Faculdade de Ciências e Tecnologia da Universidade de Coimbra

Reprogramming strategies for cord blood cd34^{+} cells with different mitochondria phenotype

João Luís Cardoso Dias Monteiro

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Supervisors: Prof. Dr. Ricardo Neves (CNC)

João Luís Cardoso Dias Monteiro

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Abstract

Induced pluripotent stem cells are an important alternative to avoid the use of embryonic stem cells. By controlling the epigenetic remodelling of a somatic cell it is possible to re-establish an embryonic like phenotype. Therefore this iPSCs (induced pluripotent stem cells) technology can be a great step in the regenerative medicine filed and drug toxicity screen. However the limited efficiency of generation of iPSCs and some differences from ESCs (embryonic stem cells) phenotype and function are major obstacles to further advance in this area.

As reprogramming depends on several stochastic events a good strategy could be the selection of an immature cell population, like the $CD34^+$ derived from cord-blood, since these cells are closer to pluripotency than fully differentiated cells. For selection of different sub-populations within the $CD34^+$-cells traditional sorting techniques require the combination of several antibodies, which is a time consuming technique and requires skilled competences. In order to overcome these constrains we explored a new method to select a primitive population of $CD34^+$ cord blood cells based on the uptake of the mitochondrial probe TMRM (Tetramethylrhodamine, methyl ester). We compared the reprogramming efficiencies of TMRM high and TMRM low populations after infection with a lentivirus vector that contained the 4 Yamanaka factors. Furthermore the oxygen environment during reprogramming was modulated using hypoxia and antimycin A.

We were able to conclude that low TMRM uptake $CD34^+$ cord blood cell population achieved better efficiencies in normoxia with higher expression of the main pluripotent genes (Oct4, Sox2 and Nanog). Both populations were characterized phenotypically and we were able to validate our sorting method to select immature cells.

Both populations after reprogramming in hypoxia show a similar behaviour in terms of reprogramming. Nonetheless the low TMRM population had still higher expression levels of the main pluripotent genes. The antimycin A treatment combined with hypoxia allowed us not only to obtain similar reprogramming efficiencies in both populations but also to level the expression of Oct4, Sox2 and Nanog.

In conclusion we were able to create a new method to select the cells from the $CD34^+$ cord blood population suitable for reprogramming studies reducing the use of antibodies and the time expended. This will not only reduce the complexity of the method but also will allow a reduction of the levels of stress imposed to the cells and a much more efficient cell sorting procedure. Furthermore there is still space for future improvements using modulatory agents as hypoxia and antimycin A.

We hope that our work may contribute to the selection of an ideal cell population for reprogramming and therefore act as a booster of the reprogramming process.

Resumo

Células pluripotentes induzidas são uma alternativa importante de forma a evitar o uso de células embrionárias. Ao controlar a remodelação epigenética de uma célula somatica é possível reestabelecer o fenótipo embrionário. Deste modo esta tecnologia (iPSC – induced pluripotent stem cell) poderá ser um grande passo no campo da medicina regenerativa e em monitorização de testes de toxicidade. No entanto a eficiência limitada na geração de iPSC e algumas diferenças em relação ao fenótipo e função comparado com as células estaminais embrionárias (ESCs – embryonic stem cells) são grandes obstáculos aos avanços nesta área.

Como a reprogramação cellular depende de vários eventos estocásticos, uma boa estratégia seria a seleção de uma população imatura, como as $CD34^+$ derivadas a partir do sangue do cordão umbilical, uma vez que estas células estão mais próximas da pluripotência do que células diferenciadas. Para seleção de diferentes sub-populações dentro das $CD34^+$ as técnicas tradicionais de sorting requerem a combinação de vários anticorpos, são demoradas e requerem técnicos competentes. De forma a ultrapassar estes problemas exploramos um novo método para selecionar uma população primitiva das células $CD34^+$ do sangue do cordão umbilical baseado na captação de uma sonda mitocondrial TMRM (Tetramethylrhodamine, methyl ester). Nós comparamos as eficiências de reprogramação das populações com elevada e baixa captação de TMRM depois de infeção com um vector lentiviral que continha os 4 factores do Yamanaka. Adicionalmente os níveis de oxigénio durante a reprogramação foram modulados usando hipoxia e antimicina A.

Fomos capazes de concluir que a população $CD34^+$ do sangue do cordão umbilical com menor captação do TMRM foi capaz de conseguir atingir melhores eficiências em normoxia e com maior expressão dos genes pluripotentes principais (Oct4, Sox2 e Nanog). Ambas as populações foram caracterizadas fenotipicamente permitindo-nos validar o nosso método de sorting para selecionar células imaturas.

Após a reprogramação em hipoxia ambas as populações demonstraram um comportamento similar. No entanto a população com menor captação do TMRM tinha níveis mais elevados de expressão dos genes pluripotentes principais. O tratamento com antimicina A combinado com a hipoxia permitiu-nos não só obter eficiências de reprogramação semelhantes em ambas as populações como também equalizar os níveis de expressão de Oct4, Sox2 e Nanog.

Em conclusão, fomos capazes de criar um novo método para selecionar as células $CD34^+$ do sangue do cordão umbilical mais adequadas para estudos de reprogramação reduzindo o número de anticorpos e tempo gasto. Desta forma não só reduzir-se-á a complexidade do método como também permitirá uma redução dos

níveis de stress impostos às células e um procedimento de sorting mais eficiente. Adicionalmente existe ainda espaço para melhoramentos futuros usando agentes moduladores como a hipoxia e a antimicina A.

Esperamos que o nosso trabalho venha a contribuir para a seleção de uma população celular ideal para reprogramação e desta forma reforçar o processo de reprogramação.

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Introduction

In modern society there is a great need to find new treatments for several disorders related with cellular dysfunction that highly compromise our quality of life and affect large groups of people.

However the ability to restore cellular function is limited and often the replacement of organs and tissues through transplants is needed. Cell therapies like bone marrow transplants have already become a standard procedure in patients with blood-related disorders but the procedures depend on availability of compatible donors. Therefore the success of these strategies is dependent on immune tolerance, is limited by the number of cells that can be harvested and has a narrow range of clinical applications.

It is in this context that pluripotent stem cells emerge as a new alternative. By allowing the replacement of damaged tissues they open new perspectives in medicine. These cells hold the capacity to differentiate in all types of cells present in the human body with the exception of the umbilical cord and placenta having therefore in theory an unlimited potential to generate any organ or tissue compromised by age, disease or injury. The most promising applications are in the treatment of cardiovascular diseases, diabetes, spinal cord injury, and degenerative and congenital diseases.[1]

There are currently two main sources for the collection of stem cells: the embryonic stem cells and the adult stem cells. Embryonic stem cells can be obtained from the inner cell mass of the blastocyst; this requires the destruction of an early embryo generating much ethical debate around it [2-4]. To overcome these problems scientists were forced to find other stem cell sources for regenerative medicine. Human cord blood is an attractive solution as a source of adult stem cells because of the great advantage of the establishment of blood banks that can store the cells for future transplants. Besides, there is also the fact that it constitutes a young tissue collected after birth being less likely to contain mutations accumulated by age, radiation and viral infections.

A target sub population within these cells is the $CD34^+$ fraction of the cord blood that is composed of hematopoietic stem and progenitor cells. It is thought that these cells have suffered fewer cell cycles and are in a more primitive state (less differentiated) than the remaining cord blood.[5] The peripheral blood is another source of CD34+ cells but with a reported efficiency 10 times lower on isolation[6] and with a lower proliferative state caused by lower telomerase activity[7].

Still, a critical problem for cord blood use in cellular therapy resides in the limited number of cells that can be collected from a single donor. Transplants of hematopoietic cells (multipotent) can be performed but require a high number of cells to restore regular function. The use of cells derived from cord blood in transplants would obligate the combination of cells gathered from different donors which could result in a high probability of immune rejection.[8] Other disadvantage resides in the small range of applications because of the limited capacity of these cells to generate other lineages, since they are only multipotent.

In an attempt to overcome the technical limitations and ethical issues raised against the current pluripotent and multipotent cell sources, science is evolving in the direction of reprogramming somatic or multipotent cells back to pluripotency – the induced pluripotent stem cells (IPSc).

Nonetheless there are still many barriers to surpass for the clinical translation of IPS-derived cells as therapeutic agents. Some of the obstacles are related with identifying the best source of cells for reprogramming; improving the efficiency of the method; controlling the maintenance of the self-renewal capability and guiding the differentiation process towards a specific cell type.

One of the main purposes of this work is to find which types of cells within the $CD34^+$ population are more effectively reprogrammable. The glycolytic metabolism of the CD34+ is another attractive feature for reprogramming strategy. As most differentiated cells rely on oxidative phosphorylation they require a metabolic shift when the cells are reprogrammed [9] . We believe that in the cord-blood $CD34^+$ population there are sub-populations that can be distinguished by their characteristics in terms of mitochondrial membrane potential. This will distinguish cells by differences

in the metabolic state and production of reactive oxygen species, which is determinant for the cell fate.

In order to address some of the main strategies of reprogramming and the main obstacles faced during this process the next pages of this introductory summary focus on basic concepts of stem cell biology.

1.1 Stem cells

Stem Cell Types

There are essentially two stem cell types that can be harvested from human donors: embryonic stem cells (ESC) and the adult stem cells. The ESC have high self-renewal ability and are pluripotent, which means they are capable to originate the three germ layer tissues: endoderm, mesoderm and ectoderm. On the other hand, the adult stem cells have a more restricted self-renewal capability and are only multipotent.

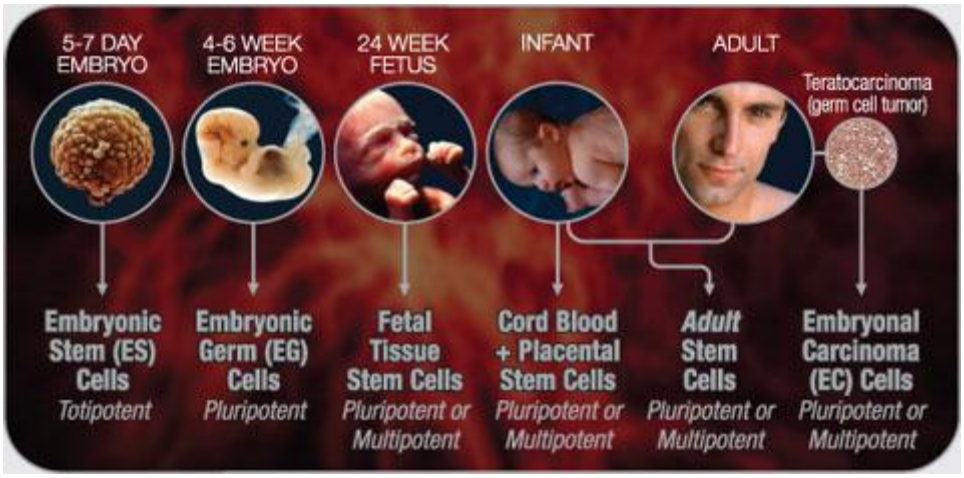


Figure 1 – Stem cells sources (adapted from http://www.sabiosciences.com/pathwaymagazine/pathways9/biology-great-promise-stem-cells.php)

Stem cells reside in highly regulated microenvironments, called niches, which maintain a constant dialogue between the cells and their surroundings and keeps a balance of self-renewal and differentiation. Niches have the ability to shield the cells from apoptosis stimulus, differentiation and over proliferation; which if it is not properly controlled may lead to cancer.

Stem cells can have a quiescent behavior or can undergo symmetric and asymmetric division. Within asymmetric division the mother cell gives rise to two different daughter cells, one that keeps the stem cell identity and other one that acquires a differentiated phenotype. On the other hand symmetric division leads to the generation of two identical daughter cells, either with a stem cell phenotype or differentiated phenotype. These different fates can be due to different

microenvironments or due to the effect of intrinsic factors that are differently segregated.

Embryonic Stem cells

In 1998 the first isolation of human embryonic stem cells from in vitro fertilization embryos was reported[2].

Embryonic stem (ES) cells are pluripotent cells (capable of originating the three germ layers: ectoderm, mesoderm and endoderm) derived from the inner cell mass (ICM) of Blastocysts (which are formed around day 5 to 6 of embryonic development), and that have great potential for differentiation.

These cells can be characterized by their unlimited proliferation in vitro (while preserving a normal diploid karyotype); capacity to form a teratoma (a tissue composed by the three germ layers) after injection subcutaneously into an immune deficient animal; and ability to originate chimeras (a single organism with two or more populations of genetically distinct cells) after fusion with an early embryo or a fertilized egg.

Characterization of these cells can be made by confirming the presence of specific markers or proteins (as shown in table 1).

Undifferentiated state marker	Mouse	Human
Cell-surface and nuclear antigens		
SSEA1 *	+	–
SSEA3/4 *	–	+
TRA1–60/81 **	–	+
TRA2-54	–	+
GCTM-2 **	–	+
TG343 **	?	+
TG30 ***	?	+
CD9 ***	+	+
CD133/prominin	+	+
OCT4	+	+
NANOG	+	+
SOX2	+	+
Enzymatic activities		
AP	+	+
Telomerase	+	+
In vitro *culture requirements*		
Feeder-cell dependent	+	+
LIF dependent	+	–
FGF4	+	–

Table 1- Comparison of cell markers and culture conditions in human and mouse embryonic stem cells. *Glycolipids. **Keratan chondroitin sulphate proteoglycans. ***Tetraspannin transmembrane proteins. AP, alkaline phosphatase; EB, embryoidbody; ESC, embryonic stem cell; FGF4, fibroblast growth factor-4; LIF, leukaemia inhibitory factor; OCT4, octamer-binding transcription factor-4; SOX2, SRY-related high-mobility group (HMG)-box protein-2; SSEA, stage-specific embryonic antigen; TRA, tumour-rejection antigen[10]

Although embryonic stem cells have desirable features for cell therapy their use in allograft transplants is associated with immune responses in the host. These cells increase their immunogenicity by gaining human leukocyte antigens during differentiation, which might increase the risk of rejection. Other problem resides on the lack of proper imprinting patterns and regulation of certain genes which might lead to spontaneous and uncontrolled differentiation resulting in developmental abnormalities[11].

On the other hand working with human embryos raises ethical issues that are still on debate. The collection of the cells implies the destruction of human embryos, which goes against many beliefs and values in our society. The commercial value over these cells and legitimacy of patents are other questions that need to be answered.[4]

As pluripotency and self-renewal are the most relevant characteristics of the embryonic stem cells, this chapter will now focus on a small review on the main agents that regulate these pathways.

Transcription factors

Transcription factors are proteins capable of recognizing specific DNA sequences and can either activate or prevent transcription. Like most proteins they are transcribed in the nucleus and translated in the cytoplasm. Their return to the nucleus where they can interact with DNA is assured by a nuclear localization signal. This signal consists in positively charged sequences of lysine or arginine residues that target proteins and allow them to bind to a importin. The complex can then move through the nuclear pore. Within the nucleus, the transcription factor is released and is now free to interact with the enhancers and promoters of the genes.

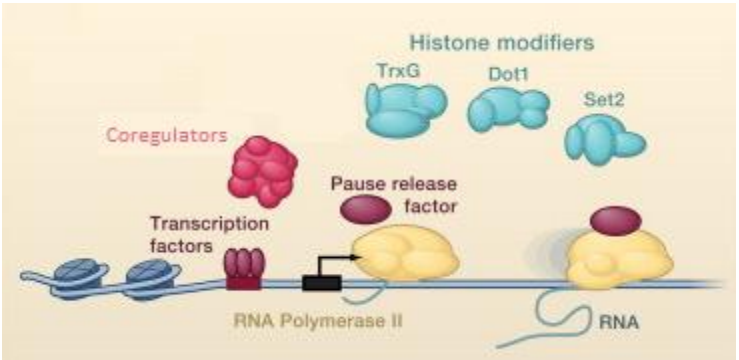


Figure 2 - On active genes, enhancers are bound by multiple transcription factors, which recruit cofactors that can linteract with RNA polymerase II. RNA polymerase II generates a short transcript and pauses until pause-release factors and elongation factors

allow further transcription. Chromatin regulators, like histone-modifying enzymes such as TrxG, Dot1, and Set2, are recruited by transcription factors or the transcription apparatus and mobilize or modify local nucleosomes.[12]

The enhancers are cis-acting DNA sequences that can greatly increase gene expression. They can be located upstream or downstream of the gene and act by allowing modifications on the DNA conformation in the presence of transcription factors. These changes will result in a better interaction between the RNA polymerase II and the promoters.

This interaction with the enhancer may result in multiple mechanisms to regulate genetic expression:

- Stabilization or blocking of RNA polymerase binding;
- Recruit co-activators or co-repressors of the transcription factor DNA complex
- Catalyse histone acetylation by promoting the activity of histone acetyltransferase (HAT) which weakens the association of the DNA with the histone, making the DNA more accessible promoting the activity of the transcription machinery;
- Catalyse the histone de-acetylation by the histone dacetylase (HDAC) activity, which participates in the opposite reaction of HAT.

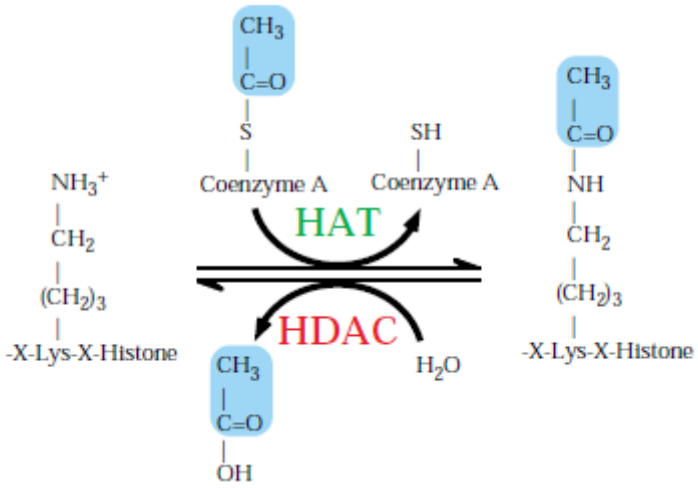


Figure 3- Acetylation reaction mediated by HAT, where acetyl coenzyme A is the donor of the acetyl group (COCH3). And opposite reaction mediated by HDAC[13]

Another important group of enzymes that are responsible for histone modifications are the: histone methyltransferases (HMTs) and histone demethylases. HMTs are usually related with gene activation/silencing by transferring a methyl group (CH3) to a specific lysine or arginine on histone 3 and 4. On the other hand, histone demethylases activate/silence gene expression by removing the methyl group.

Key transcription factors in pluripotency - Oct4, Sox2 and Nanog

Oct4 (also known POU5F1) is considered a key regulator of pluripotency. For this reason it is often used as a reprogramming factor and its expression is an evidence of pluripotency. Its expression starts right from the 2 cell stage especially prior to 8 cell stage until the blastocyst stage where it is strongly expressed in the inner cell mass (ICM). As differentiation occurs, the levels of this protein decrease being almost absent in most somatic cells. The activation of this gene is vital for embryogenesis since embryos with Oct4 knockout, fail in the development of ICM and die after blastocyst stage[4, 14]. Additionally, this factor has been reported as crucial to promote survival of germ line cells as disruption of its expression leads to apoptosis[15].

Oct4 can bind the downstream promoters (AGTCAAAT) activating or suppressing the expression of several genes. Among the targeted genes regulated by this protein are important mitochondria enzymes like the pyruvate carboxylase (Pcx) and ATP synthase (Atp5d). The products of these genes are considered to be responsible for the decision of the metabolic behaviour of the cell.[16, 17]

Down-regulation of genes may also occur by the formation of heterodimers with other factors like Sox2 (SRY (sex determining region Y)-box 2). This is another important transcription factor that is also expressed in the ICM and in precursor cells of the nervous system. By combining with Oct4 controls the expression of genes such as Fgf4, UtF1, NANOG, Fbx15 and Oct4 itself.

The third main pluripotent gene is Nanog. The important role of Nanog relies on the maintenance of self-renewal and undifferentiated state by modulation of the transcription factors Oct4 and Sox2. Other targets of this factor consist in:

- Foxd3, a transcriptional repressor important for the maintenance of the inner cell mass;
- Setdb1 gene which encodes an enzyme that methylates histone H3 on lysine 9 (H3K9) and is crucial for repression of a subset of genes encoding developmental regulators in ES cells[18].
- Rif1 activation which associates with dysfunctional telomerases and has a role in the S-phase checkpoint in the response to DNA damage

An interesting feature of these three transcription factors resides in their auto regulatory loop by targeting the promoters of their own genes as shown in figure 3. This mechanism is believed to promote stability of gene expression and reduce response time to an external stimulus.

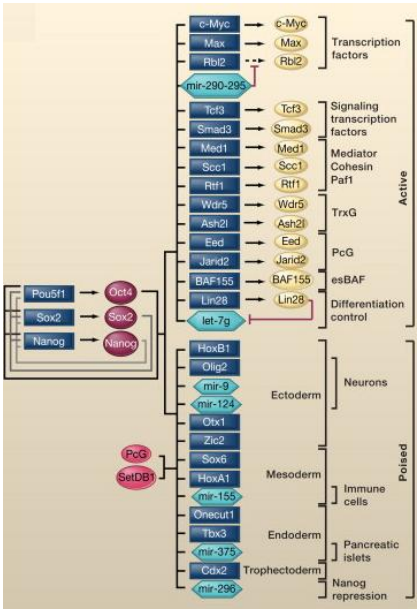


Figure 4- Selected Components of ESC Core Regulatory Circuitry and Its Disruption during Differentiation. The genes are dark blue rectangles; the gene products are yellow circles, the hexagons are miRNA promoters. Lin28 inhibits the maturation of the Let-7g miRNAs wich are required for differentiation. Oct4, Sox2, and Nanog are also associated with SetDB1- and PcG-repressed protein-coding and miRNA genes that are poised for differentiation[12].

The interruption of this auto regulatory cycle results in down-regulation of the main transcription factors and the loss of the pluripotent state as shown in figure 5.

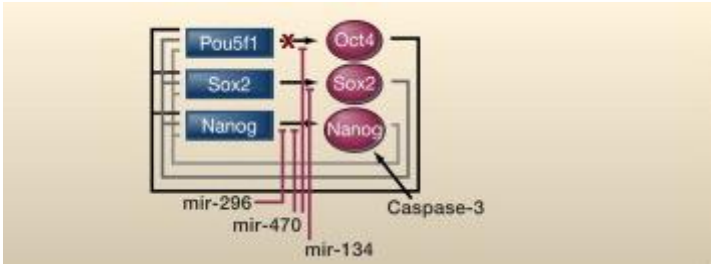


Figure 5 - The loss of pluripotent state during differentiation involves the silencing of the Pou5f1 gene, the proteolytic destruction of Nanog by caspase-3, and miRNA-mediated reduction in Oct4, Nanog, and Sox2 mRNA levels[12]

Other transcription factors – Lin28, Klf4, c-Myc

Although not so important as the pluripotent genes mentioned above, other transcription factors have an important role in the maintenance of several features of pluripotent cells.

c-Myc is an important transcription factor that is involved in the regulation of transcription of about 10% of the genome[19]. c-Myc is also a component of active chromatin by associating with various histone acetyl-transferase complexes (HAT), such as p300 and TRRAP, and thus having an important role in opening chromatin conformation through histone acetylation and favour the access of Oct4 and Sox2 to their targets. Another important effect of c-Myc is the inhibition of p21 promoter an important regulator of DNA repair, preventing its activation by p53. The result is an increase in proliferation since cell cycle will not be interrupted at the G1-S checkpoint[20]. On the other hand, a sustained expression of c-Myc will increase the apoptosis in a p53 mediated way[14]. This gene is believed to promote mitochondria biogenesis[21].

Klf4 importance resides in the control of proliferation and apoptosis. It has been reported that this transcription factor has an important effect in balancing the action of c-Myc. At the same time that counters the apoptotic effects of c-Myc by repressing p53 it also inhibits proliferation by activating p21[14, 22].

Lin28 protein is localized both in the nucleus and cytoplasm and its role in pluripotency is essentially related with inhibition of let-7 family miRNAs by diminishing their maturation. The lin28/let-7 axis is an important regulator of cell proliferation, since let-7 miRNAs are responsible for blocking the expression of pro mitogenic factors such as c-Myc and Ras. For this reason lin 28 can replace the use of c-Myc in reprogramming studies[23]. Lin28 also interacts with mRNAs of genes involved in cell cycle progression such as cdk4, cyclins A and B and contributes for the maintenance of the pluripotent state[24]. Additional functions are related with the stabilization of the transcription of mRNA from genes in the pluripotent transcriptional network and expression of the Oct 4 gene at post transcriptional level[25].

Adult stem cells

Although ESCs seem to be the perfect candidates for clinical trials and platforms for studies of new drugs or pathways their use as been controversial because of ethical concerns. For this reason the use of adult stem cells became a very important matter of study.

Hematopoietic stem cells (HSCs) from bone marrow are responsible for the creation of all the blood cell types and are currently being used in transplants. Recently, other adult stem cells have been found in a variety of different tissues. These findings have generated a great deal of excitement for expanding the use of stem cells for treatment of diseases. The adult tissues that have been reported to contain stem cells include the brain, bone marrow, peripheral blood, blood vessels, skeletal muscle, skin, and liver[3]. In addition to these lineage-restricted adult stem cell types, mesenchymal stem cells are a less differentiated form of stem cell that have the ability to mature into a more diverse variety of cells.[26]

There are however several obstacles restraining the uses of adult stem cells in research and clinical therapies namely the problem related with the procedure of isolation and culture of these cells. Although there can be several sources to obtain adult stem cells, there is a limit for them to maintain their self renewal capability for long periods in culture without expecting a significant differentiation. The same problem is observed in stem cells derived from umbilical cord blood.[5]

Other issues concerning the use of adult stem cells can be[27]:

- the possibility that the cells have accumulated mutations caused by age or radiation;
- the limited capacity of differentiation;
- the risk of immune response after allogenic transplants;
- the amount of cells that can be harvested and that can prove to be insufficient for the desired applications;

Human Cord blood hematopoietic stem cells

Human cord blood is an important accessible source to obtain hematopoietic stem cells. Preservation of these cells is currently being made for blood and autoimmune disorders as these cells contain some differentiation potential. Their ability to generate immune system cells, adipocytes [28], cardiomyocytes [29], endothelial cells [30],

fibroblasts[31], liver cells [32], osteochondrocytes [33], and pancreatic cells [34] has been reported.

