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Mestrado em Biologia Molecular e Genética

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We animals are the most complicated
and perfectly-designed pieces of
machinery in the known universe.
Put it like that, and it is hard to see why anybody studies anything else!

Richard Dawkins in *The Selfish Gene*, 1976

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Abstract

Somatic cell reprogramming has been extensively studied over the last years and opened new perspectives in the use of pluripotent cells for regenerative biomedical purposes. Spontaneous cell fusion has been suggested to be involved in regenerative processes *in vivo.* Strong evidences support the hypothesis that the reprogrammed hybrids resulting from the fusion between a pluripotent cell and a somatic cell exhibit pluripotent characteristics and may provide a source for cell therapy in the future. Previous evidences show that tetraploid hybrid cells are originated after the fusion event and that both *in vitro* and *in vivo* these cells can give rise to diploid cells by mitotic processes that are not fully understood. This "ploidy reduction" is the focus of this project.

The fate of the hybrid cells was characterized by addressing the karyotype of the reprogrammed cells originated after fusion between Embryonic Stem cells and multipotent Neural Stem cells. We identified stable tetraploid and diploid clones that resisted the selection system after fusion and exhibit pluripotent characteristics. Furthermore, we showed that the obtained diploid cells have a fusion origin and are not a result of transdifferentiation or resistant Embryonic Stem cells. This study shows that ploidy reduction could be a consequence of fusion-mediated reprogramming corroborating the results published by other research group. We hypothesize that fusion-derived diploid cells might have been ignored in other studies or confounded with transdifferentiation events.

The characterization of ploidy reduction is important to understand the role of cell fusion-mediated reprogramming during tissue regeneration and to uncover how these hybrids proliferate to eventually repopulate the damaged area.

Key words: Reprogramming; Cell fusion; Regeneration; Ploidy reduction; Multipolar mitosis.

Resumo

O desenvolvimento de um organismo desde a fecundação do óvulo até à formação de um organismo adulto foi durante muito tempo considerado um processo unidireccional e irreversível. A ideia de que uma única célula é capaz de originar um organismo adulto através de uma sucessiva e organizada sequência de eventos explica a progressão ao longo das diferentes fases do desenvolvimento e também mecanismos de diferenciação que ocorrem já na fase adulta. Exemplo disso é a participação de algumas células estaminais adultas em processos de reparação de tecidos. Em geral, durante o desenvolvimento de um mamífero, dá-se uma progressiva especialização das células embrionárias em outras cada vez mais diferenciadas. Existe assim uma perda sucessiva de plasticidade, ou "potência", celular ao longo do desenvolvimento das linhagens celulares. Assim, células especializadas numa determinada função não podem regressar a um estado que lhe permita dar origem a células de outras linhagens.

No entanto, ao longo dos últimos 20 anos, diversos estudos defendem a possibilidade de que células somáticas adultas podem ser induzidas a regressar a um estado de maior plasticidade através de reprogramação do seu genoma. Durante este processo, uma célula somática é induzida a tornar-se mais indiferenciada ganhando capacidade de se diferenciar em outras células.

O primeiro método desenvolvido com o objectivo de reprogramar o genoma de uma célula somática para um estado de maior plasticidade foi a transferência nuclear de células somáticas (SCNT). Os investigadores revelaram que era possível originar células pluripotentes através da transferência de um núcleo de uma célula somática para um oócito. Obteve-se assim a primeira evidência de que o genoma presente numa célula adulta é suficiente para reverter o seu destino a um estado de pluripotência e de que os genes de pluripotência não são irreversivelmente inactivados durante o desenvolvimento.

A partir destes estudos, vários grupos de investigação tentaram identificar quais os factores que podem induzir a reprogramação de um núcleo somático a um estado pluripotente. Vários factores de transcrição, como Oct4, Sox2, Klf4 e c-Myc, foram identificados em células estaminais embrionárias, tendo sido demonstrado que eram fundamentais para manter o seu estado de pluripotência. Assim, foi sugerido que após a indução deste genes em células somáticas, onde normalmente não são expressos, estas poderiam adquirir características típicas de células pluripotentes à medida que se reprogramam e se tornam mais indiferenciadas. O desenvolvimento desta técnica abriu novas possibilidades para a aplicação

de terapia genética e regenerativa, visto que através deste método se podem originar células de todas as linhagens e se evita a utilização de oócitos ou de células embrionárias.

Outro método pelo qual se podem reprogramar células somáticas é através de fusão celular. Diversos grupos demonstraram independentemente que a fusão de uma célula diferenciada com uma célula estaminal embrionária resulta na formação de um híbrido tetraplóide no qual é inibida a expressão de genes específicos da célula somática. Assim, o híbrido contém informação genética de ambas as células, mas comporta-se como uma célula embrionária podendo dar origem a todos os órgãos e tecidos. Estes híbridos sofrem mudanças epigenéticas passando a expressar genes específicos de células pluripotentes, apresentam grande capacidade de auto-renovação e podem diferenciar-se em todos os tecidos originados pelas três camadas germinativas.

De maior importância foi a descoberta *in vivo* de que células originárias da medula óssea podem contribuir para a regeneração de diversos órgãos através de fusão com células danificadas. Apesar de grande parte dos fenómenos de regeneração em mamíferos se deverem à activação de células estaminais adultas, existe a possibilidade de que processos de fusão celular possam contribuir também para a reparação de tecidos. Assim, certas células podem fundir-se com células de tecidos afectados e promover a reparação desse tecido através da formação de híbridos com capacidade de proliferação.

Um dos maiores problemas que resulta da formação de híbridos através de fusão é o facto de a célula híbrida ser tetraplóide e potencialmente tumorigénica. Assim, o estudo destes híbridos é importante no sentido de saber se constituem uma opção viável para uma futura aplicação biomédica. Vários estudos, maioritariamente num modelo de regeneração de hepatócitos, mostraram que um híbrido tetraplóide é capaz de proliferar e originar células funcionais após mitose. Contudo, estudos recentes demonstram que apesar de estes híbridos formarem maioritariamente células tetraplóides após divisão, uma percentagem das mitoses origina também células diplóides. Os autores destes estudos apresentam a hipótese de que após a fusão celular, alguns híbridos podem passar por um processo de redução de ploidia e originar células diplóides.

Assim, o objectivo deste projecto é caracterizar os híbridos originados por fusão de células somáticas com células estaminais embrionárias no sentido de identificar possíveis evidências de redução de ploidia neste modelo de reprogramação.

Para isso, usámos um protocolo de fusão previamente descrito para promover *in vitro* a fusão espontânea entre células progenitoras neuronais, que apenas podem originar células de

linhagem neuronal, com células estaminais embrionárias. As células neuronais usadas contém um promotor Oct4 que controla a expressão de GFP (Proteína Verde Fluorescente) e de uma proteína que confere resistência à puromicina. Assim, apenas resistirão a um meio de cultura suplementado com puromicina se forem reprogramadas para um estado de pluripotência e passarem a expressar o factor de transcrição Oct4. Os clones seleccionados apresentam uma morfologia e capacidade de proliferação semelhante a células estaminais embrionárias. Como esperado, identificámos após selecção clones reprogramados que contêm células tetraplóides. Contudo, células diplóides foram também identificadas nestes clones o que nos levou a pensar que as células híbridas tetrapóides poderiam ter sofrido um fenómeno de redução de ploidia.

Para confirmar a origem destas células diplóides, separámos através de citometria de fluxo as células reprogramadas após fusão (células positivas para a expressão de GFP). No momento da análise, todas as células positivas para GFP eram tetraplóides e assim seguimos esta população em cultura ao longo das semanas seguintes. De acordo com a nossa hipótese, uma população de células diplóides foi identificada, o que nos possibilitou confirmar que células diplóides podem originar-se a partir de células tetraplóides após fusão.

De seguida, confirmámos também que as células diplóides identificadas não eram resultado de contaminação ou de transdiferenciação, visto que células isoladas foram capazes de proliferar independentemente e originar clones. Identificámos clones constituídos apenas por células diplóides, o que demonstra que as células diplóides provenientes dos híbridos são reprogramadas e expressam o factor de transcrição Oct4. Foram também identificados clones, provenientes de apenas uma célula, que contêm células diplóides e tetraplóides. Esta observação confirma que a redução de ploidia é um fenómeno que pode ocorrer após reprogramação mediada por fusão celular.

Contudo, não nos foi possível identificar o processo pelo qual uma célula tetraplóide pode originar células diplóides. Estudos anteriores identificaram mitoses multipolares como possível explicação para o fenómeno. Esta opção parece válida visto que a célula tetraplóide originada por fusão pode conter mais do que dois centrossomas e assim originar mitoses com mais de dois fusos mitóticos, re-distribuindo os cromossomas por mais de duas células-filhas. No entanto, outra hipótese é possível: os híbridos tetraplóides podem entrar em mitose sem passar pela fase S do ciclo celular e assim distribuir igualmente os cromossomas em duas células-filhas diplóides. Outros estudos necessitam de ser desenvolvidos de modo a que este mecanismo seja caracterizado em pormenor.

A caracterização de fenómenos de redução de ploidia é importante para entender o papel da reprogramação mediada por fusão celular durante a regeneração de tecidos e para revelar como as células híbridas proliferam para eventualmente repovoar a área danificada.

Palavras-chave: Reprogramação; Fusão Celular; Regeneração; Redução de ploidia; Mitose multipolar.

LIST OF ABBREVIATIONS

AP – Alkaline Phosphatase

bFGF – basic Fibroblast Growth Factor

BIO – 6-bromoindirubin-3'-oxime

BM – Bone Marrow

DIC - Differential interference contrast

DMEM – Dulbecco's Modified Eagle's Medium

DMF - N,N-Dimethylformamide

EGF – Epidermal Growth Factor

ERK – Extracellular-signal-regulated Kinase

ES cells – Embryonic Stem cells

FACS – Fluorescence-Activated Cell Sorting

FAH – Fumaryl Acetoacetate Hydrolase

GFP – Green Fluorescent Protein

GSK-3 – Glycogen Synthase Kinase 3

ICM – Inner Cell Mass

iPS cells – induced Pluripotent Stem cells

Klf4 – Krueppel-like factor 4

LIF – Leukemia Inhibitory Factor

MAPK – Mitogen-activated Protein Kinase

NFB – Neutral Formalin Buffer

NS cells – Neural Stem cells

Oct4 – Octamer-binding Transcription Factor 4

PBS – Phosphate Buffered Saline

PEG – Polyethyleneglycol

PI - Propidium Iodide

R26R – Rosa 26 reporter

SCNT – Somatic Cell Nuclear Transfer

Tcf3 – T-Cell Factor 3

I. INTRODUCTION

1. Stem Cells and Development

In mammals, the development of a fertilized egg into an adult organism has been considered a unidirectional route towards more committed and differentiated cell types. This process involves the progressive loss of pluripotency by the blastocyst-derived cells as specific cell functions become available. Stem cells are characterized by their self-renewal capacity and the ability for differentiation in mature progeny [1] and represent an important cell population during the development of mammals. Firstly, by being present in the inner cell mass (ICM) of the blastocyst a population of pluripotent stem cells (from which Embryonic Stem (ES) cell lines are derived [2]) has the capacity to generate all the specialized cell types in the body including terminally differentiated cells [3,4]. These pluripotent cells can originate each of the three germ layers – endoderm, mesoderm and ectoderm – from which all the specific cell lineages are derived.

Later during development, a tissue-specific stem cell population is formed in mature tissues and originates adult (or somatic) stem cells. Adult stem cells are present in restricted tissue regions known as stem cell niches and by definition can only give rise to a subset of cell lineages within the tissue they reside in [5]. Adult stem cells are for that reason considered multipotent cells. Their presence has been confirmed in most of the organs including skin [6], lungs [7], heart and even in the brain [8]. These stem cells are involved in organ formation during early development and respond to tissue damage even during the adult developmental stages.

2. Nuclear Reprogramming by three different approaches

Interestingly, during the last years, the unidirectional view of stem cell commitment during development has been challenged, as several research groups showed that the reverse path is possible through the “reprogramming” of differentiated somatic cells into a pluripotent stem-like state (pluripotent reprogramming). Besides, the fate of a committed adult cell can be switched directly to a distinct differentiated state (lineage reprogramming) by being exposed to a different microenvironment that promotes this change of identity [9].

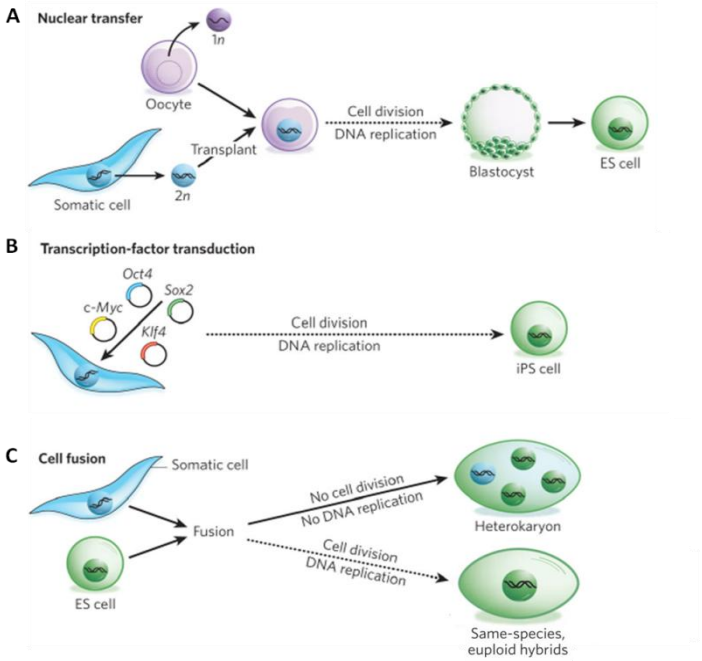

Figure 1. Different strategies to induce pluripotent reprogramming in somatic cells. Reprogramming of a somatic cell into an ES-like state may be achieved by the transfer of a somatic nucleus into an enucleated oocyte generating a diploid cell that can give rise to all the tissues of the body (A); by the transduction of specific transcription factors (like Oct4, Sox2, Klf4 and c-Myc), generating induced Pluripotent Stem (iPS) cells (B); or by the fusion of a somatic cell with a more undifferentiated cell, such as an ES cell, resulting in non-proliferative, short-lived heterokaryon hybrids or in tetraploid synkaryon hybrids that are able to divide (C). [adapted from reference 9]

The process of pluripotent reprogramming can be seen as the "de-differentiation" of somatic cells as they lose their specific features and expression patterns and become phenotypically similar to more "potent" cells [10]. Interestingly, epigenetic factors are hypothesized to be involved in nuclear reprogramming: the activation of specific molecular pathways induces epigenetic changes that can reactivate the expression of genes typical of another cell type (either a pluripotent cell or another differentiated cell) and the repression of genes expressed in the original cell [11, 12].

