Comprehensive Study of the Stability of Genomic Imprinting during Mouse iPSC Reprogramming

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Abstract: Reprogramming of the differentiated state into an Induced Pluripotent Stem Cells (iPSCs) involves major epigenetic rewiring, which is highly susceptible to errors.

Genomic Imprinting, an epigenetic phenomenon that renders genes monoallelically expressed, is frequently disrupted in iPSCs. Moreover, disruption of imprinting compromises pluripotency of iPSCs. However, up to now, no systematic monitoring of imprinting has been performed during reprogramming.

In this project, we used a reprogramming system whereby the donor cell contained both an integrated inducible reprogramming cassette and DNA polymorphisms to distinguish both parental alleles to assess imprinting maintenance of the *Peg3*, *Igf2-H19* and *Dlk1-Dio3* diagnostic imprinted *loci* during reprogramming (with or without ascorbic acid – AA) by allele-specific methylation analysis. For the *Peg3 locus*, imprinted defects were not found in the presence of AA. For *Igf2-H19 locus*, a non-allelic methylation assay showed that, at most, imprinting is labile early during reprogramming, but resolves on itself resulting in miPSCs with normal imprinting. For *Dlk1-Dio3*, evaluation of imprinting status could only be evaluated for early stages of reprogramming, where errors were found as previously reported.

Our preliminary results points out that imprinted defects do occur, varies from *locus* to *locus* and might occur from early stages of reprogramming. Genome-wide assessment of imprinting status will be required to fully understand the extent to which imprinting defects occur. We believe this knowledge is valuable for future development of new reprogramming protocols aiming at preventing imprinting disruption and guarantee the safety of use of iPSCs in their various applications.

Key Words: Induced Pluripotent Stem Cells (iPSCs); Genomic imprinting; DNA Methylation; Bisulfite Treatment; Reprogramming; *Peg3*; *Igf2-H19*; *Dlk1-Dio3*

Introduction

The differentiated state of a somatic cell was for a long time considered to be stable and irreversible. However, seminal work from Takahashi and Yamanaka (2006) proved that this epigenetic barrier could be surpassed by simple addition of defined transcription factors[1]. Indeed, they accomplished the reversal of somatic cells into stem-like cells known as Induced Pluripotent Stem Cells (iPSCs).

iPSCs can be obtained by transduction of four TFs: Oct3/4, Klf4, Sox2, and c-Myc (OKSM)[2]. Initial reprogramming protocols relied on retroviral and lentiviral transfections. Both systems have the handicap of having multiple sites of integration and possibility of re-activation of the OKSM, including the proto-oncogene *c-Myc*. New protocols have been devised, such as systems where the OKSM cassette is integrated at a single site and its expression is controlled by Doxycycline (Dox). In this respect, reprogrammable strains of mice have been generated [3,4]. Such reprogrammable strains, as, for example, the i4F-Bl6 mouse[3] represent an easy way to obtain and reprogram virtually any differentiated cell, in a controlled fashion by simple addition/removal of Dox. Therefore, they constitute an amenable model to study the dynamics of the reprogramming process.

Despite protocol improvements, using mainly mouse iPSC (miPSC), low efficiencies prevail and reprogrammed cells are still likely to present genetic and epigenetic errors[5]. The origin of such anomalies is still poorly understood, as well as their effects on pluripotency and differentiation to specific lineages. Among epigenetic defects,

epigenetic processes such as genomic imprinting are particularly sensible to the massive epigenetic rewiring and prone to accumulate errors. Therefore, genomic imprinting is an important read-out of appropriate reprogramming, which has not deeply considered and studied.

Genomic imprinting is an epigenetic phenomenon that leads to differential expression according to parental origin of the allele[6], being only observed for a small subset of genes. Imprinted genes have been associated with growth and development as well as in brain function and post-natal physiology. Accordingly, dysfunction of imprinted genes is related to human imprinted disorders related to developmental, neurodevelopmental and metabolic phenotypes. Angelman Syndrome is an example of such conditions, being characterized by an imprint disruption at *Snrpn-Ube3a*. Patients with this condition present developmental delay, speech impairment, poor motor control or seizures, among other symptoms.

The list of imprinted regions already discovered in the mouse genome contains around 150 genes that are mostly physically linked or clustered in the genome[7]. Genetic dissection of these imprinted clusters led to the discovery that Imprinting Control Regions (ICRs) are essential for imprinting regulation, presenting different epigenetic marks in the two parental alleles and carrying an "imprint" originally established in the germline[7].