Characterization of the hematopoietic population has been associated with expression of $CD34^+$ marker[35]. However this is a very heterogeneous group with major differences in terms of clonogenic capacity, suggesting the existence of different populations with variable gene expression and metabolic status.

The induction of pluripotency – reprogramming to iPSC (induced pluripotent stem cells)

The limitations in the use of the different stem cell sources in cell therapy lead researchers to look for alternative ways to generate cells that could fulfil the need for high cell number and differentiation potential. This search reached its goal with the advent of the reprogramming technology. Reprogramming consists in the de-differentiation of cells which have undergone some degree of differentiation. This re-establishing of the pluripotent capacity will avoid ethical issues involving the use of embryos. Furthermore the problem with immune response will also be diminished because of the possibility of use of cells from the patient.

As shown in the figure 6 this process consists in the introduction of reprogramming factors in a differentiated cell. The reprogramming factors which can be transcription factors or micro RNAs will induce activation of the regulatory loop of the main pluripotent genes. This will lead to the activation of the pluripotency cascade and the repression of the machinery responsible for cell differentiation (as shown above in figure 4).

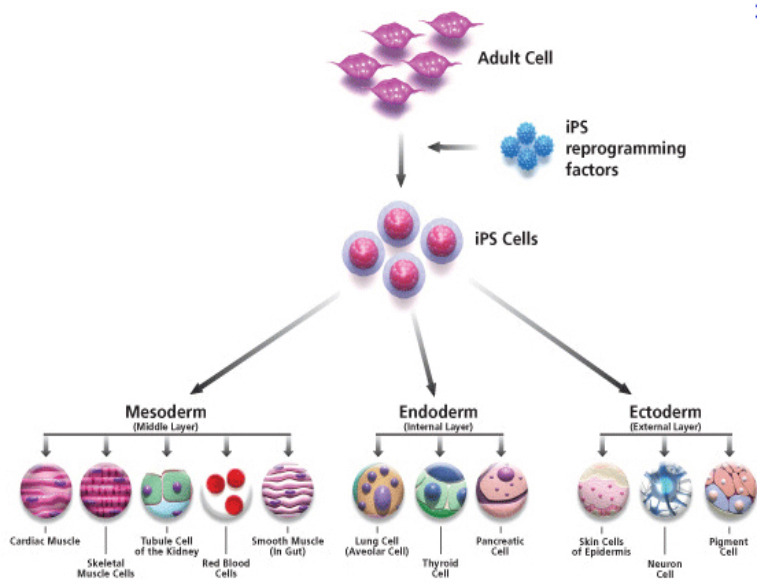


Figure 6 – Schematic representation of the reprogramming process (adapted from http://flipper.diff.org/app/pathways/info/4475)

Reprogramming strategies

The first attempts to generate induced pluripotent stem (IPS) cells were made by using nuclear transfer. This method consists in the fusion of a nucleus, removed from a somatic cell, with an egg cell which had its nucleus removed. Through this approach the somatic nucleus can be reprogrammed by the factors that are present in the egg cell. After stimulation by an electric shock the cell begins to divide by mitosis and originates cells with practical identical DNA of the donor of the somatic cell. The great promise of this technique was based on the capability of the oocyte to provide an ideal environment for reorganization of gene expression. As all the crucial intervenients would already be present in the correct proportion the number of stochastic events would surely decrease.

This technique is however hard to be applied in mammals due to the small size of the eggs which would result in a low efficiency process. The main problem that restrains its use in humans is however the ethical concern about the possible creation of human clones if the cells were implanted in a uterus during the blastocyst stage. Furthermore there is also the risk of immunogenicity caused by the mitochondrial DNA inherited from the oocyte[36, 37].

The problems mentioned above could not be ignored and forced science to find new alternatives to artificially create pluripotency. Since then several strategies have been explored in order to improve efficiency and safety while aiming to make it a standard practice in medical procedures. Figure 7 summarizes the main strategies used until now.

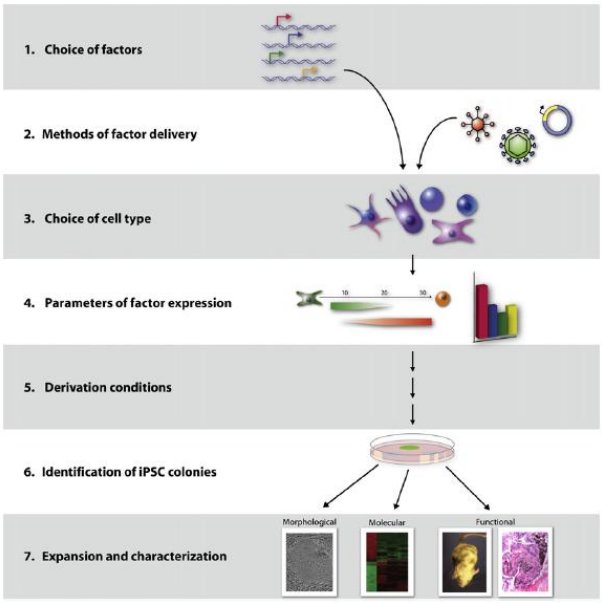


Figure 7 – Main differences between reprogramming strategies. [36]

We will now focus on an overall review about the first two parameters of the table as they represent the most critical points in this type of study. We will describe the relevant work in this area while addressing the main advantages and obstacles that have been developing in this still young technology.

2.1 Choice of reprogramming factors

Viral vectors were the first successful method for generation of IPS cells. Shinya Yamanaka in 2006 showed that is possible to generate induced pluripotent stem cells by artificially reprogramming Mouse Embryonic Fibroblasts (MEF). By using a retrovirus system Yamanaka's group reversed the differentiation process in somatic cells and confirmed the presence of embryonic features. [4] They selected c-Myc, Oct4, Sox2 and Klf4 as the best reprogramming factors between several candidates because of the similar morphology and proliferative characteristics of the reprogrammed cells with ES cells.

Later Thomson *et al* (2007) demonstrated successful reprogramming of human foreskin fibroblasts with the transcription factors Sox2, Oct4, Lin28 and NANOG also based in a viral system (lentivirus). The c-Myc transcription factor, which enhances reprogramming efficiency by interacting with chromatin and allowing Sox2 and Oct4 to bind to their targets, was not used due to its induction of ES differentiation and apoptosis[23, 38].

Most of the work in this field uses a combination of these 2 sets of factors. Oct4 and Sox2 are the most critical since they have proven in some cases to be sufficient to induce pluripotency[39].

2.2 Reprogramming Factor Delivery

Viral systems as vectors to generate pluripotency

The main goal of creating a viral vector for gene delivery is to achieve genomic expression with high efficiency. Viruses are excellent at doing this; although one should be careful to minimize the risk of mutations and the transfection of unwanted genes or the risk of creating competent viruses that represent a danger not only to the host, if the cells would be transplanted, but also for the operator.

Retroviruses were the traditional method used for gene delivery. There were however several disadvantages in the use of these vectors:

- Random insertion of genes within the genome, which can activate oncogenes;
- Possible transgene reactivation upon cell differentiation;
- Requirement of proliferating cells for infectivity;
- Poor in vivo delivery and transfection resulting in low reprogramming efficiency[40].

Among retroviruses there is a group that deserves special attention, the lentivirus, which can enter the nuclear envelope and thus are capable of replicating in non-dividing cells, making the reprogramming process more efficient and faster.

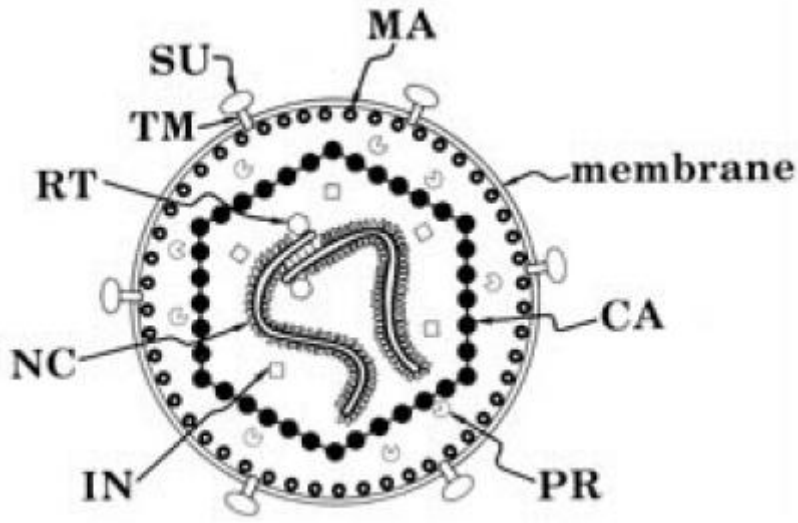


Figure 8 – Schematic representation of a lentivirus. (adapted from http://www.vcu.edu/oehs/chemical/biosafe/lentiviralvectors.pdf)

Denotation	Protein Name	Function
CA	Capsid	gag gene; protects the core
IN	Integrase	pol gene; needed for integration of the provirus
MA	Matrix	gag gene; lines envelope
NC	Nuclear Capsid	gag gene; protects the genome and forms the core
PR	Protease	Essential for gag protein cleavage during maturation
RT	Reverse Transcriptase	Reverse transcribes the RNA genome
SU	Surface Glycoprotein	The outer envelope glycoprotein; major virus antigen
TM	Trans Membrane Protein	The inner component of the mature envelope glycoprotein
Accessory Proteins (HIV)	Ex: Nef, Vif, Vpu, Vpr	Play a role for infectivity and pathogenicity of HIV

Table 2 – Function of lentiviral proteins. (adapted from http://www.vcu.edu/oehs/chemical/biosafe/lentiviralvectors.pdf)

The infection starts with the binding of the viral surface protein to the receptor of the host cell. After this the capside of the virus disassembles by a product of the pol gene and the reverse transcription takes place forming a double stranded DNA. The double stranded DNA together with other viral proteins like MA, IN and VPR (Table 2) will form a preintegration complex (PIC), which has the ability to enter the nucleus of a non-dividing cell. MA and IN are recognized by importin α, this complex then binds to importin β to be transported to the nucleus. Accessory proteins like VPR act by increasing the affinity with importin α and/ or by acting as analogues of importin β. IN is responsible for the cleavage of the DNA at both ends allowing it to be integrated in the host genome without a preferential integration site[41].

Ribosomes of the host cell will then translate viral mRNA that codifies the proteins for the formation of a new virus. This step requires that the viral protease cleaves the viral proteins in smaller mature proteins which can form a new virus.

Another interesting feature in the use of these vectors is the silencing of the transfected genes by DNA methylation as reprogramming proceeds[14].

Although the efficiency achieved with this method is reasonable when compared to the current alternatives there are still obstacles that need to be overcome. One of these is the problem of random DNA insertion of the transgenes. This could lead to changes in the transcription pattern of genes located in the genomic vicinity of the integration site. This effect is especially problematic if it occurs close to oncogenes or tumour suppressor genes giving origin to cancer.[42]

Evolution of viral reprogramming vectors

The risk of generation of a competent virus (capable of replication) is also preventing clinical applications.[43] To address this problem several changes have been made in the construction of the viral vectors. First generation vectors used three plasmids. One containing all the genes of the HIV-1 virus except the envelope protein and a CMV (Cytomegalovirus) promoter with the packaging sequence removed.[44] A different plasmid was used for the envelope protein. And on an additional plasmid was the cDNA of the genes to be transfected, two long terminal repeats (LTR´s) containing regulatory elements for gene expression, elements for gene packaging and reverse transcription. Traditionally the packaging genes are separated in different plasmids for security measures. As all of these genes are necessary to generate a competent virus and the only promoter is in the plasmid carrying the protein coding genes it would be very difficult to generate a replication competent virus because it would be necessary to have as many recombinant events between the plasmids as the number of packaging plasmids. This would be very improbable but still possible.

On the second generation all the accessory viral genes (*vif*, *vpr*, *vpu* and *nef)* were deleted from the packaging plasmid diminishing the number of HIV-1 genes present from the packaging vector and retaining only Tat and Rev.[45]

The third generation vectors were characterized by replacement of Tat promoter for other heterologous promoters and the delivery of Rev on an additional plasmid.[46]

Additionally other important modification was the deletion of 3'U3 promoter. Lentiviruses have two promoters, one in the U3 region of the 3' side and another in the U3 region of the 5' side. During reverse transcriptase the promoter in the 5' side is deleted. Reverse transcriptase uses the promoter in the 3' for the creation of the double stranded DNA. By exploring this mechanism researches deleted the promoter in the 3' region making impossible for the virus to create viral mRNA with packaging sequence. Another change introduced was the replacement of Tat by stronger promoters in the 5' side of the cDNA.[47]

Integration free techniques

Since viral integration constitutes a major risk to the host, there are several approaches that try to overcome this problem by avoiding genome integration or through the use of excisable techniques.

Adenoviruses are one of these approaches currently being studied as a possible alternative in gene delivery. Instead of incorporating the transfected genes into the host's genome, the genes are directly expressed from the viral genome[48]. The main disadvantage of this strategy is the low efficiency obtained, most likely because the expression of the reprogramming transcription factors is not maintained during sufficient time to allow epigenetic remodelling[49]. Nonetheless, although less likely, the possibility of DNA integration is not fully discharged[50].

Other alternative being explored is the Cre-loxP system. In this technique the transfected gene is flanked on both sides by a sequence named loxP[51, 52]. Transient expression of the enzyme Cre recombinase will then allow the excision of the exogenous gene. A problem with this method is the possible remaining of a loxP site and few bases external to the loxP sites on the cell host genome. These could still affect the expression of the endogenous genes leading to an aberrant gene expression.

The Piggy Back transposon system is another viable option. A transposon is a genetic element that can change its position within the genome of a cell. Through this mechanism it is possible to deliver a particular gene without depending on viral vectors. The transposon can be integrated in the host chromosome in the presence of the enzyme Piggy Back transponase that targets the sequence TTAA. The gene can be later removed by the action of the same enzyme. The TTAA integration sites can be repaired after excision. The presence of this enzyme can be assured with the use of a helper plasmid with the respective coding sequence, mRNA sequences or the enzyme itself[53].

A major advantage of the Piggy Back system is the size of the genetic cargo that can be delivered. Lacoste and co-workers (2009) reported a Piggy Back system capable of delivering 18 Kb of genes in HESC[54] while the typical viral system only allows a size of about 10 kb.

Alternatively gene delivery can be replaced by the use of recombinant proteins that can be directly incorporated in the cell target[55]. The Oct4, Sox2, Klf4 and c-Myc proteins can be purified and combined with a poly-arginine peptide tag which allows them to pass through the plasma membrane of the cell after addition to the cell medium. This strategy was used on MEFs but had however low efficiency and required the use of the histone deacetylase (HDAC) inhibitor valproic acid.

Strategies to improve the efficiency of reprogramming

The reprogramming process is a very stressful event for the cell and can trigger DNA damage response resulting in cell cycle arrest and senescence. Several studies have reported an increase in efficiency when the DNA repair machinery was suppressed[56-58]. This may be one of the major causes that are on the basis of the low efficiencies achieved. However this is also a crucial step that helps to prevent chromosomal anomalies and aberrant gene expression. Additionally, the oncogenic nature of the transcription factors as agents in the induction of pluripotency is another problem[59-61].

A possible way to reduce the number of transcription factors without compromising the epigenetic remodelling during the process is the use of small

molecules that affect the access to chromatin. Huangfu and colleagues (2008) were able to reprogram human fibroblasts with only Sox2 and Oct4, using valproic acid (VPA)[62]. Other molecule with interesting features is BIX-0129, a G9a histone methyltransferase inhibitor. Shi et al, (2008) were able to reprogram neural progenitor cells (NPC) with only Sox2, Klf4 and c-Myc, by introducing this molecule in the culture medium[63]. The G9a histone methyltransferase is responsible for Oct4, Nanog and Dnmt3l repression during differentiation[64, 65].

Bay k 8644, a calcium channel agonist, has also been reported as having a positive effect at improving efficiency of cell reprogramming in MEF´s[66]. However the mechanism of action remains uncertain.

Metabolism and reprogramming

After this brief review of the agents that control gene expression in embryonic stem cells this thesis will address the impact of mitochondria and metabolism in pluripotency. The fundamental purpose of this work relies on the selection of a population of cord blood based on the mitochondrial phenotype. Therefore it is important to describe how metabolism is stemness related and how the signalling pathways related with mitochondria are changing during reprogramming.

Recent findings have confirmed the important role of a metabolic adaptation as a requirement for the cells to undergo reprogramming to pluripotency[67]. Somatic cells rely mostly on oxidative phosphorylation for energy production while embryonic stem cells have a glycolytic metabolism. Therefore differentiated cells need to go through a metabolic shift during the reprogramming process.

There are several metabolic features that are characteristic of embryonic stem cells:

1. Embryonic stem cells are gathered from the inner cell mass of the blastocyst of pre implantation embryos. This means that they reside in a hypoxic environment without adequate levels of oxygen for oxidative phosphorylation.

2. These cells have high sensitivity to the damage caused by reactive oxygen species (ROS). Since the cells will generate every tissue in the adult body, damage caused in the genetic material during this stage would be disastrous since would be perpetuated in the daughter cells.
3. The absence of complete oxidation of glucose during the TCA cycle (or Krebs cycle) allows an increase of important substrates for biosynthetic pathways like amino acids, nucleotides and fatty acids.[68] An example is the citrate used for synthesis of cholesterol and fatty acids.

These properties need to be acquired during the reprogramming process for successful generation of iPSCs. Indeed, Andre Terzic and collaborators (2011) reported that the use of inhibitors of glycolysis (2-deoxy-glucose and 3-bromopiruvyc acid) lowered the efficiency or impaired the induction of pluripotency in mouse embryonic fibroblasts. Consistent with these results, the stimulation of mitochondrial function by piruvate dehydrogenase kinase inhibitor dichloroacetate also lowered the efficiency of the reprogramming process. Additionally, they reported that the metabolic shift was accompanied by an increase in mitochondrial membrane potential.[17]

Mitochondria and metabolic pathways in pluripotency

Mitochondria are organelles with a central role in metabolism as they are responsible for an increase in the production of energy derived from glucose through the process of oxidative phosphorylation. However the electron transport chain leads to the production of reactive oxygen species (ROS), which are important molecules that are a by-product of this pathway and have a major impact on cell structure integrity; the DNA in particular is very sensitive to oxidation. To avoid this, embryonic stem cells exhibit important differences in their mitochondria and when compared with differentiated cells they have higher antioxidant defences. Because they rely less on the oxidative phosphorylation pathway they have a more primitive mitochondrial phenotype, showing fragmented mitochondria, in small numbers with undeveloped cristaes and peri-nuclear localization. They have low amounts of mitochondrial DNA and low membrane potential[67].

Recently, the role of mitochondria in reprogramming strategies has been gaining some attention from researchers. Important changes occurring during the reprogramming event have been documented. The important relationship between a glycolytic phenotype or low oxidative capacity and an increase in reprogramming efficiency has been observed.[9] Indeed, as shown by Varum and co-workers (2011) (Figure 9), human IPS cells have a mixed phenotype between ESCs and somatic cells. Nonetheless, their metabolic state is closer to the ESCs.

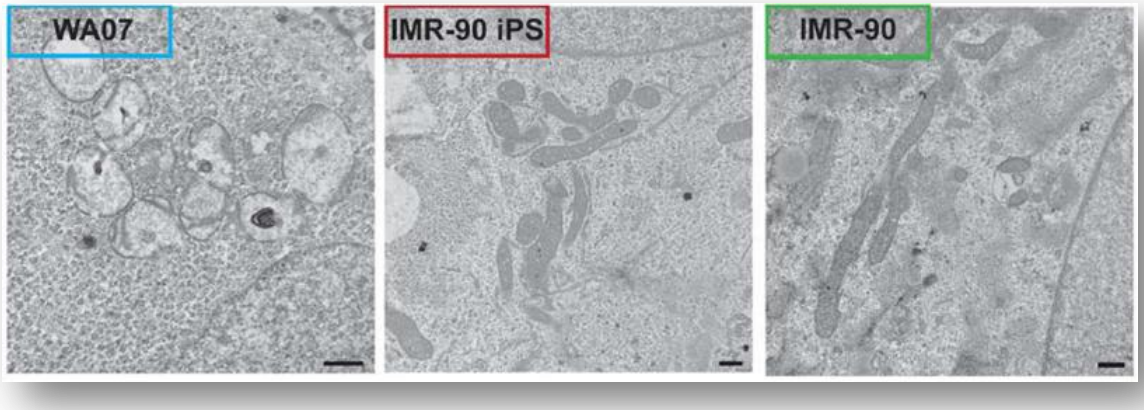


Figure 9 – Morphologic features of mitochondria obtained by transmission electron microscopy in hESCs (WA07 line), IPSCs (IMR-90 IPSC line), and fibroblasts cells (IMR-90)[69]

This metabolic shift is being described as crucial for cellular reprogramming. Therefore this part of the introduction will describe some of the metabolic pathways present in human cells that may be modulated during the reprogramming process. We give some examples of how these pathways impact in ESC cell biology and embryonic development.

mTor/IGF-1 Pathway

The mTor pathway has a relevant role in the control of pluripotency since it integrates signals from nutrients, hypoxia, energy status and the growth factor IGF-1. It is a complex network that allows the cells to adapt their activity based on changes in concentrations of substrates and avoiding to compromise critical cellular functions. (figure 10)

Rapamycin is a drug that became known because of its ability to interfere with this important pathway that regulates metabolism, autophagy and ribosome

biogenesis as shown in figure 10. By binding to the FKBP12 protein it is able to perturb the functions of the mammalian target of Rampamycin (mTOR). The mTor protein forms two complexes: mTor complex1 (mTORC1) composed of mTor, Raptor, AKTS1 and mLST8 proteins and mTor complex2 (mTORC2) composed of Rictor, Sin1 and mLST1, which is not sensitive to rampamycin inhibition and interacts with the IGF-1/AKT pathway.

In an environment rich in energy (ATP) the mTORC1 binds to two proteins p70S6 kinase 1 (p70S6K1) and 4E-BP1 in order to activate RNA translation. S6K1 activity leads to an increase of the cell size. The phosphorylation of 4EBP1 by raptor-mTORC1 prevents inhibition of eIF-4e, which is important for translation of mRNA during cell division. The mTORC1 also inhibits autophagy, a mechanism responsible for the production of energy by catabolic breakdown of cellular components during starvation that is also important to remove damaged organelles.

When the glucose, aminoacids and ATP levels are low LKB1 tumour repressor inhibits raptor-mTor by phosphorylating and activating AMPK. This mechanism is used to promote energy conservation and glucose uptake when the cells are exposed to a metabolic stress, typical in cells with a proliferative state. The AMPK activates a GTPase, the TSC2 protein, resulting in down regulation of Rheb protein and preventing it from activating mTORC1.

In a hypoxic environment the hypoxia inducible factor 1 (HIF-1) induces the expression of REDD1, which in turn releases the TSC2 protein. This protein is responsible for mTORC1 inhibition.

The mTORC2 has an important interaction with AKT/PKB, which is a key component of the PI3K pathway. PI3K pathway promotes cell survival and proliferation through inhibition of transcription factors of the FOXO class. Insulin Growth factor (IGF) is a hormone produced in the liver by stimulation of the growth hormone. When IGF-1 binds to its receptor, the IGF-1R, the result is the activation of the PI3 kinase (PI3 K) and an increase of PIP3 in the plasma membrane. PIP3 will then activate PDK1 and mTORC2. Both these molecules will activate AKT, which will phosphorylate several proteins responsible for cell survival.

Several transcription factors including AFX, FKHR, FKHRL1 from the Forkhead family, CREB and p53 are direct or indirect targets of Akt kinase. As members of the

Forkhead family they are implicated in the regulation of the cell cycle. The overexpression of AFX, FKHRL1, or FKHR causes growth suppression in a variety of cell lines[70].

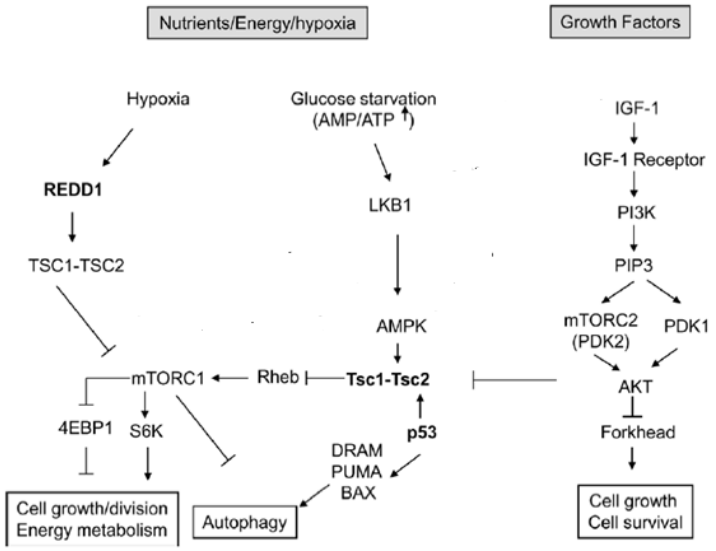


Figure 10 – Signaling of the two branches the mTOR pathway by nutrients, hypoxia and growth factors. (adapted)[71]

This pathway has been described as critical in mice embryonic development. The mTOR deficient mice die in the utero due to impaired proliferation.[72, 73]

New insights point that the use of mTOR inhibitors (like PP242, rapamycin and resveratrol) may improve both speed and reprogramming efficiency. This mechanism accelerates the bioenergetic shift promoting mitophagy .[74, 75] This is consistent with previous studies that suggest that an activation of p70s6k1 increases the levels of protein translation when pluripotent cells decide to undergo differentiation.[76] Young et al 2009 have shown that autophagy starts with deactivation of the mTOR signal.[77]

On the other hand, the overexpression of exogenous transcription factors (mediated by retrovirus) is thought to activate p53, leading to cell cycle arrest and apoptosis.[78] This may also be prevented by mTOR inhibition (increased mitotophagy) which can be an important mechanism to reduce apoptosis and cell cycle arrest.[74] The protein p53 mediates the apoptotic cascade and is capable to induce autophagy through induction of DRAM (damage-regulated autophagy modulator), a lysosomal protein, PUMA and Bax[79, 80].

