimental groups were carried out as a result of joining the different egg ageing times tested (0 h, 1h and 2h post- ovarian extraction) and the best electric treatments obtained previously (B2=L3x3 and B3=D3x3, see results). In addition, three control groups of eggs activated in system water at 0 h, 1h and 2 h after their ovarian extraction, but without pulsing, were established in parallel with their respective experimental groups.

Statistical analysis

In all experimental groups from each Experiment (A, B or C), a minimum of three replicates were done in alternative sessions. Results were analysed using the Chi-square test. When a single degree of freedom was involved, Yates' correction for continuity was performed.

RESULTS

From preliminary assays

Results showed that voltages of 8.3 V and higher were not suitable for electroactivation assays. The reason was the high degree of internal damage caused in eggs, observed as a non-clear delimited region between the animal and vegetal egg poles, once the ooplasmic segregation ended. Moreover, from 11.4 V onwards, lysis of

egg plasma membrane was also observed (8.3 V: 4 eggs damaged from 8; 11.4 V: 5 eggs lysed from 9; 13.8 V: 2 eggs lysed from 3; results not shown in tables).

From Experiment A

Results are presented in Table 1. As shown, the differences in the percentage of activated eggs only raised levels of significance between the D3 (5.4x3) group when compared with Control (only activated in system water) and the L1 (2.76x1) group.

From Experiment B

In this experiment, the electrical treatment was extended to a sequence of three pulse stimuli distributed equally for 20 min (one pulse every 10 min) to maintain the MPF levels low until completion of the zygotic stage. The stimuli applied were adjusted to the two most extreme combinations of voltage and number of pulses (L1 (2.76x1) vs D3 (5.4x3)) established in experiment A (Table 2). First, it was observed that the number of pulses applied negatively affected the percentage of damaged and lysed eggs significantly. In this sense, the combination of voltage and number of pulses in the B4 group produced a multiplicative effect that damaged or lysed all treated eggs.

In another sense, and in relation to egg activation rates, the B2 group was the only one that showed positive and significant differences with Control, reaching a percentage of 43 % activated eggs. On the other hand, groups B1 and B3 did not differ significantly from the control, although differences were relevant in the case of group B3 due to the high percentage reached (36 %, Table 2).

From Experiment C

Results are presented in Table 3.

In this experiment, with respect to the damaged/lysed eggs, differences were not observed among the three control groups of different egg ageing times, as well as among their activation rates.

The activation efficacy of B2 and B3 treatments was lower as egg age increased. Despite this, group B3 maintained the activation rates in 1 h aged eggs but declined significantly at 2 h.

DISCUSSION

The definition of an egg electroactivation procedure involves the combination of several and different electrical parameters and conditions, mainly the electric field intensity and the number of pulses applied.

Whatever the case, all of them pursue a rational similarity with the sperm-activating stimuli aimed at achieving a better activation response in eggs. In this respect, the selection of an appropriate activation medium is important. In mammals, the use of non-ionic media in electroactivation processes is common (Chang *et al*., 1992) although the ionic media are also efficient (Rickords and White, 1992; Elsheikh *et al*., 1995). In contrast with this, to our knowledge the only information available in fish refers to Bubenshchikova *et al*. (2007) and Wakamatsu (2008). In those cases, an ionic medium was used (a balanced salt solution medium designed for medaka) for the electroactivation of medaka fish eggs. Unfortunately, no further bibliography was found. So, initially, we also used an ionic medium (system water in our case) as the starting point of our electroactivation assays in zebrafish eggs.

Recent studies by Siripattarapravat *et al*. (2009a) reported that no significant differences were found in the initial dropping of MPF activity in zebrafish eggs activated by either fertilization or water. Nevertheless, they observed that MPF levels from 10 min to 30 min post-fertilization/activation were different between both activation procedures. Indeed, MPF values in eggs activated parthenogenetically by water increased slightly from 10 min post-activation, which coincided with the extrusion of second polar body, and showed a slightly greater peak difference at 20 min post-activation, in contrast with those eggs activated by fertilization. It is known in mammals that egg MPF activity can be maintained at low levels by the application of electrical pulses at different time intervals after their first artificial activation, as normally occurs after normal egg activation by fertilization. The treatment tested in the second experiment pursued an artificial maintenance of MPF activity at low levels

by means of the temporal distribution of electric pulses throughout the post-fertilization period up to the corresponding time of the first mitotic cleavage. Results obtained in the second experiment indicated the general improvement upon the activation rates achieved in the first experiment. Here, the B2: L3x3 ([2.76x3]x3) and B3: D1x3 ([5.4x1]x3) treatments were established as the most efficient.

The third experiment evaluated the activation ability of eggs stored (aged) for up to 2 h. Under our storage conditions (Cardona-Costa *et al*., 2009a), this was the maximum time in which eggs were still able to be fertilized.

The results obtained in this last experiment showed a clear advantage in the activation capability of fresh eggs compared with those partially aged (2 h). This result is contrary to that observed in mammals, where oocyte ageing improves their activation rates, whether normal or parthenogenetic, and is explained as a greater feasibility of aged oocytes to maintain reduced their MPF levels (Escribá and García-Ximénez, 1999). However, in this case the subsequent embryo development can also be penalized (Kaufman, 1981; Stice and Robl, 1988; Collas and Robl, 1990; Tanaka and Kanagawa, 1997). It is possible that the degree of ageing tested in present experiment was somewhat excessive.