ICR are, usually, a Differentially Methylated Region (DMR), and this differential methylation between parental alleles determines the allelic-specific imprinting expression of the genes in the whole cluster.

Epigenetic state of iPSCs has been a concern and has been analysed on several cell lines, where imprinting errors, in the form of aberrant methylation patterns, have been identified in both mouse iPSC (miPSCs) and human iPSCs (hiPSCs). These errors could affect several imprinted clusters, however the affected regions could vary greatly among independent miPSC lines.

Examples of imprinted defects have been documented, for example, at the *Peg3 locus,* defects were associated with hypomethylation, while at the *Igf2-H19 locu*s imprint disruption was less frequent[8]. Imprinted defects at the *Dlk1-Dio3 locus* are very prominent in miPSCs and are caused by hypermethylation of the maternal allele[6,8,9]

Similarly to miPSCs, hiPSCs are also prone to present epigenetic errors in imprinting regions. In fact, imprinting disruption in hiPSCs has also been found to be variable among cell lines[5,8], with some parallels with the miPSCs. Imprinting regions and their respective disorders affect correct cell function and overall development. Thus, upon reprogramming cells, a process that forces intense epigenetic remodelling, imprinting status must be considered and assessed before real applications of iPSCs can be devised.

This issue is of key importance when defining safety and correct physiology of reprogrammed cells, hence, its whole purpose and importance.

Imprinting errors exist in miPSCs, however, the extent to which they occur, and when, during reprogramming is poorly characterized, as most of cell systems used lack enough DNA polymorphisms essential to distinguish the two parental alleles.

Here, we assessed thoroughly the imprinting status of three diagnostic imprinting clusters (*Peg3*, *Igf2-H19* and *Dlk1-Dio3*) during reprogramming using an allelic-specific methylation assay using donor cells derived from a cross between the reprogrammable i4F-B mouse (with an integrated inducible reprogramming cassette) and the outbred *Mus musculus castaneus* (allowing for the existence of DNA polymorphisms in most genes and regulatory regions).

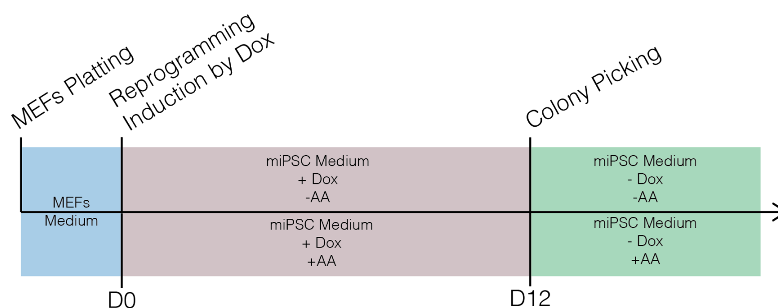


Figure 1 - Schematic representation of the two independent reprogramming protocols used

Such a system will allow us to understand the frequency and the timing of imprinting defects throughout the process of reprogramming.

Materials and Methods

Crossings and Embryo Collecting

Time-mated breedings were established using the reprogrammable mouse (i4F-Bl6) originally from the C57BL6 strain containing one Dox-inducible cassette (i4F-Bl6), which was a kind gift from Dr. Manuel Serrano (CNIO/Madrid), and the *Mus Musculus Castaneus* (Cast) strain.

Pregnancies were carried out until day 13.5, after which the female mice were sacrificed and Mouse Embryonic Fibroblasts (MEFs) isolated.

Genotyping for the Yamanaka cassette and Gender

The assessment of the presence of Yamanaka's cassette was done with a PCR reaction using primers that flanked the region where the cassette is inserted within the *Pparg* gene. The primers used were described by Abad, M. *et al.* (2013)[3].

To assess gender of the embryos, PCR to amplify simultaneously the *Ube1X* and *Ube1Y* sequences[10] was performed. The first fragment is present in X chromosome, while the second is present in the Y chromosome, yielding PCR products of different lengths and allowing distinguishing male from female genders.

iPSC Reprogramming

Female MEFs containing the Yamanaka cassette were selected for reprogramming. To induce reprogramming, cells were cultured in miPSC medium with LIF containing 1 μg/mL of Dox (BDClontech).