Nuclear reprogramming was, so far, achieved by three distinct experimental approaches: nuclear transfer of a somatic nucleus into oocytes, over-expression of transcription factors and cell fusion (Figure 1).

2.1 Somatic Cell Nuclear Transfer (SCNT)

The first successful attempts to reprogram somatic nuclei were performed by the transfer of a somatic nucleus into an enucleated oocyte by Briggs and King in frogs [13] (Figure 1A).

These early studies demonstrated the concept of cellular reprogramming by pluripotent-inducing factors present in oocytes. Somatic cell nuclear transfer (SCNT) was only achieved in mammals in the late 1990's, when Wilmut et al. transferred the nucleus of a sheep mammary cell into an oocyte giving rise to a viable animal [14]. This study proved the existence of specific factors present in oocytes that can promote the maintenance of nuclear pluripotency. This finding showed that the genome of adult cells does not undergo irreversible modifications that make them unsuitable for the development of live-born animals after reprogramming. These experiments have definitively shown that all the genes required for the development of an entire organism are present in the nucleus of specialized cells.

SCNT gave the first contribution to the study of pluripotency factors and reprogramming. However, its application in Biomedicine branches such as regenerative medicine would require a ready supply of oocytes and would be both technically difficult and expensive. Thus, other methods aiming to obtain similar reprogramming without SCNT became possible as a result of investigations on reprogramming activities of Embryonic Stem cells.

2.2 Direct Reprogramming by expression of defined factors

Since the discovery of SCNT the scientific community has tried to identify the transcription factors that are able to reprogram somatic nuclei and also tried to understand which cells normally express them. Several factors were shown to be essential for the maintenance of pluripotency in ICM-derived ES cells and therefore these factors were hypothesized to be able to induce nuclear reprogramming in somatic cells.

The studies developed by Takahashi and Yamanaka, first in a mouse model [12] and later using human cells [15], revealed for the first time the possibility to reprogram fibroblasts after the transduction of retroviral vectors expressing key ES factors. The authors identified four ES factors that sufficed to induce pluripotency in fibroblasts – Oct4, Sox2, Klf4 and c-Myc (now know as the "Yamanaka factors") (Figure 1B). After fibroblasts were transducted and the selection system was applied, the authors observed resistant cells that exhibited similar characteristics to ES cells as they turned on the expression of pluripotent cell markers. These reprogrammed cells, named induced Pluripotent Stem (iPS) cells, could differentiate into all three germ layers *in vitro* and formed teratomas after injection [12,15]. This confirmed their pluripotent characteristics. These studies showed that the over-expression of the three "Yamanaka factors" can induce pluripotency in somatic cells, indicating that Oct4, Sox2,

Klf4 and c-Myc regulate the developmental signaling network necessary for ES cell pluripotency and are sufficient to revert the developmental fate of differentiated cells.

Moreover, the transgenes that encode these factors only need to be expressed when iPS cells are being generated. After the cells are established, the endogenous genes become activated and the pluripotent capacity is only dependent on the endogenous gene expression, suggesting that iPS cells have undergone an almost complete epigenetic reprogramming. Other recent studies showed that reprogramming of mouse adult Neural Stem cells (multipotent stem cells) to an iPS state is possible using only Oct4 and Klf4 [16] or Oct4 alone [17, 18]. Direct reprogramming of adult stem cells by one single factor is important as it avoids expression of the oncogenes Klf4 and c-Myc.

A better understanding of the reprogramming processes and how epigenetic memory is established can reduce the chances of generating tumorigenic cells and consequently enhances the utility of iPS cells for a future use in cell therapy.

2.3 Cell fusion-mediated reprogramming

Cell fusion is an important physiological process to development and organ formation [19]. Fusion between two identical cells (homotypic fusion) has been shown to occur during the formation of myotubes [20], osteoclasts and placenta [21]. Also, multinucleated cells are generated by cell fusion during immune responses against chronic infections [19] and during other pathological processes.

The first evidence that silenced genes can be activated in differentiated mammalian cells by cell fusion was obtained by Blau et al. in 1983 who produced fusion-derived multinucleated cells which did not proliferate [22, 23] (Figure 1C). These hybrid cells, named heterokaryons for containing two genetically distinct nuclei, were obtained by fusing mouse muscle cells and human amniotic cells. By promoting the fusion between cells of different species it was possible to distinguish the gene products of each nucleus independently and therefore nuclear reprogramming could be assessed. The observations led to the conclusion that several human muscle proteins were expressed in the resulting hybrids indicating that muscle genes were activated in non-muscle cells [22]. Further studies using the same model confirmed that DNA replication was not required for the reprogramming process [24]. In contrast, the DNA methylation status was proved to be crucial for the reprogramming outcome, giving the first evidence for the involvement of epigenetic changes in the process.

These initial studies with heterokaryon hybrids provided evidence for the role of cell fusion in changing the expression profile of a differentiated cell and presented very clear evidence for nuclear plasticity. However the formation of heterokaryons does neither allow the study of long-term changes in the expression pattern of the hybrids nor the study of their fate after reprogramming. Also, these reports focused on lineage reprogramming mechanisms but did not address pluripotency reprogramming. Thus, several other research groups focused on the fusion between two cells of the same species in order to form hybrids in which the nuclei fuse to form a tetraploid synkaryon cell (Figure 1C).

Tada et al. initially showed *in vitro* that after the fusion between thymocytes from adult mice and Embryonic Germinal cells the resulting synkaryon hybrid does no longer express the somatic specific markers [25]. It was therefore postulated that a reprogramming event had happened. Furthermore, the fused tetraploid cells were pluripotent as they could contribute to the three germ layers in chimaeric embryos. This was the first demonstration of nuclear reprogramming of somatic cells in proliferative hybrids after cell fusion.

The same research group went on to demonstrate that somatic cells could be reprogrammed into a pluripotent state after being fused with ES cells [26]. The authors found that after hybrid formation between female mice thymocytes and ES cells, the genes on the inactive X chromosome were turned on as well as the Oct4 promoter. This shows that the somatic genome has been reprogrammed to a pluripotent state. Further epigenetic studies revealed that the reprogrammed somatic genome has the typical epigenetic markers of a pluripotent cell confirming that the epigenome was converted to a pluripotent state [27]. The extension of this research to human cells was achieved by Cowan et al. [28].

Furthermore, studies developed by Terada et al. using mouse Bone Marrow (BM) cells showed that after cell fusion with ES cells, BM cells adopt the phenotype of pluripotent cells [29] and form tetraploid hybrids that can differentiate into different lineages (Figure 2A). In a similar study, Ying et al. were able to reprogram multipotent Neural Stem (NS) cells, in the form of neurospheres, by cell fusion with ES cells [30]. The NS cells used held a construct carrying the GFP (Green Fluorescent Protein) and the puromycin resistance genes under the control of an Oct4 promoter. As Oct4 is not expressed in multipolar cells such as NS cells, only if fusion is followed by reprogramming do the hybrids survive (Figure 2B). The authors found that the cells surviving to the selection express GFP, resemble ES cells in their expression profile pattern and also carry a transgenic marker derived from the ES cells. They conclude that the altered phenotype does not arise by the direct conversion of NS cells to ES cells but rather by the generation of synkaryon hybrids [30]. By fusing either karyoplasts or

cytoplasts of ES cells with neurosphere cells, a further study by Do et al. showed that the Oct4 reprogramming capacity resides only in the karyoplasts of ES cells and that cytoplasts lack this ability [31].

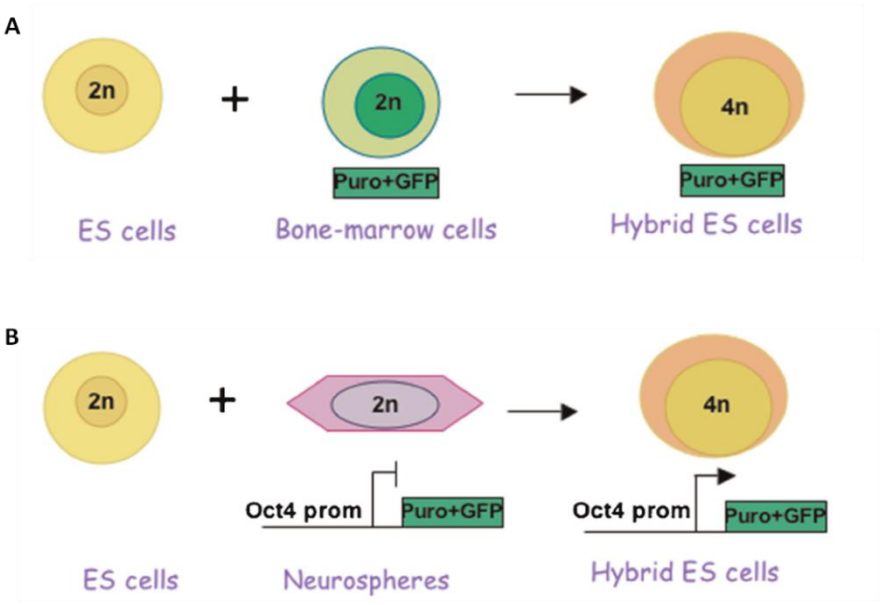

Figure 2. Experimental procedures used to demonstrate reprogramming through cell fusion *in vitro*. Co-culturing of ES cells with Bone Marrow cells expressing GFP and the puromycin resistance gene, promotes tetraploid reprogrammed hybrids formation, which can be detected after selection (A). Fusion between ES cells and neurospheres, that contain the Oct4 promoter (turned off) regulating the expression of GFP and puromycin resistance genes, leads to the activation of Oct4 in the neuronal genome. These hybrids exhibit pluripotent characteristics and are resistant to puromycin selection (B). [adapted from reference 32]

Through a cell fusion-mediated mechanism the genome of the somatic cell will be reprogrammed back to a more undifferentiated developmental state in a process that involves transcription regulation, re-activation and downregulation of specific genes and epigenetic changes [29, 30, 33]. As explained before, several studies have reported that the tetraploid hybrid cells which result from a fusion between an ES cell and a somatic cell have the typical characteristics of ES cells although they also carry the somatic genome. These hybrids express stem-specific molecular markers (such as Oct4, Nanog and Sox2) but not the somatic cell specific genes, they have a continuous self-renewing capacity and are able to differentiate into all the tissues originated by the three different germ layers, among other features [see reference 34 for extensive review].

Several research groups have tried to depict the signaling pathways and genes that enhance reprogramming of somatic cells after cell fusion. Silva et al. demonstrated that overexpression of Nanog substantially enhances nuclear reprogramming by cell fusion [35]. More recently,

Lluis et al. showed that the activation of the Wnt/β-catenin pathway in ES cells enhances the ability of these cells to reprogram different somatic cell types by a Polyethylenoglycol (PEG)-mediated fusion [33] (Figure 3). In addition, the ERK/MAPK pathway, which is known to regulate the pluripotency of ES cells [36], was also shown to play an important role during the reprogramming process [37]. Furthermore, the deletion of the Tcf3 gene in ES cells was shown to enhance the reprogramming efficiency of Neural Stem cells [38]. Tcf3 is known to be a repressor of β-catenin target genes and therefore acts as a negative regulator of pluripotency pathways [39]. Consequently the number of reprogrammed cells could be increased if ES cells in which the Tcf3 gene was knocked out were used for the fusion experiments [38].

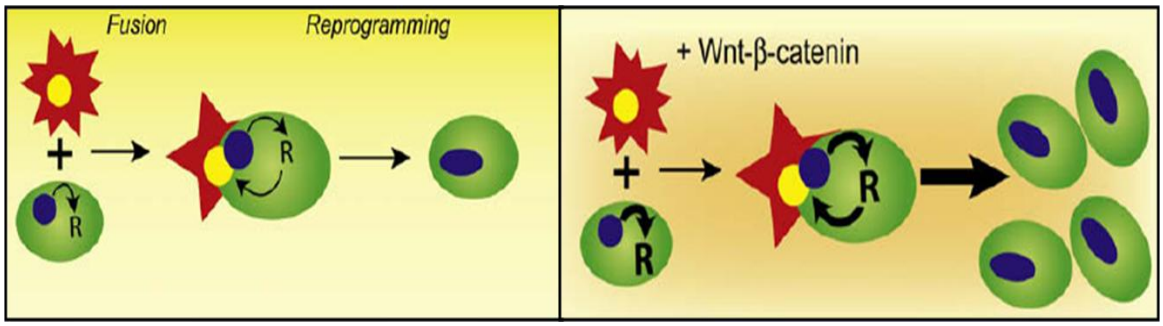

Figure 3. Enhancing reprogramming by the activation of the Wnt/β-catenin pathway. The reprogramming capacity (R) of Embryonic Stem (ES) cells (green cell) can convert the fate of a somatic differentiated cell (red cell) into a pluripotent state after fusion (left panel). The activation of the Wnt/β-catenin pathway in the ES cells, directly with Wnt or with the GSK-3 inhibitor BIO, enhances this capacity, generating pluripotent cells with higher efficiency (right panel). [adapted from reference 40]

The three experimental models used to assess nuclear reprogramming provide evidence that highly specialized somatic cells in mammalian tissues retain all the genetic information that is needed to revert their fate to an ES-like state and that these genes have not been permanently inactivated during development.