In general, in this work we observed a positive relation in the use of either higher voltage or number of pulses (higher stimulation degree) and the percentage of activated eggs. So, it can be proposed that the best activation treatment for intact (non manipulated) zebrafish eggs concerns sequence B3: an electrical pulse sequence of 20 min, where 1 pulse of 20 µs of 5.4 V was applied at 0 min post-activation in system water, at 10 min and finally at 20 min (3 pulses in total). This treatment was able to activate 66 % of fresh and aged (for 1 h) eggs.

None of the activation stimuli proposed here induced parthenogenetic development of eggs until the second mitotic cleavage, with four well organized cells (Lee *et al*., 1999). This fact represents a notable difference compared with mammals (Elsheikh *et al*., 1995; Escribá and García-Ximénez, 2000), although in this species the intensity and duration of electrical stimuli are also essential for the oocyte activation and parthenogenetic developmental rates. Until now, in zebrafish, these events (parthenogenetic development) have only been possible via genetically inactivated

sperm (Nüsslein-Volhard and Dahm, 2002). However, the activation stimulus proposed here could serve for improving the embryo developmental rates in procedures which involve nuclear injection techniques in zebrafish such as NT (Siripattarapravat *et al*., 2009b; Huang *et al*., 2003; Pérez-Camps *et al*., 2009 or ICSI (Poleo *et al*., 2001) as occurs in mammals (Machaty, 2006). Moreover, in fish, the electrical (and/or chemical) stimuli would be the only ones available in these procedures once the egg has already been activated by water.

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Table 1. Percentage of activated eggs under 1, 2 or 3 consecutive electric pulses stimulus at two voltage levels (2.76 V and 5.4 V). In Control, eggs were only activated in system water.

Group	Control	L1	L2	L3	D1	D2	D3
Voltage x Pulses		2.76x1	2.76x2	2.76x3	5.4x1	5.4x2	5.4x3
Initial	65	81	80	63	92	96	60
Activated	14%[a]	10%[a]	17%[ab]	19%[ab]	21%[ab]	19%[ab]	32%[b]
	(9)	(8)	(14)	(12)	(19)	(18)	(19)

Data in columns with different superscripts are statistically different (P<0.05)

Table2. Percentage of activated eggs and damage-lysis rates as result of applying an electrical treatment of 20 min duration, consisting of a sequence of three equal electrical stimuli (B1=L1(2.76Vx1)x3; B2=L3(2.76Vx3)x3; B3=D1(5.4Vx1)x3; B4=D3(5.4Vx3)x3). In Control, eggs were only activated by system water.

Groups (Voltagexpulses)x3	Control	B1 (L1x3)	B2 (L3x3)	B3 (D1x3)	B4 (D3x3)
Initial	62	44	58	44	30
Damaged/lysed	0%[a]	0%[a]	17%[b]	2%[a]	100%[c]
	(0)	(0)	(10)	(1)	(30)
Activated	18%[b]	18%[b]	43%[c]	36%[bc]	0%[a]
	(11)	(8)	(25)	(16)	(0)

Data in columns with different superscripts are statistically different (P<0.05)

Table 3. Percentage of activated eggs and damage-lysis rates in B2 and B3 groups (B2=L3(2.76Vx3)x3; B3=D1(5.4Vx1)x3) previously aged for different times (0 h, 1 h and 2 h). In Controls, eggs were stored in CH medium at 8 ºC and activated in system water.

Egg ageing time	0 h			1 h			2 h		
Groups	Control	B2	B3	Control	B2	B3	Control	B2	B3
	0h			1 h			2 h		
Initial	76	92	57	101	110	62	142	132	66
Non activated	92%	40%	23%	88%	70%	27%	91%	86%	47%
	(70)	(37)	(13)	(89)	(77)	(17)	(129)	(113)	(31)
Damaged/lysed	0% ^{ac}	4% ^{ab}	9% ^{b}	2% ^{abc}	3% ^{abc}	7% ^{ab}	0% ^{c}	2% ^{abc}	35% ^{d}
	(0)	(4)	(5)	(2)	(3)	(4)	(0)	(3)	(23)
Activated	8% ^{a}	56% ^{c}	68% ^{c}	10% ^{a}	27% ^{b}	66% ^{c}	9% ^{a}	12% ^{a}	18% ^{ab}
	(6)	(51)	(39)	(10)	(30)	(41)	(13)	(16)	(12)

Data in columns with different superscripts are statistically different (P<0.05)

STUDY VI: Comparison of different activation stimuli efficiency in zebrafish nuclear transplant.