Two independent reprogramming protocols were used which only differ by the absence or presence of 500 ng/mL Ascorbic Acid (AA) in the culture media. (Figure 1)

Fluorescence Activated Cell Sorting of Reprogramming Intermediates

Reprogramming intermediates were obtained using Fluorescence Activated Cell Sorting (FACS). Pre-miPSC intermediates were sorted with the cell surface signature Thy1.2- SSEA1+- (Thy1.2 is characteristic of differentiated mouse fibroblasts; while SSEA-1 is a marker associated with mouse pluripotent stem cells[11]). Sorting was performed at day 6 (D6) and at day 12 (D12) of reprogramming using FACSAria IIu (BDBiosciences).

Allelic Specific Imprinting Assessment

Genomic DNA was treated with bisulfite ion (HSO_3^-), which converts unmethylated cytosines into uracils, but not methylated cytosines. Bisulfite treatment was performed with the EZ DNA Methylation-Gold™ kit (Zymo Research), following the manufacturers' instructions.

The chosen regions of interest were: *Peg3*, *Igf2-H19* and *Dlk1-Dio3*.

In order to amplify these regions' ICRs, and due to the high content in thymines of DNA after bisulfite treatment, which could compromise specificity of downstream PCRs, a Nested PCR approach was devised. For the two rounds of PCR, primers were chosen taking into account that they included the regions under study and allow SNP detection.

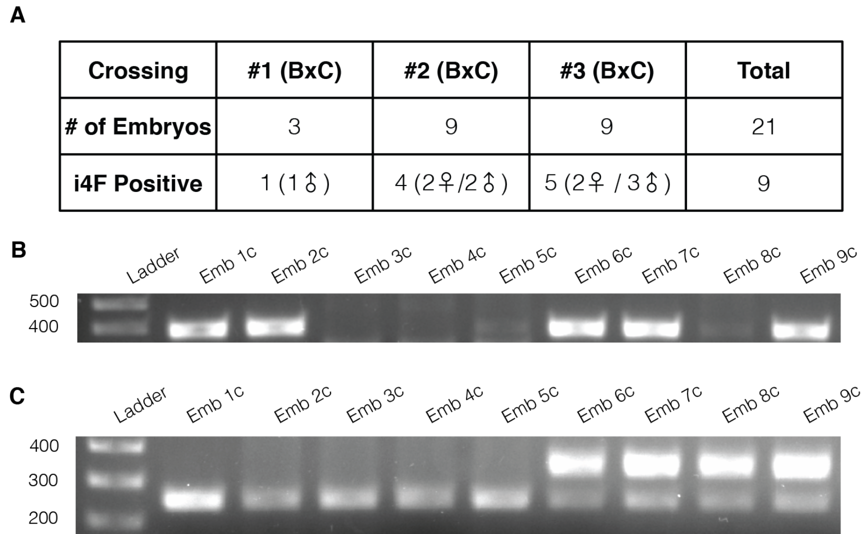
Combined Bisulfite Restriction Analysis

Combined Bisulfite Restriction Analysis (COBRA) uses restriction enzymes to target bisulfite-treated DNA, taking advantage of the fact that action of such enzymes is influenced by the original methylation status of the sequence.[12]

A COBRA assay was devised for the *Igf2-H19 locus*. Restriction digest with the *Bsu*15I of purified bands should cut the sequence CG/CG which will cut the PCR product into fragments of 87 bp and 335 bp, This sites are only kept after bisulfite treatment if originally methylated, but not if they were originally unmethylated.

Bisulfite Sequencing

To clone purified PCR fragments, adenine overhangs at 3', resultant of *Taq* polymerase activity, were taken into account. Fragments were ligated to a plasmid using pGEM®-T Easy Vector System (Promega). DH5-α competent cells were transformed with the ligation products using standard heat shock.



Crossing	#1 (BxC)	#2 (BxC)	#3 (BxC)	Total
# of Embryos	3	9	9	21
i4F Positive	1 (1♂)	4 (2♀/2♂)	5 (2♀ / 3♂)	9

Figure 2 - Time-mated breedings between i4F-Bl6 and Cast mice; A) Summary of successful crossings between i4F-Bl6 females and Cast males; B) PCR for Yamanaka cassette for Crossing #3 embryos; C) Gender assessment for Crossing #3 embryos

After overnight incubation at 37 ºC, colonies were picked and incubated overnight in 3 mL of LB with 100 µg/mL of ampicillin in a shaking incubator at 37 ºC. Plasmid DNA was then purified using NZYMiniprep Kit (NZYTech) following manufacturer's instructions.