3. Reprogramming and Regeneration

As mentioned above, adult stem cells are the main contributors for tissue repair and can be activated from their dormant state to repopulate the tissue. Yet it is also possible that transdifferentiation and/or cell fusion events occur that contribute to organ regeneration if the adult stem cells resident in the affected tissue are compromised and are not able to progress

into a regenerative process [32]. For instance, BM-derived cells can reach affected areas and transdifferentiate into the required cell type or fuse with the damaged cells leading to reprogramming and subsequent differentiation [41] (Figure 4).

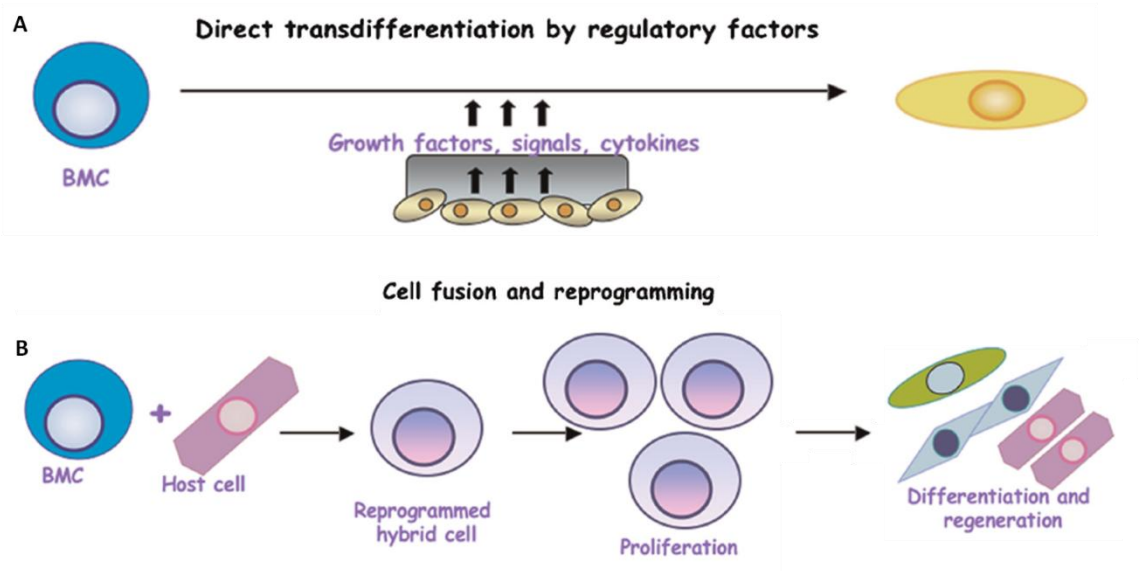

Figure 4. Direct transdifferentiation *versus* cell fusion during regeneration. A tissue-specific adult cell, such as a Bone Marrow Cell (BMC) can directly transdifferentiate into a somatic cell of a distinct lineage by the action of growth factors or cytokines or by the activation of signaling pathways. The transdifferentiated cell may contribute to a repair mechanism in an affected organ (A). On the other hand, adult stem cells of a specific lineage (BMC for instance) can fuse with somatic cells (host cell) in a damaged organ and reprogram their genome back to a more "potent" state, generating tetraploid hybrid cells. These can proliferate and differentiate into distinct cell types possibly leading to the regeneration of the affected tissue (B). [adapted from reference 32]

3.1 Transdifferentiation and Regeneration

Transdifferentiation consists in the conversion of a cell type of a given lineage into a cell of a distinct lineage. During the process, the expression of tissue-specific markers of the original cell type is lost and the cell acquires the markers and features of the new transdifferentiated cell type [1]. Therefore it is considered that the genome of the initial cell type is reprogrammed to be able to express the features of the transdifferentiated one [42].

According to the hypothesis that links transdifferentiation to organ regeneration, after damage the regulatory factors that are released in the affected organ may trigger the recruitment of a cell from a different compartment into the affected tissue. Being present in a different environment, this cell could change its fate, transdifferentiate and therefore contribute to the regeneration of that organ (Figure 4A). Several studies have addressed this

hypothesis by showing that BM cells can transdifferentiate into neurons, skeletal and cardiac muscle cells [43] and also into liver and kidney epithelial cells [44-46]. Moreover, the transdifferentiation of exocrine pancreatic cell into beta-cells by the induction of three specific factors was shown to occur without reversion to a pluripotent stem cell state [42]. This last study provides an important advance in the regeneration field as it shows a direct transdifferentiation mechanism into insulin-producing cells.

3.2 Cell fusion and regeneration

Another process that is proposed to contribute to tissue repair is cell fusion. As explained before, cell fusion is an important physiological process that is involved in several development processes [19-21]. In the case of organs such as muscle and liver, cell fusion happens between two cells from the same lineage (homotypic fusion) and is involved in the development and repair of these tissues [47]. Remarkably, it was also demonstrated that two cells from different lineages can fuse (heterotypic fusion) and that the resulting hybrid acquires new characteristics that could be more similar to one of the fusion partners [22, 23]. This shows that one of the original cells has a dominant phenotype over the other [48]. This exciting feature has also been shown to happen between a fully differentiated somatic cell and a less differentiated, more plastic cell type such as a multipotent cell (like a BM stem cell) or a pluripotent stem cell [26-33, 34 for review].

These latter studies reported that the genome of the multipotent or pluripotent cell can be dominant over that of the somatic cell after fusion. This results in a reprogrammed hybrid that, although containing the genomic information of the somatic cell, is phenotypically similar to a more plastic cell type and thus is capable of differentiating in distinct cell types (Figure 4B).

Even though it is still not clear that cell fusion-mediated reprogramming can occur in a physiological environment during tissue regeneration in mammals, over the last years new findings suggest that some cells have the capability to reprogram affected cells in damaged tissues by a cell fusion mechanism [49-58].

Gibson et al. initially suggested that many cases thought to be due to transdifferentiation could instead be cell fusion-related [49]. The authors implanted mouse dermal fibroblasts into mice that lack dystrophin. After a few weeks, dystrophin-positive fibers were identified in the muscle and the conversion of dermal fibroblasts into hybrid myogenic cells was confirmed by the analysis of host and donor markers [49]. In other studies, BM cells were injected in mice

in which muscle degeneration was previously induced. These cells seemed to be recruited to the damaged area where they underwent fusion restoring the muscle function [50]. BM cells have also been shown to contribute to the regeneration of other organs by fusing with cells such as the intestinal epithelium cells [51], perycites and cardiomyocytes in the heart [52, 53] and even Purkinje neurons in the brain [54,55].

To demonstrate the contribution of BM cells through cell fusion, Alvarez-Dolado et al. developed a Cre-loxP system that allowed to clearly distinguish transdifferentiation from cell fusion events [54]. In this study, BM cells expressing the CRE recombinase and GFP were transplanted into irradiated R26R mice, which express the LacZ reporter only when there is the excision of the loxP sites. This allows to distinguish fused cells ($LacZ^+/GFP^+$) from transdifferentiated ($LacZ^-/GFP^+$) cells. Spontaneous cell fusion of BM cells with hepatocytes, Purkinje cells and cardiomyocytes was detected [54]. Strikingly, the results obtained with Purkinje cells were confirmed by Weimann et al. who showed that human BM cells can fuse with neurons in patient affected brains [55]. In further studies using a mouse model, it was proposed that hematopoietic cells can form heterokaryons with Purkinje cells under chronic inflammation conditions, leading to the activation of endogenous neuron-specific genes in the hematopoietic nuclei [56]. This observation is consistent with the hypothesis that after fusion, the nuclei of BM-derived cells can undergo reprogramming to a neuron fate.

Cell fusion has also been shown to be an important mechanism for hepatocytes regeneration. Most of the available studies focused on a model of mouse liver regeneration in which the host hepatocytes lack the Fah gene, which encodes a fundamental enzyme (FAH - Fumaryl Acetoacetate Hydrolase). The mice were transplanted with Fah+/+ hematopoietic cells and fusion events between donor and host cells were recognized, forming hybrids that exhibited an expression profile similar to functional hepatocytes [57, 58]. These reprogrammed hybrids were able to synthesize the FAH enzyme and could repopulate other livers.

Even though cell-fusion mediated reprogramming may play a role in tissue regeneration, the mechanism by which the cells fuse and the actual trigger of the fusion process are still largely unknown. However, according to results obtained with cardiomyocytes [59] and with Purkinje cells [56], pro-apoptotic factors may increase the rate of reprogramming after cell fusion.

In conclusion, it has to be considered that both cell fusion and transdifferentiation processes contribute to the regeneration of damaged tissues and also that these processes can either take place independently or one after the other. For example, BM-derived cells may

translocate to a damage area, fuse with resident cells, reprogram their genome and consequently confer the ability to transdifferentiate. In this way the resulting hybrids could gain a proliferative capacity to repopulate the tissue [60].

4. Fate of Reprogrammed Hybrids

Either *in vitro* or *in vivo* one of the main questions related to the nature of the tetraploid hybrid cells upon cell fusion-mediated reprogramming is how do they proliferate and how their mitosis are regulated. The study of the reprogrammed cells fate is important in order to have a good characterization of the hybrids and to assure that their proliferative features are compatible with cell therapy approaches.

Some studies have addressed *in vivo* this question mainly on a liver regeneration model by following hepatocytes lacking the FAH enzyme (in Fah-/- mice) after transplantation of Fah+/+ cells [58, 61, 62]. By transplanting female Fah+/+ LacZ BM cells into male Fah-/- mice it is possible to identify the hybrids because only the fusion-derived hepatocytes have a functional FAH enzyme allowing them to repopulate a secondary liver [61]. It is also possible to identify the hybrids by the presence of host and donor markers. Since normal liver cells have a tendency to be polyploid (4n, 8n, etc) it is expected that after the injection of BM cells in the liver, fusion-derived hepatocytes are minimally tetraploid. Interestingly the authors found that among the expected tetraploid (4n, XXXY) and hexaploid (6n, XXXXYY) cells, there were also diploid cells (2n, XY) that had the cellular markers of both parental cells (Figure 5). To facilitate the genetic analysis of fusion-derived hepatocytes, the authors performed the same experiment using the Cre-loxP system. They transfected R26R Fah+/+ Bone Marrow cells in Fah-/- recipient mice expressing the Cre recombinase exclusively in hepatocytes (Alb-Cre mice). In this case β-gal is only expressed if fusion occurs. The authors confirmed the previous observations and revealed that a fraction of the diploid cells expressed both β-gal and FAH [61]. This led to the conclusion that fusion-derived hepatocytes undergo ploidy reduction events generating diploid hepatocytes that express markers from the two parental cells.

It was then hypothesized that some of the tetraploid hybrid cells may undergo a specific mitosis, called “reduction mitosis”, which originates stable diploid cells [61]. Similar to their tetraploid counterparts, these diploid cells have a functional FAH enzyme re-establishing the normal activity of the hepatocytes. The described reduction mitosis of the tetraploid cells is a mechanism through which instead of the typical bipolar mitosis, the cells likely undergo a

multipolar mitosis due to their excessive number of centrosomes and chromosomes. In case the resulting cells have a correct set of chromosomes they can continue to proliferate.

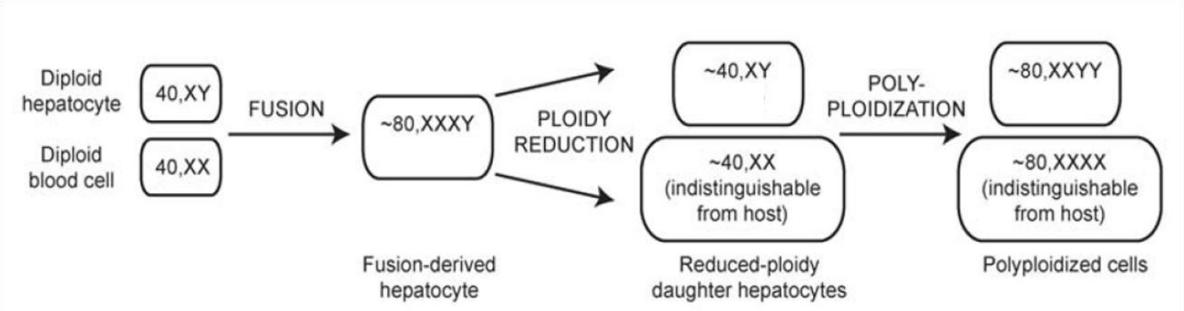

Figure 5. Ploidy reduction of hepatocyte-BM cell hybrids. Fusion between a female diploid BM cell and a male host diploid hepatocyte (40 chromosomes each) leads to the formation of a tetraploid hybrid that can undergo ploidy reduction events and originate diploid cells (40, XX and 40, XY). These reduced-ploidy daughter hepatocytes can then polyploidize and form again tetraploid cells (80 chromosomes) [adapted from reference 61]

Although it is still unclear what the mechanisms underlying the formation of fusion-derived diploid cells are, recent results from the same authors provided some viable hypothesis. Through live-imaging experiments they demonstrated that nearly 90% of tetraploid hepatocytes divided in a bipolar manner originating viable daughter cells. Among these, several tetraploid cells transitioned, before division, from a multipolar intermediate to a standard bipolar configuration [62]. This observation is in agreement with previous work by Ganem et al. on multi-centromeric cancer cell lines, who hypothesized that multipolar spindles could represent a transitory step in mitosis before the organization of a bipolar spindle [63]. Nevertheless, in addition to standard divisions, Duncan et al. observed tripolar divisions (around 3% of total) and rare double divisions originating four distinct diploid nuclei by two synchronized mitosis [62]. Thus it appears that there are different possibilities by which a tetraploid cell can undergo ploidy reduction, originating viable cells with half the DNA content. The results also suggested that reduced-ploidy daughter cells arise through a single mitotic event that happens one or two cell cycles after fusion.