On the other hand, autophagy seems to be related with reports of accumulation of mid bodies in ESC and iPSC cells.[81] Mid bodies are organelles formed during cytokinesis required for cell separation. After division they can be degraded by autophagy or accumulated and is suggested that they may have additional roles in determining cell fate. Apparently, these organelles accumulate in the daughter cell with the older centrosome during asymmetric division. This accumulation has been associated with the maintenance of stemness after division and with the reprogramed iPSC cells. Additionally induction of autophagy with rapamycin treatment led to the reduction of mid bodies in tumor cell lines (HeLa cells).[82]

It is clear that the modulation of this pathway can be an important procedure that will benefit induction of pluripotency and the culture of iPSCs.

HIF

Human stem cells reside in environments with low concentrations of oxygen this may explain their high vulnerability to ROS. These reactive molecules may damage the DNA of the undifferentiated cell, which can result in a perpetuation of the lesions by cell replication. The modulation of the oxygen environment can therefore be an important factor in reprogramming technology.

In response to hypoxia there is a change in gene expression mediated by hypoxia-inducible factor (HIF)-1, that has an impact on cell survival, proliferation, apoptosis and glucose metabolism[83] (Figure 11). This heterodimeric transcription factor is constituted by two proteins: the HIF-α, which has a oxygen dependent degradable domain, and HIF-β or ARNT which is insensitive to oxygen. Regulation of HIF activity is mediated through the stabilization of the alpha subunit. Under conditions of abundant oxygen HIF1-α protein is translated but is rapidly degraded. As oxygen levels decrease HIF1-α is stabilized and binds to constitutively expressed ARNT (HIF-β) subunits in the nucleus. The transcription and translation of HIF1-α remains unchanged by this mechanism, the difference in expression in hypoxia is only due to the stabilization of this protein that otherwise has a half-life of 5 minutes. The complex will bind to DNA and activate transcription through interactions with co-activators.

HIF-1a activation may also be induced by non-hypoxic stimuli. A possible mechanism involves the increase of translation of the HIF-1α protein through the mTOR/p70S6K and PKC (diacylglycerol-sensitive protein kinase C) pathways. PKC leads to gene transcription of the gene Sp1, which regulates the expression of HIF-1α. In this mechanism the rate of translation is increased while the degradation is unchanged.

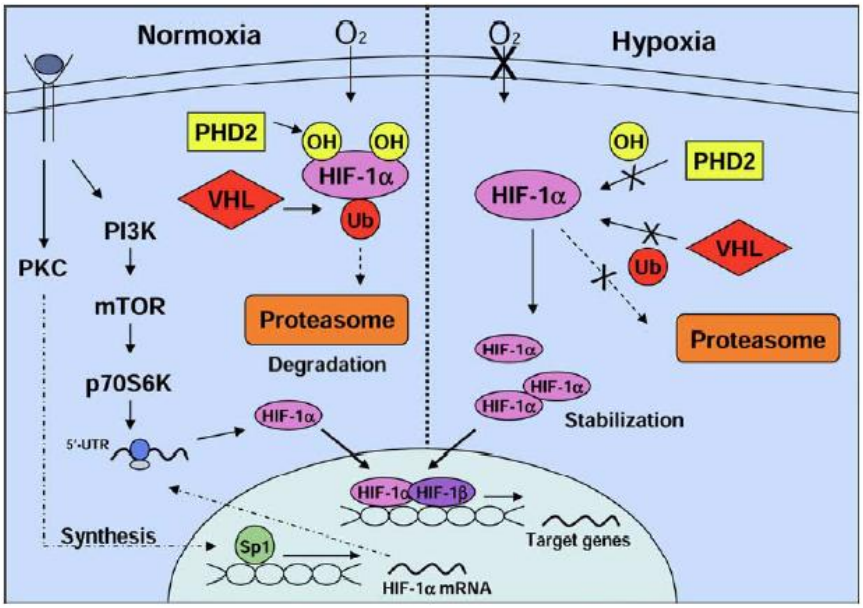


Figure 11 - Oxygen-dependent and independent regulation of HIF-1. In normal oxygen conditions, HIF-1_ is hydroxylated by PHD2, ubiquitinated by the pVHL complex and rapidly targeted to degradation by the proteasome. Non-hypoxic stimuli increase the transcription of HIF-1 gene by stimulation of PKC. The HIF-1 mRNA is then translated by increased activation of the PI3K pathway, which results in the increased HIF-1. Most non-hypoxic stimuli activate the PKC and PI3K pathways following binding with their associated receptors, which may explain the cell-type specific activation of HIF-1.[83]

HIF-1 is responsible for the increase of many enzymes of the glycolytic pathway (table 2), as well as the glucose transporters 1 and 3 (GLUT1, GLUT3)[84]. Therefore it is expected to have a critical role in determining cell metabolism.

Function	Gene (abbreviation)	Reference
Erythropoiesis/ iron metabolism	Erythropoietin (EPO)	Semenza et al., 1991
	Transferrin (Tf)	Rolfs et al., 1997
	Transferrin receptor (Tfr)	Bianchi et al., 1999
	Ceruloplasmin	Lok and Ponka, 1999
Angiogenesis	Vascular endothelial growth factor (VEGF)	Levy et al., 1995
	Endocrine-gland-derived VEGF (EG-VEGF)	LeCouter et al., 2001
	Leptin (LEP)	Grosfeld et al., 2002
	Transforming growth factor-β3 (TGF-β3)	Scheid et al., 2002
Vascular tone	Nitric oxide synthase (NOS2)	Melillo et al., 1995
	Heme oxygenase 1	Lee et al., 1997
	Endothelin 1 (ET1)	Hu et al., 1998
	Adrenomedulin (ADM)	Nguyen and Claycomb, 1999
	α_{1B}-Adrenergic receptor	Eckhart et al., 1997
Matrix metabolism	Matrix metalloproteinases (MMPs)	Ben-Yosef et al., 2002
	Plasminogen activator receptors and inhibitors (PAIs)	Kietzmann et al., 1999
	Collagen prolyl hydroxylase	Takahashi et al., 2000
Glucose metabolism	Adenylate kinase-3	O'Rourke et al., 1996
	Aldolase-A,C (ALDA,C)	Semenza et al., 1996
	Carbonic anhydrase-9	Wykoff et al., 2000
	Enolase-1 (ENO1)	Semenza et al., 1996
	Glucose transporter-1,3 (GLU1,3)	Chen et al., 2001
	Glyceraldehyde phosphate dehydrogenase (GAPDH)	Graven et al., 1999
	Hexokinase 1,2 (HK1,2)	Mathupala et al., 2001
	Lactate dehydrogenase-A (LDHA)	Semenza et al., 1996
	Pyruvate kinase M (PKM)	Semenza et al., 1994
	Phosphofructokinase L (PFKL)	Semenza et al., 1994
	Phosphoglycerate kinase 1 (PGK1)	Semenza et al., 1994
	6-phosphofructo-2-kinase/gructose-2,6-bisphosphate-3 (PFKFB3)	Minchenko et al., 2002
Cell proliferation/survival	Insulin-like growth factor-2 (IGF2)	Feldser et al., 1999
	Transforming growth factor-α (TGF- α)	Krishnamachary et al., 2003
	Adrenomedullin (ADM)	Cormier-Regard et al., 1998
Apoptosis	Bcl-2/adenovirus EIB 19kD-interacting protein 3 (BNip3)	Carrero et al., 2000
	Nip3-like protein X (NIX)	Bruick, 2000

Table 3 - Target genes of HIF-1 and their function[85]

An important gene activated in this pathway is the *PDK* gene, which encodes the pyruvate dehydrogenase (PDH) kinase (Table 3). PDK phosphorylates and inhibits PDH, the enzyme that converts pyruvate into acetyl coenzyme A (AcCoA) in the mitochondrial citric acid cycle, which generates reducing equivalents that are donated to the electron transport chain. As a result there is a decrease in the substrate for oxidative phosphorylation causing reduced ATP synthesis. By the other hand, there is an increase in glucose uptake via glucose transporters and increased conversion of glucose to lactate by the activity of glycolytic enzymes and lactate dehydrogenase A, all of which are encoded by HIF-1 target genes.

This pathway is an important mechanism that adapts the synthesis of ATP in an environment with variations in oxygen concentration and without increasing the production of reactive oxygen species, being therefore relevant in reprogramming studies where a metabolic shift is essential.

Numerous studies have reported benefits of culturing ESCs under low oxygen concentrations by reducing spontaneous differentiation, supporting self-renewal and decreasing chromosomal aberrations.[86-89] Yoshida et al 2009 demonstrated that low oxygen pressure enhances the number of induced pluripotent stem cells generated in human and mouse cells.[90]

Forristall et al 2010 have reported that ESCs cultured at 3-5% oxygen tension have an increase of Oct4, Sox2 and Nanog at mRNA levels when compared with the same

cells cultured at 20% oxygen tension. In ESC with HIF-2-α knockdown (a sub-unit of the HIF family) they described that the cells lost the capacity to mantain pluripotency, showing signs of differentiation with less clear borders and with areas with no expression of the pluripotent markers TRA-1-60 and POUF51. Additionally they also reported that cells with all the three subunits of HIF-α (HIF-1-α; HIF-2-α and HIF-3-α) family knocked-down failed to generate colonies and survive in culture. [91]

All these insights point to the importance of this pathway to achieve/maintain pluripotency, suggesting its modulation is a tempting strategy to improve the efficiency of reprogramming.

Motivation of this project

As discussed before it is of great interest to select the population of cells more prompt for reprogramming. This is a fundamental step that lacks optimization in order to increase efficiency. A connection between the metabolism and mitochondrial phenotype in the achievement of pluripotency has been reported in fibroblasts; the mitochondrial status and cell immaturity seem to be indicative of reprogramming plasticity[67]. Our goal consists in finding if this connection holds true in cord-blood, by evaluating a possible relationship between the mitochondrial membrane potential and the kinetics and efficiency of reprogramming. In this work it is assumed that the mitochondrial potential is also related with mitochondrial mass[92]. Based in this criteria we segregated two different populations of umbilical cord blood $CD34^+$ cells based on mitochondrial membrane potential using TMRM, a cationic mitochondrial probe. We then monitored the number of colonies generated after delivery of Yamanaka's transcription factors using a lentiviral system. Additionally, we also tested the effect of low oxygen concentration (hypoxia) in the development of iPS colonies. We evaluated the use of Antimycin A (inhibitor of complex III of the mitochondrial respiratory chain) to avoid reoxygenation each time that the cells were brought to normoxia to change the culture medium. Finally the colonies were characterized not only based on their morphology but also on gene expression, at the mRNA and protein level.

Methods

Plasmid amplification

The amount of DNA available for transfection was amplified in bacteria. The amplification was done separately for each plasmid. First 35µl (per transformation) of the bacteria Stbl3 *Escherichia coli* Chemically Competent were thawed on ice for 20 minutes. Next we put 1 to 100ng of the plasmid to amplify in contact with 15µl of the bacteria solution. We applied a heat shock for 45 seconds at 42^{o}C and 2 more minutes on ice. The heat shock was important to make the bacteria internalize the plasmid. We added 450µl of LB medium with ampicilin (100µg/ml) to the vial with bacteria and incubate them for 1 hour at 37^{o}C on a shaker at 220 rpm (rotations per minute). The bacteria cells were put on the incubator at 37^{o}C on a pre-warmed Petri dish with LB medium and ampicilin (100µg/ml) and agar to grow for 18 to 20 hours. As the plasmids contained a gene that conferred resistance to ampicillin, only the cells that internalized the plasmid survived.

We inoculated a single colony and put them on an Erlenmeyer with ampicillin (100µg/ml) and 150 to 200ml of LB medium. The solution stayed on a shacking incubator at 37^{o}C and 220 rpm (rotation per minute).

After 18 hours we collected the solution and centrifuged for 20 minutes at 4000g and 4^{o}C.

DNA extraction

The extraction of the plasmid from the bacteria was performed using a HiSpeed Plasmid Purification kit from Quiagen, as shown on figure 12. The pellet was resuspended with a vortex on 6ml of P1 buffer, a resuspension buffer that contains LyseBlue rom. The lysis of the bacteria was accomplished with addition of 6ml of P2 buffer followed by manual agitation. This buffer has a detergent the SDS that binds to phospholipids and proteins of the bacteria membrane. As this buffer reacts with LyseBlue by turning the solution blue, this confirmed the homogeneity of the solution. After 5 minutes we added buffer P3 that neutralized the previously reaction and avoided plasmid denaturation.

The lysate was incubated 20 minutes at room temperature while forming a floating precipitate. We filtered the solution using an extraction kit filter and used buffer QC to wash the DNA contaminants from the bacteria lysis. The collection of the DNA was performed using buffer QF to a new sterilized falcon for DNA elution. We added 3,5ml of isopropanol and incubated the solution for 10 minutes allowing the DNA to precipitate. Finally we passed the solution by a precipitator (QIA precipitator) also provided by the kit and collected the plasmid using mili Q water. The quantification of the DNA collected was done using a nanodrop device.

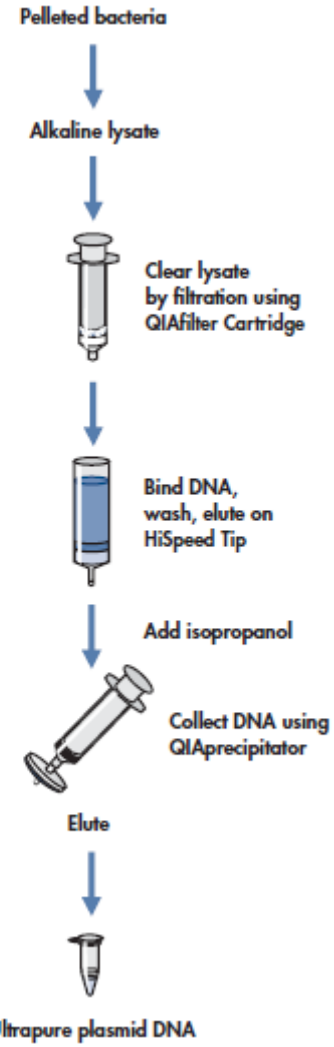


Figure 12 Schematic representation of extraction of the DNA plasmids from a bacteria culture (adapted from QIA mini spin kit QIAGEN handbook)

Viral packaging

The production of the third generation viral system was made by transfecting 293T cells with 4 plasmids. We used 293T (Human Embryonic Kidney) cells because these cells are easy to grow and transfect.

The packaging set was divided on 3 plasmids to decrease the probability to generate a competent virus as a safety measure. As only plasmid with the transcription factors had the promoter that assured the reverse transcription it would be necessary a recombination of the 4 plasmids to generate a virus capable of replication.

This vector construction was based on (Warlich, E. et al 2011)[93].

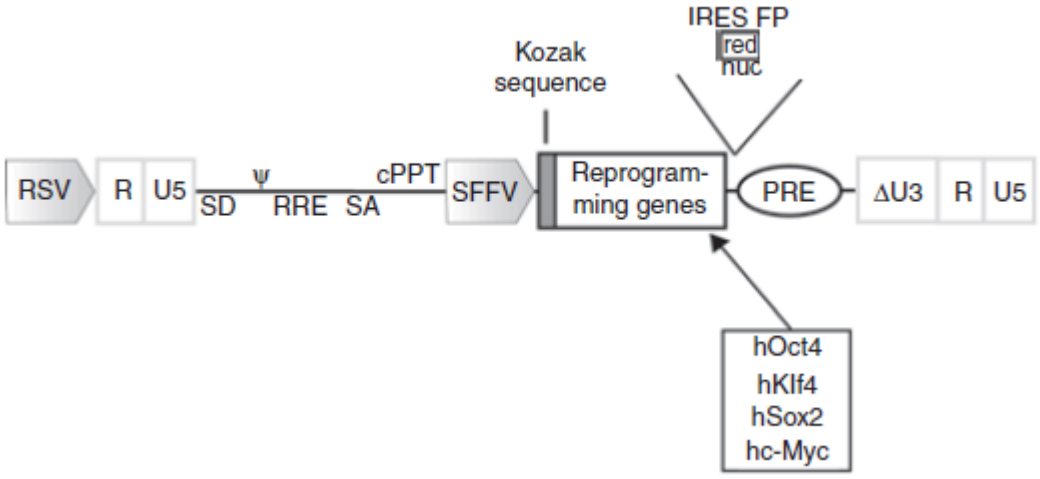


Figure 13 -Modular configuration of the self-inactivating (SIN) vector backbones for expression of the human (h) reprogramming factor (RF) Oct4, Klf4, Sox2, and c-Myc,. Δ marks the SIN configuration with partially deleted U3 of the 3' long terminal repeat. cPPT, central polypurine tract; FP, fluorescent protein dTomato;) IRES, internal ribosomal entry site; nuc, nuclear membrane-localized derivative; PRE, post-transcriptional regulatory element; RRE, rev-responsive element; RSV, Rous sarcoma virus U3 promoter; SA, splice acceptor; SD, splice donor; SFFV, spleen focus-forming virus promoter; ψpackaging signal. (adapted from Warlich, E. et al 2011)[93]

IRES allows the recruitment of ribossomes to the middle of the mRNA molecule. This will result on alternative splicing which is the ability of a single mRNA to code multiple proteins with different function and sequence. The RSV promoter is naturally silenced since the virus is SIN (self inactivating), only the SFFV promoter is involved in the expression of the reprogramming genes. During the reprogramming the cell undergoes an epigenetic remodelling leading to silence of the SFFV promoter. Consequently the exogenous pluripotent genes and the gene coding for d'Tomato fluorescent red protein will cease to be expressed which can be monitorized on a fluorescent microscope. For this reason it is possible to correlate the fluorescence of the colonies with induction of pluripotency.

The main plasmid expressed the 4 Yamanaka's transcriptional factors (Oct4, Sox2, c-Myc and klf4) and a d'Tomato protein as a fluorescent marker. The promoter used was SFFV (spleen focus forming virus). In case of epigenetic remodelling the promoter would be recognized as exogenous to the cell being silenced which results in loss of fluorescence by the Tomato protein. This was a practical tool to help characterization of the colonies. The other 3 plasmids were used for packaging in order to create a self-inactivating replication incompetent virus: pcDNA3.GP.4xCTE expressing HIV-1 gag/pol; pRSV-Rev (enconding both a nuclear localization signal, for the pre integration complex, and a nuclear export signal for the viral RNA exit the nucleus before degradation); and pMD.G encoding the vesicular stomatitis virus glycoprotein (VSVG).

The cryovials with 293T cells were thawed, and the cells were grown in t75 flasks with a specific medium: DMEM (Dulbecco's Modified Eagle´s Medium from GIBCO/Alfagene), 10% FBS (Fetal Bovine Serum from GIBCO/Alfagene) and PenStrep (Penicillin-Streptomycin Mixture from Lonza/VWR). We only proceed with the transfection after the t75 flasks have reached about 80% confluence.

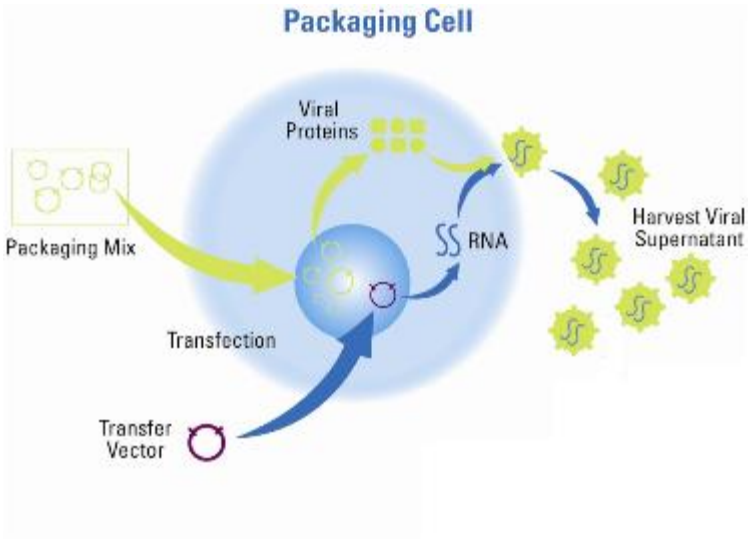


Figure 14 - Schematic representation of the viral production on 293T cells. (adapted from https://www.openbiosystems.com/Viral%20Packaging/TransLentiviral%20Packaging%20Syst/)

We used different concentrations of each plasmid as shown on table 4:

Plasmid	Concentration
pRSV-Rev	5µg
pcDNA3.GP.4xCTE	13 µg
pMD.G	3,75 µg
Lentiviral vector	13 µg

Table 4– Plasmids concentration used on transfection.

To insert the plasmids on the 293T we used the calcium phosphate method. First we mixed our plasmids with calcium chloride (2,5M). Then we dropped the solution drop by drop on a tube containing HBS (HEPES-buffered saline) (1M). The mixture formed a precipitate of calcium phosphate and DNA. As the DNA and the cell membrane have a negative charge, this method allows overcoming the charge repulsion. As the solution was put in contact with the 293T cells the precipitate attached to the cell membrane and entered the cell by endocytosis[94].

After 5 hours we changed the medium. At 72 hours after the transduction we collected the medium containing the virus. The medium was filtered using a 0,45µm low binding protein filter and was centrifuged 3h at 22500 rpm at 10^{o}C for concentration. We made aliquots of the virus diluted on 20 µl of DMEM and store them at -80^{o}C.

Viral titration

We calculated the number of infectious units (IU) per ml on our virus. To access this number we plated 293T cells on a 6 well plate that was incubated at 37^{o}C overnight. On the next day we changed the medium and infected each well with different volumes of our stock virus: 2,5µl; 5µl; 10µl; 20µl and 50µl. Cells were incubated at 37^{o}C for 18 hours after which we washed the cells 2 times with PBS and added new medium. We incubated cells at 37^{o}C for 48 hours. Finally we counted the

number of infected cells on fluorescent microscope. The number of IU was calculated by the following formula:

$$IU = (\frac{initial\ \ nr\ cells \times \%\, positive\ \ cells)}{uL\ \ stock\ \ virus\ used})$$

CD34$^+$ isolation

We isolated the CD34$^+$ cells from human umbilical cord blood with a protocol optimized in our laboratory. First we diluted the blood in the same volume of a dilution medium (2mM of EDTA, in PBS (Phosphate-buffered saline) with 7,2 pH). The EDTA in this solution is used to avoid cell aggregation and coagulation of the blood. We put the mixture in a falcon with one third of the final volume in Lymphoprep. Lymphoprep is used to achieve a density gradient separation during centrifugation.

We centrifuged the sample at 400g during 35minutes, with no brakes at 20^{o}C. After this step the solution was separated in several layers. (Figure 15)

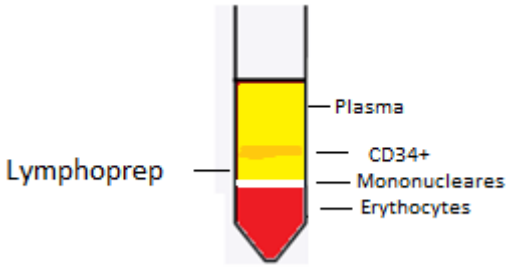


Figure 15– Schematic representation of the separation in layers through density gradient.

We aspirate the layer containing the CD34+ cells and discharged the remaining layers. Cells were centrifuged at 300g for 10 minutes at 20^{o}C and the supernatant was discharged. To completely remove the platelets we resuspended the cells on 50ml of MACS buffer (0,2 mM of EDTA, 0,5% BSA, and 5,7mM of NaOH in PBS with 7,5 pH)and centrifuge the cells 450g for 15 min at 20^{o}C. We repeated this step one more time.

To isolate the cells with CD34 marker we used magnetic assorted cell sorting (MACS).

The cells were resuspended on 320µl of the MACS buffer and it was added 100µl of CD34 MicroBeads and 100µl of FcR blocking agent. The MicroBeads are attached to an antibody specific to the CD34 membrane receptor. The FcR blocking agent is used to eliminate unspecific interactions between the MicroBeads like beads aggregation.

The cells stayed in contact with the beads for 30 minutes at 4^{o}C with mixing every 10 minutes. We centrifuged the cells for 10 minutes 300g at 4^{o}C and resuspended them on 3ml of MACS buffer.

We calibrated a magnetic column with 3ml of the dilution buffer and pass the solution with cells three times through the column. Only the cells that had the MicroBeads attached to the cell membrane remained on the column as the others were washed to a recover tube.

In the end we remove the column from the magnet and recovered the CD34+ cells with 5ml of the dilution buffer.

We diluted and counted the cells with a hemocitometer. The cells were centrifuged 300g for 10 minutes at 4^{o}C and were left overnight on StemSpan medium (bovine serum albumin (BSA), recombinant human insulin, iron-saturated human transferrin, 2-mercaptoethanol and supplements in Iscove's Modified Dulbecco's Medium (IMDM)) with cytokines (SCF (100ng/ml), Flt-3(100ng/ml)). The StemSpan is a medium specific for hematopoietic stem cells. The cytokines used stimulate the proliferation of these cells[95, 96].

Fluorescence-activated cell *sorting* (FACS) with TMRM

On the following day of the CD34+ cells isolation, we proceeded with antibody labeling. We used TMRM (Tetramethylrhodamine, Methyl Ester, Perchlorate from Invitrogen), a lipophilic cationic mitochondria probe that is sequestered by the mitochondria according to the membrane potential. Cells that rely on oxidative phosphorylation have a higher mitochondria membrane potential which we hoped to correlate with differences in the reprogramming efficiency. For maximum purity the cells were also labelled with anti CD34 antibody (10µl; miltenyi biotec).