ABSTRACT

The oocyte activation is one of the most important step for the nuclear transplant (NT) technique outcome. In previous works, we used sperm fertilization and water as activating agent and established an activation technique by electric pulses using non manipulated eggs. To day, the only way by which haploid larvae can be obtained is using radiated sperm as activating agent. Firstly, in the present work, we adapted the UV radiation dose to be applied to *gold* sperm for using it as activating agent in NT experiments. Secondly, we compared the embryo survival rates and the developmental degree derived from the transplant of adult fibroblast nuclei before or during egg activation associated to four activating stimuli (fresh sperm, radiated sperm, electroactivation and water). Unfortunately the electroactivation technique established for non manipulated eggs lysed all eggs when it was associated to the NT. Results from the other three stimuli together with the two NT methods showed higher embryo survival rates when the activation was by sperm (24h larvae: fresh sperm 31% and 17%; radiated sperm 20% and 11%; water 0% in both). Regarding the developmental degree, embryos activated by fresh sperm reached the free stage (5 days), those activated by radiated sperm the 24h stage and embryos activated by water only reached the MBT stage wherever the NT method.

KEYWORDS: Activation, Haploid development, Nuclear transplant, Sperm, Zebrafish.

INTRODUCTION

The oocyte activation is a critical step for the outcome of the nuclear transfer (NT) technique, both in mammals and in fishes.

In fishes, the stimulus of the spermatozoa is required to obtain a complete activation, although the initial contact with fresh water initiates the process. In zebrafish, Perez-Camps *et al*. (2009) developed a technical method for performing the NT using metaphase II eggs. In such study, fresh sperm was used as activating agent both after and simultaneous to the nuclear transplant. The sperm as activating agent in NT into non enucleated eggs supposed an initial tetraploid condition that favours a better chromosomic status for like-clones obtaining, as occurs by diploidising eggs in medaka (Bubenshchikova *et al*., 2007). However, the sperm used as activation agent prevented to know if the inserted nucleus in the conditioned embryonic and early larval developmental ability.

However, the water as the only activating agent resulted less efficient than the sperm stimulus, obtaining only embryos at mid blastula transition stage (Pérez-Camps *et al*., 2009*).

If radiated sperm, the only way to activate partenogenetically zebrafish eggs to date (Westerfield, 2007), is used as activation stimulus of non enucleated metaphase II eggs after the NT or during the transplant, the participation of the spermatozoa nucleus would not exist so, a hypothetic triploid condition could be generated when non enucleated eggs were transplanted. This condition supports an embryo development in a lot of fish species. However, in zebrafish, as the female pronucleous seems to degenerate in the first cleavages after the transplant (Li *et al*., 2000; Li *et al*., 2003), it may be possible to obtain a high activating stimulus without the participation neither the receptor nor spermatozoa nuclei.

As a possible alternative to the use of inactivated sperm, an electroactivation method recently developed in our laboratory which improved the activation response of non manipulated zebrafish eggs (Cardona-Costa *et al*., 2009**) can be proposed to be associated to the NT. This fact would also allow a reinforcement of other primary stimuli, as the water, in the NT experiments whatever the egg status.

So, the aim of the present work is to compare the efficiencies of activation and embryo development reached when the four activation stimulus (fresh water, radiated

sperm, non radiated sperm and electric pulses) are associated to two methods of nuclear transplantion, before or simultaneously to activation.

MATERIAL AND METHODS

Non activated eggs and sperm collection.

Eggs and sperm were collected after evaluating the sexual behavior of both *gold* strain males and females at dawn. Eggs were obtained by gentle extrusion of the ovary and sperm was recovered from the genital pore in individually glass microcapillaries (Westerfield, 2007). Then, non activated gametes were kept in egg medium at 8ºC (Cardona-Costa et a., 2009a).

Nuclei donor cells.

Somatic cells used as nuclei donor came from *wild* zebrafish caudal fin primary cultures. The tissue was obtained and treated as Pérez-Camps *et al*. (2009a) described and cultured in Leibovitz medium supplemented with 20% of FBS and 0.036 g/L of glutamine (L15-FBS) at 28.5ºC (Westerfield, 2007).

Somatic cell nuclear transplant.

The two somatic cell nuclear transplant techniques, as well as the equipment employed were described by Pérez-Camps *et al*. (2009). Method A: nuclear transplant prior to the egg activation: The somatic cell nucleus was inserted in the central region of the egg. To prevent egg activation, the transplant was performed in a handling drop composed of egg medium and the micromanipulation area was cooled down to 8 ºC. Method B: nuclear transplant simultaneously to the egg activation: the donor nucleus was injected in the incipient animal pole, just where zygote nucleus was being constituted. The handling drop was composed by fresh water so that the eggs became the activation and the micromanipulation area was not cooled (room temperature).

Activation by sperm fertilization.

The activation was carried out as Pérez-Camps *et al*. (2009a) described. When the method A was used, non activated transplanted were individually in vitro fertilized and cultured at 28.5ºC in fresh water. In the case of using the method B, non activated eggs and sperm were previously mixed and kept at 8ºC, next they were individually deposited in the handling drop containing system water so that gametes were activated and fertilized.

Activation by fresh water.

The nuclear transplant before or during egg activation by only fresh water was described by Pérez-Camps *et al*. (2009*). Non activated eggs were activated by fresh water after the NT when the method A was used. Regarding the method B, eggs were already activated in the transplant handling medium (see above).