Reduction mitosis is a rarely described and poorly understood phenomenon that was initially shown by Lindahl in 1953 [64] and later by Martin et al. [65] and Pera et al. [66]. The process may be similar to “somatic meiosis” which was already shown in non-mammal organisms [67]. However, ever since these initial studies, there have been very few publications directly concerning ploidy reduction.

Since reprogramming by cell fusion can constitute an essential mechanism at the basis of tissue regeneration, it is of utmost interest to study whether ploidy reduction is a consequence

of all fusion-mediated reprogramming processes or a rather random event. Finally, in a biomedical regeneration context, it is important to understand if cell fusion-mediated reprogramming can be an efficient mechanism to regenerate affected tissues and to further investigate ways to enhance the capacity of multipotent stem cells, such as BM stem cells, to reprogram terminally differentiated somatic cells.

II. OBJECTIVES

The main objective of the present study is to characterize the fate of tetraploid hybrid cells originated by cell fusion-mediated reprogramming and to describe the mitotic events that these cells undergo. To reach this objective, the following goals are addressed:

1) to reprogram Neural Stem cells back to a pluripotent state by promoting spontaneous cell fusion with Embryonic Stem cells;

2) to analyze the chromosome content of the fusion hybrid cells by cytogenetic methods and depict their pluripotent characteristics;

3) to investigate the subsequent behavior of the hybrids in culture and study their karyotype to look for evidences of ploidy reduction.

The characterization of fusion-derived hybrid cells, including their destiny and regenerative capacity, is an important step to understand the role of cell fusion-mediated reprogramming during tissue repair and may contribute to future biomedical applications.

III. MATERIALS AND METHODS

Cell Culture

Neural Stem (NS)-Oct4-puro cells which carry the regulatory sequences of the mouse Oct4 gene driving GFP and puromycin resistance genes were a gift from Dr. A. Smith. They were cultured in T25 flasks pretreated with 0,01% poly-ornithine (Sigma) and 1mg/ml laminin (Sigma) and grown in ESGRO medium (Millipore) supplemented with 0,05µg/ml EGF and 0,01µg/ml bFGF according to previous works [68].

Embryonic Stem (ES) cells were cultured on gelatin (0.1% in PBS) in knockout Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% heat inactivated fetal bovine serum (HyClone), 1X penicillin-streptavidin (Sigma), 1,2mM Sodium Pyruvate (Gibco), 2,4mM L-Glutamine (Gibco), 1X MEM non-essential amino acids (Gibco), 0,1mM 2-mercaptoethanol (Gibco) and 1000 U/ml mLIF ESGRO (Millipore). ES cells where the Tcf3 gene was knocked out (ES Tcf3-/-) were a gift from Dr. B. Merrill.

Both cell lines were kept in incubators at 37ºC and 5% CO_2.

Fusion Experiments

Fusions between NS-Oct4-puro and ES wild-type (wt) or ES Tcf3-/- cells were performed as follows: 1 x 10^6 NS cells were plated into T25 flasks with NS medium; after 2 hours 1 x 10^6 ES cells were plated onto them, allowing ES cells to attach to the NS cells; after a further 2 hours the medium was removed and replaced by ES complete medium; finally the cells from each T25 flask were trypsinized and re-cultured in 5 p100 pre-treated dishes to have a diluted population in each p100. In order to select the reprogrammed hybrids, 1µg/ml puromycin was added after 72 hours directly into ES medium.

The fusions between ES wt and NS Oct4-GFP-puro cells were performed with or without the pre-activation of the Wnt/β-catenin pathway in ES cells by 6-bromoindirubin-3'-oxime (BIO) before co-culturing. Around 250.000 ES cells were plated in pre-gelatinized p60 dishes and cells were treated for 48, 24 and 12 hours with 1µM BIO. Before fusion, cells were trypsinized, counted and 1x10^6 ES cells were co-cultured with NS cells. The fusion protocol was followed as described before.

Alkaline Phosphatase staining

Seven days after selection, p100 dishes were washed once with cold PBS 1X and cells were fixed in 10% Neutral Formalin Buffer (NFB) for 15 minutes at 4ºC. Then, the dishes were rinsed with distilled water and kept with water for further 15 minutes. The dishes were incubated for 45 minutes at room temperature with a solution consisting of: 0,005g Naphthol AS MX-PO_4 (Sigma), 200µl DMF (N,N-Dimethylformamide) (Fischer Scientific), 25 ml Tris-HCl pH=8,3, 25ml distilled water and 0,03g Red Violet LB salt (Sigma). Finally, the dishes were rinsed again in distilled water 3-4 times and left in distilled water until the staining started to be visible and Alkaline Phosphatase-positive colonies could be counted.

Clone selection

The dishes were observed regularly and some days after fusion the ES-like clones were picked individually, trypsinized and finally plated into gelatinized 96-wells plates so that each clone could grow independently. The clones were always grown in ES medium supplemented with 1ug/ml puromycin to maintain the selective pressure onto the reprogrammed hybrids.

In order to obtain a pool population of hybrids from each fusion experiment, the clones in one p100 were trypsinized, re-cultured into a new p100 and kept in ES medium with 1ug/ml puromycin over the subsequent passages.

Karyotype Analysis

Cells were trypsinized and around 1 x 10^6 cells were plated in a p60 gelatinized dish. The next day the medium was changed and after 3 hours cells were arrested in metaphase by adding 0,15µg/ml colcemid (Gibco) for 3 hours at 37ºC, 5% CO_2. Then the cells were trypsinized, collected in medium, pelleted, resuspended for 6 minutes at room temperature in 5ml of pre-heated KCl (6,4g/L) added dropwise and then fixed in cold 3:1 methanol/acetic acid. Finally cells were dropped on slides to create chromosome spreads. Slides were stained with 4% Giemsa (Fluka) in K_2HPO_4 (3,4g/L, pH=6,8) for 15 minutes, rinsed twice with distilled water, let dry and assessed with light microscopy. The images were then analysed by Adobe Photoshop CS3 (Adobe).

FACS analysis and sorting

To FACS (Fluorescence-Activated Cell Sorting) sort the reprogrammed GFP-positive cells the pool population was trypsinized and collected in ES medium. After centrifugation the pellet of cells was resuspended in ES medium at a concentration of 2 x 10^6 per ml. Cells were then filtered before FACS acquisition to exclude aggregates from the analysis. The sorting was performed in a FACSAria II SORP (Becton Dickinson). The gating strategy is described on Annex III. Briefly, doublets and aggregates were excluded from the analysis on the basis of pulse width; dead cells were excluded on the basis of 1µg/ml Propidium Iodide (PI) incorporation; the GFP signal was identified using a 488 nm laser and a 525/50 nm bandpass filter; the purity of the sorted populations was determined after each sorting and only high purity populations (>99,9% purity) were used for subsequent experiments. The populations of interest were sorted and collected in complete ES medium in order to plate the cells or directly on ethanol 70% to perform a PI staining and analyze the cell cycle profile (see below).

Single-cell FACS sorting

In order to obtain clones derived from single cells, after fusion cells were trypsinized, resuspended in ES medium and single-cell sorted into pre-gelatinized 96-wells plates using a FACS AriaII SORP (Becton Dickinson). Only alive cells (PI-negative) were sorted as shown in the gating strategy (Annex V). In this way each well contains only one cell. The clones obtained were grown in ES medium supplemented with 1µg/ml puromycin (except on the first day, when the concentration was 0,5µg/ml to avoid high stress conditions).

Cell cycle profile

To analyze the cell cycle profile, the population of interest was trypsinized and 1 x 10^6 cells were centrifuge and then resuspended twice in PBS 1X. After the last centrifuge the pellet was resuspended with 300 µl of PBS 1X and 700 µl of absolute Ethanol added dropwise. The solution was kept on ice for 2 hours. Finally the cells were centrifuge at 4.4rpm, washed twice with PBS and the pellet was resuspended in a working solution which consists of 970 µl of PBS 1X, 15 µl of RNAse (20mg/ml) and 15 µl of Propidium Iodide

(1mg/ml). The solution was kept at 4ºC over night and then the cells were examined in a FACScan analyzer (Becton Dickinson) with a 488nm laser and a 585/42 bandpass filter.

Live-cell imaging

GFP-positive fusion derived hybrids were FACS-sorted using the same gating strategy as before (Annex III). After sorting, cells were plated on a pre-gelatinized coverslip (WillCo Dish) with complete ES medium and two hours later the plate was placed under the microscope. Live-cell imaging was performed with an automated widefield fluorescence microscope Zeiss Cell Observer HS with controlled environment chamber (37ºC, 5% CO_2). Images were collected every 15 minutes over sessions lasting 8 to 12h. Cellular structures were visualized with DIC (0,05s exposure) and chromosomes visualized with Hoechst 33342 (0,8s exposure). Only isolated cells were considered for the analysis. Cells were stained with 15µg/ml Hoechst 33342 (30 minutes of incubation at 37ºC) prior to the sorting so they could keep the staining during cell culture. The focal plane was set at the beginning of each imaging session in each plate position. Images were treated and time-lapse movies were formatted using ImageJ 1.43 and exported in QuickTime (Apple) for further visualization.

IV. RESULTS AND DISCUSSION

1. Reprogramming of Neural Stem cells by fusion with Embryonic Stem cells

Fusion between Embryonic Stem (ES) cells and Neural Stem (NS-Oct4-puro) cells was performed as previously described [30, 33]. In our fusion experiment, we used NS cells carrying the GFP (Green Fluorescent Protein) and puromycin resistance genes under the control of an Oct4 promoter. Since NS cells do not express Oct4, the promoter is normally turned off. NS cells will only express puromycin resistance if their genome is reprogrammed to a pluripotent state meaning that the Oct4 promoter was turned on.

Therefore, to exclude the non-fused parental cells and to exclusively select reprogrammed hybrids, puromycin was added to the ES culture medium. Furthermore, both fetal bovine serum and LIF (Leukemia Inhibitory Factor), components of the ES culture medium, induce differentiation and growth arrest on NS cells and both NS and ES cells do not resist in medium with puromycin. On the other hand, fused cells that do not undergo reprogramming and do not activate the NS Oct4 promoter should also die shortly after fusion. The only option for a cell to resist in these conditions is if fusion between a NS and an ES cell results in reprogramming of the NS genome and consequent activation of the Oct4 promoter (see Annex I).

ES cells were able to reprogram NS cells originating Alkaline Phophatase (AP)-positive clones, an early marker of pluripotency (Figure 6). Furthermore, fusions using wild-type (wt) ES cells in which the Wnt/β-catenin pathway was activated with the glycogen synthase kinase-3 (GSK-3) inhibitor 6-bromoindirubin-3'-oxime (BIO) result in a higher number of reprogrammed clones compared to the non-treated ES cells. GSK-3 is a known repressor of β-catenin that promotes its degradation in the cytosol and inhibites its translocation into nucleus. Thus the effect of BIO is to activate the Wnt/β-catenin pathway by preventing β-catenin degradation. As shown in Figure 6A and 6B, the higher percentage of reprogramming, addressed by the number of AP-positive clones, is obtained in fusions in which the ES cells were pre-treated with BIO for 24 hours. Namely there is a 9-fold increase in the number of reprogrammed clones with respect to the control (non-BIO treatment). This observation resembles the results obtained by Lluis et al. [33] and shows that the periodic activation of the Wnt/β-catenin pathway by BIO increases the ability of these cells to reprogram NS cells after fusion.

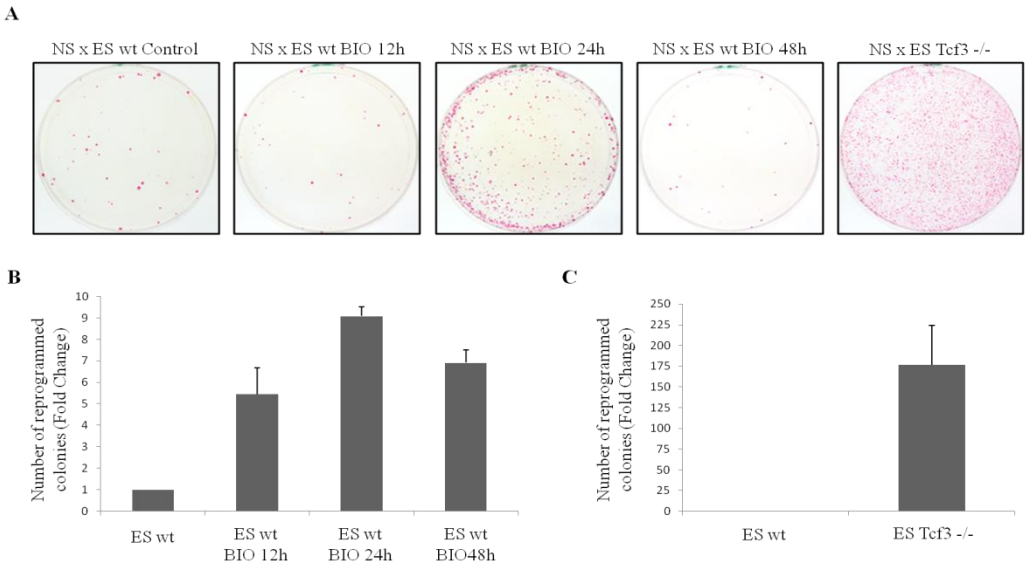

Figure 6. Fusion between ES and NS cells originates reprogrammed clones. Representative plates of hybrid colonies from fusion of NS-Oct4-puro cells with ES wt cells pre-treated with BIO, as indicated, or with ES Tcf3-/- cells. Colonies were stained for AP expression (A). Quantification of the AP-positive colonies (as fold increase in the number of colonies) counted after fusion of NS-Oct4-puro with ES wt cells pre-treated with BIO as indicated (B) or with ES Tcf3-/- cells (C) (average ± SEM; n=2).