We prepared a stock solution of TMRM in DMSO. We diluted the TMRM solution in PBS, in order to obtain a final concentration of 2,5nM on the medium containing the $CD34^+$ cells. Cells were put in contact with the dye and were incubated for 10 minutes at 37^oC. In order to proceed with the CD34 labeling we centrifuged the cells at 300g for 5 minutes, washed 1 time with PBS and resuspended them on 0,1ml of PBS with 10% FBS (Fetal Bovine Serum). We added 5nM of $CD34^+$-FiTC antibody and incubated the cells for 10 minutes at 4^oC. Finally we resuspended the cells on 10 ml of PBS (with 10% FBS).

We also prepare the following controls:

Cells only
Cells with CD34+ antibody only
Cells with TMRM only

Table 5 – Sample conditions

As the CD34-FiTC emits around 525 nm the gate was set in fl3 channel to see this fluorescence while the TMRM was analysed on the PE channel. The sorting was performed on a FACSAria sorterfrom BD Biosciences.

After sorting the cells were collected in StemSpan medium and stayed overnight at 37^oC in a 6 well plate.

Characterization of high and low TMRM populations – CD34, CD15, CD45 and CD133 markers

On a parallel experiment we compared the expression of different markers to confirm if the different uptake of the TMRM probe was sufficient to correlate with different maturation stages of the cells. We marked cells with the antibodies: CD34 Percp cy5.5 BD (10µl; BD Biosciences), CD15-FITC (2,5 µl; BD Biosciences), CD45 Pacific Orange (2,5 µl; Invitrogen)and CD133 –APC (10µl; miltenyi biotec).

Viral transfection

After sorting we had two populations with distinct membrane potential which was correlated with the mitochondria membrane potential.

The method of transfection was based on (Giorgetti, A., N. Montserrat, et al. 2010)[96]. First we coated 24 well plates with retronectin. This procedure improves viral infection by attaching the viral particles to the bottom where the cells were seeded. We putted retronectin (15µg/cm^2) on each well of the 24 wells plate and incubated it for 2 hours at room temperature. We remove the retronectin and putted 500µl of PBS with 2% BSA (Bovine serum albumin, from Sigma-Aldrich) on each well and incubated for 30 minutes at room temperature. BSA is used as a blocking agent to make sure the virus only attaches on a coated surface. We removed the BSA solution and washed one time with PBS.

We thawed aliquots of the virus in Dulbecco's Modified Eagle Medium DMEM (500µl for each well with retronectin) and added 1µl of polybren. The polybren interacts with the viral protein increasing the charge at its surface. As the cell membrane is negatively charged the transport through the membrane is facilitated in molecules positively charged. We centrifuged the plate for 1 hour at 2000g and 32^{o}C. This step was required for the attachment of the viral particles on the retronectin coat. The viral supernatant was removed and washed with 1ml of PBS taking care that the virus bound to the retronectin did not dried out.

On the first experiment we collected the CD34+ cells from each condition and put about 108000 cells with the high mitochondrial potential condition (high population) in a well of a 24 well plate and 160000 of the low mitochondrial potential (low population) on a similar well, both with a retronectin coating. We infected the cells a second and a third time with 12 hours between each infection (Giorgetti, 2010). On the last two infections we carefully removed the 500µl supernatant to avoid cell removal and replaced it with 500µl of thawed virus solution and the 1µl polybren.

Inactivation of MEFs (Mouse Embryonic Fibroblasts)

Culture of Human embryonic stem cells requires a feeder layer that can provide β-FGF to the medium. This factor is involved on wnt and Activin/Nodal pathways, inhibiting differentiation[97] and stimulating pluripotent genes like Oct4[98].

MEFs were cultured and expanded in t75 flasks coated with 0,1% gelatin (0,1g/100ml PBS) in MEF's medium: DMEM (Dulbecco's Modified Eagle´s Medium from

GIBCO/Alfagene), 10% FBS (Fetal Bovine Serum from GIBCO/Alfagene) and 0,5% PenStrep (Penicillin-Streptomycin Mixture from Lonza/VWR) (10000 U). When the flasks became confluent, the cells were inactivated with mitomycin C at 37^{o}C for 2 hours. Mitomycin C is a DNA crosslinker that suppress cell division.

Inactivated MEFs were plated in a gelatin coated 6 well plate (0,1%) on the day of the last infection of the CD34^{+}.

IPS cells culture (normoxia)

The CD34^{+} cells were passed to inactivated MEFs on a pre treated gelatine coated 6 well plate 24 hours after the last infection with 2ml of HES medium: Knockout Dulbecco's Modified Eagle Medium (78,3%, from invitrogene); Knockout serum (20%), Non essential aminoacid solution (1%); 2-mercaptoethanol (0,2%); L-glutamine (0,5%); bFGF (0,05%) and 0,5% PenStrep (Penicillin-Streptomycin Mixture from Lonza/VWR) (10000 U). After 48 hours the HES medium was replaced daily with careful to not resuspend the cells. On the first experiment we seeded about 108000 cells of the high TMRM uptake and about 160000 of the low TMRM uptake on a 6 well plate with MEFs. About 10000 cells were seeded per well on a 6 well plate on the following experiments. The morphology of the colonies was observed at the optical microscope after the daily medium change.

On the first experiment after 7 days in culture with MEFs the wells were confluent and were passed from 1 to 3 wells with MEFs pre inactivated with gelatine coating. On the following experiments that involved conditions in normoxia the cells were not passed.

IPS cells culture (hypoxia)

The Low and high cells were seeded on a 6 well plate (10000 cells per well) with matrigel (from BD Biosciences) 24 hours after last infection and supplemented with conditioned medium in a 0,5% O2 and 5% CO2 environment. Conditioned medium is HES medium used in culture of MEFs for 24 hours. This medium contains important

factors that help avoid hESC to differentiate such as FGF2 and BMP inhibitor GREMLIN 1.[99, 100]The medium was changed on day 8 and 14 after the last infection.

On the last experiment to access the effect of an mitochondria respiratory chain inhibitor, we added 2nM of antimycin A (Sigma-Aldrich, ref: 8781) to the conditioned medium of both samples high and low.

Monitorization of the reprogrammed colonies

Both conditions were analysed using photographies taken with a fluorescent microscope during 12 days after the last infection. We evaluate the efficiency of reprogramming in both populations by comparing the number of non fluorescent colonies with the number of infected cells 48 hours after the last infection. Furthermore the average size and the number of fluorescent colonies were also estimated using ImageJ software.

RT - PCR

To access the mRNA of the colonies was done a reverse transcription reaction to obtain cDNA. We extracted mRNA from the cells using trizol. The isolation of the mRNA was done with an extraction kit (QiAGEN RNAesy®).

After purification, the mRNA was used to produce the cDNA, using Taq – TaqMan kit from invitrogen. the enzyme reverse transcriptase, water, random oligonucleotides, $MgCl_2$, RNA inhibitor, dNTPs and the RNA template in a termocycler for 45 minutes: 10 minutes 25^{o}C; 30min 48^{o}C and 5min 95^{o}C.

To access the gene expression was performed a real time polimerase chain reaction (RT-PCR) in Fast Real-Time PCR System from Applied Biosystems. The conditions of the real time PCR cycle are presented in table 7.

We compared the expression of Sox 2, Oct 4 and Nanog in both populations using as housekeeping gene, the GAPDH gene expression. The primers used are listed below:

	Volume used (µl)
Primer forward	2,5
Primer Reverse	2,5
Mix-Power SYBR Green Master (life technologies from applied biosystems)	12,5
H_2O	5
cDNA	2,5

Table6 – Volumes of each component added to perform the RT-PCR

Cycle	**Cycle conditions (time and temperature)**
Denaturation of the DNA chain step (1 time)	94–98 °C for 5minutes
Annealing step (40 times)	94 °C for 30 seconds, 60°C for 30 seconds and 72°C for 33 seconds
Elongation step (1 time)	72 °C or 7 minutes
Final elongation (1 time)	95 °C for 15 seconds, 60°C for 1 minute and 95°C for 15 seconds

Table 7 – Cicle conditions during the RT-PCR

Oligo name	**sequence (5'-3')**
NANOG-For (Forward Nanog)	TCTCCAACATCCTGAACCT
NANOG-Rev (Reverse Nanog)	GCGTCACACCATTGCTAT
SOX2-For (Forward Sox2)	GATGGTTGTCTATTAACTTGTTCA
SOX2-Rev (Reverse Sox2)	TCTCTCCCTTTCTTTCTCTCT
OCT-4-F-Con (Forward Oct4)	GTGGAGGAAGCTGACAACAA
OCT-4-R-Con (Reverse Oct4)	CTCCAGGTTGCCTCTCACTC
GAPDH-F (Forward GAPDH)	AGCCACATCGCTCAGACACC
GAPDH-R (Reverse GAPDH)	GTACTCAGCGCCAGCATCG
FH2_PFKM (Forward Human 2 PKFM)	TTTATTCCTGAATGTCCACC
RH2_PKFM (Reverse Human 2 PKFM)	CAGATTCTTGATGTCTTCTGAG
FH1_LDHA (Forward Human 1 LDHA)	CACCATGATTAAGGGTCTTTAC
RH1_LDHA (Reverse Human 1 LDHA)	AGGTCTGAGATTCCATTCTG
FH2_HK2 (Forward Human 2 HK2)	GAAAGCAACTGTTTGAGAAG
RH2_HK2 (Reverse Human 2 HK2)	CAATGTCTGAGATGTCTTTGG
FSLC2A1 (Forward Glut1)	GAGACACTTGCCTTCTTC
RSLC2A1 (Reverse Glut1)	GCTTTGTAGTTCATAGTTCG

Table 8 – Primers RT PCR for low and high population (obtained from SIGMA- ALDRICH).

We evaluated the mRNA content of Oct4, Sox2, LDHA, HK2, PKFM, Glut1 and NANOG on high and low populations cultured in normoxia on the first experiment.

We also checked if the metabolic footprint of CD34+ cells could be correlated with the TMRM uptake. This was done by evaluating the mRNA of genes on which high expression is associated with increased glycolysis rate (HK2, LDHA and PKFM). HK2 (hexokinase II) is a gene coding an enzyme that is responsible for the glucose uptake by the cell. PKFM codes for a crutial step on glycolysis where fructose-6-phosphate is converted to fructose-1-6-bisphosphate. LDHA is an important gene that codes an enzyme convert piruvate in lactate regenerating NADH and preventing it from entering on krebs cycle.

Western Blot

We evaluated gene expression on high and low cells of the first experiment cultured on normoxia conditions at the protein level to confirm the increased transduction of the pluripotent genes in the reprogrammed cells.

After the extraction we quantified the levels of protein in each sample using Protein Standard, Micro Standard (BSA quantification Kit) from Sigma-Aldrich, using BSA as protein standard for calibration purposes. This step was important to make sure that all the samples had the same amount of protein before running the gel. The gel used to run our samples was NuPAGE 4-12% Bis Tris Gel 1.0 mmx10 well (ref: NP0321PK2) from novex by life technologies. We accessed the levels of Oct4 and Sox2, using β actin as a reference protein. The antibodies and incubation conditions were the following:

- Oct4 (primary antibody - mouse) – Oct3/4 (C-10): catalogue sc-5279 from Santa Cruz Biotecnology, inc. (1:500, incubated for 16 hours)
- Sox2 (primary antibody – rabbit) – catalogue AB5603 from Millipore (1:1000, incubated for 16 hours)
- The secondary antibodies belonged to the kit ECF Western Blotting Reagent Pack (mouse and rabbit) 2nd Antibody - GE HealhCare. (1:5000, incubated for 1 hour).

Results and Discussion

Since the beginning of this work we were interested in exploring new approaches that could help to improve the results of reprogramming studies. We focused our strategy in developing an alternative method to efficiently select the cells within the cord-blood population that were more suitable for this purpose.

Our line of thought was based on the idea that the metabolic phenotype could be directly correlated with cell maturity. Using the mitochondrial probe TMRM (reporter of mitochondrial membrane potential) we segregated two different populations within the cord blood $CD34^{+}$ population. By delivering the Yamanaka genes (OSKM) in a lentiviral vector we successfully achieved reprogramming with distinct efficiencies. We confirmed that the sorted populations were indeed different with respect to their stemness after phenotypic characterization of the 2 populations with cell surface antibodies. Additionally we tested reprogramming in hypoxic conditions and the use of a respiratory chain inhibitor in an attempt to further increase efficiency. It was interesting to find that both hypoxia and the treatment with the mitochondrial inhibitor not only increased the reprogramming efficiency of the less immature population but also equalized efficiencies and gene pattern expression between the high and low TMRM populations.

Cell sorting

The cells with normal size, internal complexity and higher expression of the CD34 marker were sorted according with the uptake of TMRM (Tetramethylrhodamine, methyl ester; invitrogen). TMRM is a cationic probe that is sequestered by active mitochondria. If the mitochondria is polarized, which happens in oxidative phosphorylation by the existence of a proton gradient, the matrix has a negative charge. As the probe has a positive charge it will be in higher concentrations inside the active mitochondria. (Figure 16)

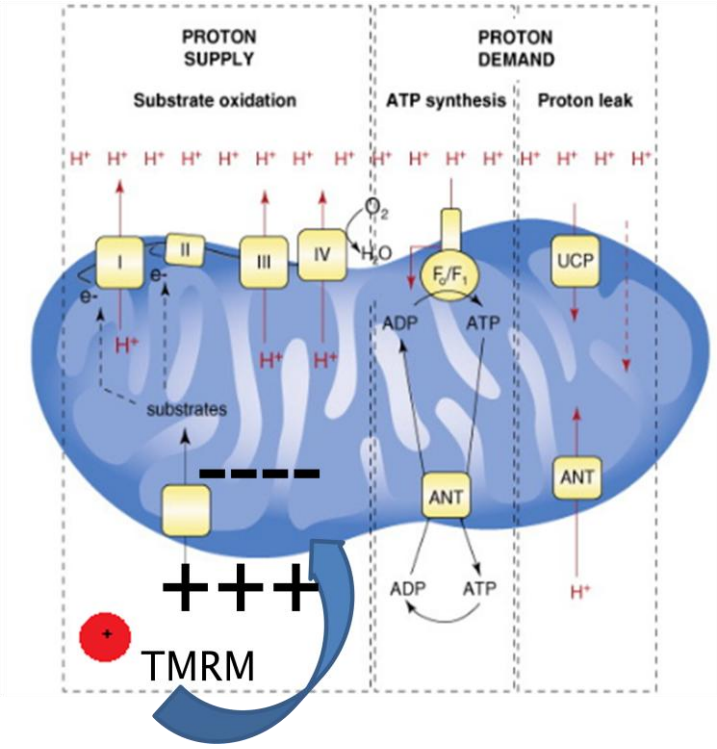


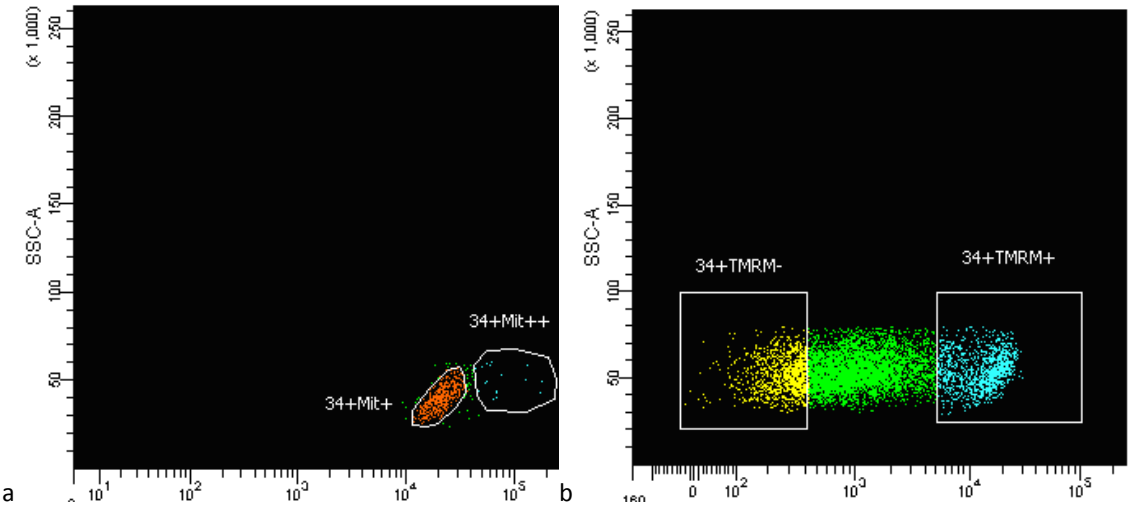
Figure 16 – Uptake of the TMRM probe according to the polarization of the mitochondrial membrane caused by the proton gradient. (Adapted from Vian Azzu et al 2010)

TMRM titration

Magnetic-bead isolation of the CD34+ cord blood cells was done using the Miltenyi technology. These cells were then sorted based on the uptake of the mitochondrial probe TMRM (Tetramethylrhodamine, methyl ester; invitrogen). This way we expected to compare the efficiency of reprogramming in 2 populations with different mitochondrial phenotypes.

In a first trial we used 25nM concentration of TMRM to label the $CD34^{+}$ cells. This caused an overload of TMRM in the cells and this unspecific uptake could not be correlated with mitochondrial membrane potential. The fact that all cells were showing a high signal was indicative that the TMRM probe had a narrow dynamic range at this concentration (figure 17a).

For determination of the optimal concentration to use in CD34+ cells we did a titration with mononuclear cells using several concentrations of TMRM (1nM; 2,5nM; 5nM; 10nM; 20nM). The concentration that showed the best compromise between positive signal and wide dynamic range was 2,5nM and this was used for the rest of the experiments (figure 16b).

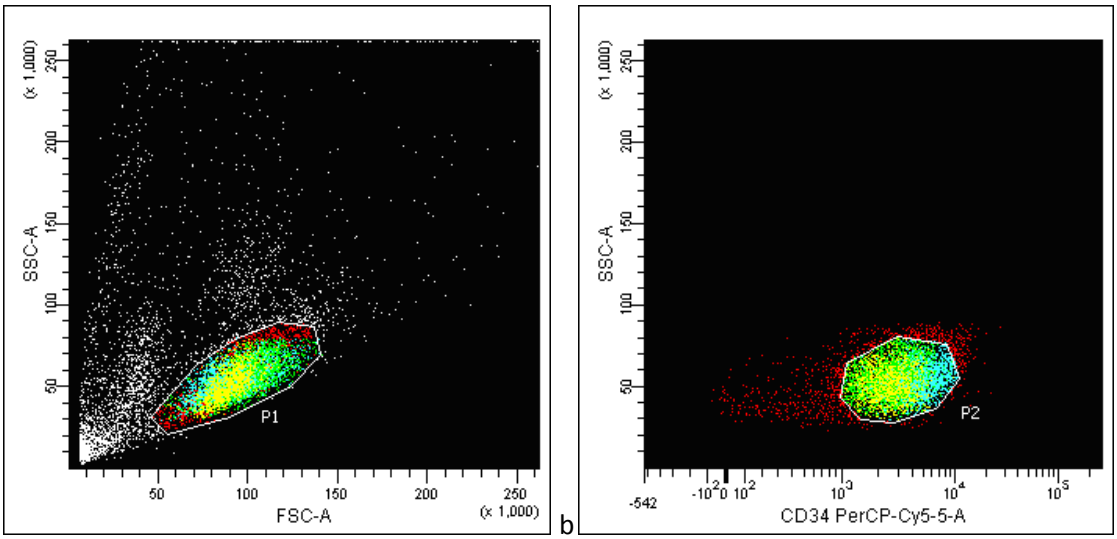


a b

Figure 17a and b – a) scatter plot of TMRM labelled CD34+ cells from cord blood before titration. Since the concentration was too high this made most of the cells to have a similar high uptake of the probe, making impossible to distinguish between the two phenotypes. b) after performing a titration and using a 10 times lower concentration it was possible to identify 2 populations with different uptakes and thus different mitochondrial phenotypes

Sorting the CD34^{+} cells

After CD34+ isolation using MACS protocol it was possible to collect 2 million cells from one blood bag. After sorting, cells with low TMRM uptake (low population) and cells with high TMRM uptake (high population) were obtained; the narrow gates used can explain this reduced number of cells. Our aim was to compare only the extremes of both phenotypes.



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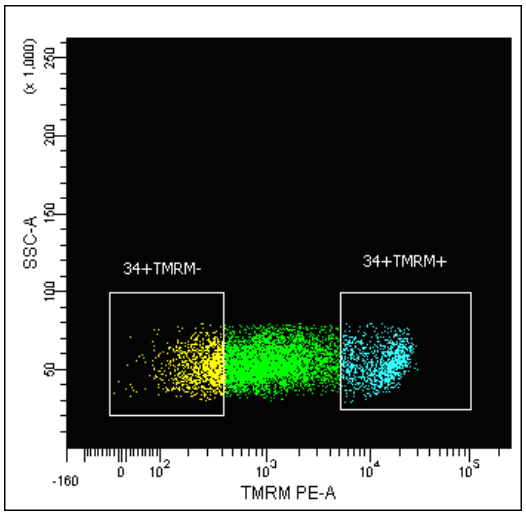

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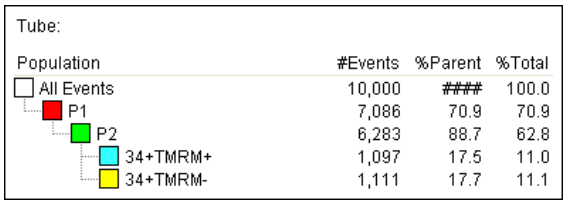


d

Tube:

Population	#Events	%Parent	%Total
All Events	10,000	####	100.0
P1	7,086	70.9	70.9
P2	6,283	88.7	62.8
34+TMRM+	1,097	17.5	11.0
34+TMRM-	1,111	17.7	11.1

Figure 17a, b, c and d- Scatter plots from flow cytometry on CD34+ cells; a) selection of a population based on the volume and internal complexity discharging contamination from beads, differentiated cells and cell debris; b) we further select the cells that had a higher expression of CD34 marker; c) The cells that had higher expression of CD34 marker were sorted based on the mitochondria phenotype: the cells that had lower uptake of the cationic probe TMRM and the cells with higher uptake. This uptake is correlated with mitochondria polarization; d) percentage of cells in each phenotype selected.

Reprogramming efficiency: TMRM Low *versus* TMRM High populations

The cells with lower uptake of the TMRM probe upon sorting were easier to be reprogrammed; this was patent by the appearance of more non-fluorescent colonies along the time in this population, when compared with the high TMRM population. To evaluate the efficiency of reprogramming, we counted the colonies that did not express the fluorescent reporter (d'Tomato protein) and compared them with number of cells infected in the beginning of the process. The viral vector used a retroviral promoter that is not expressed in human hematopoietic stem cells. During the epigenetic remodelling that occurs upon reprogramming the promoter is recognised as strange by the cellular machinery and silenced.[93] This event assures that the transcription factors expressed in non-fluorescent colonies have an endogenous origin, being activated by the auto regulatory loop of the main pluripotent genes.

The lower uptake of TMRM seems to be correlated with a more primordial phenotype of the mitochondria, with slightly polarized membranes. These cells rely mostly on glycolysis to generate ATP and this explains their low membrane potential; the opposite phenotype is characteristic of more differentiated cells where the mitochondrial membrane is more polarized due to the proton gradient of oxidative phosphorylation.[69] Indeed, human embryonic stem cells are also glycolytic and also

show this mitochondrial phenotype. Since reprogramming seems to be affected by stochastic events, choosing a population within cord-blood that more closely resembles the embryonic stem cell characteristics seems to be an important step to increase final efficiency.

These results seem however contradictory with the report of Folmes et al (2011), where the increase in mitochondrial membrane potential correlated with a higher efficiency of reprogramming. We believe that the metabolic profile of the cells before induction of pluripotency can explain these discrepancies. The rise in TMRM uptake in mouse embryonic fibroblasts (MEFs) described by these researchers is probably related with the metabolic shift that these cells must undergo during reprogramming. MEFs rely on the mitochondrial electron transport chain to produce ATP while the ESC and iPSC use a glycolytic metabolism. In order to undergo this metabolic shift MEFs require the shutdown of the mitochondrial electron transport chain machinery. This will let them exposed to a high concentration of ROS produced by electron leakage from the electron transport chain. These ROS should be the ones responsible for the increase of membrane potential reported.

On the other hand, hematopoietic stem cells are mostly glycolytic so they do not require a metabolic shift to reprogram. Thus it is not expected, in this case, the correlation between higher polarization of mitochondria and reprogramming efficiency.

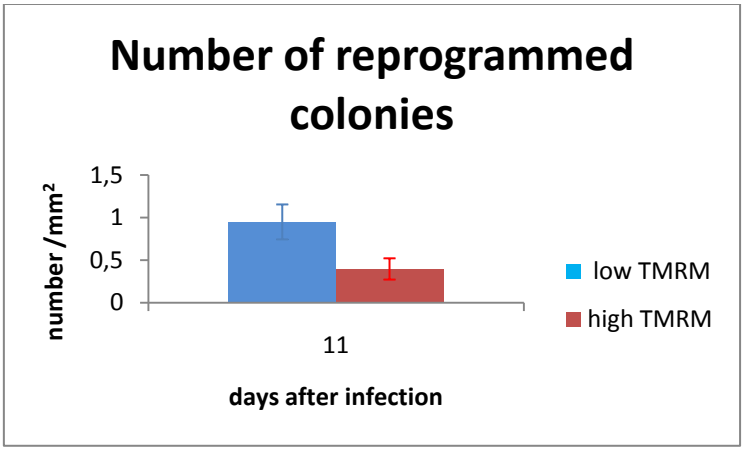


Figure 18 – Number of reprogrammed colonies per mm^2 on high and low populations. The number of reprogrammed colonies was approximately the double in the low TMRM population.