***Activation by UV radiated sperm*.**

We were looking for the optimal UV radiation dose of *gold* fresh sperm to be used as activation stimulus in NT, since a effect of UV radiation was proven to be strain dependent in zebrafish. Once determined this time, the two NT methods (A and B) were carried out in a similar way than the activation by non radiated sperm (see above).

A sample of 100 μm of the sperm diluted in Hanks' balanced solution (Cardona-Costa *et al*., 2009) was radiated using a UV 85 germicide lamp (General 86 Electric, 30W). Irradiation was carried out at 62 cm of focus-object distance. The radiation dose applied was 0.529 mW/cm2 87 and was measured by a USB 4000 (Miniature 88 Fiber Optic Spectrometer; Ocean Optics Inc. First In Photonics, USA). A vortex (MS1-92 IKA, Wilmington, CA, USA) at 200 rpm was used with the aim of homogenising the radiation area during UV 93 exposure.

Preliminary assays determined the maximum time of sperm radiation by which non activation signals (motility) were observed. This time was established in 18 minutes. So, 4 groups were irradiated (2, 5, 10 and 15 min) from 15 min, as a maximum time, to the 2 min, the time used by other authors (Westerfield, 2007) for the partenogenetic haploids production with *wild* sperm.

The survival rates were evaluated at MBT stage, 24 h and 5 days were, as well as the abnorlamity and degeneration rates at 24h. Abnormality was evaluated by the haploid syndrome. Haploid embryos have a characteristic syndrome. The body is shorter and thicker than a diploid; the brain is less clearly sculptured; the ears are variable in number; and the heart beats in a swollen pericardial cavity. Haploid cells are smaller than diploid cells and there are problems with organogenesis (Westerfield, 2007).

Electroactivation.

The electroactivation equipment consisted of an Electro Cell Manipulator (ECM 2001; BTX) complemented with a digital oscilloscope from Tektronix. The pulsing chamber used was the model 453 from ECM. With the aid of a Pasteur pipette, previously stretched and fire polished, eggs were directly transferred from the NT plate to the the pulsing chamber, containing system water as electroactivation medium. Immediately, the electric pulse predefined by Cardona-Costa *et al*. (2009**) in non manipilated eggs was applied. This pulse, which activated the 66% of the non manipulated eggs, consisted in 20 µs of 5.4 V electric pulses in a sequence of 20 min (0 min post-NT, at 10 min and finally at 20 min). The pulse was systematically verified through the oscilloscope.

Embryo culture.

Transplanted and activated eggs were incubated in system water at 28.5 ºC (Pérez Camps *et al*., 2009).

RESULTS AND DISCUSSION

Electroactivation of NT eggs

The electric pulse applied on transplanted eggs was established on non manipulated oocytes in a previous work (Cardona-Costa *et al*., 2009**). Unfortunately, this electroactivation treatment resulted in the lysis of all eggs previously transplanted. Most of the eggs were lysed even before the second pulse was applied. Such results led us, in a following assay, to test the minimum electric pulse assayed in our previous

work. This lower electrical stimuli pulse consisted in a 20 µs pulse of 2.7 V applied 10 min post-activation in fresh water. This second assay resulted in the lysis of 15 from 17 transplanted eggs and the 2 surviving eggs showed only abortive cleavages. These results indicate that the electric parameters previously defined to activate non manipulated eggs were not useful for the electroactivation of transplanted eggs. So, the experimental electroactivation group initially proposed to be compared with the other was ruled out. Moreover, the results obtained in the assays of electroactivation forces us to develop, in future work, an activation treatment specific for zebrafish NT experiments. However, the definition of an electric pulse associated to the NT in zebrafish exceeded the objective of the present work.

UV radiation of gold sperm for egg activation in NT

Although, it is widely established the parthenogenotes obtaining by sperm or egg radiation in *wild* zebrafish (Nüsslein-Volhard and Dahm, 2002; Westerfield, 2007), the use of UV radiated *gold* sperm to activate *gold* eggs after or simultaneously to the NT has not reported to date. After finding sensibility differences to the UV radiation between *wild* and *gold* embryos (Francisco-Simão M *et al*., 2009) the aim of this experiment was to establish the optimal UV radiation for *gold* sperm to achieve the higher fecundation ability but the lower embryo development and the high abnormality and degeneration rates. Fresh *gold* sperm was radiated for 2, 5, 10 and 15 minutes.

Significant differences were observed among the four groups in the embryo survival at MBT stage. The number of embryos that reached this stage was decreasing when the radiation times was increasing (2 min: 67%; 5 min: 45%; 10 min: 18%; 15 min: 4%; $p<0.05$). Differences between survival rates were significant neither at 24h nor at 5 days post-fertilization. This result, together with the high degeneration and abnormal rates at 24h in the group of embryos fertilized with sperm radiated for 2 min, led us to establish in 2 min the optimal radiation time of *gold* sperm to be used as activation stimulus in NT.