Furthermore, we performed the same experiment using ES cells in which the Tcf3 gene is knocked out (ES Tcf3-/-). The TCF3 protein is known to be an inhibitor of the Wnt/β-catenin pathway targets and it was previously shown that the deletion of the Tcf3 gene in ES cells enhances the reprogramming ability after fusion with NS cells *in vitro* [38]. Our results sustain this observation, as fusions between ES Tcf3-/- and NS cells result in a 170-fold increase in the number of reprogrammed clones (AP-positive clones) compared with the fusions between ES wt and NS cells (Figure 6A and 6C). Since these results resemble the ones obtained by Lluis et al. [33, 38], we decided to follow our study using reprogrammed clones from fusions between NS and ES Tcf3-/- cells in order to have a higher number of hybrid clones to work with.

2. Clone selection and characterization

Fusions between ES Tcf3-/- and NS-Oct-puro cells were performed as before. After the selection system was applied, surviving clones that resembled ES clones were picked individually and grown independently. ES-like clones proliferate as ES cells in culture, have similar morphologic characteristics than ES cells and express the Oct4-GFP-puro transgene (Figure 7A). Fusion derived clones retain pluripotent characteristics in culture and remain

highly proliferative. Signals of senescence were still not observed. However, there was morphological variation among the reprogrammed clones (Figure 7B, 7C and 7D). While some clonal lines had a morphology similar to ES Tcf3-/- cells, characterized by rounded colonies with well defined outlines (Figure 7D), others resembled ES wt cells as their outline was more irregular and had more tendency to differentiate (Figure 7C). These differences may reflect the reprogramming level that each clone has undergone, although this was not further investigated.

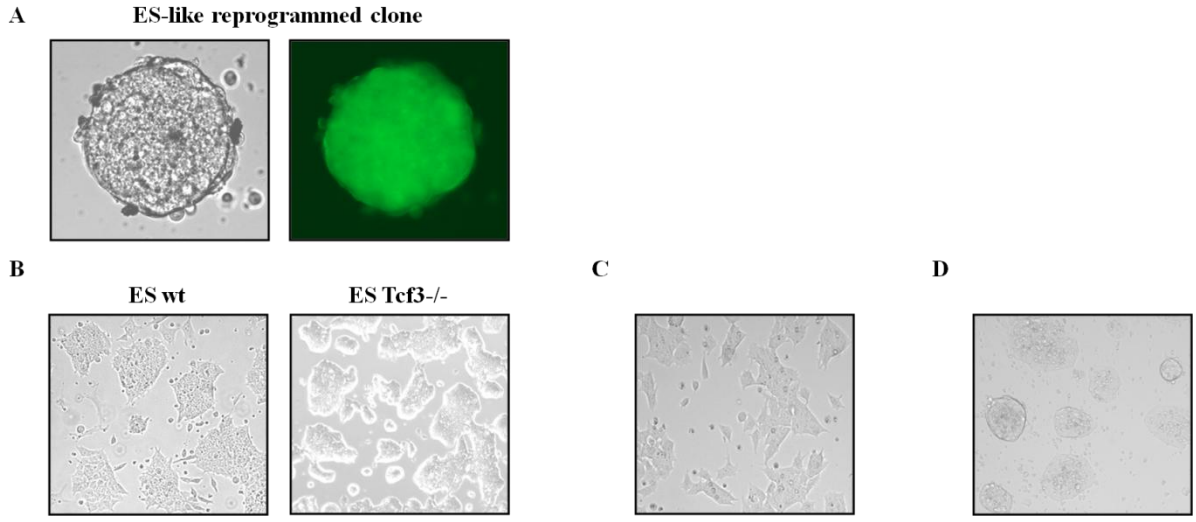

Figure 7. Morphology of reprogrammed hybrids. ES-like reprogrammed clone and GFP expression one week after selection (40x magnification) (A). Representative pictures of colonies formed by ES wt and ES Tcf3-/- in culture (B) and by two reprogrammed clones (C and D) (20x magnification).

3. Karyotype analysis of reprogrammed clones

After some passages the karyotype of the cells in each clone was analysed. For this analysis, we considered reprogrammed clones from three independent fusion experiments between ES Tcf3-/- and NS-Oct-puro cells. As shown in Figure 8 and Annex II, all clones contained tetraploid (80 chromosomes) cells proving their fusion origin. Interestingly, diploid (40 chromosomes) cells were also identified in 26 of 27 clones analysed. No correlation was found between the percentage of diploid cells in a clone and its morphological features. In addition, aneuploidy events were identified both with loss and gain of chromosomes with respect to diploidy and tetraploidy. This observation is in agreement with previous studies in tetraploid cells [69] and well as in ES cells [70]. Actually we observed that the percentage of aneuploidy in tetraploid cells is higher when compared with that of diploid cells, as seen by the high percentage of cells that contain between 76 and 79 chromosomes.

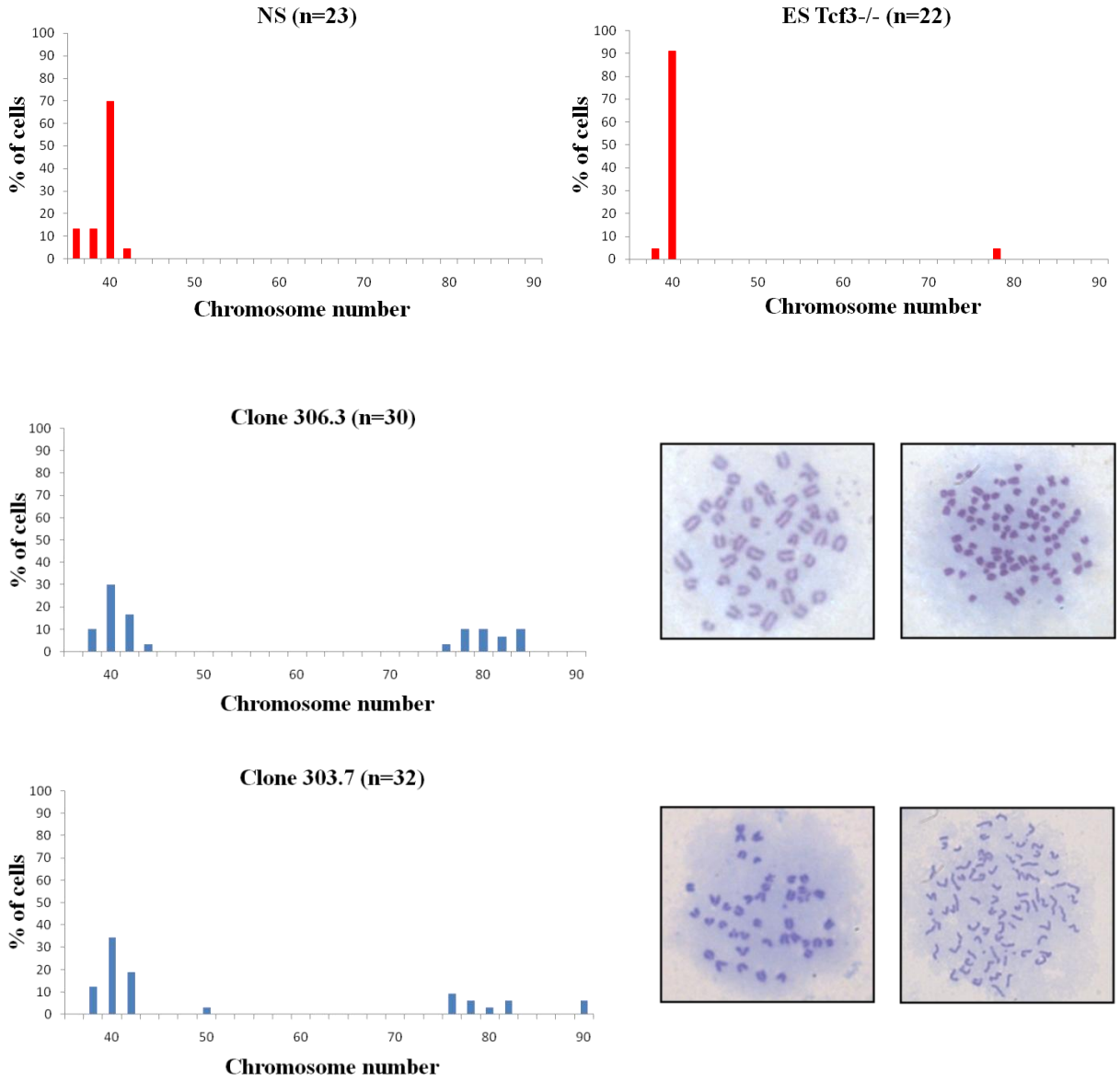

Figure 8. Karyotype analysis of reprogrammed clonal lines. The number of chromosomes was counted for each population after preparation of chromosome spreads. Histograms indicate the percentage of cells with a specific chromosome number from NS and ES Tcf3-/- parental lines (red bars) and from the representative hybrid lines 306.3 and 303.7 (blue bars). Mouse diploid cells contain 40 chromosomes while tetraploid cells contain 80 chromosomes. n is the number of individually scored chromosome spreads analysed for each cell line. Representative pictures are also presented (630x magnification). See Annex I for karyotypes of other clonal lines.

More significantly, we did not observe cells with an intermediate number of chromosomes, between 40 and 80, in 25 out of 27 analysed clones (Table 1). This observation is important as it suggests that diploid cells that arise from fusion-derived cells are not originated by a successive loss of chromosomes experienced by the tetraploid cells. On the contrary, it seems that diploid cells may originate from fusion-derived tetraploid hybrids through a single event, namely a non-conventional mitosis, which would result in more than two daughter cells. If

tetraploid hybrids experience aneuploidy events towards diploidy we would expect to identify more cells with a chromosome number between 40 and 80. As this was observed only in 2 out of 27 clones, we focused our approach in the study of the mitotic strategy adopted by the hybrid cells after fusion.

Table I. Analysis of the chromosome distribution in the reprogrammed clones. (1)

	Only 2n	Only 4n	Between 2n and4n	2n and 4n	Total
Number of Clones identified	0	1	2	24	**27**

(1) Clones were categorized depending on the ploidy of their cells in: clones exclusively containing diploid cells (Only 2n) or tetraploid cells (Only 4n), clones containing more than 10% of cells with an intermediate number of chromosomes (Between 2n and 4n) and clones containing both diploid and tetraploid cells (2n and 4n).

4. Sorting of reprogrammed hybrid cells

In order to confirm that diploid cells can arise from reprogrammed hybrid cells we decided to separate the reprogrammed cells, that is, the cells that express GFP, by Fluorescence-activated cell sorting (FACS) technology. This was done shortly after the selection of puromycin resistant clones. To do this we collected all the surviving clones to form a pool population 8 days after the addition of puromycin to the medium and we analysed the GFP expression of these cells.

When compared to the GFP-negative control (ES Tcf3-/- cells) we observed 91% of GFP-positive cells in the pool population (Figure 9A). The most likely explanation for the existence of GFP-negative cells is that these cells may represent hybrids that are not fully reprogrammed and that start to lose their GFP expression when the Oct4 promoter is no longer activated. Since the turning off of the Oct4 promoter is not immediate, these GFP-negative cells do not die instantly but would probably not survive if maintained further under puromycin selection.

Taking this into account, we decided to follow the proliferation of reprogrammed (GFP-positive) cells in culture in an attempt to identify the presence of diploid cells. Around 500.000 GFP-positive cells were sorted from the pool population according to the gating strategy depicted in the Annex III and their cell cycle profile was followed in the following weeks (Figure 9B-D and Annex IV). The medium was regularly supplemented with

puromycin. Immediately after sorting, all the GFP-positive cells were tetraploid as seen by the Propidium Iodide (PI) staining (Figure 9B).

In agreement with our hypothesis, diploid cells started to be observed 2 weeks after sorting although at first they did not exhibit the typical G1-peak in the cell cycle profile. In that moment, these cells only accounted for 2,83% of all the cells (Figure 9C). On the following weeks the percentage of this diploid population increased up to 3,74% at 4 weeks, reaching 14,64% after 10 weeks of culture (Figure 9D). At this point we could observe a clearer diploid population characterized by a typical G1-peak. This result confirms that diploid cell can indeed be derived from reprogrammed tetraploid cells.