After plasmid purification, digestion was performed to confirm whether the plasmid contained the PCR product. For that, *Not*I Fast Digest (Thermo Fisher Scientific) was chosen since it cuts at both sides of the inserted PCR product.

Positive samples were sent to Sanger sequencing at GATC Biotech, using plasmid DNA and the pUC/M13 Reverse and/or Forward primers.

Sequences was analysed by the QUMA (Quantification tool for Methylation Analysis) online tool for analysis of bisulfite sequence (http://quma.cdb.riken.jp/), which allows both assessment of methylation status and multiple alignment of sequences for SNP detection.

Results and Discussion

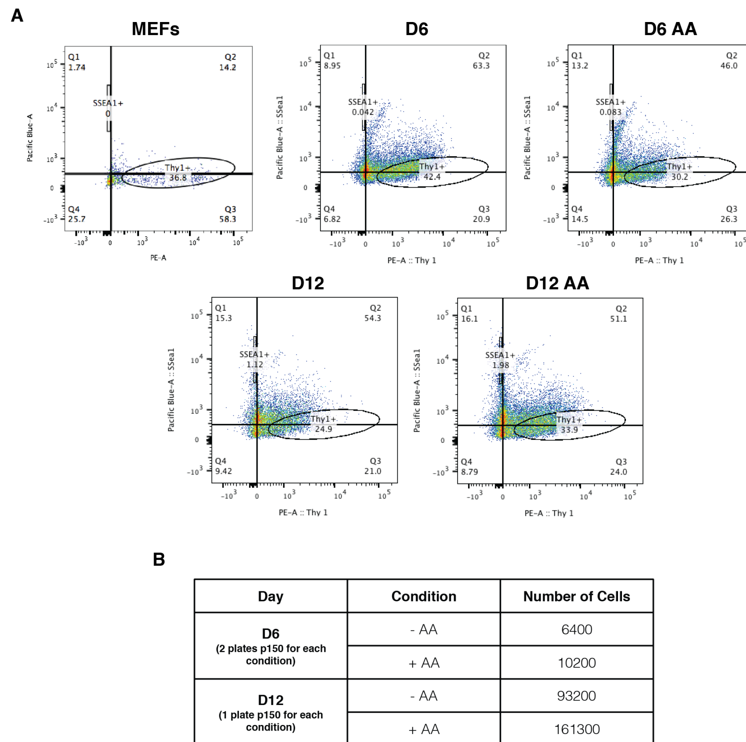
Time-Mated Breeding

Time-mated reciprocal crossings were established between two strains of mice: i4F-BL6 and *Mus Musculus Castaneus* (*Cast*). These strains were used due to their phylogenetic distance, which allows the generation of progeny and simultaneous detection of Single Nucleotide Polymorphisms (SNPs). From the reciprocal time-mate crossings established, only the crossings between i4F-Bl6 females and Cast males produced progeny.

Figure 2 A summarizes the progeny of the 3 successful crossings. Out of the 3 crossings, we were able to obtain 21 embryos. Due to the fact that i4F-Bl6 mouse are heterozygous and Cast strain does not have the cassette, we needed to genotype the offspring for the presence of the Dox-inducible cassette. To do so, we performed PCR for the *Pparg* region in which the cassette is inserted[3].

After selecting the embryos from the crossing #3, gender was verified through PCR for the *Ube1X* and *Ube1Y* regions. (Figure 2 B and C)

From these crossings, we chose MEFs from the 2c female embryo, which is positive for the Yamanaka cassette. Female cells were chosen since female cells have been associated to accumulate more imprinted errors than male cells during reprogramming.



Day	Condition	Number of Cells
D6 (2 plates p150 for each condition)	- AA	6400
	+ AA	10200
D12 (1 plate p150 for each condition)	- AA	93200
	+ AA	161300

Figure 3 - Isolation of early and late intermediates of the reprogramming process; A) FACS of pure MEFs populations and D6 and D12 of culture populations in the absence and presence of AA; B) Summary of FACS yields for the different time-points

Isolation of Early and Late Reprogramming Intermediates

MEFs were reprogrammed by addition of miPSC medium supplemented with Dox. Two different reprogramming settings were used to dedifferentiate cells, a first one, with miPSC medium plus Dox and the second one, which was supplemented with Ascorbic Acid (AA) (Fig. 1). The rationale behind the use of AA was because it was previously shown to have a protective role in maintaining correcting imprinting at the *Dlk1-Dio3 locus*, an imprinted cluster where imprinting defects are very frequent in miPSCs.