Comparison between activation stimuli

In the present work, three activation stimuli have tested in reconstructed eggs obtained by two NT methods in zebrafish, NT before or during egg activation (Table 2). Related to the two NT methods, no significant differences were observed when the three activation stimulus (fresh sperm, radiated sperm or fresh water) were compared at MBT stage, epiboly stage, 24h and 5 days. Regarding the NT performed before the activation (method A), it has to point out that the methods of activation based in sperm, radiated or not, were in general more efficient than the activation by only fresh water. When the NT was carried out during the egg activation (method B), no differences in the survival rates were observed between the three activation stimulus neither at MBT stage nor at 24h. However, these results must be taken cautiously into account due to the low number of embryos that reached at these stages.

There are different degrees of oocytes response depending on the activating stimulus. Viable larva were only obtained when the activation were performed using fresh sperm as stimulus with rates of 15% for the method A (NT before activation) and 13% for the method B (NT during activation), values that were not significantly different (Table 2). By using radiated sperm, the reconstructed embryos by the two methods of NT reached the 24h stage but they could not continue their further development.

When non inactivated sperm fertilizes the recipient oocytes and participates in the embryo and larval development, zygotic nuclei derived may help the somatic nucleus integration through a nuclear helper role (Howlett *et al*., 1987; Eakin and Hadjantonakis, 2006) and they may form diploid chimaeras (Bubenshchikova *et al*., 2007). On the other hand, when the sperm nucleus is inactivated, there is not a nuclear helper role, but there are sperm derived factors which are responsible for triggering the calcium transient in the activation of a lot of species from sea urchin and ascidian oocytes (Dale *et al.*, 1985; Dale, 1988) to human (Tesarik *et al.*, 1994, Dale *et al.*, 1996). However, zebrafish egg activation does not involve the need of any sperm-derived factors (Swann, 1993) since when eggs come in contact with water they activate (Hart and Yu, 1980; Hart and Fluck, 1995; Sakai *et al*., 1997), even in absence of sperm, they undergo some abortive cleavages (Lee *et al*., 1996). In the present work, parthenogenetic development was not observed, neither using non motile

radiated sperm (with derived-factors) as activating agent (results not shown) nor applying an electroactivation protocol to non activated eggs (Cardona-Costa *et al*., 2009**). In fact, to date, the only way to activate zebrafish eggs to accomplish full parthenogenetic development is by radiated but motile sperm. So, it can be proposed that in NT experiments, the sperm-oocyte interaction, rather than the sperm factors, induces the activating response. The limited embryo and larval development using water or radiated spermatozoa could be ascribed to the inability to maintain embryo development by the scarcely reprogramming of transplanted somatic nucleus.

The major problem when radiated sperm is used is the indetermination regarding the sperm nucleus inactivation or not. It has noted that haploid larvae can be easily detected by the presence of haploid syndrome characteristics (Westerfield, 2007), but it is obvious that, when the NT is associated, the reconstructed embryo lacks these characteristics.

To date, only one reference of egg activation by electric pulses associated to the NT has found in medaka (Bubenshchikova *et al*., 2007). Recently, Cibelli and collegues (Siripattarapravat *et al*. 2009) in a NT method developed in zebrafish refer to an activation protocol, but the fact is that they activate reconstructed eggs by "egg water" (60µg ml^{-1} sea salt), equivalent to the usual system water employed.

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Table 1. Embryo survival rates after *in vitro* fecundation of *gold* eggs by *gold* sperm radiated for 2, 5 10 or 15 min. (Radiation dose: 0.529 mW/cm2)

Developmental stages	2 min	5 min	10 min	15 min
MBT/initial	92/138 **67%^{a}**	73/163 **45%^{b}**	18/102 **18%^{c}**	3/74 **4%^{d}**
24h/MBT	27/92 **29%**	32/73 **44%**	8/18 **44%**	2/3 **67%**
5days/MBT	27/92 **29%**	28/73 **38%**	6/18 **33%**	2/3 **67%**
Abnormal and degenerated/24h	22/27 **81%**	22/32 **69%**	4/8 **50%**	2/2 **100%**

Data in columns with different superscripts are statistically different (p≤0.05)

Table 2. Survival rates of reconstructed embryos derived from nuclear transplants, before (method A) or during (method B) egg activation by fresh sperm, radiated sperm or water as activating agents.

	Transplant before egg activation (Method A)			Transplant during activation (Method B)		
	Activation by fresh sperm	Activation by radiated sperm	Activation by water	Activation by fresh sperm	Activation by radiated sperm	Activation by water
Initial number	26	20	25	23	19	25
MBT/initial	11/26 42%^{a}	8/20 40%^{abc}	3 12%^{bc}	8/23 35%^{abc}	9/19 47%^{a}	5/25 20%^{abc}
epiboly/initial	8/26 31%^{a}	6/20 30%^{a}	0 0%^{bc}	4/23 17%^{abc}	7/19 37%^{a}	0/20 0%^{b}
24h/initial	8/26 31%^{c}	4/20 20%^{abc}	0 0%^{ab}	4/23 17%^{abc}	2/19 11%^{abc}	0/20 0%^{b}
5days/initial	4/26 15%	0 0%	0 0%	3 13%	0 0%	0/20 0%

STUDY VII. Reconstruction of heteroparental gynogenetic diploid condition by nuclear transplant in zebrafish: preliminary results.