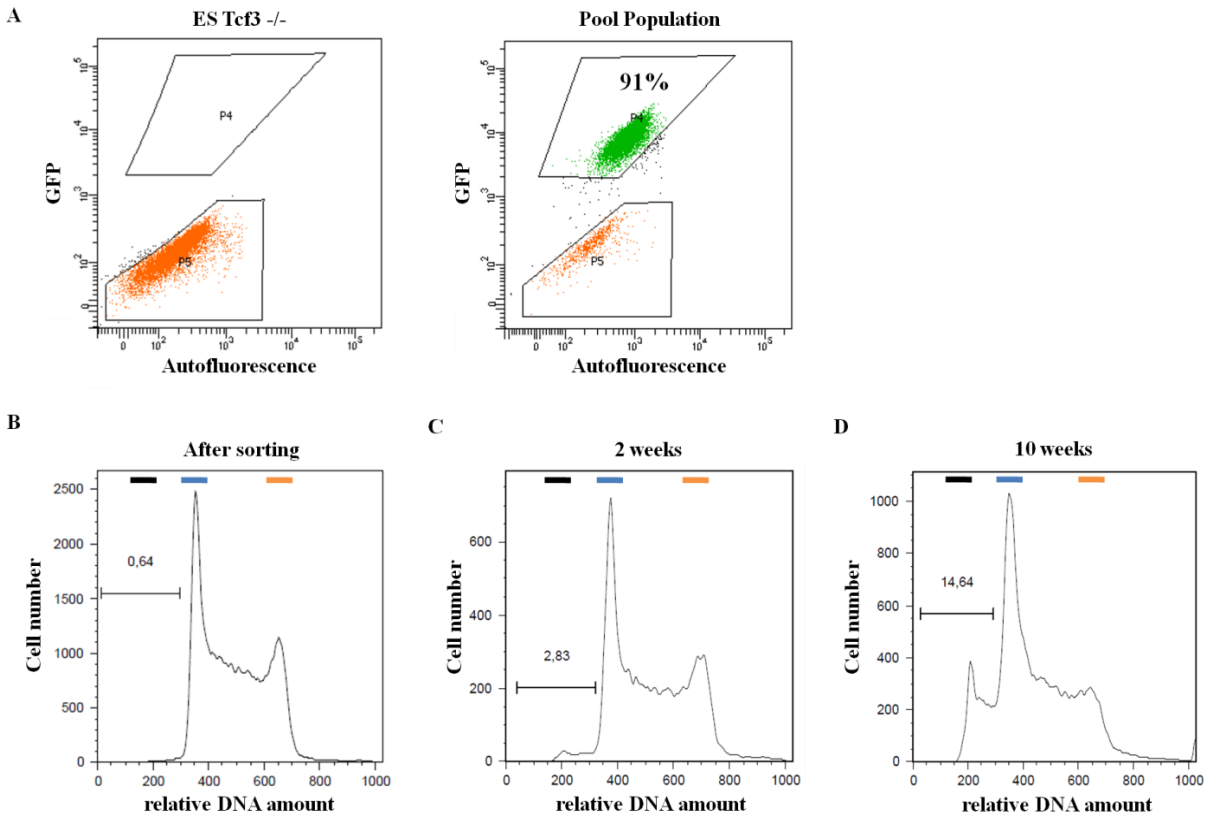

Figure 9. Sorting of reprogrammed hybrid cells. FACS analysis of GFP (Green Fluorescent Protein) expression in the ES Tcf3-/- control population and in the pool population (A). GFP-positive cells (green dots) were FACS-sorted from the pool population according to the gating strategy depicted on Annex II. DNA ploidy shown by the cell cycle analysis immediately after sorting (B), two weeks after sorting (C) and ten weeks after sorting (D) (See Annex III for other time points). Horizontal bars indicate the amount of DNA that corresponds to 2n (black), 4n (blue) and 8n (red) ploidies. Percentages of the marked regions are shown in the plots.

The reason why we could see a diploid population only 2 weeks after sorting could be that at the time of the FACS-sorting the tetraploid cells were already stabilized and could be less

prone to undergo ploidy reduction. Therefore we hypothesize that if ploidy reduction happens it must be a very early event which happens shortly after cell fusion and reprogramming. Further studies are needed to determine the moment when ploidy reduction events begin to occur.

5. Establishment of reprogrammed sub-clonal lines

In order to confirm the previous result we used a FACS-sorting approach to obtain hybrid clones from single cells. In this way, it is possible to analyze what is the progeny of each single reprogrammed cell and what is the ploidy of its daughter cells after several rounds of division. From two independent fusion experiments between ES Tcf3-/- and NS-Oct4-puro cells, we sorted single cells from a pool population around 4 weeks after fusion. Each cell was dropped in an individual well so it could proliferate independently. The pool population was obtained as explain before and at the moment of the sorting contained at least 19% of diploid and 43% tetraploid cells (Figure 10A). PI-positive cells were not collected to exclude dead cells (see sorting strategy in Annex V.A). In this way we created independent "sub-clonal" lines that were kept under puromycin selection.

After growing the surviving single cell-derived sub-clones we analysed their cell cycle profile by PI staining (Figure 10B, 10C and Annex V.B). As expected, we found sub-clones which only consisted of diploid cells and others which only consisted of tetraploid cells consistent with clones resulting from single-diploid and single-tetraploid cells, respectively. The cell cycle profile of these sub-clones is similar to what is expected for ES-like cells, with most of the cells in S phase and very short G1 and G2 phases. This profile was observed in both diploid and tetraploid sub-clones. In total we analysed 90 sub-clones from which 31 (34,4%) were "stable" diploid and 25 (27,8%) were "stable" tetraploid (Figure 10D). We considered a sub-clone as "stable" diploid if at least 95% of the cells were diploid and a similar interpretation was made for tetraploid sub-clones. This limit was set based on the fact that ES cell lines have a percentage of post-G2 (tetraploid) cells which ranges from 2% to 5% (data not shown).

It is important to keep in mind that tetraploid cells normally have a higher rate of aneuploidy than diploid cells. This may result in a higher genomic instability and cell death in the tetraploid cell lines, especially if a tetraploid cell is growing isolated, as was the case here. Thus, this could be the reason why the number of tetraploid sub-clones was lower than what was expected considering the distribution of ploidy in the pool population (Figure 10A).

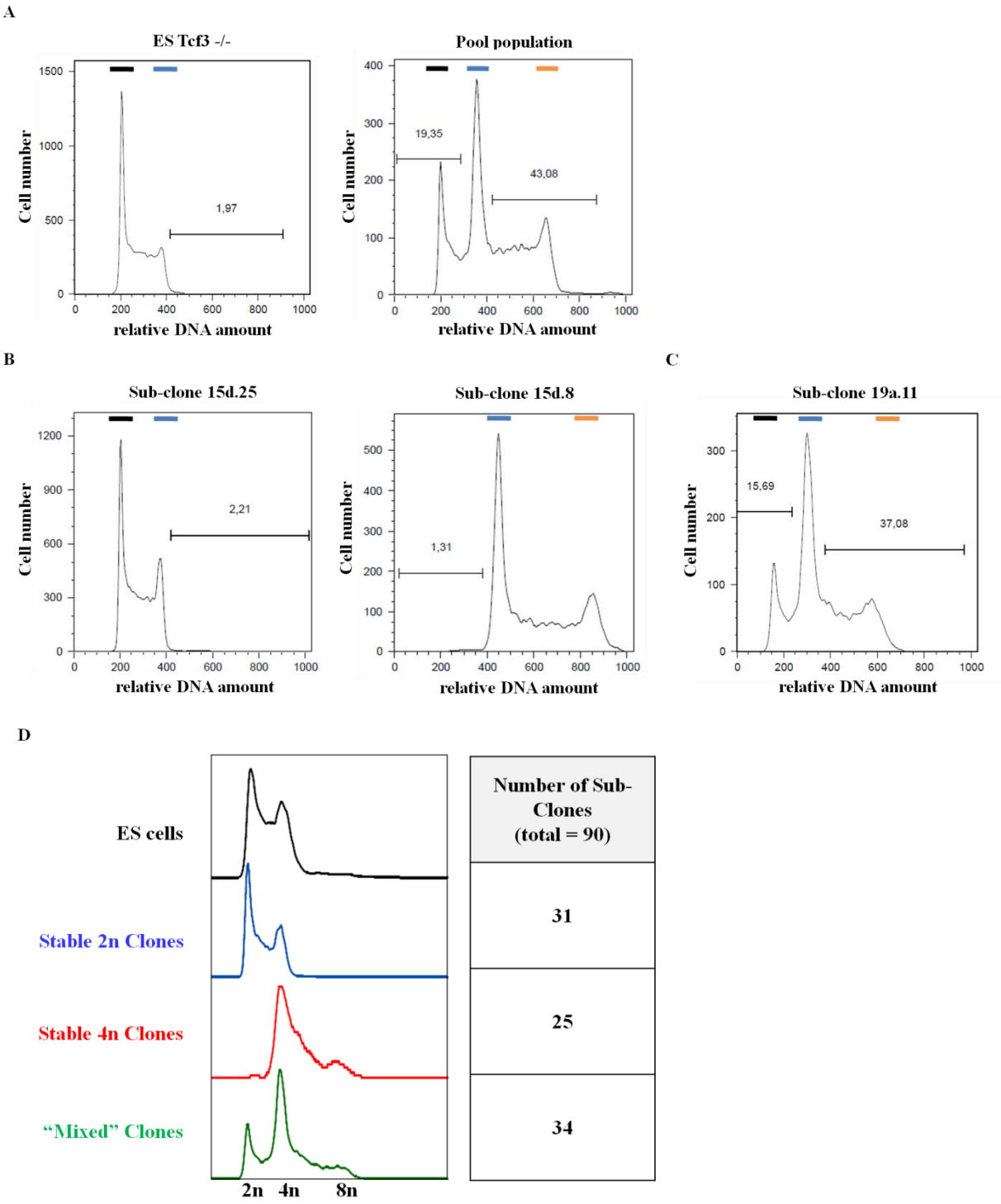

Figure 10. Analysis of the ploidy of the reprogrammed sub-clonal lines. DNA ploidy shown by the cell cycle analysis of ES Tcf3-/- cells and of the pool population before the single cell FACS-sorting (A). Cell cycle of representative sub-clones – stable diploid sub-clone (15d.25) and stable tetraploid sub-clone (15d.8) (B). Example of a sub-clone containing both diploid and tetraploid cells (C). Sub-clonal lines were categorized in stable diploid (2n), stable tetraploid (4n) and "mixed" (2n and 4n); the total number of sub-clones of each category is shown (D). Horizontal bars indicate the amount of DNA that corresponds to 2n (black), 4n (blue) and 8n (red) ploidies (B, C and D). Percentages of the marked regions are shown in the plots. See Annex IV for ploidy analysis of other sub-clonal lines.

Although “stable” diploid and tetraploid sub-clones represent 62,2% of the total number of sub-clones analysed, there is a significant percentage (37,8%) of single cells that formed “mixed” clones containing both diploid and tetraploid cells (Figure 10C and 10D). Since FACS-sorting is a very reliable method for single cell separation, we interpret the appearance of these sub-clonal lines as being originated from one single tetraploid cell and not as a sorting error. Probably this cell, or its progeny, underwent ploidy reduction originating diploid cells which continued to proliferate. As most of these “mixed” sub-clones have a higher percentage of diploid cells compared to the percentage of tetraploid cells, we hypothesize that diploid cells tend to divide faster than tetraploid cells and can, for that reason, become predominant in the sub-clone.

Another reason why sub-cloning was fundamental was to assure that the cells identified after selection were indeed reprogrammed cells and not resistant cells that did not come from fusion events. Although puromycin selects only for reprogrammed cells containing the NS chromosome that carries the puromycin resistance, there is a small chance that natural non-resistant cells (like the ES cells) survive in a medium in which there are resistant cells by the exchange of the resistance mechanism. By doing a single-cell sorting and collecting the cells in medium with puromycin, we can be sure that all the surviving single cells capable of forming clones are resistant to the selection system.

Furthermore, we performed another control to show that ES Tcf3-/- cells cannot survive after single-cell sorting in ES medium supplemented with puromycin. In order to do this we sorted PI-negative single cells from an ES Tcf3-/- population collecting one cell per well. As expected, we did not observe any clone formation in a total of 96 sorted cells (data not shown).

6. Live-imaging of tetraploid cells division

In order to depict in which way tetraploid hybrid cells divide we performed live-imaging experiments on tetraploid cells in culture. Since we confirmed that 8 days after the addition of puromycin all the reprogrammed GFP-positive cells were tetraploid, we decided to sort only the GFP-positive cells for the live-imaging experiments. 7500 cells were sorted and plated in a gelatinized coverslip to be accessed by light microscopy. To label the DNA, cells were incubated with the vital DNA-binding dye Hoechst 33342 before the FACS-sorting. Each cell was assessed by taking sequential pictures every 15 minutes in sessions lasting 8 to 12 hours.

As fusion-derived tetraploid cells should in principle contain more than two centrosomes we would expect to observe multipolar mitosis in some of the cells. This would result in the formation of a multipolar spindle during mitosis and in more than two daughter cells. Surprisingly, in the analysis of a total of 60 image sequences, we were only able to detect tetraploid cells undergoing bipolar mitosis with a normal spindle pole organization (Figure 11 and Annex VI). We hypothesize that we could not detect multipolar mitosis due to the fact that most of the tetraploid cells were already stable in the moment of the sorting, meaning that they were in a stage where they no longer can experience ploidy reduction events. One of the possibilities is that these cells can overcome the problem of extra centrosome number and generate bipolar mitosis. Previous studies reported that tetraploid cells can aggregate or exclude extra centrosomes in order to form only two microtubule organization centers during mitosis, originating only two daughter cells [63]. Even though we could not confirm this hypothesis, it seems plausible that ploidy reduction events may happen very early after the reprogramming of the somatic nucleus and that some tetraploid cells may become stable after some cell cycles.