The first non-fluorescent colonies (reprogrammed) were documented in the low TMRM population. As there was no fluorescence of the Tomato protein we

concluded that the viral promoter was already silenced and the embryonic features were maintained by the expression of the endogenous factors. The bigger number of colonies that underwent epigenetic remodelling was achieved in the low TMRM population (figure 18).

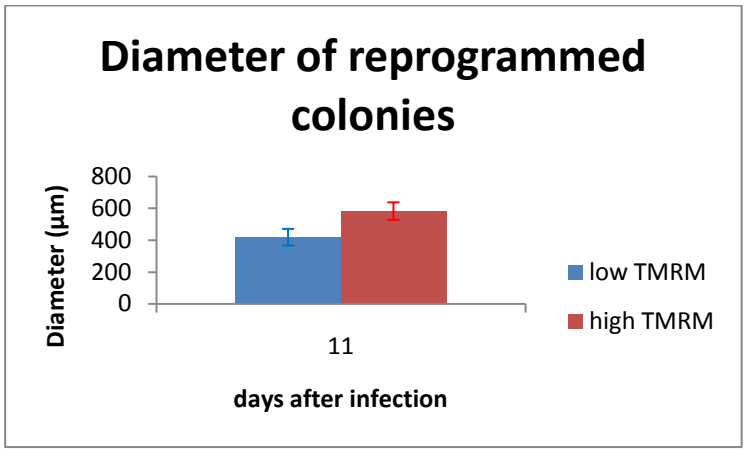


Figure 19– Diameter of the reprogrammed colonies 11 days after infection. The difference was not statistically relevant.

The diameter of the colonies was practically the same in both conditions indicating similar proliferative rates (figure 19).

Morphology of the colonies

Human ESCs (Embryonic Stem Cells) colonies are characterized by high nucleus/cytoplasm ratio, round shape, compact appearance, and with well defined borders as shown in figure 20.[2, 101] The colonies generated from the low TMRM population (figure 21) had closer morphology to ESCs than the cells from the high population (figure 22).

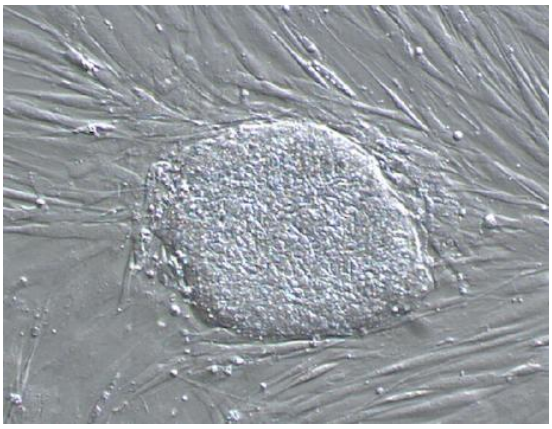


Figure 20 - Human embryonic stem cell colony. (adapted from http://www.stemcellresearch.umich.edu/news/photos.html).

On the other hand the colonies from the high population showed sign of disintegration and the exogenous genes were not fully silenced as shown by the presence of cells expressing the red fluorescence reporter. Both conditions showed however some degree of differentiation at the borders.

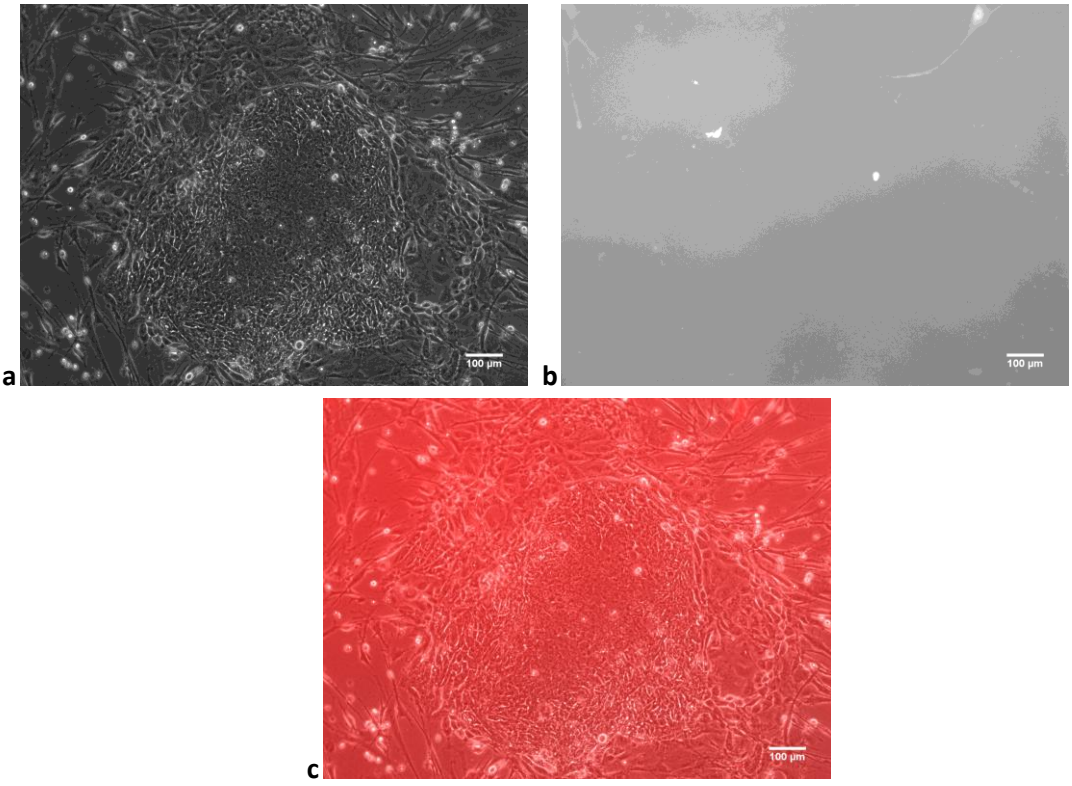


Figure 21 a) b) Non fluorescent colonies 12 days after the third infection. a) Cells of the low TMRM population showing some differentiation at the borders of the colony but compact and round shape at the centre. b) Fluorescence emission channel photo showing no signs of d'Tomato expression. c) Merge of the photo a and b.

Nevertheless, it is important to report that in this first experiment we encountered a major obstacle in our analysis method. We started with a cell density per well that was higher than optimal (~100000 cells per well of 6-well plate) and

because of that during proliferation of the colonies we noticed that they started to touch neighbour colonies. Faced with the dilemma of the passage of the colonies, we decided to pass them to avoid differentiation. As our purpose was to compare the number of colonies generated in both conditions, the passage of the cells may have led to disintegration of some colonies compromising final analysis. (figure 22).

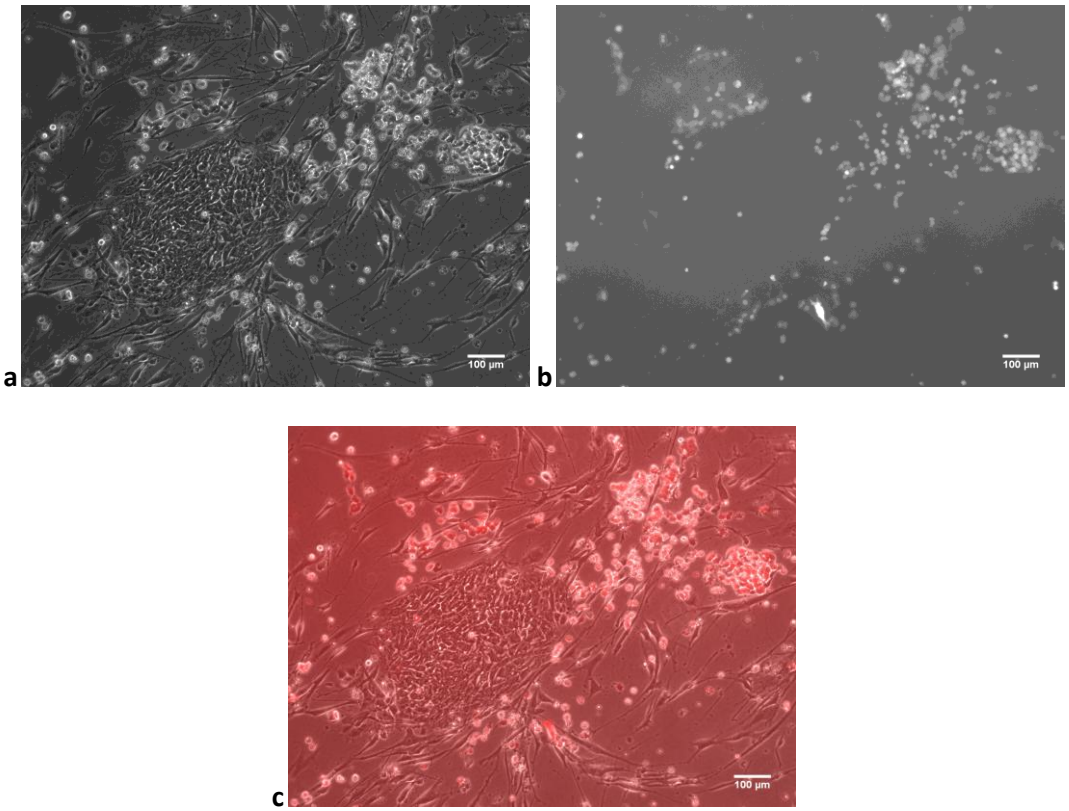


Figure 22 a) and b) Cells of the high TMRM population 12 days after the third infection. a) the cells are less compact, more heterogeneous and show signs of disintegration. B) the fluorescent channel show signs of expression of the exogenous genes on some cells pointing to failed reprogramming . c) Merge of the photo a and b.

Efficiency – Low TMRM population was more efficient

Our evaluation of the efficiency was made by comparing the number of reprogrammed colonies (not expressing our reporter) with the initial number of infected cells on the day following the infection using the fluorescent microscope (58000 on the low TMRM uptake and 56000 on the high TMRM uptake condition). This way we estimated from our photos how many cells that had the exogenous genes initially integrated on their genome and were able to give origin to colonies with ESC like morphology and with the exogenous genes silenced.

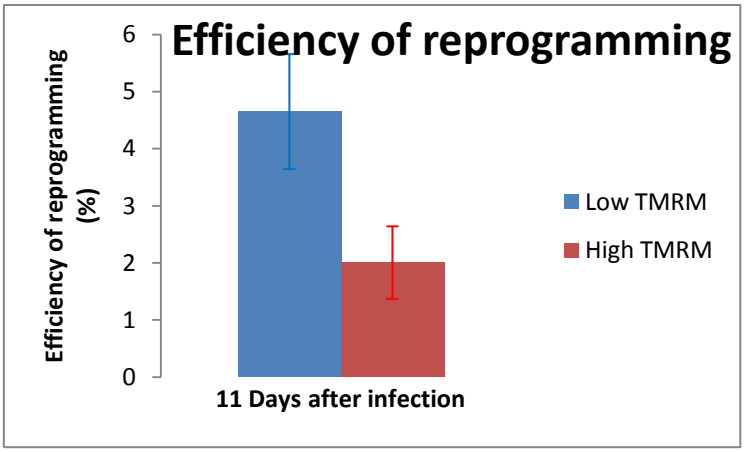


Figure 23 – Efficiency of reprogramming on both populations 11 days after the last infection. The error in the graphic is the standard error.

The efficiency of the low TMRM population was practically the double of the high population on the last day of reprogramming. (figure 23)

We were able to achieve a better efficiency than the ones reported in the papers where we based our strategy in[93, 96]. An obvious explanation for this discrepancy is the type of cells used for reprogramming. As $CD34^+$ cells are more naive than MEFs they require less epigenetic remodelling to achieve pluripotency. Other important reason is related with the use of a polycistronic virus that contained all 4 Yamanaka's factors instead of only 2 (Oct4 and Sox2) as in (Giorgetti, A. Et al, 2010), reinforcing the importance of c-Myc and klf4 to improve the efficiency of the process.

Another point has to be taken in account as the initial number of infected cells was counted only 2 days after infection instead of the following day. This could have interfered in the calculi of the final efficiency because of cell proliferation in culture. Finally it is important to note that we could be introducing a considerable error by using our method to count colonies. As we only took 10 photos using fluorescent microscope for each well. After this we calculated the average number of colonies per photo and with this number we estimated the number of colonies per mm^2.

Phenotypic characterization of the low and high TMRM populations

Since the best results were obtained in the low TMRM cells we decided to further characterize this population. Although we had assumed that there should be a

relationship between cells with lower uptake of TMRM and a primordial mitochondria phenotype we needed to confirm the immaturity of these cells. We cannot exclude the possibility that the signal obtained by the TMRM probe can be affected by mitochondrial mass, as cells with more mitochondria and less mitochondrial membrane potential can display similar levels of TMRM than cells with less mitochondrial mass but higher mitochondrial membrane potential. On the other hand, the immature cells of the $CD34^+$ population could be more effective at "pumping out" the TMRM probe affecting the correlation between mitochondrial phenotype and TMRM uptake. Indeed, the staining of $CD34^+$ cells with rhodamine mitochondrial probes (Rh-123) was found to be more effective using P-glycoprotein (P-gp) inhibitors. P-gp is an efflux pump that is highly expressed in hematopoietic progenitors and Rh-123 along with other dyes is substrate for transport mediated by P-gp through the membrane. Higher expression was associated also with cells displaying characteristics of pluripotent stem cells.[102]

We immunolabelled cells with CD133, CD45 and CD15 antibodies to see if TMRM signal was somehow correlated with different phenotypic sub-populations of CD34+-cells. The CD133 is a primitive hematopoietic marker and is expected to have higher expression in the most immature population.

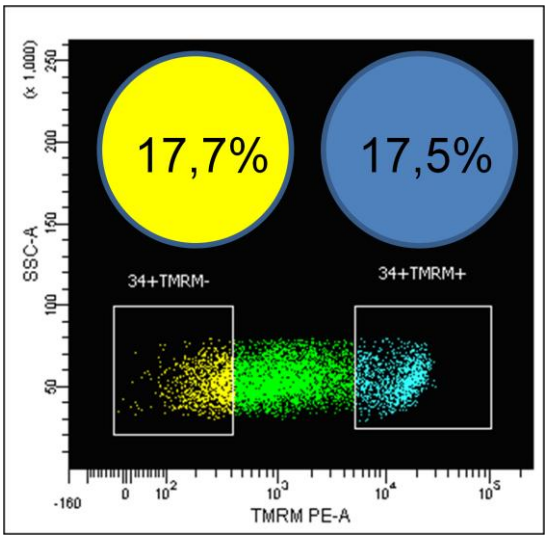


Figure 24 – Scatter plot from flow cytometry of the CD34+ cells. The cells were marked with TMRM probe and two different populations were selected according with their uptake. We isolated 17,7% of the cells of the $CD34^+$ pool with low TMRM uptake and 17,5% with high uptake. The yellow cells have low uptake (low population) and the blue have high uptake (high population).

The CD133^{-} CD34^{+} cells consist mainly on B-cell progenitors and erythroid progenitors [103]. On the other hand the CD15 and CD45 markers are related with differentiation commitment. While the CD45 marker is associated with myeloid commitment (granulocytes, basophiles, eosinophils)[104] the CD15 is highly expressed in the neutrophile population[105].

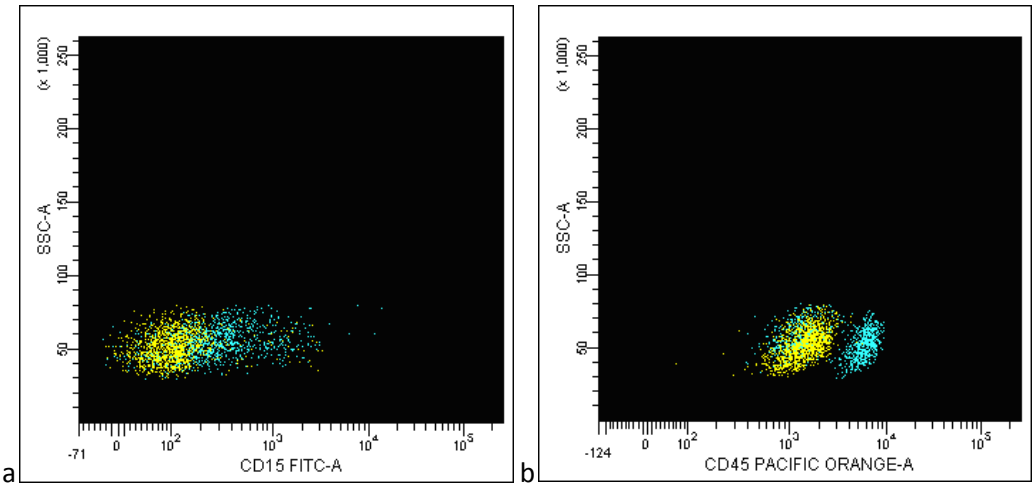


Figure 25 – scatter plot from flow cytometry. a - Expression of the CD15 marker on the CD34+ population. The low yellow cells have low TMRM uptake and the blue cells have high TMRM uptake. The higher expression of this marker in the high TMRM population is a sign of neutrophile commitment. b- Expression of the CD45 myeloid marker. Although both populations show a great expression of this marker, it is again possible to confirm a higher differentiation commitment within the high TMRM population.

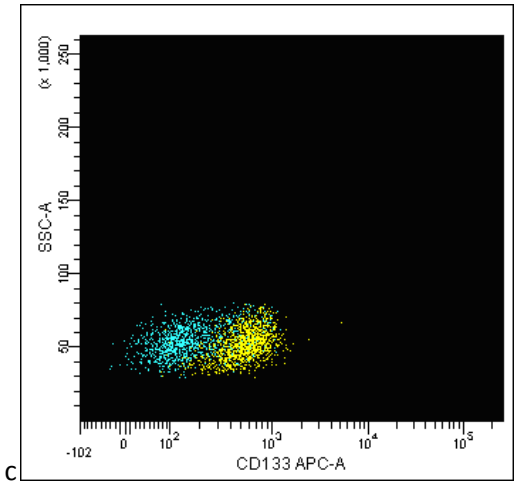


Figure 25c – Expression of the primitive marker CD133. In accordance with the previous results the primitive marker CD133 is more expressed in the low population.

The results obtained by flow cytometry analysis were clearly showing the existence of a different degree of stemness between high and low TMRM populations (figures 24 and 25). It was also possible to confirm that the low population has a more

primitive phenotype (higher CD133), which is in accordance with the results obtained in terms of reprogramming efficiency.

Gene expression characterization of the reprogrammed colonies

RT-PCR confirmed the higher expression of the pluripotent genes Nanog, Oct4 and Sox2 in the reprogrammed colonies of the low TMRM population.

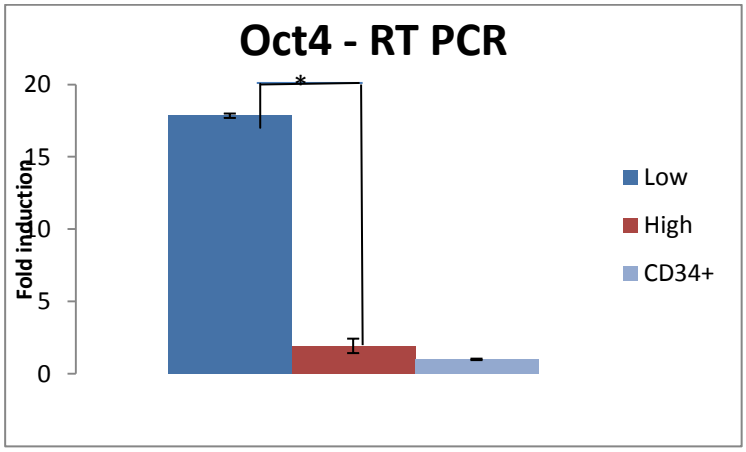


Figure 26 – Graphic showing the fold induction of Oct4 gene of 11 days reprogrammed colonies of high and low samples. Low cells have 18 times more Oct4 mRNA than CD34+ cells and 9 times the level on the high population. Fold induction was normalized with $CD34^{+}$ population. *P<0,05 (P= 0,001106)

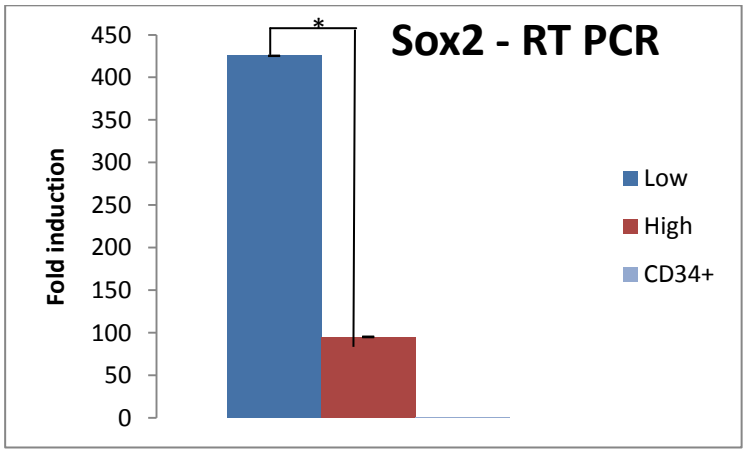


Figure 27 – Graphic showing the fold induction of Sox2 gene of 11 days reprogrammed colonies of high and low TMRM samples. The difference off Sox2 fold induction was the greatest of all the pluripotent genes reaching in the low population 425 times the levels expressed in CD34+. Fold induction was normalized with CD34+ population. *P<0,05 (P= 0,009662)

Figures 26, 27 and 28 show the fold induction of Oct2 SOX2 and Nanog on the reprogrammed colonies of high and low populations. This value is correlated with the

amount of mRNA of each pluripotent gene and allowing to confirm if the reprogramming process successfully increased gene expression transcription.

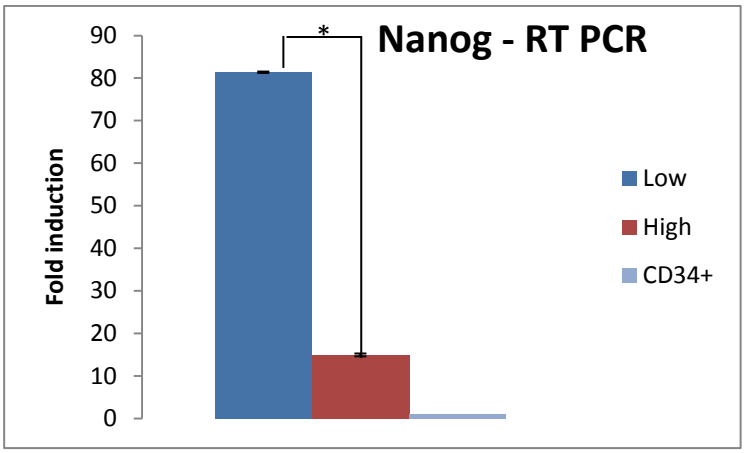


Figure 28– Graphic showing the fold induction of Nanog gene of 11 days reprogrammed colonies of high and low samples. In accordance with the previous graphics, the best results are shown in the low population. Fold induction was normalized with $CD34^{+}$ population. *P<0,05 (P=6,95047E-06)

The graphics on figure 26, 27 and 28 show greater increase in expression of the main pluripotent genes at mRNA transcript level in the low TMRM population when compared to the high TMRM population.

It is important to note the big increase in terms of Nanog expression in the low TMRM population, especially because it was not one of the genes delivered by the lentiviral vector used in these experiments. This result clearly shows that the exogenous genes were able to activate the auto regulatory loop involving the 3 main pluripotent genes (Oct4, Sox2 and Nanog).

Protein expression analysis by western blot confirmed the RT-PCR results showing higher Sox2 and Oct4 expression in the low TMRM population than in the high TMRM population.

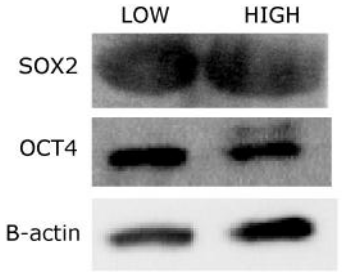


Figure 31 – Western blot images comparing Sox2 and Oct4 protein expression in reprogrammed colonies at 11 days. ECF reagent and a secondary antibody containing alkaline phosphatase were used in this western blot.

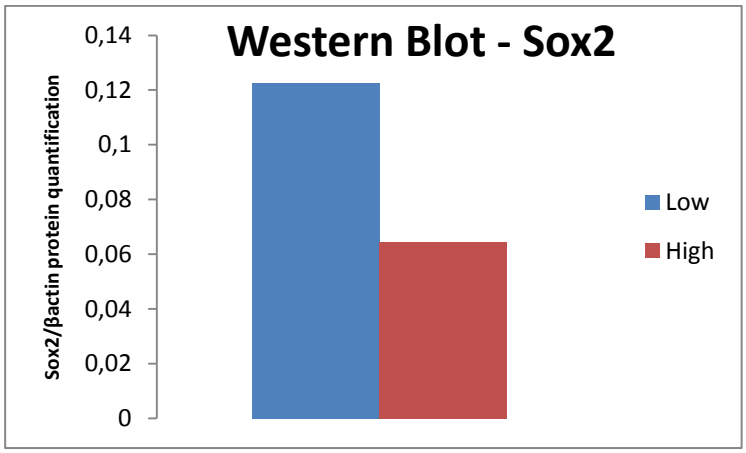


Figure 29 – Western blot results on Sox2 protein on reprogrammed colonies high and low with 11 days . These values are relative to the quantity of βactin protein that serves as housekeeping protein. It is clear the increase of Sox2 on the low population.