ABSTRACT

The genomic imprinting has commonly studied in mammal. However, the limited parthenogenetic development reached by these species make difficult the use of partenogenetic embryonic cells as donor nuclei in nuclear transplant experiments. The parthenogenetic developmental ability in zebrafish allows obtaining, as well as haploid embryonic cells, haploid somatic cells. So, the aim of this work was the reconstruction, in zebrafish, of the heteroparental gynogenetic diploid condition by nuclear transplant of haploid somatic nuclei into metaphase II oocytes and early zygotes. Haploid somatic cells from primary cultures derived from gynogenetic larvae at 24h stage were obtained. The 19% of the reconstructed embryos reached the 100% epiboly stage when early zygotes were used as recipient. From them, 5 embryos developed to 24h and 2 survived to 5 days, although they showed morphologic abnormalities. The preliminary results obtained from this work allow suggesting the zebrafish as model system for imprinting studies.

KEY WORDS: Parthenogenetic, Genomic imprinting, Hemicloning, Zebrafish.

INTRODUCTION

Since the 1940's, an incipient interest in studying the parthenogenetic development in diverse species, especially in mammals, emerged (Surani and Barton, 1983; Fundele *et al*., 1989). Associated to this type of development, a specific pattern of genetic expression in the germ line, called "genomic imprinting" was observed, which conditioned the development depending on the paternal or maternal embryonic cells origin (Norris *et al*., 1990; Surani, 1991). Genomic imprinting is a form of epigenetic gene regulation that results in expression from a single allele in a parent-of-origin dependent manner. In addition, imprinted genes undergo to wide de-methylation that involves the erasure of the epigenetic marks in the early embryo, and the novo acquisition of imprints in both male and female gametes.

The imprinting phenomenon has been commonly studied in mouse (Howlett *et al*., 1989; Surani *et al*., 1990; Ferguson-Smith and Surani, 2001). However, some limitations exists in it use due to the low haploid embryo developmental ability (only few cells), both in androgenote and gynogenote embryos, which does not reach preimplantation stage. This fact makes difficult, in this specie, the heteroparental diploid embryos reconstruction from parthenogenetic nuclei transplanted in into partially enucleated zygotes.

In our lab, rabbit parthenogenetic embryo development until more advanced stages (32-64 cells) were achieved, so these haploid cells were suitable nuclei donors to reconstruct the heteroparental diploid condition by electrofusion with a hemienucleated zygote, resulting even in alive and normal offspring (Escribá and García-Ximénez, 2001).

In zebrafish, haploid parthenogenetic larvae (even 5 day stage) can be easy obtained (Nusslein-Volard and Dahm, 2002). So, zebrafish points to be a good model for evolution studies of genomic imprinting.

There are routine techniques for androgenetic and gynogenetic haploid zygotes (thus haploid larvae) based on inactivating eggs and sperm respectively.

Zebrafish nuclear transplant (NT) techniques have been recently developed in our lab, using somatic cells (adult fibroblasts) as nuclei donors. The NT technique opens the possibility to reconstruct the heteroparental diploid condition using nuclei

from cells derived from zebrafish haploid somatic cells. In this context, the development of this technique will therefore allow studying: (i) all combinations between resident and donor nucleus (gynogenote recipients-gynogenote donor; gynogenote recipients-androgenote donor; androgenote recipients-gynogenote donor; androgenote recipients-androgenote donor); (ii) the different epigenetic status of the donors nuclei imprinting in the different developmental stages, from embryonic stage to 5 days of haploid development.

There seems to be indications about the genomic imprinting phenomenon throughout the zebrafish development which concerns to relevant genes expression as specifically occurs in mammals among other species (McGowan and Martin, 1997; Hahn *et al*., 2005; Tsalavouta *et al*., 2009).

The availability of this methodology will allow deepening in the imprinting mechanisms in this species. To this end, this work pursued the reconstruction of the heteroparental gynogenetic diploid condition by nuclear transplant in zebrafish, as a first methodological step, using metaphase II oocytes and recently activated oocytes as recipients and nuclei from primary cell cultures derived from haploid gynogenetic larvae as donors.

MATERIAL AND METHODS

Recipient eggs.

Eggs were collected after evaluating the sexual behavior of both *gold* strain males and females at dawn. Eggs were obtained by gentle extrusion of the ovary (Westerfield, 2007). Then, non activated eggs were kept in egg medium at 8ºC (Cardona-Costa *et al*., 2009a).

Haploid somatic cells.

Gold male sperm was recovered from the genital pore in individually glass microcapillaries (Westerfield, 2007). Then, non activated gametes were kept in egg medium at 8ºC (Cardona-Costa *et al*., 2009a).

A sample of 100 µm of the *gold* sperm diluted in Hanks' balanced solution (Cardona-Costa *et al*., 2009) was radiated using a UV 85 germicide lamp (General 86

Electric, 30W). Irradiation was carried out at 62 cm of focus-object distance. The radiation dose applied was 0.529 mW/cm2 87 and was measured by a USB 4000 (Miniature 88 Fiber Optic Spectrometer; Ocean Optics Inc. First In Photonics, USA). A vortex (MS1-92 IKA, Wilmington, CA, USA) at 200 rpm was used with the aim of homogenising the radiation area during UV 93 exposure. The time of sperm radiation was 1 min.