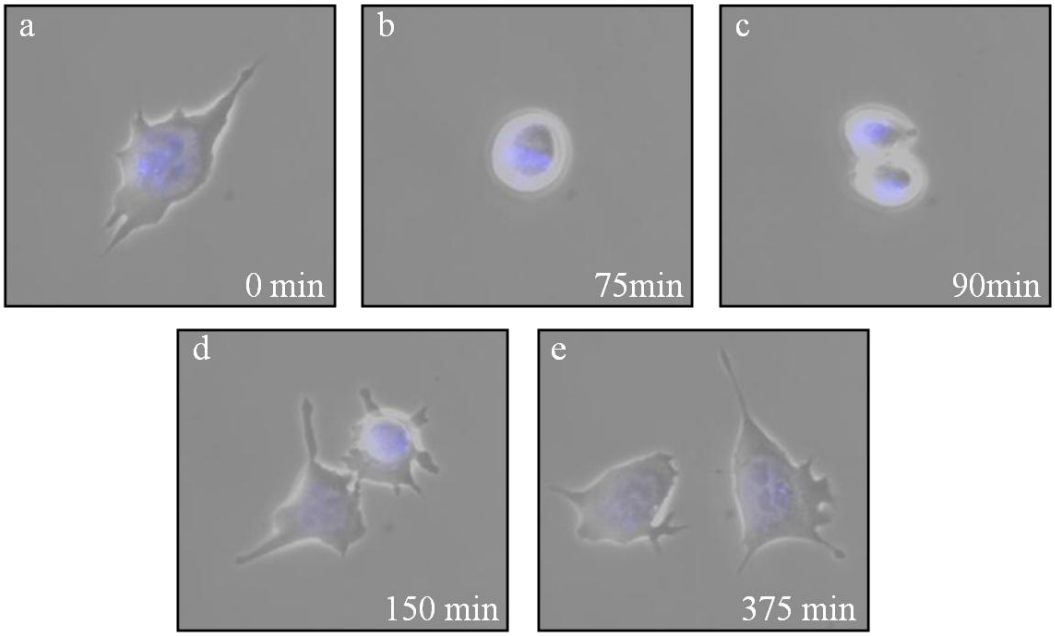

Figure 11. Bipolar mitosis of a tetraploid fusion-derived hybrid. Live-imaging pictures of a single mononucleated (a) tetraploid hybrid cell in different mitotic stages. After chromosome condensation in metaphase in a bipolar fashion (b), the cell enters in anaphase and telophase (c) and finally originates two independent cells (d and e). Cell structures were tracked with DIC and DNA was stained with Hoechst (pseudocolored blue). Time of picture capture is indicated. (400x magnification)

V. CONCLUSIONS AND FUTURE PERSPECTIVES

The work described here studies the fate of the tetraploid cells generated after reprogramming by cell fusion. Since ploidy reduction events have been observed after cell fusion in another system [61, 62], we hypothesized that hybrids resulting from the reprogramming of somatic cells by fusion with Embryonic Stem (ES) cells could experience a similar phenomenon.

We applied a previously described method for the reprogramming of adult somatic cells to a pluripotent state. This method consists on the spontaneous fusion of adult Neural Stem cells, containing the Oct4-GFP-puro construct, with pluripotent ES cells [30, 33]. Using this method it is possible to select reprogrammed clones in a simple and fast way by adding puromycin to the culture medium. We were able to select reprogrammed clones that consisted of tetraploid but also diploid cells (Figure 8 and Table I). This first observation encouraged us to investigate how diploid cells could appear from diploid-diploid fusion hybrids. As we did not see a significant percentage of cells with an intermediate number of chromosomes (between diploidy and tetraploidy), we suggest that if tetraploid hybrids undergo ploidy reduction events towards diploidy, this must happen through a single step event and not through an aneuploidy phenomenon. This was also suggested by Duncan et al. who were able to identify diploid cells a few cell cycles after the formation of fusion-derived polyploid hepatocytes [62].

In order to clearly confirm that tetraploid cells could originate cells with half the DNA content, we decided to separate reprogrammed cells using a Fluorescence-Activated Cell Sorting (FACS) approach one week after the selection system started to be applied. We chose this time point in order to be sure that all non-fused partners were not considered for the future analysis. In that moment, all GFP-positive cells were tetraploid (Figure 9B) and so we followed this tetraploid population over time to look for the presence of derived diploid cells. Indeed a diploid population started to be observed after 2 weeks although a clear diploid G1-peak was only identified 10 weeks after sorting (Figure 9D). Further experiments need to be set up in order to determine when ploidy reduction starts to occur and if there is a temporal relation between the reprogramming events and the reduction of ploidy.

Furthermore, we showed that both individual diploid and tetraploid single cells can survive and proliferate to form sub-clones under selective conditions (Figure 10). This result proves that the single diploid cells are indeed reprogrammed and can proliferate as ES cells *in vitro*. 34,4% of the identified sub-clones consisted exclusively of diploid cells and 27,8% of

tetraploid cells. Moreover, 37,8% of the single cell-derived sub-clones were neither “stable” diploid nor “stable” tetraploid. As these sub-clones were originated from one single cell, we confirm that indeed a single tetraploid cell can proliferate to give rise to diploid progeny. Since live-imaging experiments did not show a direct reduction of ploidy, we hypothesize that ploidy reduction of tetraploid hybrids may be a rare event that likely happens very early during the reprogramming process.

According to Duncan et al., there are some explanations to how diploid cells are generated from tetraploid fusion-derived reprogrammed hybrids (Figure 12). The first possibility is that after cell fusion a binucleated cell is formed, the two nuclei remain separated and the cell undergoes cytokinesis forming two diploid cells (Figure 12A). In our case, this would mean that in the binucleated cell the pluripotent ES cell would reprogram the genome of the adult NS cell even without nuclear fusion. After cytokinesis the reprogrammed NS cell would be the only one to survive in the presence of puromycin. Although we cannot exclude completely this hypothesis, this seems unlikely since we identified reprogrammed tetraploid cells in the surviving clones (Figure 8), meaning that synkaryon formation has occurred.

The second possibility is ploidy reduction via multipolar mitosis, which leads to the random segregation of chromosomes from both fusion partners in more than two daughter cells (Figure 12B). Tetraploid fusion hybrids are expected to have an increased number of centrosomes and therefore multipolar spindle poles may be formed during mitosis. The resulting cells would receive a random set of chromosomes and thus daughter diploid cells may be originated. In our system we selected for one NS chromosome that carries the resistance to puromycin, so if tetraploid cells undergo multipolar mitosis, only one cell would receive this chromosome and survive. It is important to note that the cell cycle phase in which the cells are at the moment of fusion, and hence the number of centrosomes present in the hybrid cell, may determine the probability that the hybrid undergoes an atypical division that results in cells with less chromosomes.

Finally, it is also possible that cell division happens in the tetraploid hybrid without DNA replication (Figure 12C), although this type of ploidy reduction was never described in mammals. According to this hypothesis, fusion derived reprogrammed cells can skip the S phase and progress directly to the G2/mitosis phase, ensuring the correct chromosome segregation to two diploid cells by pairing homologous chromosomes. In our case, reprogramming may happen after nuclear fusion even without DNA synthesis as it was shown in previous studies [24, 31]. However, only one daughter cell would receive the NS chromosome that would confer it resistance to puromycin. Furthermore, this model could

explain the existence of both stable tetraploid and diploid reprogrammed cells and is a strong possibility for the fate of tetraploid reprogrammed hybrids.

The mechanism of ploidy reduction needs to be clarified in order to better describe what are the mitotic strategies adopted by fusion-derived tetraploid cells and to show if any of the previously described hypothesis are valid after reprogramming by cell fusion. Previous studies on fusion mediated-reprogramming have very rarely identified fusion-derived diploid cells. Thus, we hypothesize that events of ploidy reduction might have been ignored or confounded with transdifferentiation events.

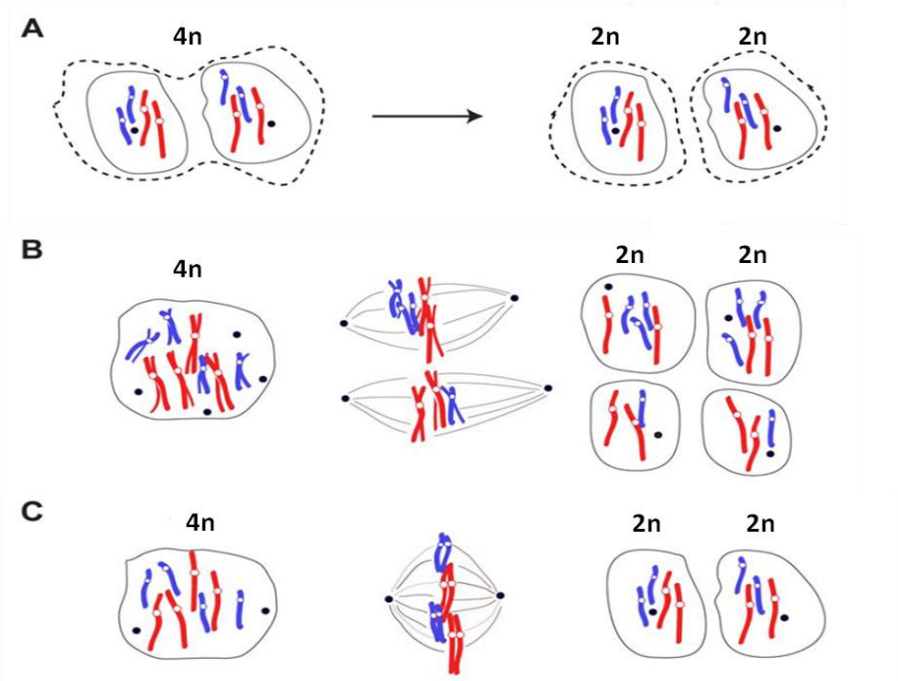

Figure 12. Ploidy reduction hypothesis According to Duncan et al., after diploid-diploid cell fusion there are mainly three options by which tetraploid cells (4n) can undergo ploidy reduction originating diploid daughter cells (2n): the hybrid may undergo cytokinesis without passing through S phase or mitosis (A); nuclear fusion and S phase may happen, followed by the formation of multipolar spindles leading to multipolar (in this case tetrapolar) mitosis (B); or the hybrid may skip the S phase and undergo mitosis by pairing the homologous chromosomes (C). [adapted from reference 61]

The present work contributes to the research field of cell fusion-mediated reprogramming by describing the fate of hybrids between ES cells and adult NS cells *in vitro*. Considering that certain cells, such as BM cells, have been shown to contribute for tissue regeneration by cell fusion, it is possible that the resulting hybrids undergo similar mechanisms of ploidy reduction than the ones hypothesized here. Further work needs to be done regarding the importance of ploidy reduction after fusion and reprogramming and whether this phenomenon is essential for the regeneration of damaged organs.