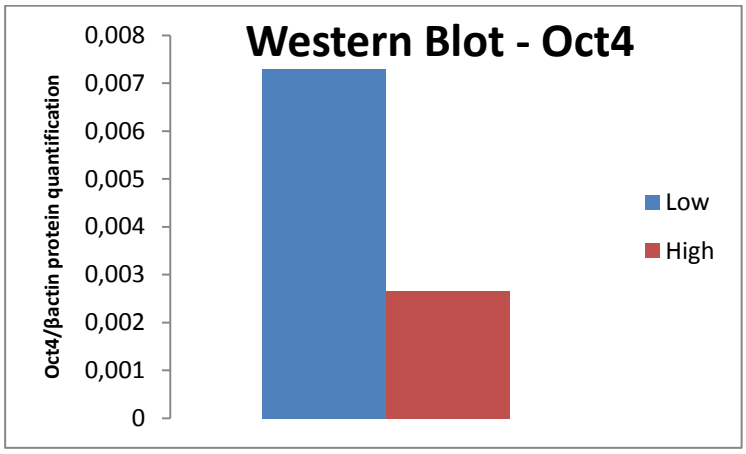


Figure 30 – Western blot analysis of Oct4 protein on reprogrammed colonies with 11 days for high and low populations. These values are relative to the quantity of βactin protein that serves as housekeeping protein. Oct4 protein levels are also increased in the low population

These evidences further support our previous results that claim that the population with low TMRM uptake is preferential for reprogramming.

RT PCR of high and low TMRM population before reprogramming

To complement our characterization of the high and low TMRM populations in terms of their stemness and metabolic profile we decided to evaluate the expression of glycolytic related genes such as Glut1, PFKM, HK2 and LDHA with RT PCR. Glut1, codes for a glucose transporter that allows glucose to cross the cell membrane through facilitated transport. The PFKM, is a phosphofructokinase isoenzyme that catalyses the phosphorylation of fructose-6-phosphate to fructose-1,6-biphosphate during

glycolysis. Hexokinase 2 (HK 2), is another important glycolytic enzyme that phosphorylates glucose to glucose-6 phosphate being the first step in glycolysis. The LDHA protein is an enzyme used by high glycolytic cells to convert pyruvate and NAD+ on NADH and lactate in the final step of anaerobic glycolysis.

The first important result of this experiment was the amount of mRNA extracted from each condition: 3,959 ng/µl in the low TMRM cells and 11,975 ng/µl in the high TMRM cells. This greater mRNA content in the high TMRM cells is an indicator of higher RNA polymerase II activity, which is characteristic of a more proliferative state.

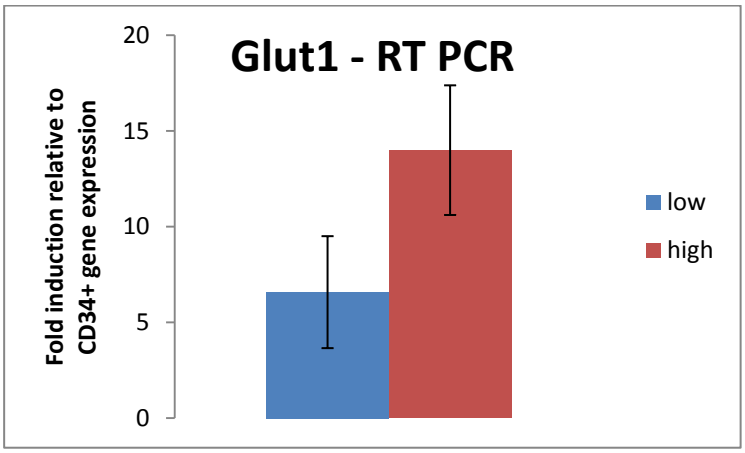


Figure 29 – Graphic showing fold induction of the glucose transporter Glut1. The high cells have higher expression of this gene than the low population. The error bars are standard error.

The results on figure 29, 30 and 32 point that the high TMRM population has glycolysis happening at higher levels than the Low TMRM cells. This can be concluded as more glucose should be transported inside the cell (figure 29), more piruvate should be converted on lactate at the end of glycolysis (figure 30) and higher levels of fructose-6-phosphate should be phosphorylated (figure 31).

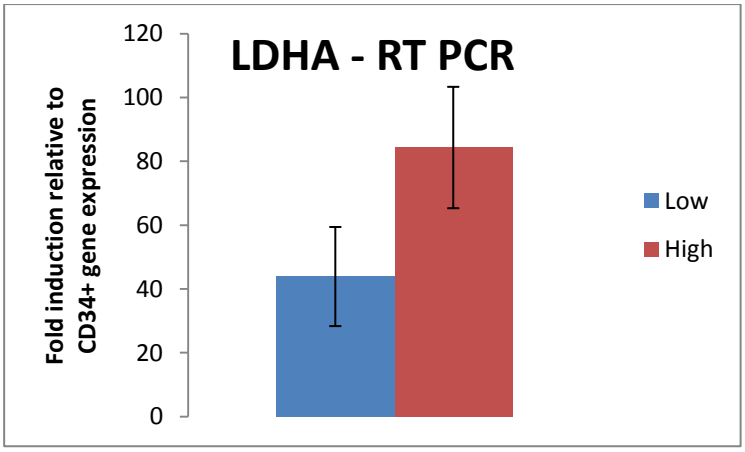


Figure 30 – Graphic showing fold induction of the anaerobic glycolysis related enzyme. There is also higher expression of this gene on the high population. The error bars are standard error.

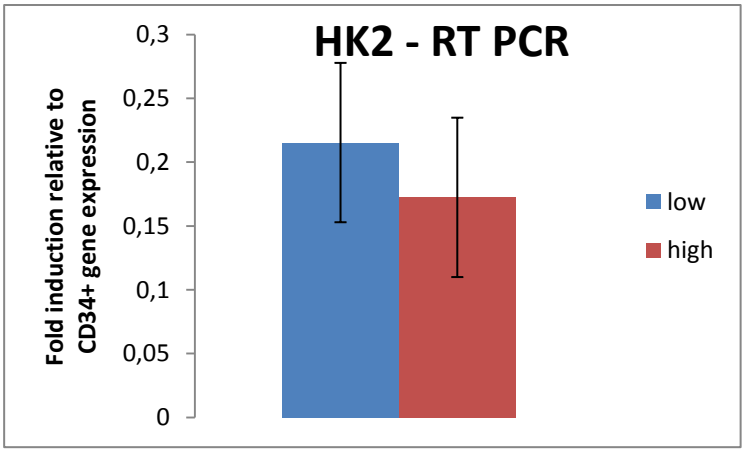


Figure 31 – The mRNA levels of hexokinase 2 are higher in the low population. The error bars are standard error.

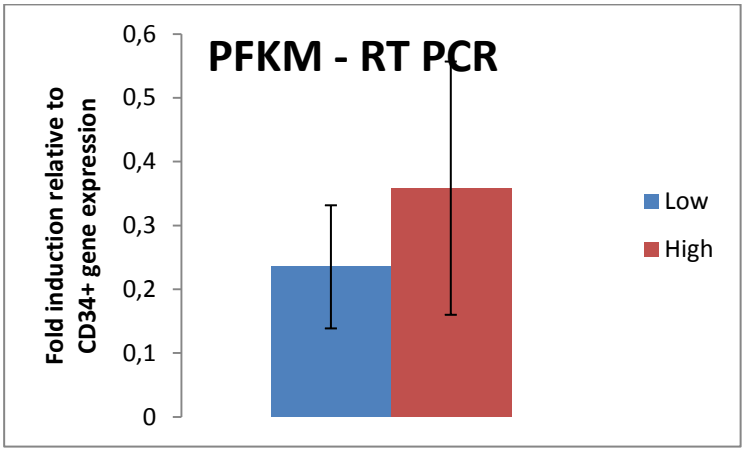


Figure 32 – Results of RT PCR for PFKM showing higher expression on the high population but very similar with the result on the population. The error bars are standard error.

We expected to see considerable differences in the expression of the glycolytic genes that pointed the low population as the one with the most glycolytic profile however interpretation of our data was not so direct. Our results show that the high population has more mRNA levels of glycolytic enzymes like LDHA and PFKM and the glucose transporter Glut1. These results apparently contradictory can make sense if we take into consideration the findings described in Pires das Neves et al (2010)[106] and Romero-Moya et al (2012)[92]. Romero-Moya et al (2012), used a mitochondrial mass specific probe, the Mito tracker green, in human $CD34^+$ cord blood cells to segregate two populations based on the uptake levels. They reported that the mitochondrial mass of the cells is correlated with mitochondrial membrane potential. Additionally, cells that had lower uptake of Mito tracker green were shown to be more primitive cells; while the cells with higher uptake were enriched in hematopoietic progenitors that have higher clonogenic capacity *in vitro*. Another important finding was that the

cells with higher Mito tracker green had higher levels of ATP and the levels in the low Mito tracker cells increased upon differentiation to similar levels of the high population. Moreover, the ATP levels in the high Mito tracker population remained constant during 15 days.

Pires das Neves et al (2010), reported a positive correlation between available ATP levels and the rate of transcription in several mammalian cells. They also stated that cells with more mitochondrial mass had shorter cell cycle, which means higher proliferation rates. By crossing our data with this information, we conclude that our low population is more quiescent and for this reason has a lower metabolic demand. This was also supported by the inferior amount of mRNA content obtained for RT PCR analysis in the low TMRM population. This indicates less activity of RNA polymerase II.

On the other hand as the high population has more hematopoietic progenitors with higher proliferation capacity they require more ATP and have higher glycolysis rate although they rely more on oxidative phosphorylation than the low TMRM population.

An important remark that should be addressed in future experiments is the necessity of studying the expression of genes related with the mitochondrial respiratory chain. This way we could safely conclude that the higher glycolytic gene expression found in the high TMRM population was related with higher metabolic rates. Also one important experiment would be quantifying the levels of ATP in the high and low TMRM populations.

Reprogramming in hypoxia

CD34+ cells are mainly glycolytic and our results suggest that the low TMRM population showed better efficiencies of reprogramming because it was mainly constituted by the most primitive (CD133+CD34+) cells. With this in mind we thought that hypoxia, together with the reprogramming conditions used in the experiments reported above, could be a potent inductor of reprogramming.[107] We were curious to

see if we would decrease or increase the gap between high and low TMRM cells in terms of efficiency of the process under hypoxia.

Hypoxia leads to stimulation of Hypoxia Inducible Factor alpha, which is known to diminish the production of reactive oxygen species and avoid the depletion of important substrates used in the synthesis of biomolecules (such as citrate, an intermediate of Krebs cycle important for production of lipids).[74, 108] These are important evidences that suggest that the reprogramming process may benefit from an hypoxic environment. The unlimited proliferation profile requires a high anabolic demand that can be sustained by discouraging the aerobic regeneration of the NAD^+.[109, 110]Additionally by downregulation of the respiratory chain the cells will be less exposed to the damage of reactive oxygen species that is associated with senescence, DNA mutations and apoptosis[111]. On the other hand, stabilization of the HIF sub units should help to stimulate the glycolytic metabolism that is critical for pluripotency induction.[84, 85]

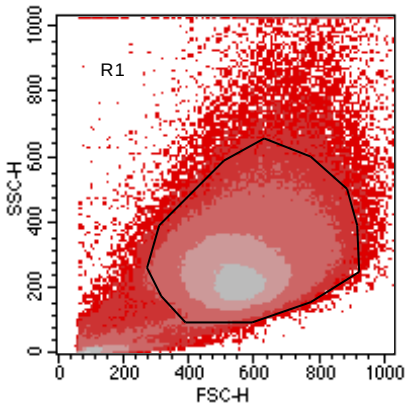
Bone marrow Hematopoietic Stem Cells (HSCs) reside in a oxygen environment that ranges from <0,1%, for the most primitive HSCs, to 7% in perivascular areas, where the most committed precursors can be found[112]. Current literature support that 0,1% oxygen levels support maintenance and return of HSCs to a quiescent state in G0;[113] while 1% and 1,5% maintain cell proliferation, self renewal and preservation of HSCs in culture.[107, 114] On the other hand, typical cultures of HSCs at 20% O_2 lead to the exhaustion of the stem cell potential.[112]

Next we describe the experiments of reprogramming under the influence of the hypoxic environment with only 0,5% oxygen. We anticipated that this concentration would allow a balance between the promotion of self-renewal and the maintenance of the primitive status.

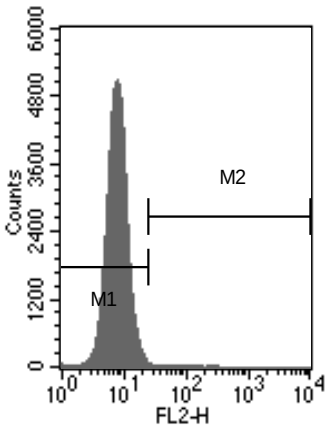
We evaluated how this new parameter can affect the efficiency of reprogramming in high and low TMRM populations.

Analysis of the percentage of cells infected with our viral vector

On the following experiments we calculated the efficiency of the infection using FACS (Fluorescence-activated cell sorting) analysis of the cells one day after the last infection. We used FACSCalibur from Becton Dickinson and Cell Quest software.



a



b

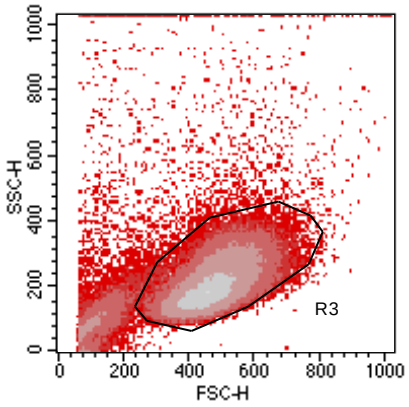
Histogram Statistic

File: cells only.001 Log Data Units: Linear Value
Sample ID: cells only Patient ID:
Tube: Panel:
Acquisition Date: 27-Jul-12 Gate: G1
Gated Events: 452400 Total Events: 500000
X Parameter: FL2-H (Log)

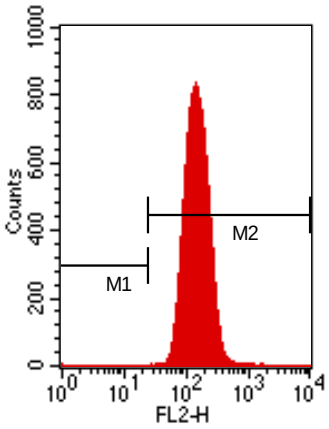
Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 991(	452400	100.00	90.48	8.13	7.57	47.60	7.57	7
M1	1, 2	450675	99.62	90.14	8.01	7.52	37.04	7.50	7
M2	25, 991(	1773	0.39	0.35	39.85	36.44	60.70	32.78	25

c

Figure 33 a,b and c - Evaluation of the fluorescence of CD34+ cells not infected used for negative control. a-Selection of the $CD34^{+}$ viable cells in our sample based on SSC (Side Scatter -cellular complexity) and FSC (Forward Scatter - volume of the cells).b – Histogram where we definded the basal fluorescence of the cells that are not infected (M1) on FL2 channel and the fluorescence of infected cells (M2). c- histogram statistic .99% of the cells selected on the gate R1 were contained on the M1 interval. We considered that the cells that had fluorescence higher than this interval were infected with the vector.



a



b

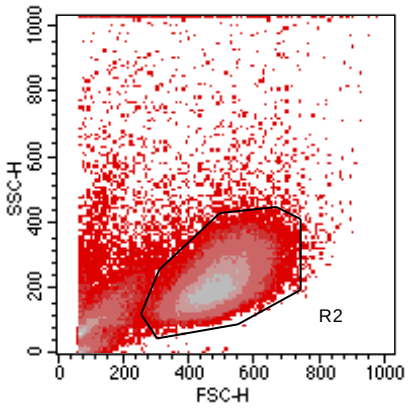
Histogram Statistic

File: Data.041 Log Data Units: Linear Valu
Sample ID: low Patient ID:
Tube: Panel:
Acquisition Date: 17-Dec-12 Gate: G3
Gated Events: 88744 Total Events: 100000
X Parameter: FL2-H (Log)

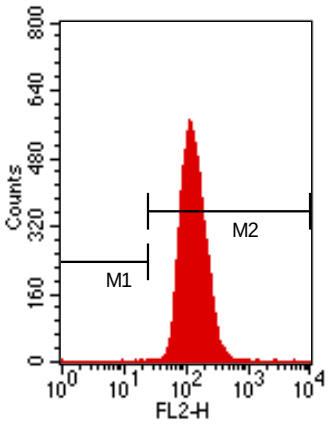
Marker	Left, Right	Events	% Gated	% Tota	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 991(	88744	100.00	88.74	149.28	138.35	41.68	137.00	138
M1	1, 2	1	0.00	0.00	1.00	1.00	***	1.00	1
M2	25, 991(	88743	100.00	88.74	149.28	138.36	41.67	137.00	138

c

Figure 34 a, b and c – Analysis of the percentage of infected cells in the low TMRM uptake population 24 hours after infection. a – selection of the viable cell population. b histogram showing the number of cells that were contained in the interval M2 defined the fluorescence of the infected cells.. c –histogram statistics. We obtained 100% of the cells with fluorescence inside the interval M2.



a



b

Histogram Statistic

File: Data.040 Log Data Units: Linear Valu
Sample ID: high Patient ID:
Tube: Panel:
Acquisition Date: 17-Dec-12 Gate: G2
Gated Events: 58840 Total Events: 74850
X Parameter: FL2-H (Log)

Marker	Left, Right	Events	% Gated	% Tota	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 991(	58840	100.00	78.61	133.63	122.02	58.50	118.64	106
M1	1, 2	12	0.02	0.02	17.52	14.45	38.81	18.60	24
M2	25, 991(	58828	99.98	78.59	133.65	122.07	58.48	118.64	106

c

Figure 35 a, b and c - Analysis of the percentage of infected cells on the high TMRM uptake population 24 hours after infection. a – selection of the viable cell population. b – histogram showing the number of cells that were contained on the interval M2 defined the fluorescence of the infected cells. c – histogram statistic. As we obtained 99,98% of the cells with fluorescence inside the interval M2 we considered 100% of efficiency of infection within this condition

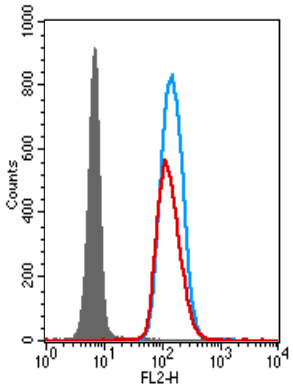
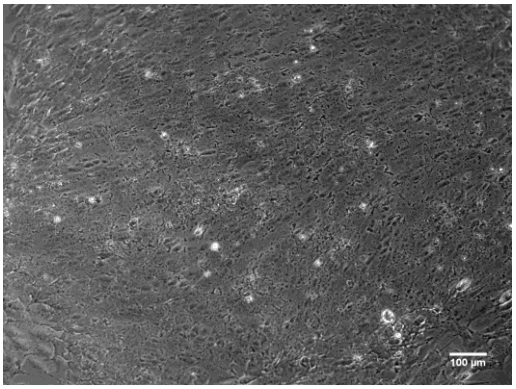


Figure 36 – Overlay of the fluorescence of the three conditions. Cells only in grey. High TMRM uptake cells in red and Low TMRM uptake cells in blue.

Morphology of the reprogrammed colonies on hypoxia

On the 13th day of reprogramming we took pictures of the colonies on both conditions using a fluorescent microscope.

a 

b 

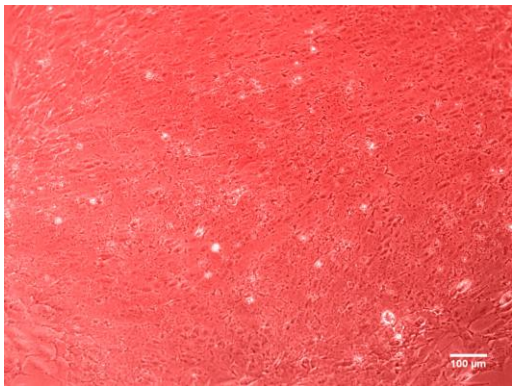
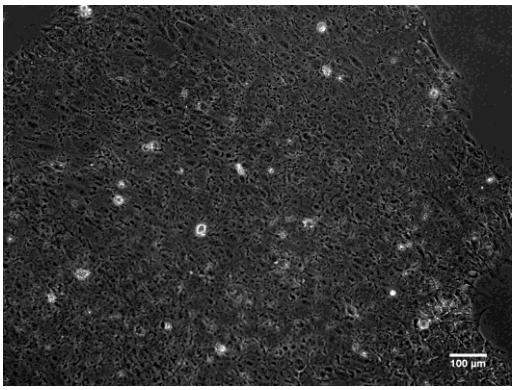
c 

Figure 37 a) and b) Reprogrammed colony on the low TMRM uptake population condition in hypoxia. Although some heterogeneity could be seen on the morphology, the colony is very compact and shows high proliferation B)There is no fluorescence of the colony indicating successful reprogramming. c) Merge of the photo a and b.

As shown in figures 37 and 38 both high and low TMRM conditions show similar response to this stimulus. The cells show high proliferative capacity and big sized colonies.

a 

b 

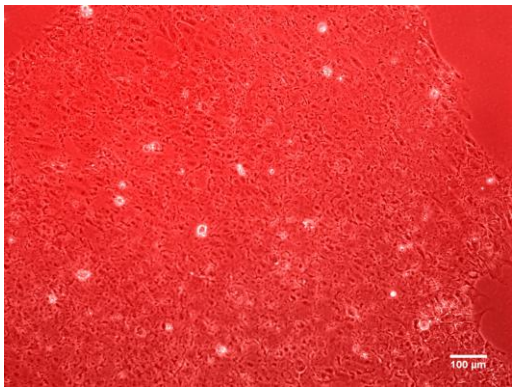
c 

Figure 38 a) and b) Reprogrammed colony on the high TMRM uptake population condition in hypoxia. The aspect of the colony resembles the colony in the low TMRM condition. c) Merge of the photo a and b.

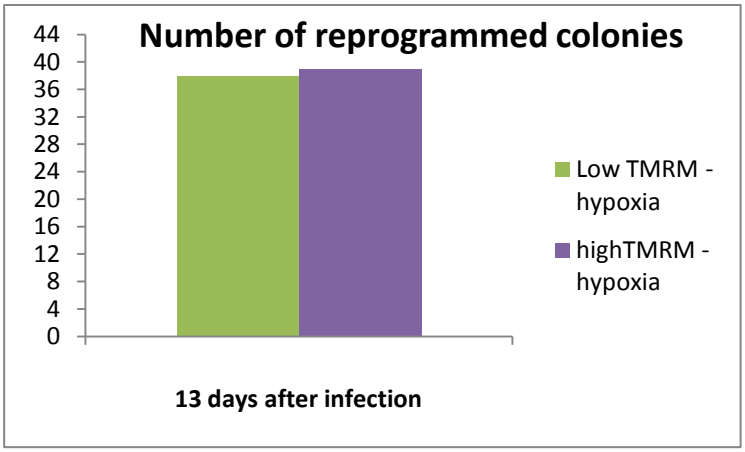


Figure 39 – Number of reprogrammed colonies in hypoxia for both populations. The number of colonies was very close in both populations.

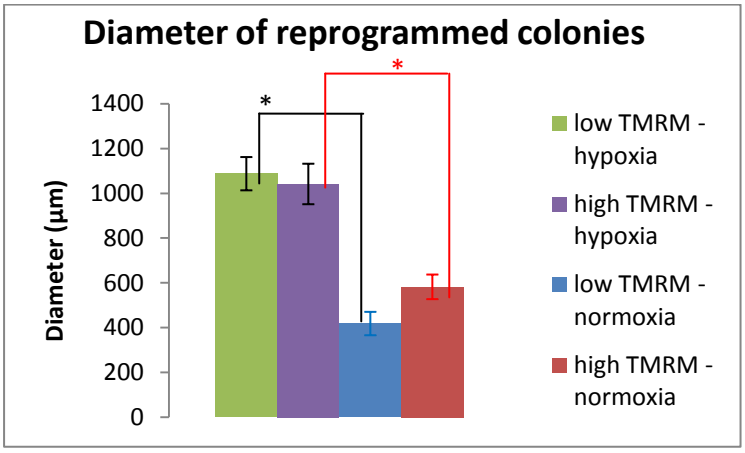


Figure 40 – Diameter of the reprogrammed colonies. There is no significant difference between the sizes of the colonies between high and low TMRM conditions. However, it is clear that there is an increase of proliferation in hypoxia when compared to the cells in normoxic condition. *P<0,05 (P=5,95832E-07 for the low TMRM uptake – normoxia vs hypoxia and P= 0,00107 for the high TMRM uptake cells – normoxia vs hypoxia)

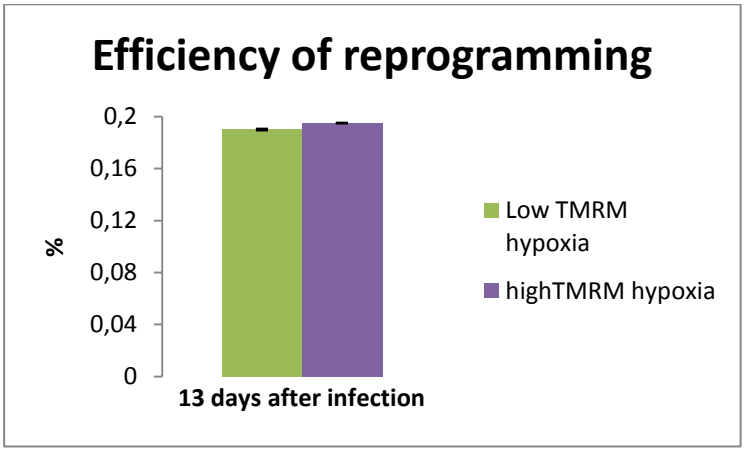


Figure 41 – Efficiency of reprogramming. These values were calculated considering the number of infected cells one day after the last infection.

We were expecting that the hypoxic environment could increase the gap in terms of efficiencies between the high and the low TMRM cells. Since the metabolism of the low TMRM cells does not rely on oxidative phosphorylation, this should be sufficient to allow a better adaptation to the new conditions.