In vitro fertilization was carried out as Westerfield (2007) describes. Non activated eggs and sperm were mixed in a 35-mm Petri-dish and both gametes activated by the addition of a 1 mL of system water. After 2-3 min, the dish was filled with system water to achieve well developing embryos. Further culture was done at 28.5 ºC.

Larvas at 24 h that showed the "haploid syndrome" (Westerfield, 2007) were selected for *in vitro* culture.

Somatic cells used as nuclei donor came primary cultures of *gold* zebrafish gynogenetic larvae at 24h stage. The tissue was cultured in Leibovitz medium supplemented with 20% of FBS and 0.036 g/L of glutamine (L15-FBS) at 28.5ºC (Westerfield, 2007).

Somatic cell nuclear transplant.

The two somatic cell nuclear transplant techniques, as well as the equipment employed were described by Pérez-Camps *et al*. (2009*). Method A: nuclear transplant prior to the egg activation: The somatic cell nucleus was inserted in the central region of the egg. To prevent egg activation, the transplant was performed in a handling drop composed of egg medium and the micromanipulation area was cooled down to 8 ºC. Non activated eggs were activated by fresh water after the NT Method B: nuclear transplant simultaneously to the egg activation: the donor nucleus was injected in the incipient animal pole, just where zygote nucleus was being constituted. The handling drop was composed by fresh water so that the eggs became the activation and the micromanipulation area was not cooled (room temperature). Eggs were activated in the transplant handling medium by the water drop.

Two experimental groups were established, transplant of haploid somatic cells into the central region of the metaphase II eggs (Method A) and transplant of haploid somatic cells into the animal pole during the egg activation (Method B).

An initial fertilizability control group was done immediately when eggs were extracted to test the egg quality, whatever the time and method of transplant. Nuclear transplant sessions were considered only when the initial fertility rates were higher than 70%. Survival rates were evaluated at MBT stage, 24h and 5 days.

At least three replicates were done in all experimental groups. Results were analysed using the Chi-square test. When a single degree of freedom was involved, Yates' correction for continuity was performed.

RESULTS AND DISCUSSION

Regarding the MBT stage developmental rates, significant differences ($p<0.1$) were observed in favour the NT performed simultaneously to egg activation (Table 1). This differences were more evident ($p<0.05$) at 100% epiboly stage. The 20% of embryos transplanted simultaneously the activation reached the 100% epiboly stage, however, embryos transplanted before activation stopped their development at MBT stage (Table 1). Five of the surviving embryos at 100 % epiboly continued developing to 24 h and only two survived until 5 days. In this case, the larva showed morphologic anomalies, although it presented heartbeat.

To this respect, it is important to point that if genomic imprinting certainly exists, the embryonic condition derived from coupling two haploid female nuclei would not be the most favourable to propiciate further embryonic development. The objective of this work was the methodologic establishment of heteroparental diploid condition by nuclear transplant in zebrafish. So it was chosen gynogenetic receptor-gynogenetic donor condition, due to its intermediate situation related to the difficulty of parthenogenetic larvae obtaining between those embryos derived from gynogenetic receptor-androgenetic donor and androgenetic receptor-androgenetic donor couplings.

It has be noted that, in previous work (Pérez-Camps *et al*., 2009*), in which diploid adult somatic cells were used as nuclei donors, reconstructed embryos only

reached MBT stage. Several reasons can be proposed for the results obtained in the present work: (i) The first more plausible explanation is the use of larval and not adult cells. In fact, in NT experiments in fish, as in mammals, the developmental rates are higher with early developmental stages donors (Lee *et al*., 2002; Kaftanovskaya *et al*., 2007; Luo *et al*., 2009; Siripattarapravat *et al*., 2009b; (ii) Other possible explanation comes from the differences in the reconstructed embryo ploidy. In first instance, the diploid nucleus transplantation into a non enucleated oocyte should result in a triploid condition, although some authors indicate that resident pronucleus degenerate after scarce cleavages (Li *et al*., 2000; Li *et al*., 2003). If same phenomenon occurs with the transplantation of an haploid nucleus, a worst embryo development should be expected. However, this is not observed after comparing the results from the present work with those obtained in the transplant of diploid adult somatic nuclei (Pérez-Camps *et al*., 2009*). In the case of transplanting haploid nucleus, we suggest that the pronucleous resident does not degenerate.

The developmental stage differences between the two NT methodologies assayed here (previous or simultaneously to egg activation) using haploid nuclei as donors, are not according with previous results obtained when diploid nuclei donors were used (Pérez-Camps *et al*., 2009). At this moment, any plausible explanation can be given.

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Table 1: Survival rates of reconstructed embryos derived from the transplant of haploid somatic nuclei into metaphase II eggs or early zygotes in zebrafish.

	NT before egg activation	NT during egg activation
MBT*	15 % [x]	31 % [y]
	8/54	16/51
100 % EPIBOLY	0 % [a]	20 % [b]
	0/54	10/51

Data in columns with different superscripts are statistically different ($p<0.05$).
* These data statistically differ at 10 % ($p<0.1$)

The main conclusions arisen from this work are the following:

Related to the objective 1,

- The embryo UV radiation previously to the manipulation into an isosmolar medium optimizes the germ-line chimaerism rate.