At day 6 of reprogramming, a total of 4 p150 plates were used, 2 for each condition. As for day 12 of reprogramming, only 1 plate of reprogramming cells was used for each condition, due to higher content of Thy1.2^{-} SSEA-1^{+} cells *per* plate. Cell populations were sorted according to their fluorescence, as it can be seen on Figure 3 A. There was an initial screening on a pure population of MEFs (see first graphic of Figure 3 A) that show that no SSEA-1+ cells were present before reprogramming has been induced.

Upon sorting of the aforementioned plates, we could obtain the results on Figure 3 B. Interestingly enough, FACS sorting yielded very different number of cells in AA- vs. AA+ conditions for both D6 and D12, revealing a higher efficiency in reprogramming in the presence of AA, as it has been published[13].

Cells were reprogrammed for 12 days in the presence of DOX in AA- and AA+ conditions. At day 12, miPSC colonies were picked and plated in feeders in iPSC medium devoid of Dox, and subsequently passaged and diluted until feeders were absent. After around 50 days after reprogramming induction, cells were collected.

Methylation Status during reprogramming

At the end, 9 cell populations were collected for DNA extraction and DNA was bisulfite-treated. The first population consisted in the initial MEFs, while the next two populations were intermediate cells undergoing reprogramming in two different stages: early intermediates (D6) and late intermediates (D12) in both presence and absence of AA (D6 and D6 AA; D12 and D12 AA). The final miPSC colonies picked were used as the last population (miPSC 1 and miPSC 2; miPSC 1 AA and miPSC 2 AA).

These different cell populations, spanning through the dedifferentiation protocol, allow systematic analysis of the methylation status.

Peg3

The *Peg3 locus* is characterized by the paternal-specific expression of the gene. Methylation of the maternal ICR, located upstream of the gene blocks the expression of *Peg3* in *cis*[14] (Figure 4 A).

The results of bisulfite sequencing can be seen on Figure 4 B. In the donor hybrid MEFs, the expected methylation status (unmethylated paternal allele and methylated allele) was observed, confirming that our approach could be used to assess the methylation status during reprogramming (Figure 4 B). For the reprogramming done in the presence of AA (AA+), we could observe correct maintenance of the imprinting at all stages analysed (for D6, we could only sequence one paternal unmethylated allele), which suggests that presence of AA is protective for epigenetic disturbances at this *locus*. In the AA- conditions, we observed a bias towards the amplification of the paternal allele at all stages of reprogramming (Figure 4 B).