Interestingly, however, we observed that both populations showed similar reprogramming results at low oxygen concentrations. With these atmospheric conditions the high population seems to be forced to shift to the glycolytic metabolism that characterizes the low population, allowing an improvement in reprogramming efficiency. On the other hand as the low population does not require oxygen there are no stimuli affecting the metabolism and thus there will be no significant change of efficiency. This increase in proliferation status was consistent with the report on Forristal et al 2010 where colonies where HIF2A and HIF3A expression had been disrupted experimented a reduction in colony size.[91]

As for the low efficiencies obtained (figure 41), these are to be expected as the cells were cultured in matrigel (from BD Biosciences) with conditioned medium instead of using MEFs as feeder cells.[115] The feeder cells provide appropriate cell contacts, various growth factors and extracellular matrix (ECM) proteins that are required for the undifferentiated growth of hPSCs.[116]

Feeder-free reprogramming in Normoxia *vs* Hypoxia

To fully prove the negative effect of culturing our iPSCs using matrigel and conditioned medium instead of a feeder cell culture we decided to repeat the hypoxic reprogramming and compare it with the efficiencies in normoxia under the same conditions. Additionally, we also reprogrammed high and low cells in normoxia using MEFs.

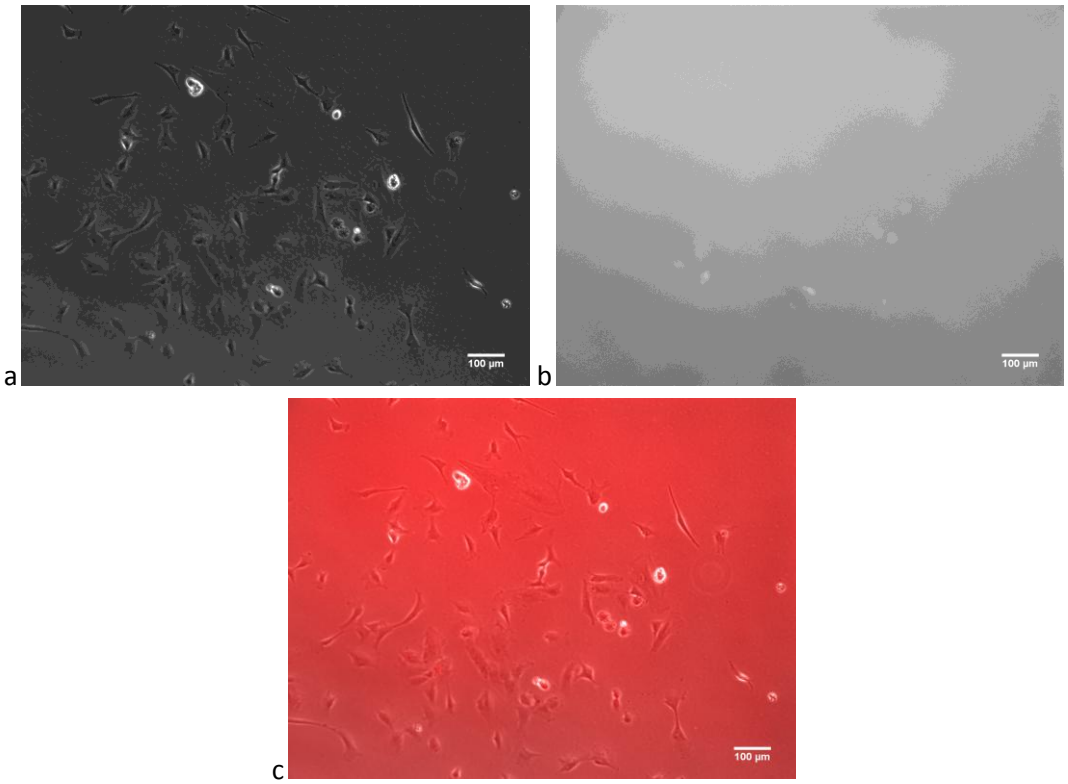


Figure 42– a) -Low TMRM cells cultivated on matrigel with conditioned medium in normoxia 15 days after last infection. b) Cells on the fluorescent channel. This condition failed to generate colonies. c) Merge of the photo a and b.

As shown in figure 42 and 43 the cells cultured in normoxia with the same feeder free system failed to generate reprogrammed colonies. The low TMRM condition did not show any colony while the high TMRM cells formed a small colony but did not have embryonic features and still expressed the fluorescent reporter.

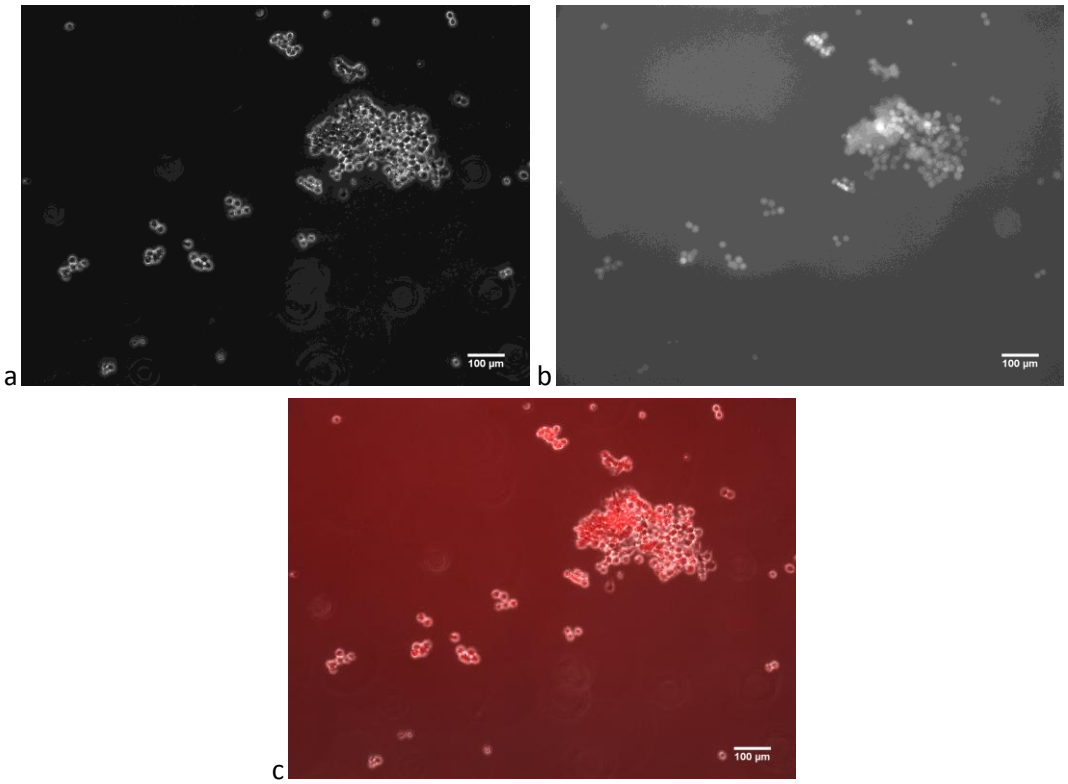


Figure 43 – a) High TMRM cells cultivated on matrigel with conditioned medium 15 days after last infection. b) These cells also failed to achieve the pluripotency state as the colonies formed still expressed d'Tomato protein, did not have round shape, without a well defined border, not compact and showed signs to be disintegrating. c) Merge of the photo a and b.

On the other hand we confirmed the good morphology of the colonies cultured in hypoxia and feeder free conditions.

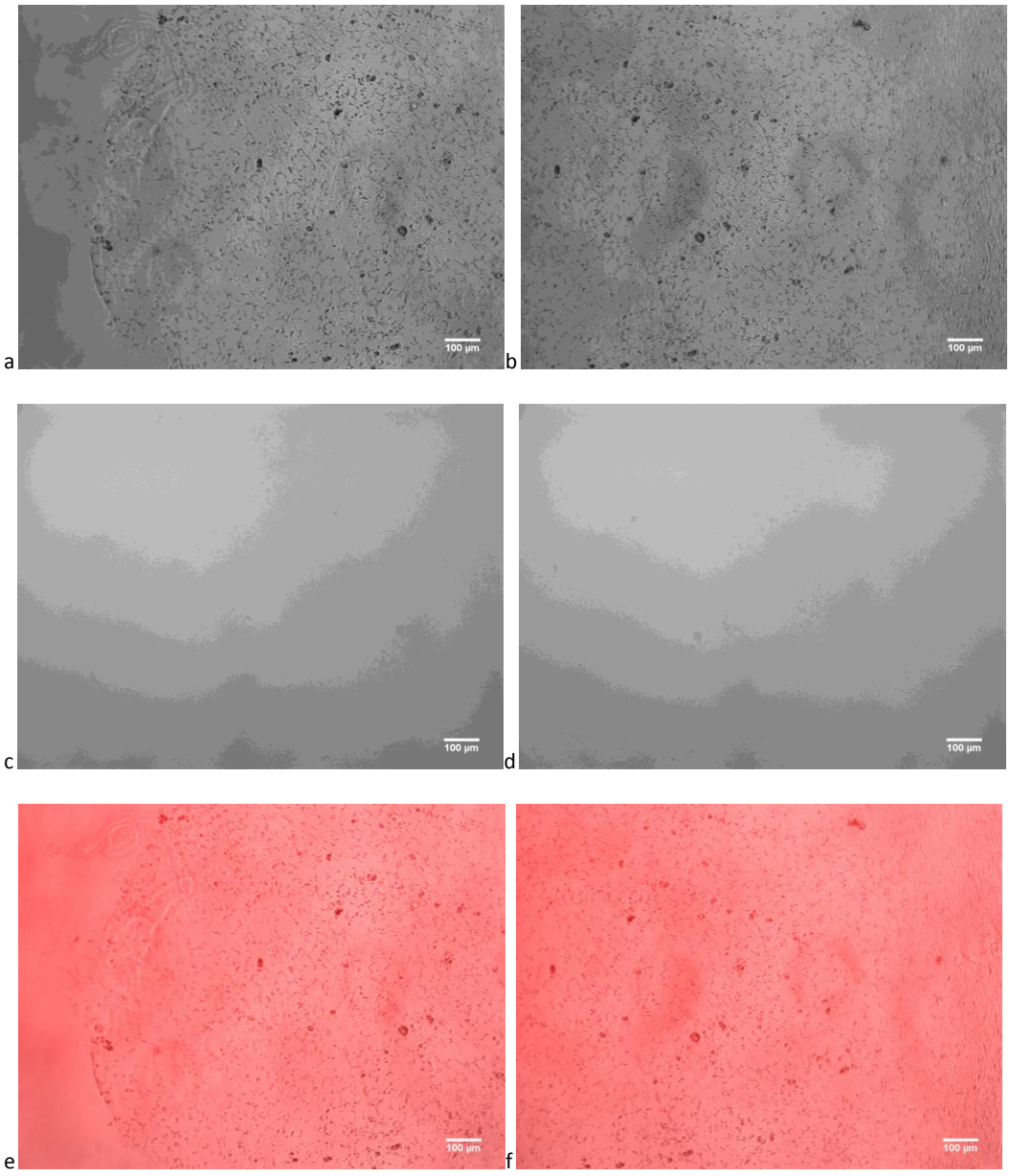


Figure 44 a),b),c),d)- Reprogrammed colony of the low TMRM condition cultured in hypoxia on matrigel with conditioned medium 15 days after last infection. As it is illustrated there was the requirement to take more than one picture to evaluate a single colony. Although the morphology of the colonies was sacrificed there was a clear improvement of proliferation of the cells. e and f) Merge of the photo a, b c and d.

In the hypoxia condition we confirmed the expected results both in high and low TMRM uptake cells (figure 44 and 45): big colonies, very compact and with cells with high nucleus/cytoplasm ratio when compared with previous data (figure 33 and 34).

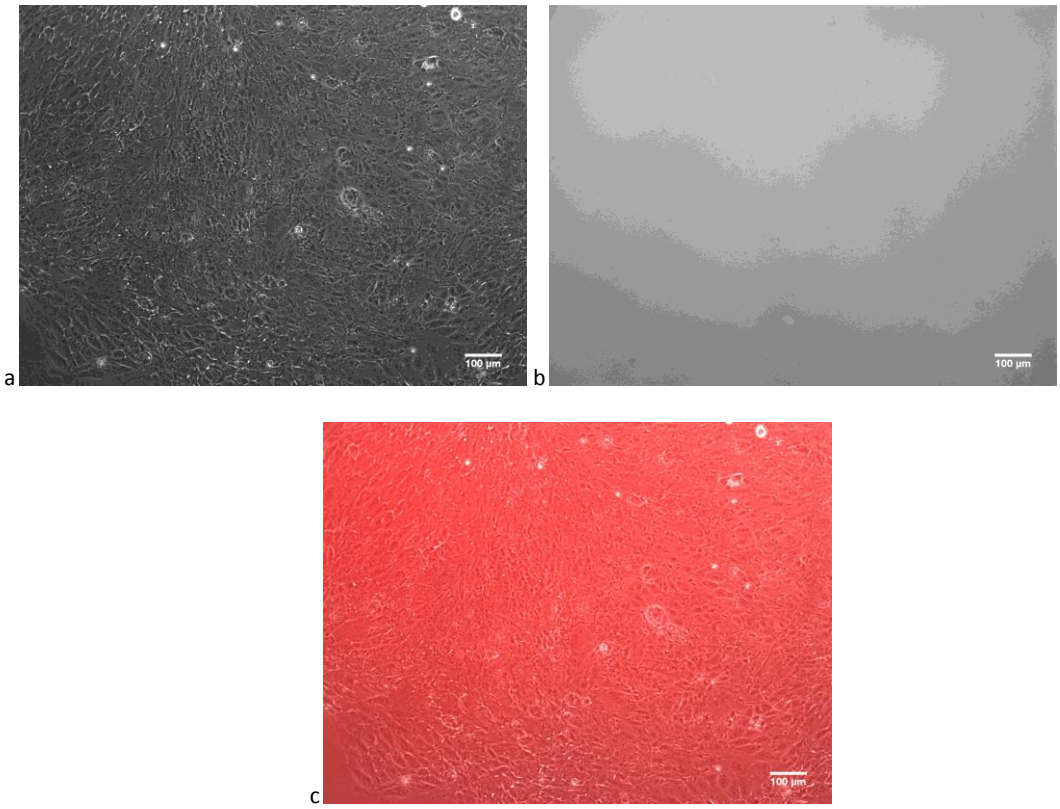


Figure 45 a) and b) Reprogrammed colony of the high TMRM condition cultured in hypoxia on matrigel with conditioned medium 15 days after last infection. The results are similar when compared to the low population on the same conditions although the colonies were smaller. c) Merge of the photo a and b.

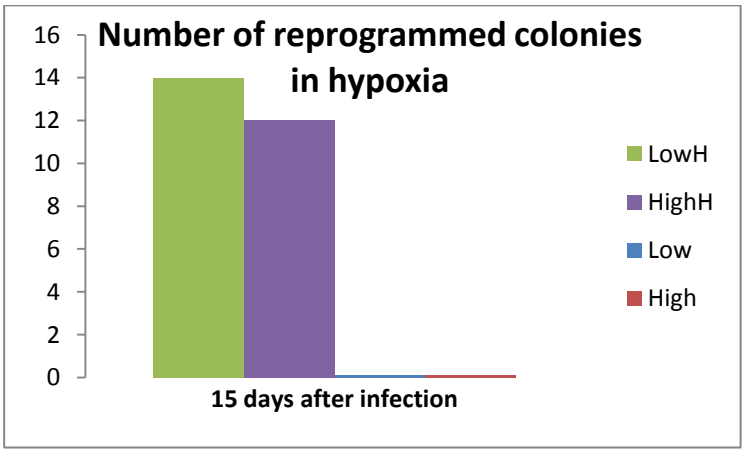


Figure 46 – The number of reprogrammed colonies was practically the same on hypoxic conditions, reinforcing the effect of hypoxia in equalizing efficiency between low and high TMRM populations. In normoxia the low and high TMRM uptake conditions failed to generate any reprogrammed colony.

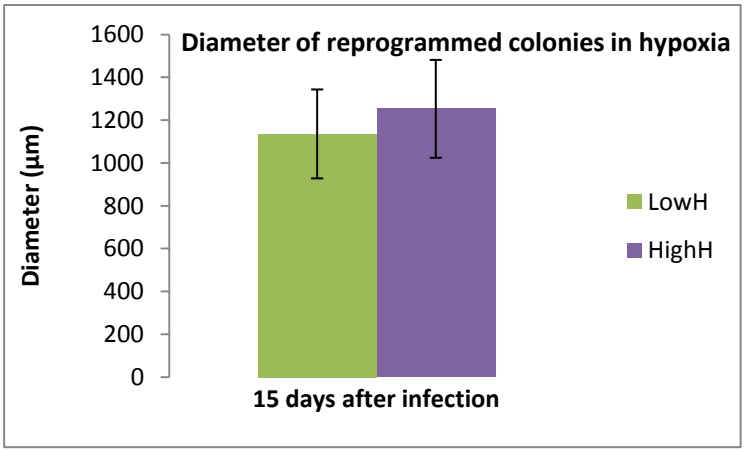


Figure 47 – The size of the colonies was also similar since the difference is contained in the error bar calculated (standard error). The error bar is the standard error.

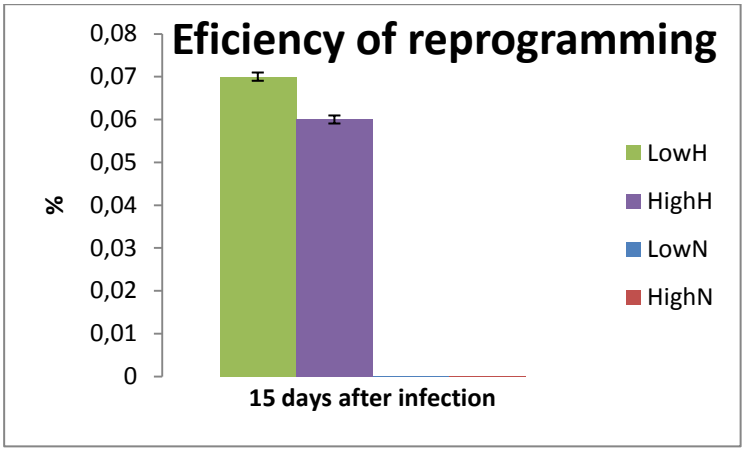


Figure 48 – The reprogramming efficiency was very close in hypoxia on both populations. There was however a reduction if we compare the results with the ones obtained on the previous study (figure 35).

There was a decrease in the efficiency (figure 48) when compared to the results obtained in our previous experiment in hypoxia (figure 35). We determined the efficiency as the number of colonies generated per infected cell. As these colonies have the tendency to grow to large sizes it could be that some of the colonies may be the result of aggregation of several smaller colonies. This would mask the real number of colonies and result in a false reduction of efficiency.

Nonetheless these results were very important to elucidate the impact of the hypoxia in boosting efficiency. Not only this stimulus allowed generation of reprogrammed colonies in both conditions, which was not possible in normoxia, but also allowed similar efficiencies in both populations.

Fine-tuning data analysis

Has it was mentioned above we had two problems in our previous experiment: the first was the possible excessive error that we introduced with the estimation of the number of colonies per area; the second was the introduction of a step of passaging of the cells during the reprogramming process. In order to overcome these problems we have started with a smaller cell density (10000 per well of a 6-well plate) and counted all the colonies present in each well. Additionally, we tried to reduce the stress imposed to the cells by passaging.

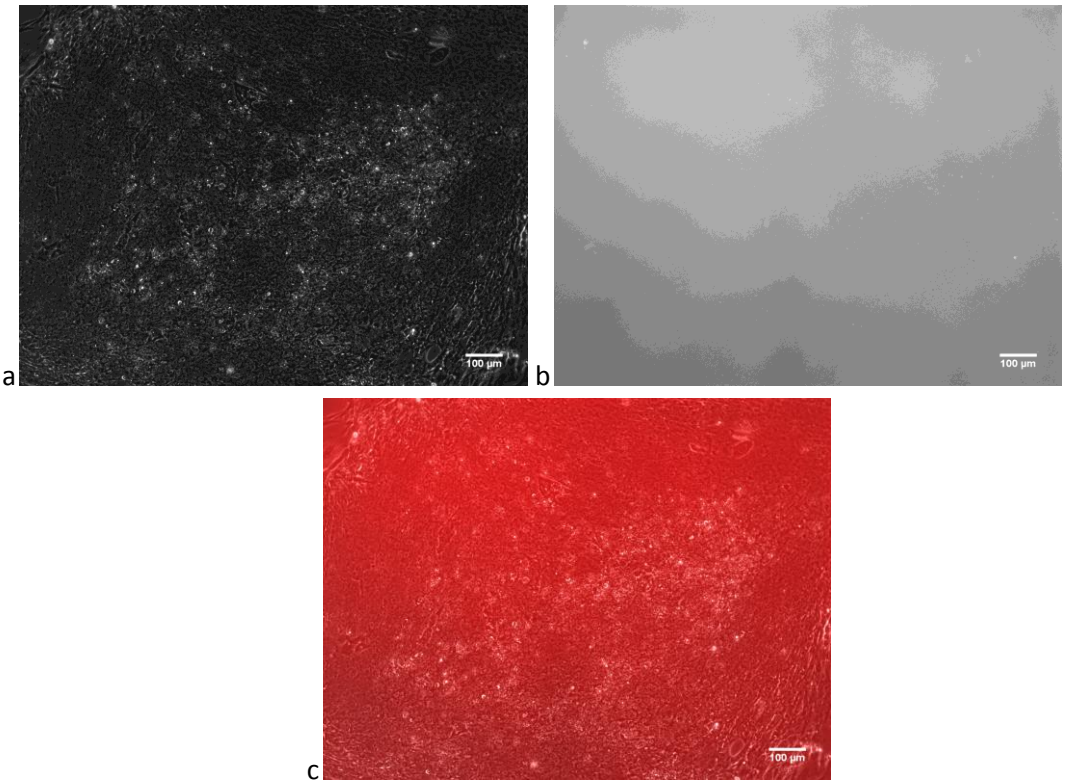


Figure 49 a) andb) Reprogrammed colony of the low TMRM condition in normoxia cultured on MEF's 15 days after last infection. a) the aspect of the colony was improved when compared with the results obtained on the first experiment as the colonies stayed in culture additional time. c) Merge of the photo a and b.

Comparing the results of this experiment (figure 49 and 50) with our first data in normoxia (figure 21 and 22) it is clear that the colonies that were not passed showed in both conditions better morphology, with greater size, round shape and well defined borders. Furthermore, the cells of the high TMRM population did not show signs of disintegration. This suggests that the stress imposed during the passages is deleterious for the reprogramming process.

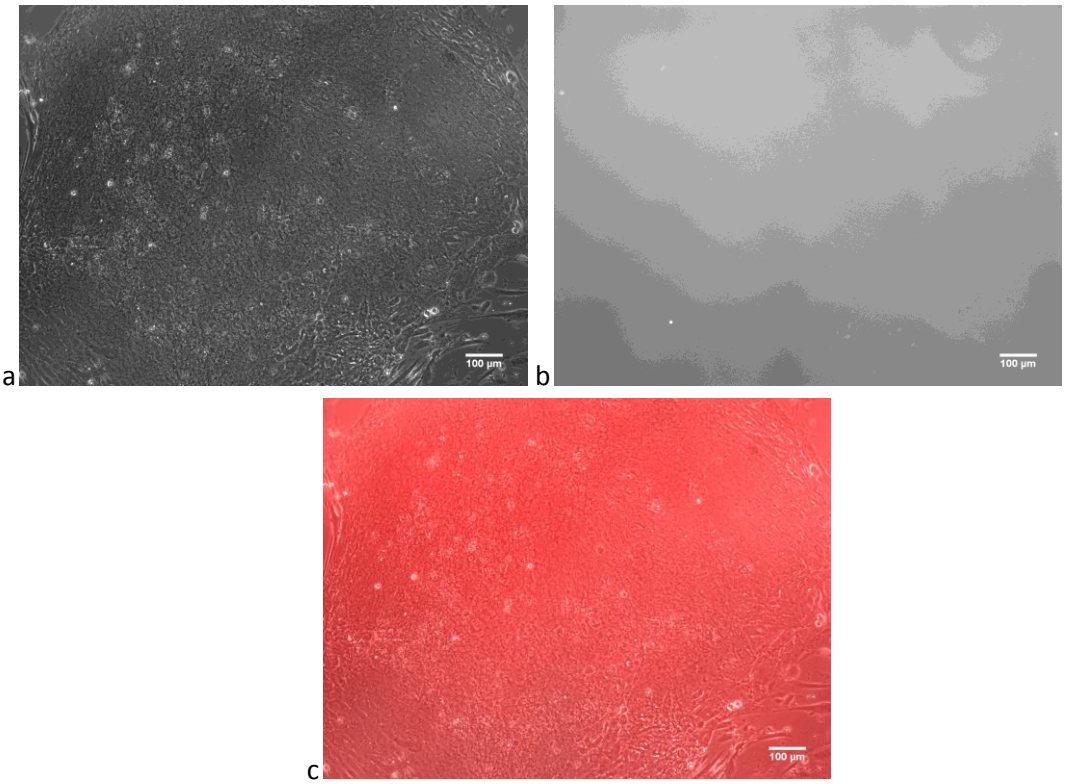


Figure 50 a) and b) Reprogrammed colony of the high condition in normoxia cultured on MEFs 15 days after last infection. c) Merge of the photo a and b.

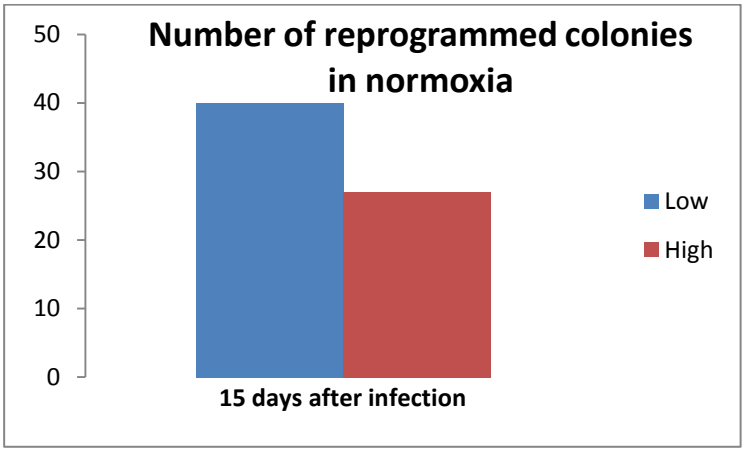


Figure 51 – Number of reprogrammed colonies in normoxia. It is possible to confirm the greater number of colonies in the low population. The error bar is the standard error.