- Neither somatic nor germ-line chimaerism was observed when blastema cells were used as donors. This result indicates the no complete dedifferentiation of the blastema cells in the regeneration process, what indicates that they are not pluripotent.

Related to the objective 2,

- *Donor nucleus location.*

- Adult fibroblast inserted in the central region of the metaphase II eggs integrates in the reconstructed embryo without the need to be them located close to the metaphasic plate through the micropyle. So, the central region of metaphase II eggs is a suitable novel place to inject the somatic nucleus in zebrafish nuclear transplant.

- There are not evident differences in the developmental stage reached by the embryos derived from the nucleus transplantation into metaphase II eggs or into early zygotes.

- *Activating stimulus.*

- The survival rates and developmental degree reached by the reconstructed embryos are maximum when the stimulus is the sperm, minimum when is the only water contact and intermediate when is the radiated sperm.

- The more efficient electroactivation stimulus defined to activate non manipulated eggs involves a complete lysis when it is applied after the NT.

- *Ploidy.*

- In the absence of the mixoploidy rate assays, developmental rates reach adult stage when the NT is associated to the fertilization (tetraploid condition). The diploid condition derived from the transplant of a diploid nucleus into non enucleated egg and non fertilized, due to the female pronucleus degeneration, leads to MBT stage when the activating stimulus is the water and to 24h larvae when it is the radiated sperm.

Related to the objective 3,

- Haploid somatic cells can be obtained from 24h gynogenetic larvae cultures.

- MBT embryos are obtained when haploid somatic nuclei are transplanted into non enucleated egg, wherever the nucleus location. However, 5 days larva stage only is reached when the nuclei transplant is done in the animal pole. This indicates that the resident pronucleus does not degenerate when haploid cells are used as donors.

- The developmental degree reached in the preliminary experiments, and with the cautions derived from the limited number of embryos accumulated to date, allow proposing that there is no total genomic imprinting in this specie, or as an alternative, that the imprinting is already erased in the partenogenetic 24h larvae used as cell donor.

IMPLICATIONS

- *Chimaerism.*

To have an efficient and repetitive chimaerism technique, as the established in our lab, opens diverse possibilities in the biomedicine field. Today, as was indicated in the Introduction, there is an objective pursued by many labs around the world: the pluripotet cells obtaining to be used in regenerative medicine. The *in vitro* evaluation of the pluripotency degree reached by the differentiated cells after the application of several reprogramming treatments has limitations. The viable specimens obtaining by chimaerism is considered as standard in the reprogramming assessments. The problem of this type of evaluation in mammals lies in that reprogramming only can be evaluated after the natural or artificial birth and only in the specimens that reach this stage. The use of zebrafish as model system, having this technique of chimaerism, allows monitoring and evaluating the whole process. The potential of this model is even more since ES cells lines were already obtained, and, at present, predictable limitations to the zebrafish iPS cells obtaining do not exist.

On the other hand, it is known that zebrafish embryos are able to integrate human tumoral cells which acquire different fates depending on their differentiation ability. It is predictable that *in vitro* induced pluripotent cells are capable to colonize different tissues so, the toti- or pluripotentialty of them can be evaluated in zebrafish.

The possibility of integrating human genes from diverse pathologies in zebrafish, together with the ES cells availability (maybe also with iPS cells in the future) which can be *in vitro* genetically modified, will allow, by chimaerism, the *in vivo* monitoring of the effects caused by those genes through the whole development. This strategy involves higher efficiencies because of transplanted transgenic cells can be previously *in vitro* selected.

- *Nuclear transplant.*

The definitive test that would show a total reprogramming of the iPS cells would be these in which, without the needed of chimaerism, iPS cells would be capable to aggregate to configure an embryo at post-MZT stage, what has not achieved yet. So, other way to evaluate the reprogramming degree lies in the use of iPS cells nuclei as donors in NT experiment. This fact would allow completing the reprogramming, as well as, the use of the NT technique as a means of testing other reprogramming treatments.

On the other hand, it is widely know the oocytes ability to reprogram cell nuclei. However, the exact mechanisms by with it is achieved are still unknown. To study, by NT, how an oocyte does it, will provide keys to improve other *in vitro* reprogramming techniques.

- *Hemicloning.*

In addition to the possibilities related to the study of the genomic imprinting that offer the hemicloning technique in zebrafish, the results from the present work (haploid somatic cells cultured and larval developments) allow considering its use in the transgenic obtaining with higher efficiencies than with the conventional cloning technique, at least in this species.

Maybe, the hemicloning technique in zebrafish will also allow studying sex linked disorders.

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*Pérez-Camps M, Cardona-Costa J, García-Ximénez F. 2009. Transplant of adult fibroblast nuclei into the central region of metaphase II eggs resulted in mid blástula transition (MBT) embryos. Zebrafish (submitted).

**Cardona-Costa J, Pérez-Camps M, García-Ximénez F. 2009 Electroactivation of zebrafish (Danio rerio) eggs. Zebrafish (submitted).

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