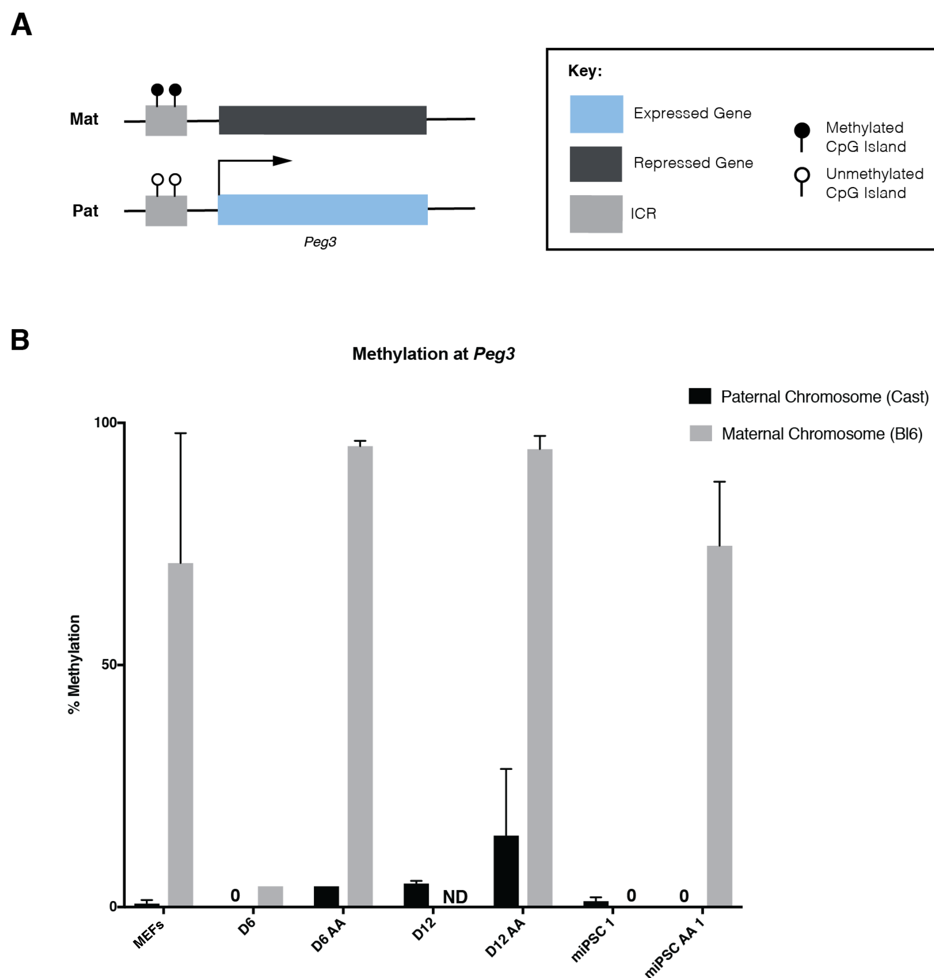


Figure 4 - Methylation Status at *Peg3* ICR during reprogramming; A) Schematic representation of the expression landscape at *Peg3 locus*; B) Percentage of mean methylation levels for *Peg3* ICR at maternal and paternal alleles during reprogramming (plotted with SEM values)

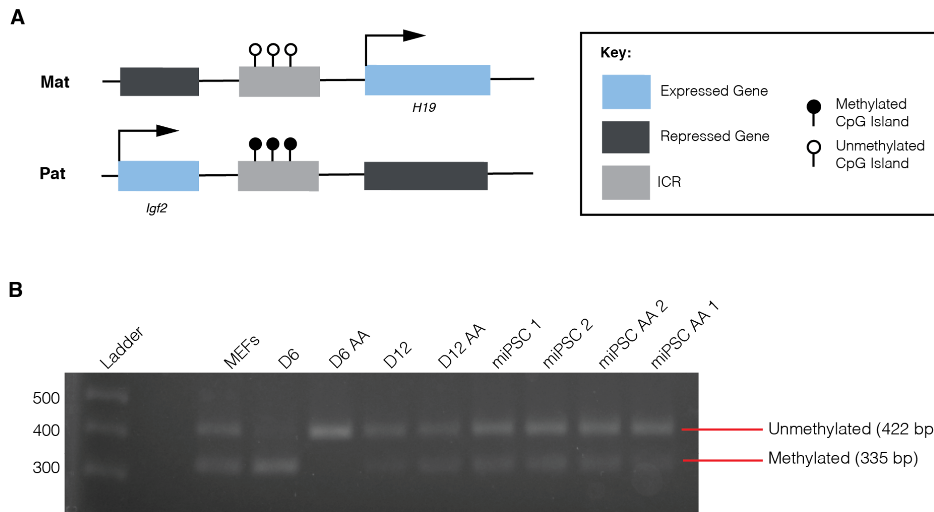


Figure 5 - Methylation status at the *Igf2-H19* ICR during reprogramming; A) Schematic representation of the expression landscape at the *Ig2/H19* imprinted region; B) COBRA for *Igf2-H19 locus* throughout reprogramming

Igf2-H19

Imprinted expression in *Igf2-H19* region is controlled by paternal-specific methylation of an ICR located between the two genes. This situation leads to a paternal expression of *Igf2*, that is upstream of the ICR, and a maternal expression of *H19*, downstream of the methylation region[15]. (Figure 5 A)

To assess methylation at *Igf2-H19*, we performed COBRA for this *locus*. (Figure 5 B) At D6 in AA-, we could only detect the band corresponding to the methylated allele, which leads to a single band with the size of the biggest cleaved product of *Bsu*15I digestion. As for D6 AA, occurs the reverse situation, only the band of the non-digested product, which represents the unmethylated allele. Although these results seem inconsistent with the rest of the samples, where correct methylation (one methylated and one unmethylated band) is seen, COBRA was repeated two more times in the same samples (technical replicates) yielding the same results (data not shown).