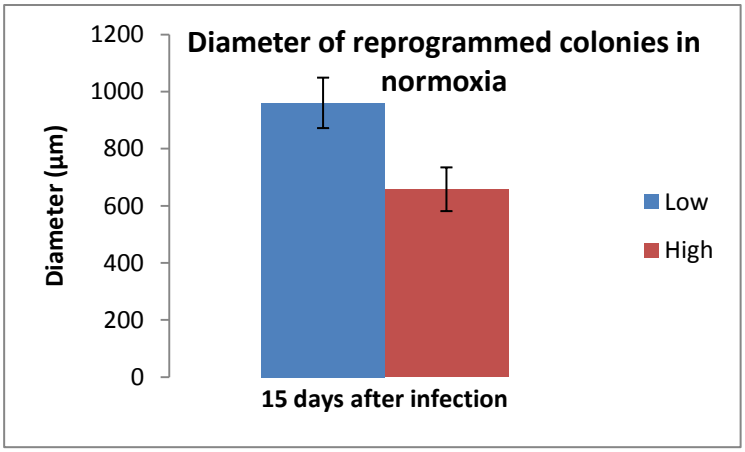


Figure 52 – The diameter of the reprogrammed colonies is considerably greater in the low population, showing greater proliferation capacity. The error bar is the standard error.

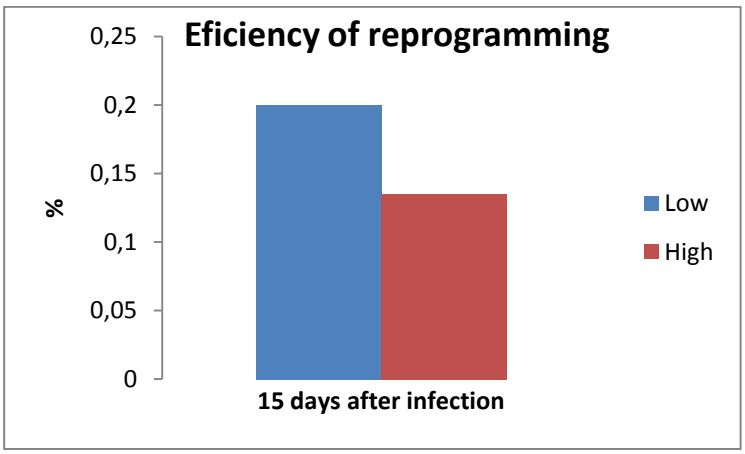


Figure 53- Although the difference on the reprogramming efficiency is smaller than the number previously estimated, there is still a clear gap between the two samples. The low population is better suited to generate more colonies.

Comparing these results obtained in normoxia (figure 53) with our previous data (Figure 18) we can see a significant reduction in the global efficiency and the gap between the 2 populations is also decreased.

Effect of antimycin A on hypoxic reprogramming

The results obtained in hypoxia were quite promising however we did not have the required equipment to conduct an experiment where cells are kept at low oxygen concentrations even while changing culture media. During this procedure it seems that there is a reoxygenation burst in the cell environment and this may induce deleterious effects in terms of cell fate and state.[117] For this reason we decided to introduce a complex III inhibitor of the respiratory chain of mitochondria. This should reduce the

oxygen impact during medium change, which could compromise the induction of pluripotency.

To test this new variable we repeated our study in hypoxia with and without antimycin A for both populations.

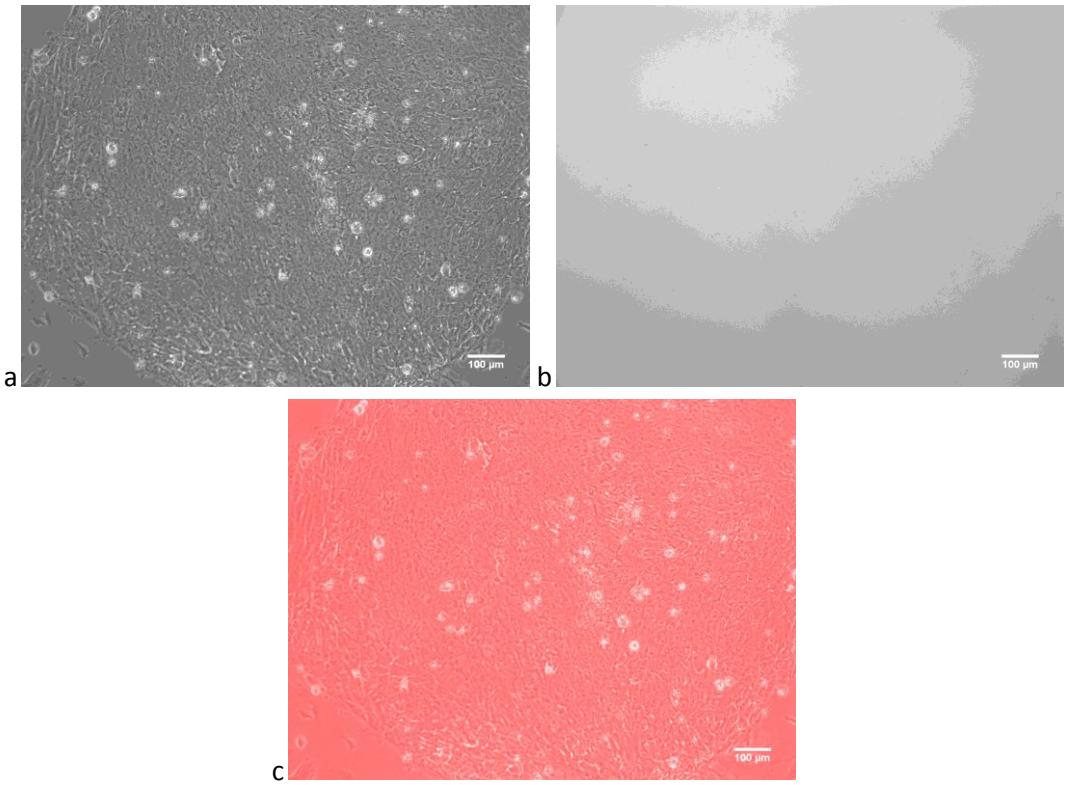


Figure 54 –a) and b) Low cells cultivated on matrigel with conditioned medium 15 days after last infection without antimycin A treatment.. These results were consistent with the data obtained on previous experiments as the morphology show some heterogeneity but with high proliferation capacity. c) Merge of the photo a and b.

The cells that were not exposed to antimycin A treatment (figure 54 and 55) showed a morphology consistent with previous results in hypoxic conditions (figures 37, 38, 44 and 45). The colonies were big, compact and they didn't express the fluorescent reporter.

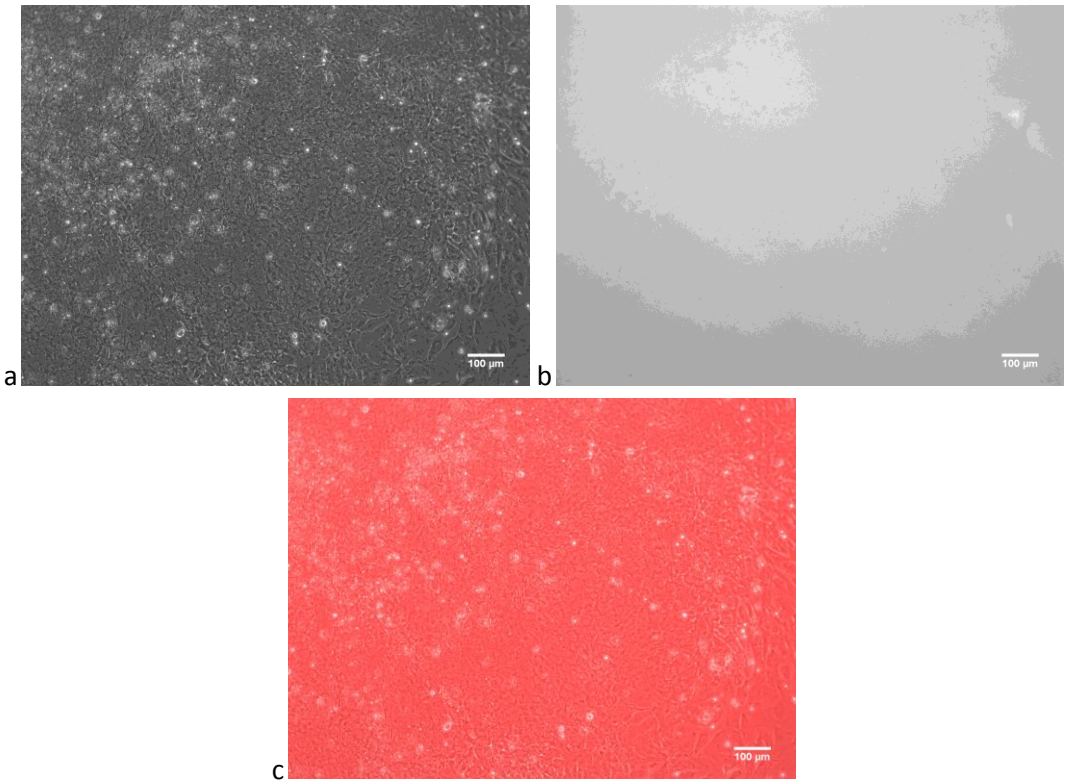


Figure 55 a) and b) High cells cultivated on matrigel with conditioned medium 15 days after last infection without antimycin A treatment.. These results resemble the low population on the same conditions . c) Merge of the photo a and b.

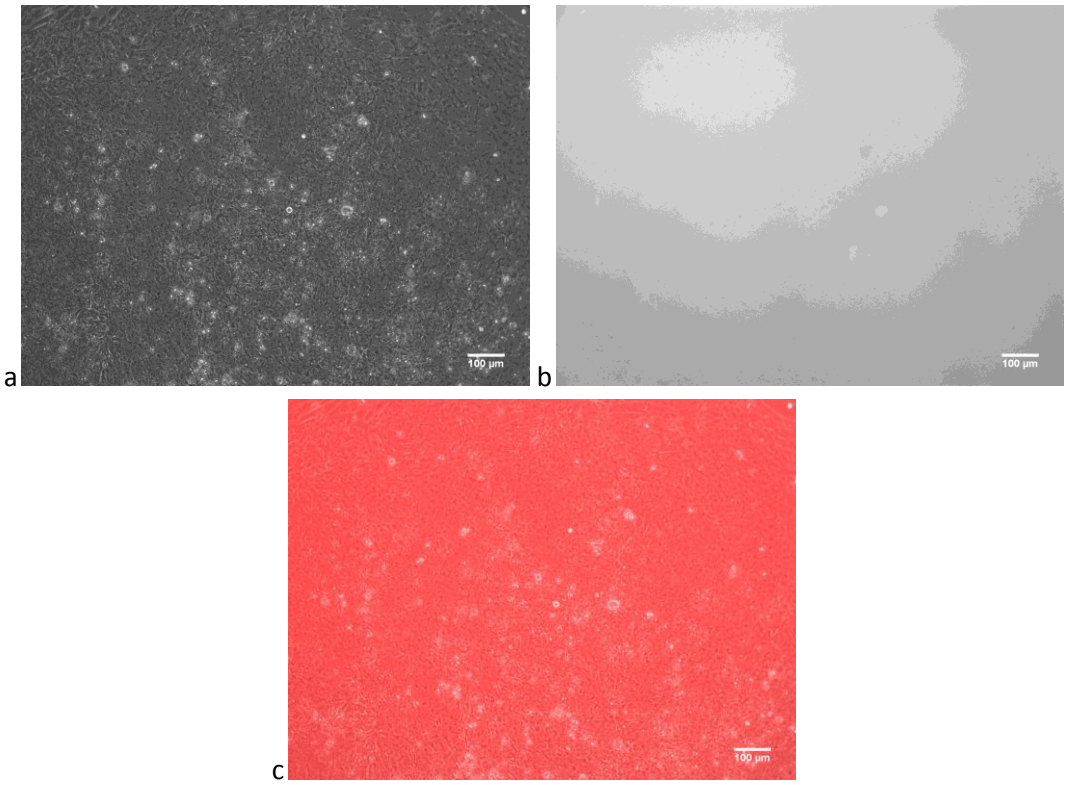


Figure 56a) and b) – Low cells cultivated on matrigel with conditioned medium 15 days after last infection with antimycin A treatment. The colonies do not show significant differences from the condition without antimycin A treatment. c) Merge of the photo a and b.

Antimycin A treatment did not reveal a visible impact in the morphology of the colonies as we can see in figures 54, 55, 56 and 57.

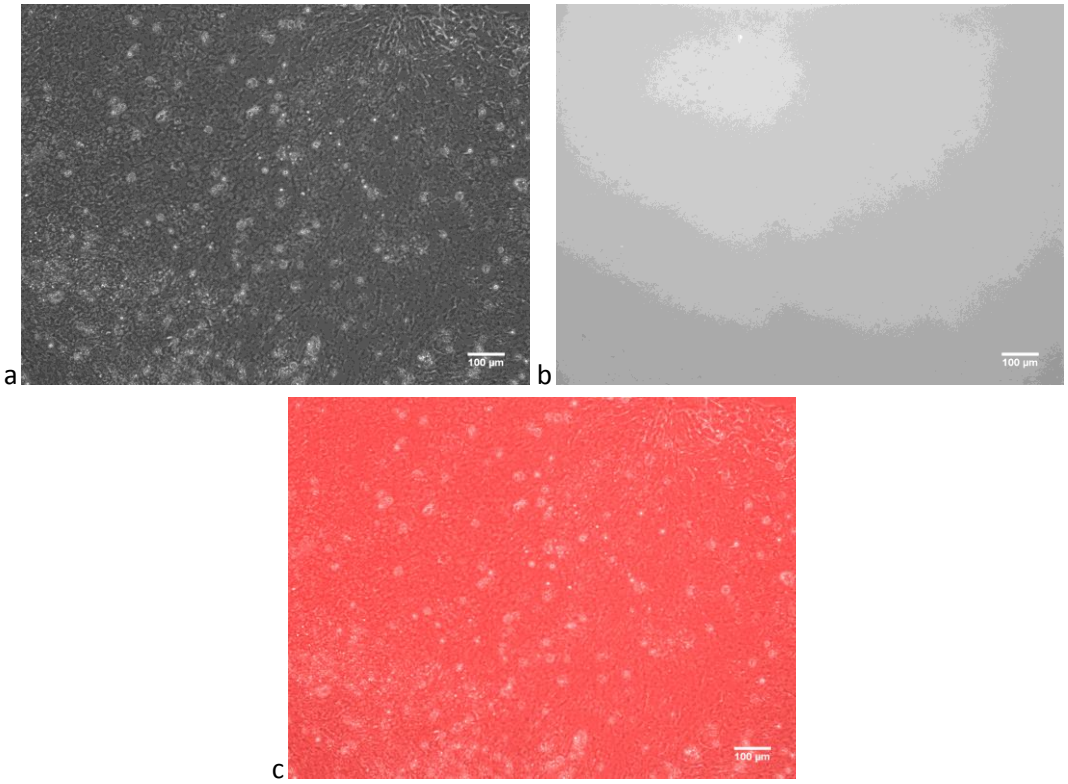


Figure 57 a) and b) High cells cultivated on matrigel with conditioned medium 15 days after last infection with antimycin A treatment. The colonies do not show significant differences from the condition without antimycin A treatment. c) Merge of the photo a and b.

In this study it was not possible to monitor the number of colonies during the experiment since we wanted to protect cells from the stress of reoxygenation that could lead to differentiation and compromise the experiment. Another issue that compromised this analysis was the big size of the colonies that raised obstacles in the identification of the borders between the colonies.

There was a high proliferation rate in the colonies. As the colonies grew they started to coalesce making it impossible to evaluate their accurate number. Taking this into account we decided to determine the area occupied by reprogrammed cells. We analysed a photo from each condition cultured in a 6 well plate and estimated the area using imageJ software.

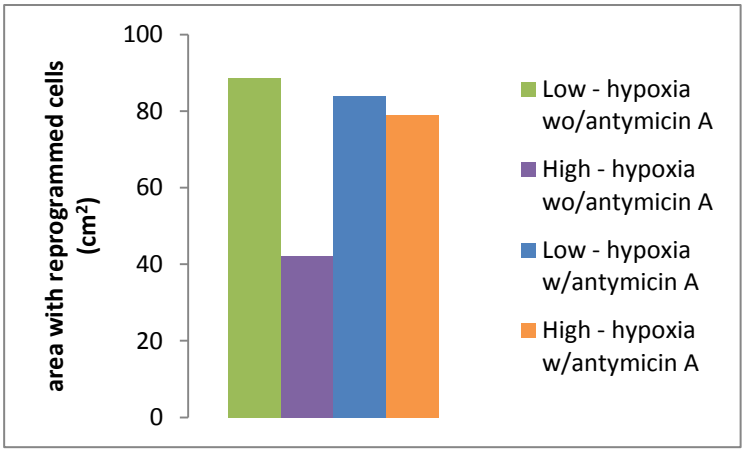


Figure 58 –Area of reprogrammed cells in cm2 in a 6 well plate in the 4 conditions in hypoxia: high with and without antimycin A treatment and low with and without antimycin A treatment.

With the introduction of the antimycin A in the culture conditions we could see that the differences between high and low TMRM populations were attenuated. This was visible both in terms of reprogramming efficiency and proliferation and by analysis of gene expression by RT-PCR of the 3 main pluripotent genes (Oct4, Sox2 and Nanog).

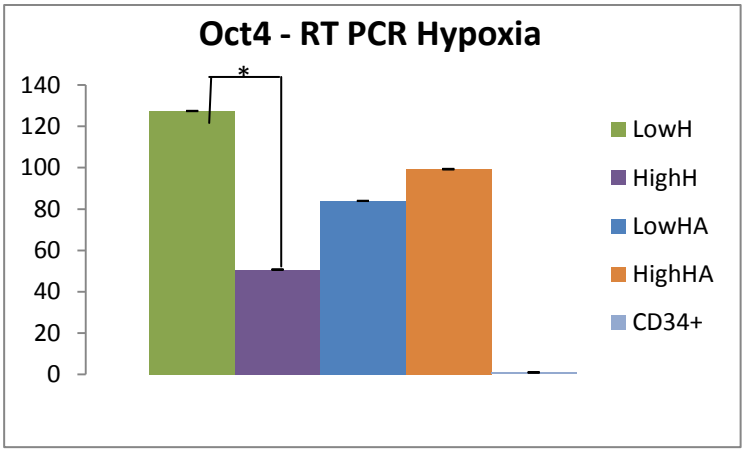


Figure 59 – Oct4 expression for all the hypoxic conditions (with and without antimycin A treatment). LowH - Low cells on hypoxia. HighH – high cells on hypoxia. LowHA – low cells in hypoxia with antimycin A treatment. HighHA – High cells in hypoxia with antimycin A treatment. The results were normalized for $CD34^{+}$ cells expression. The higher epression is shown on low population without antimycin treatment. The antimycin A improve the fold induction of the pluripotent gene on the high population (which depended more on oxidative phosphorylation). The fold induction on the low population was decreased. *P<0,05 (P=0,000269 for Antimycin A non treated cells; P=0,574554 in Antimycin A treated cells). The difference is only statistically significant on the cells that did not had the inhibitor.

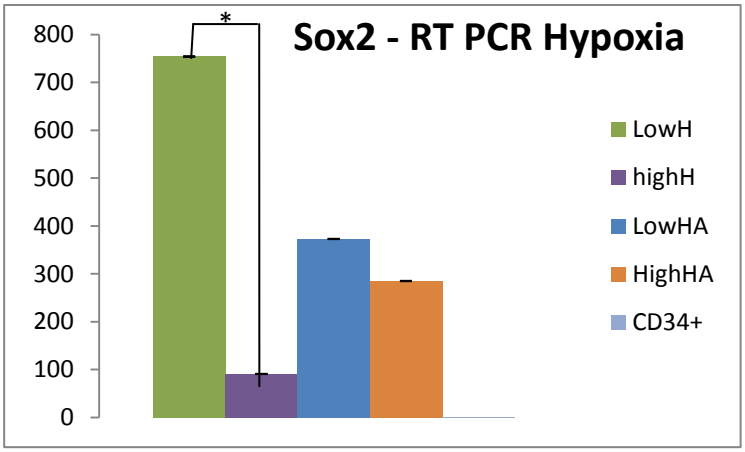


Figure 60 – Sox2 expression for all the hypoxic conditions (with and without antimycin A treatment). LowH - Low cells on hypoxia. HighH – high cells on hypoxia. LowHA – low cells in hypoxia with antimycin A treatment. HighHA – High cells in hypoxia with antimycin A treatment. The results were normalized for $CD34^{+}$ cells expression. The results for the fold induction of this gene were consistent with the Oct4 results. The higher fold induction was seen in the low population without antimycin A treatment. The use of the inhibitor made both population to have a similar behaviour on the expression of the pluripotent gene. The error bar is the standard error. *P<0,05 (P= 0,00216 for Antimycin A non treated cells; P=0,384989 in Antimycin A treated cells). The difference is only statistically significant on the cells that did not have contact with the inhibitor.

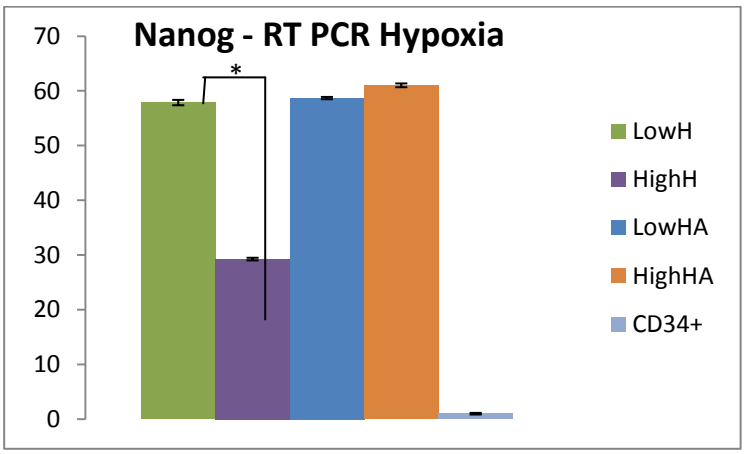


Figure 61– Sox2 expression for all the hypoxic conditions (with and without antimycin A treatment). LowH - Low cells on hypoxia. HighH – high cells on hypoxia. LowHA – low cells in hypoxia with antimycin A treatment. HighHA – High cells in hypoxia with antimycin A treatment. The results were normalized for $CD34^{+}$ cells expression. The low population expressed more quantity of Nanog mRNA than the high population without antimycin A treatment. However the inhibitor improved fold induction of this gene and equalizing the result on both conditions. The error bar is the standard error. *P<0,05 (P=0,037867101 for Antimycin A non treated cells; P=0,35843945 in Antimycin A treated cells). The difference is only statistically significant on the cells that did not have contact with the inhibitor.

Although the efficiency of reprogramming could not be improved in the low TMRM population by using this inhibitor there was a positive effect in making both populations to have a similar behaviour and with a reasonable expression of the pluripotent genes. The fact that there was a drop in expression of Sox2 and Oct4 (figure 59 and 60) in the Low TMRM population with antimycin treatment did not have parallel in the expression of Nanog (figure 61) or in the proliferation (figure 58) of the reprogrammed cells. We are not aware of the reason for this effect. Future

experiments should focus on this effect to try to validate the use of antimycin to generate iPSCs under hypoxia conditioning where a full hypoxic system is not available in the laboratory.

Conclusion

As reprogramming consists in a stochastic process, a good way to increase efficiency is lowering the number of events required to achieve pluripotency by selecting the less differentiated population.

Our work successfully developed a new method to select the cells within the cord blood $CD34^+$ population with more potential for pluripotency induction. We managed to correlate the mitochondrial phenotype with the maturation of the cells by analysing the membrane potential with TMRM. The results clearly point the cells with low mitochondrial membrane potential as the best candidates to achieve pluripotency. The cells with high mitochondrial membrane potential showed practically half of the reprogramming efficiency. Additionally surface marker characterization confirmed a more naïf stage with less expression of lineage commitment markers for the low TMRM population. The colonies of the low population that no longer expressed the genes delivered by the viral vector had higher expression of the pluripotent genes Nanog, Oct4 and Sox2; confirmed by mRNA analysis using RT-PCR. Also, western Blot analysis of Sox2 and Oct4 protein was consistent with RT-PCR data.

On the other hand, the culturing conditions were shown to have an important impact in the efficiency of pluripotency induction. The hypoxic environment improved proliferation in both conditions and allowed to obtain reprogrammed cells without requiring the use of feeder cells with similar efficiencies.

Furthermore the use of antimycin A has proven to be a mean to homogenize the behaviour within the CD34+ cell population. This mitochondrial complex III inhibitor improved the efficiency of reprogramming in the high TMRM population, which was confirmed with RT PCR gene expression.

While the traditional method to isolate an immature population within the cord blood is very complex, time and resource consuming, requiring the use of several antibodies, our method offers a new cheap alternative to perform isolation.

As the reprogramming process demands a delicate balance between several variables, where time is a very critical factor, our method reduces sorting time several fold and this seems to be crucial to increase efficiency. By selecting the cells according with the mitochondrial phenotype instead of marker expression we optimized this step

reducing the amount of stress imposed to the cells. We believe this may diminish cell death and differentiation.

Other important aspect is related with the complexity of the method. The use of antibodies that label surface markers requires the coordination of the wavelength emission of the channels and the use of several control samples to reduce the interference of the different markers. This interference leads to additional time required to distinguish false positives, higher number of ambiguous events discarded and the requirement of a highly skilled technician to collect the cells and interpret the data. Also, reducing the number of antibodies for sorting is also important for cost reduction of the overall process and incubation time of the samples.

We hope our work may contribute to increase the efficiency of other reprogramming studies. We also expect to introduce a new methodology of research for the segregation of primitive cells of the umbilical cord blood as we show quite promising results in our selected population.

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