This being said, the results on Figure 5 B seem to indicate that, while early intermediates are prone to imprint errors, those errors, somehow, resolve themselves during the progression of reprogramming.

PCR bias cannot, obviously, be ruled out, and further bisulfite sequencing is needed to confirm such results. If that is not the case, the hypermethylation observed in early intermediates indicates that, at early stages, *Igf2-H19* ICR is very sensitive to reprogramming epigenetic changes.

Dlk1-Dio3

The ICR at the *Dlk1-Dio3 locus* is paternally methylated and is known as IG-DMR (Intergenic - Differentially Methylated Region). This epigenetic state leads to paternal expression of *Dlk1* and *Dio3*, coding genes and maternal expression of the *Gtl2* lncRNA[16]. (Figure 6 A)

Amplification of *Dlk1-Dio3 ICR* was attempted with a Nested PCR optimization for primers from Ferron et al. (2011). However, this attempt did not worked, as it can be seen on the left side of Figure 6 B. The reasons for this failure could not be determined. To solve this issue, we designed a new nested PCR for this ICR, using the first PCR round of the initial method and combining it with a second round with the primers described by Nakamura et al. (2007)[17] to the same region By doing so, the region of interest for D6, D6 AA, D12 and D12 AA samples was successfully amplified (left side of Figure 6 B).

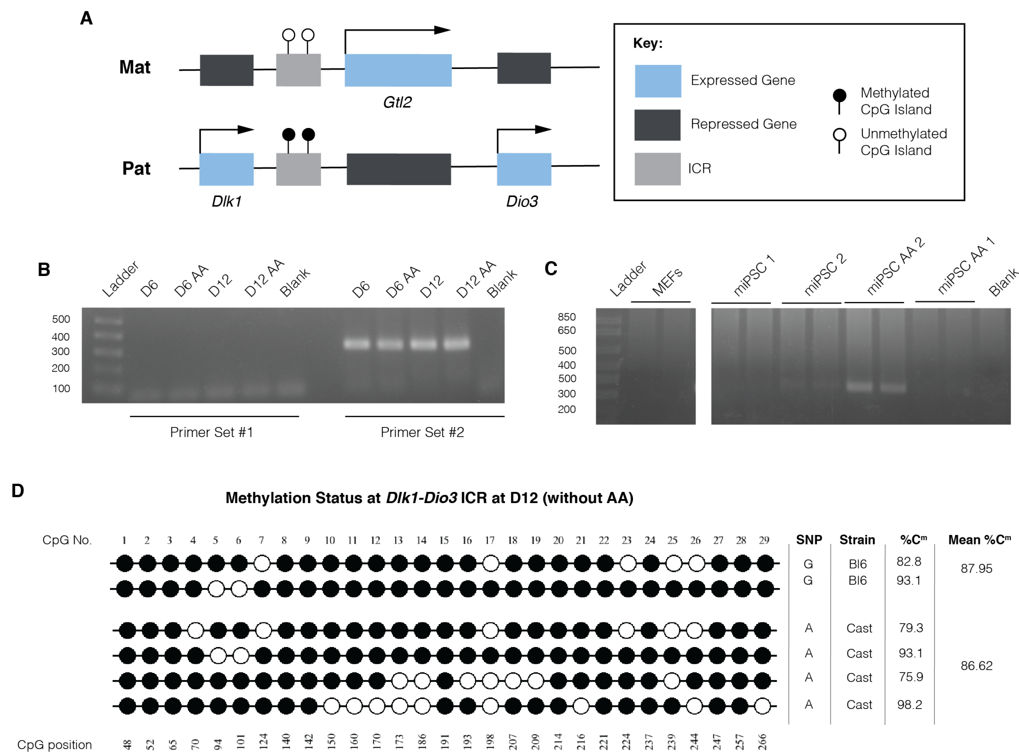


Figure 6 - Methylation status at *Dlk1-Dio3* IG-DMR region; A) Schematic representation of the expression landscape at the imprinted *Dlk1-Dio3* gene cluster; B) Nested PCR results for the *Dlk1-Dio3* ICR with Primer Set #1 and Primer Set #2 for D6, D6 AA, D12 and D12 AA samples; C) Nested PCR results for *Dlk1-Dio3* ICR with Primer Set #2 for MEFs, miPSC 1, miPSC 2, miPSC AA 1 and miPSC AA 2 samples; D) Bisulfite sequencing results for the *Dlk1-Dio3* ICR at D12 in –AA conditions

When the same strategy was applied to the remaining samples, it failed to amplify properly the fragment, except for miPSC AA 2 sample (Figure 6 C). Different parameters as DNA concentration for PCR and bisulfite treatment or PCR conditions were used to try to isolate the region, but such optimization pursuits failed to accomplish the desired goal. (Data not shown) Therefore, we could not find nor solutions nor explanations for this problem, and were unable to obtain *Dlk1-Dio3* PCR products for all the samples.

Even though it was not possible to do a full analysis of the methylation status of the ICR of *Dlk1-Dio3*, we decided to perform bisulfite sequencing for the purified PCR products available on the reprogramming intermediates (D6, D6 AA, D12 and D12 AA). Figure 6 D shows the methylation status observed for this *locus* for D12. As it is observed both the maternal (Bl6) and paternal alleles (Cast) are highly methylated. Similar results were observed for the other time points regardless of the presence of AA (data not shown).

Even though the evolution of the methylation status cannot be drawn, these intermediate results seem to point out to the reported hypermethylation for this *locus* upon reprogramming.

Final conclusions at this *locus* could only be made once we have the results from the original MEFs and the final miPSCs to understand whether imprinting defects at intermediates stages of reprogramming were not present in the original donor cells and if they persist or not in the final miPSCs.

Conclusions and Future Perspectives

The work developed in this project devised methylation assay protocols during reprogramming for 3 imprinted regions: Peg3, *Igf2-H19* and *Dlkl1-Dio3*. Among others, these *loci* have been reported to undergo imprinting disruption during cell reprogramming. Due to the importance of imprinting on cell physiology and animal development and growth,

understanding what happens and when it happens it is of utmost importance when thinking about iPSCs function and future applications.

Aiming at unveiling the reasons behind imprinting defects during reprogramming, we monitored those 3 *loci* during reprogramming of MEFs.

These MEFs were reprogrammed taking advantage of a Dox-inducible cassette encoding OKSM, which allows controlled induction of the Yamanaka factors. Apart from this, to obtain the MEFs, mouse time-mated crossings were established between i4F-Bl6 and Cast, so that SNPs could be detected and parental alleles identified.

These *loci* were analysed during reprogramming at four different stages using bisulfite treatment and subsequent amplification. Two of those stages were intermediate time-points, which required the use of FACS to isolate cells undergoing reprogramming.

As for the results for the methylation status during reprogramming, the data obtained for *Peg3* pointed out to a preservation of imprinting in the presence of Ascorbic Acid (Figure 4 B). However, the imprinting status in the absence of AA, it was not possible to gather enough information to assess the effect of epigenetic remodelling that happens during dedifferentiation. (Figure Table Sum up) Further bisulfite sequencing is needed to complete this image and allow comparing protocols regarding the effect of AA.

Next, for *Igf2-H19*, the preliminary results from COBRA seem to indicate a disruption in the imprinting status of that region that is corrected afterwards (Figure 5 B). The next logic step for this *locus* would be bisulfite sequencing, that would give allele-specific information, clarifying the present results.

Lastly, for *Dlk1-Dio3* ICR, methylation status could not be assessed thoroughly, since the two different protocols that were optimized to evaluate the imprinting status at this *locus* failed to yield PCR products that allowed further methylation analysis. New amplification strategies must be designed so that *IG-DMR* can be amplified and sequenced.

Apart from DNA methylation assessment, it is also important to study the expression profile of these *loci*, since imprinting does not only rely on methylation of CpG dinucleotides.

These results are important, not only due to the information they reveal, but also because they point towards a significant need to further develop protocols for methylation analysis of imprinted regions. Such *loci* play important roles in cellular function and the maintenance of their correct status of different epigenetic marks is highly significant for the success of iPSCs in medical research. Additionally, knowing the behaviour of imprinted regions during reprogramming can also provide useful insight for future reprogramming protocols that can overcome such flaws and present higher efficiencies for cell reprogramming. The use of compounds to tackle such abnormalities, like epidrugs, might also be an interesting approach to tackle imprint disruption, since these compounds modulate the epigenetic phenomena, possibly protecting imprints from reprogramming remodelling.

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