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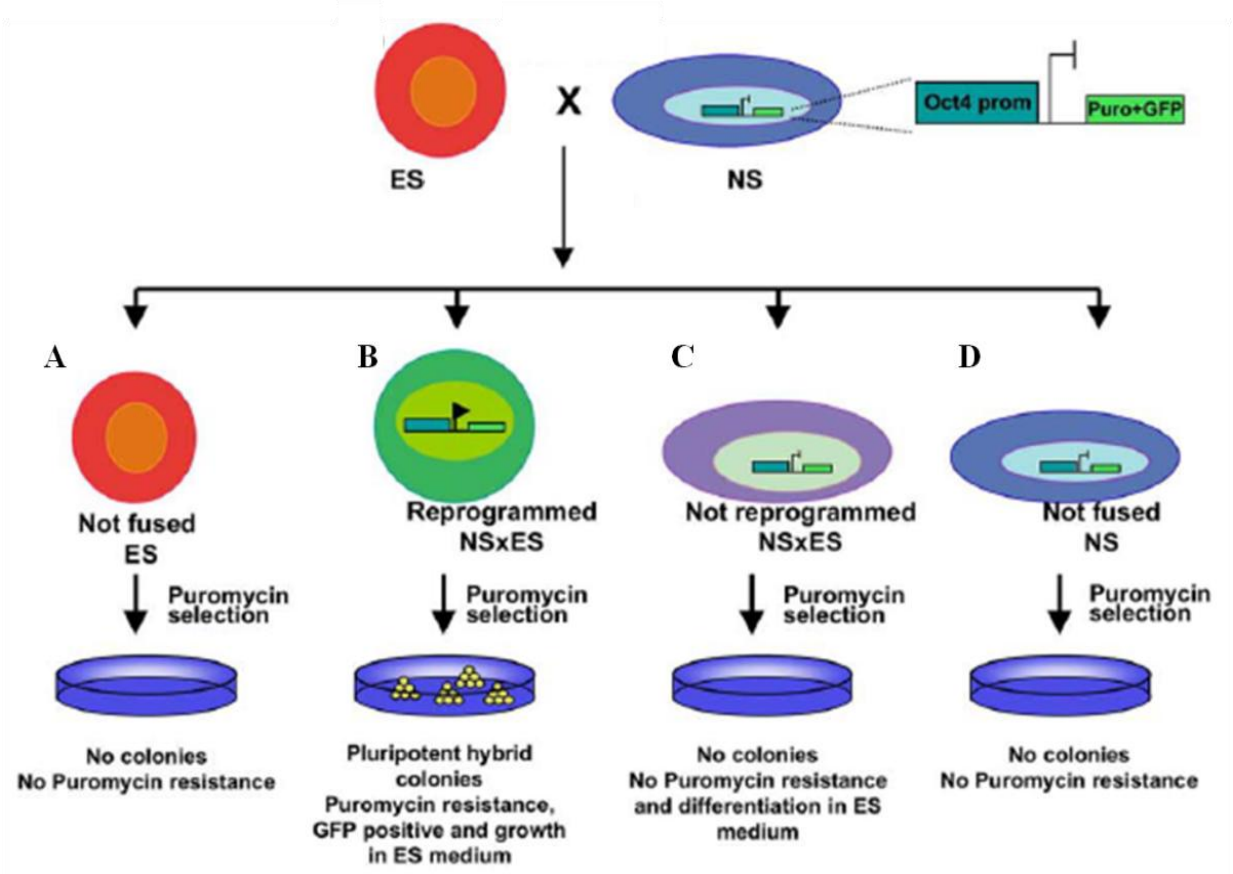
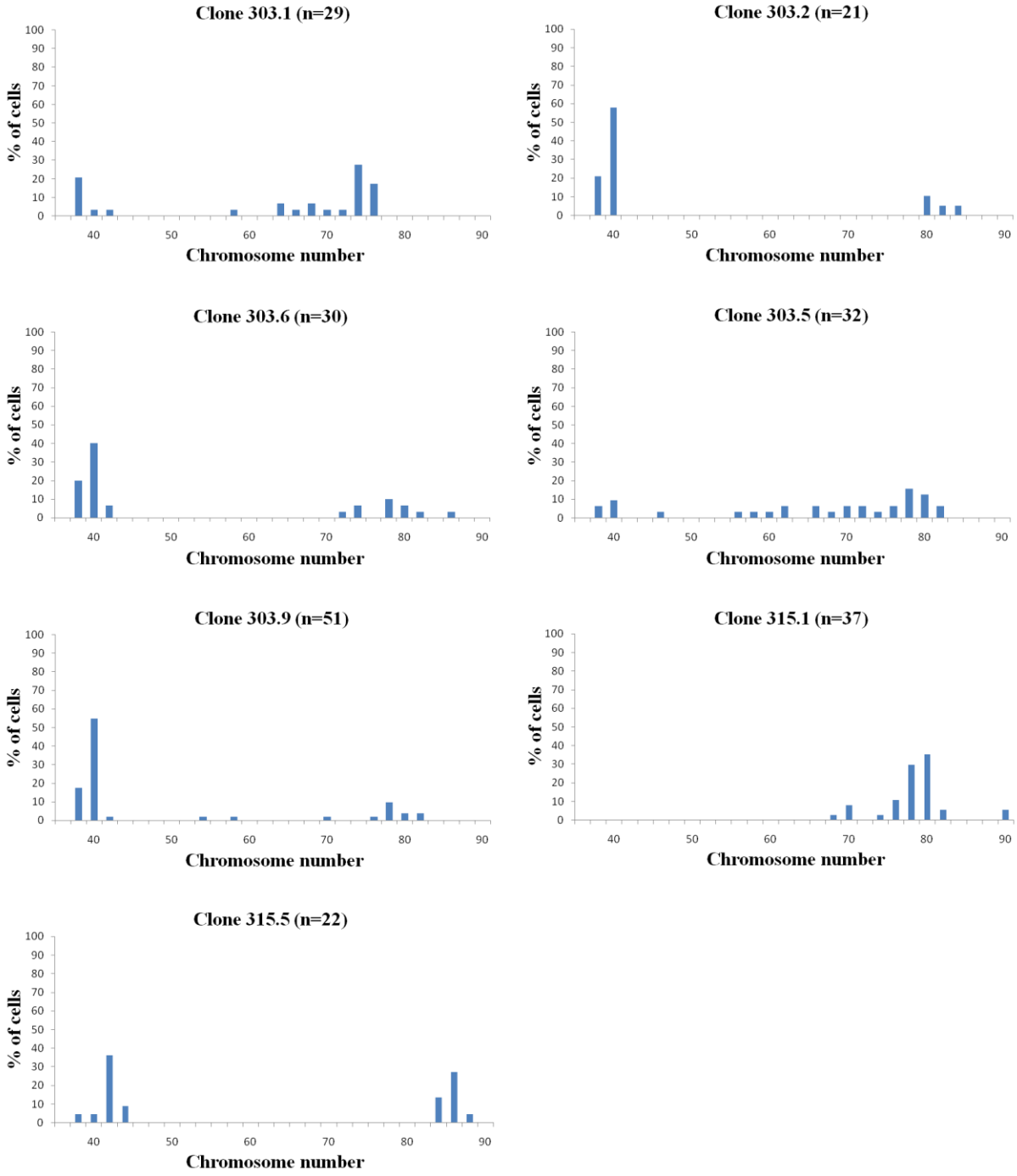
VII. ANNEXES

ANNEX I. Schematic overview of the experimental system



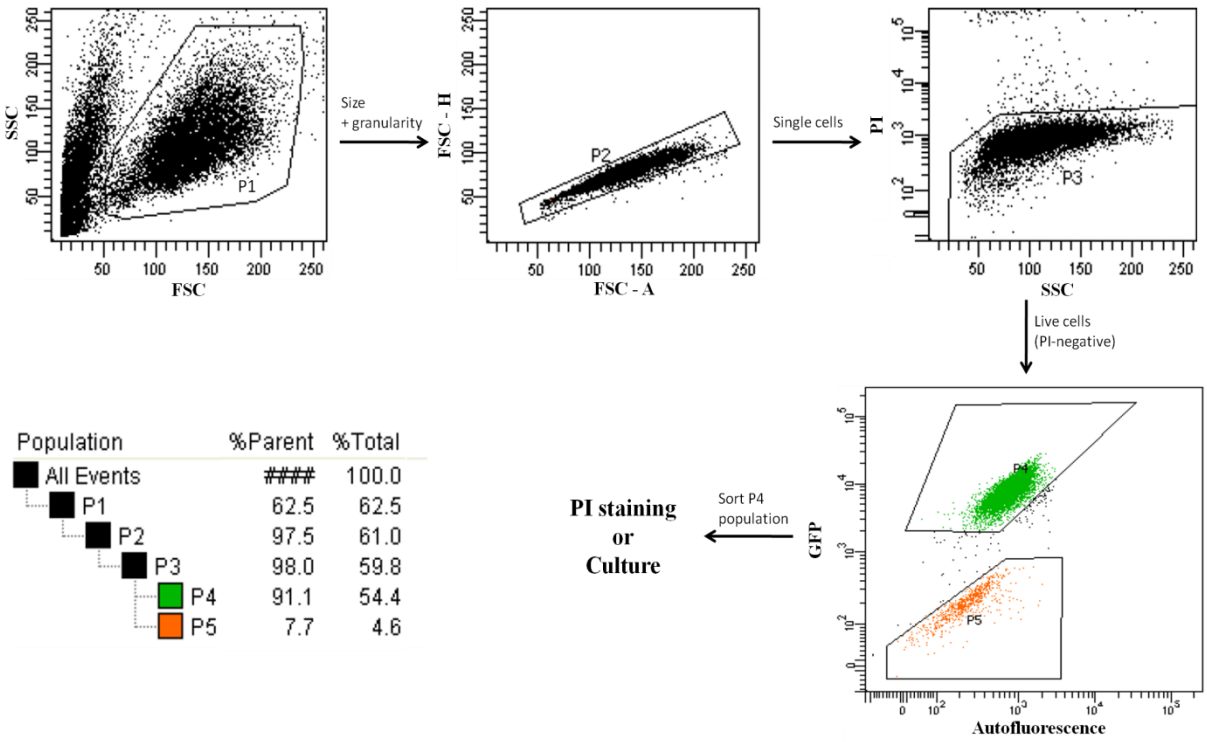
Embryonic Stem (ES) cells were fused with Neural Stem (NS) cells containing the Oct4-GFP-puro transgene. Oct4 is not expressed in NS cells and so the only NS cells that will resist in a medium supplemented with puromycin are those in which the Oct4 promoter is turned on, meaning that their genome was reprogrammed. This is possible because: non-fused ES and NS cells will not resist to puromycin and will not form colonies (A and D); fused, non-reprogrammed hybrids will not express puromycin resistance and hence will differentiate in ES medium (C); only reprogrammed NS cells, expressing Oct4 and the transgenes for GFP and puromycin resistance, will form colonies and grow in ES medium (B). [adapted from reference 33]

ANNEX II. Karyotype analysis of representative reprogrammed clonal lines.



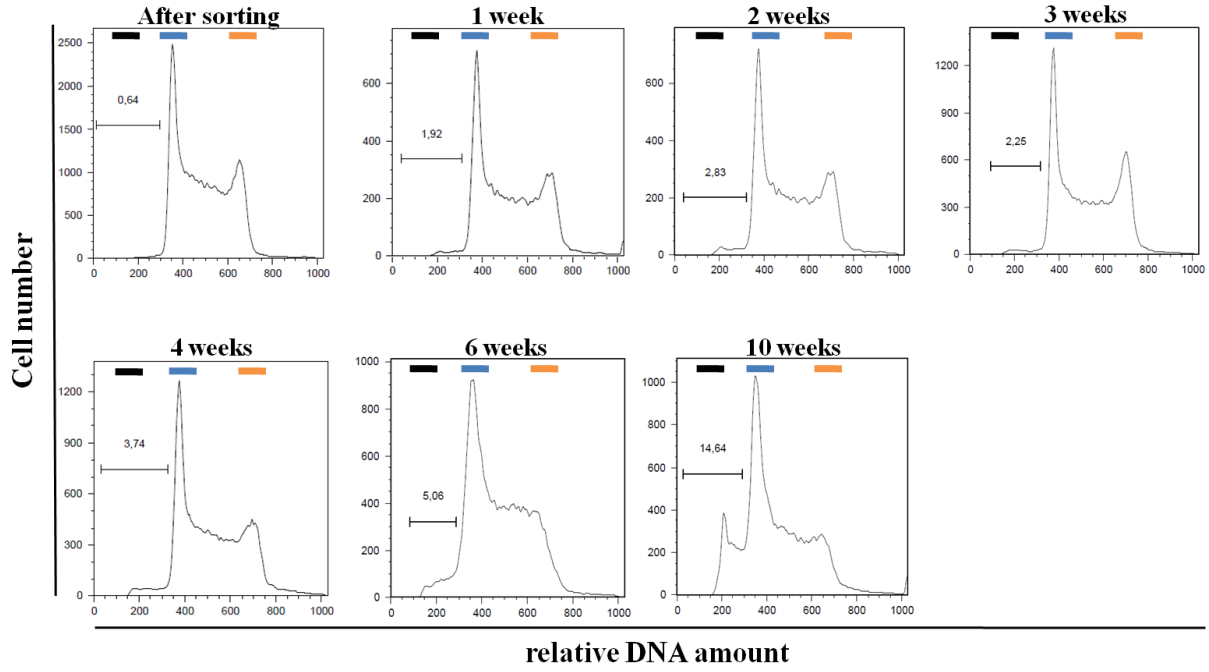
Histograms indicating the percentage of cells with a specific chromosome number from representative clonal lines. Note clone 303.5 – which contains more than 10% of cells with chromosome number between 40 and 80 – and clone 315.1 – which almost exclusively contains tetraploid cells. Mouse diploid cells contain 40 chromosomes while tetraploid cells contain 80 chromosomes. n is the number of individually scored chromosome spreads analysed for each cell line.

ANNEX III. Gating strategy used for the sorting of GFP-positive cells



GFP-positive cells were sorted according to the following gating strategy: firstly, cells were analysed according to the Forward Scatter (FSC) and Side Scatter (SSC) channels in order to sort for the right size and granularity, respectively. Then cells adhering to each other (that is, doublets) were eliminated, keeping only the single cells. Dead cells were excluded on the basis of Propidium Iodide (PI) incorporation. GFP-positive cells (green dots) were identified (comparing with the GFP-negative control – see Figure 9A) and sorted (P4).

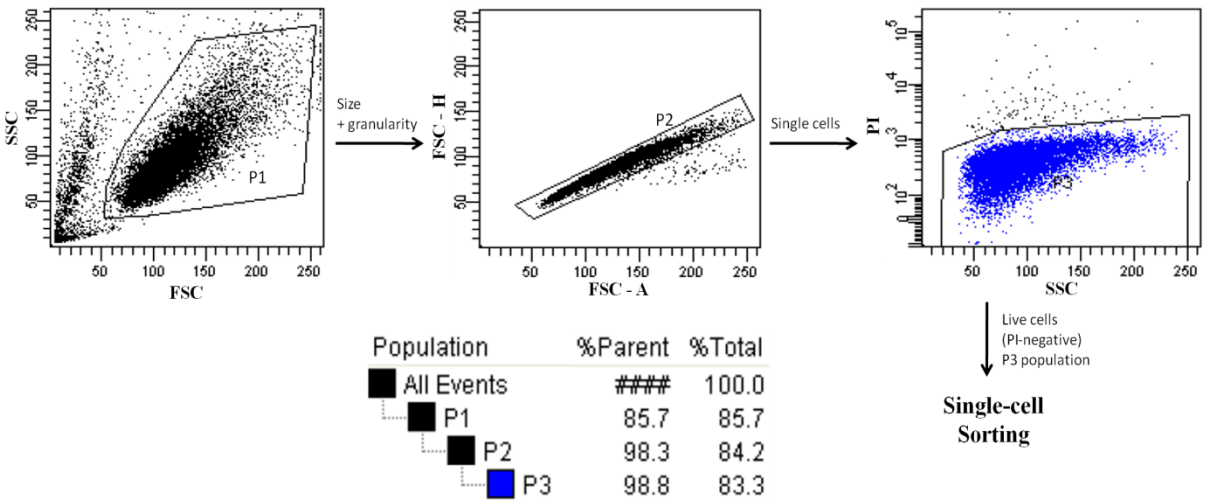
ANNEX IV. Ploidy of the reprogrammed hybrid cells



The ploidy of the reprogrammed hybrids (GFP-positive cells) was followed by cell cycle analysis over time, as indicated. Horizontal bars indicate the amount of DNA that corresponds to 2n (black), 4n (blue) and 8n (red) ploidies. Percentages of the marked regions are shown in the plots.

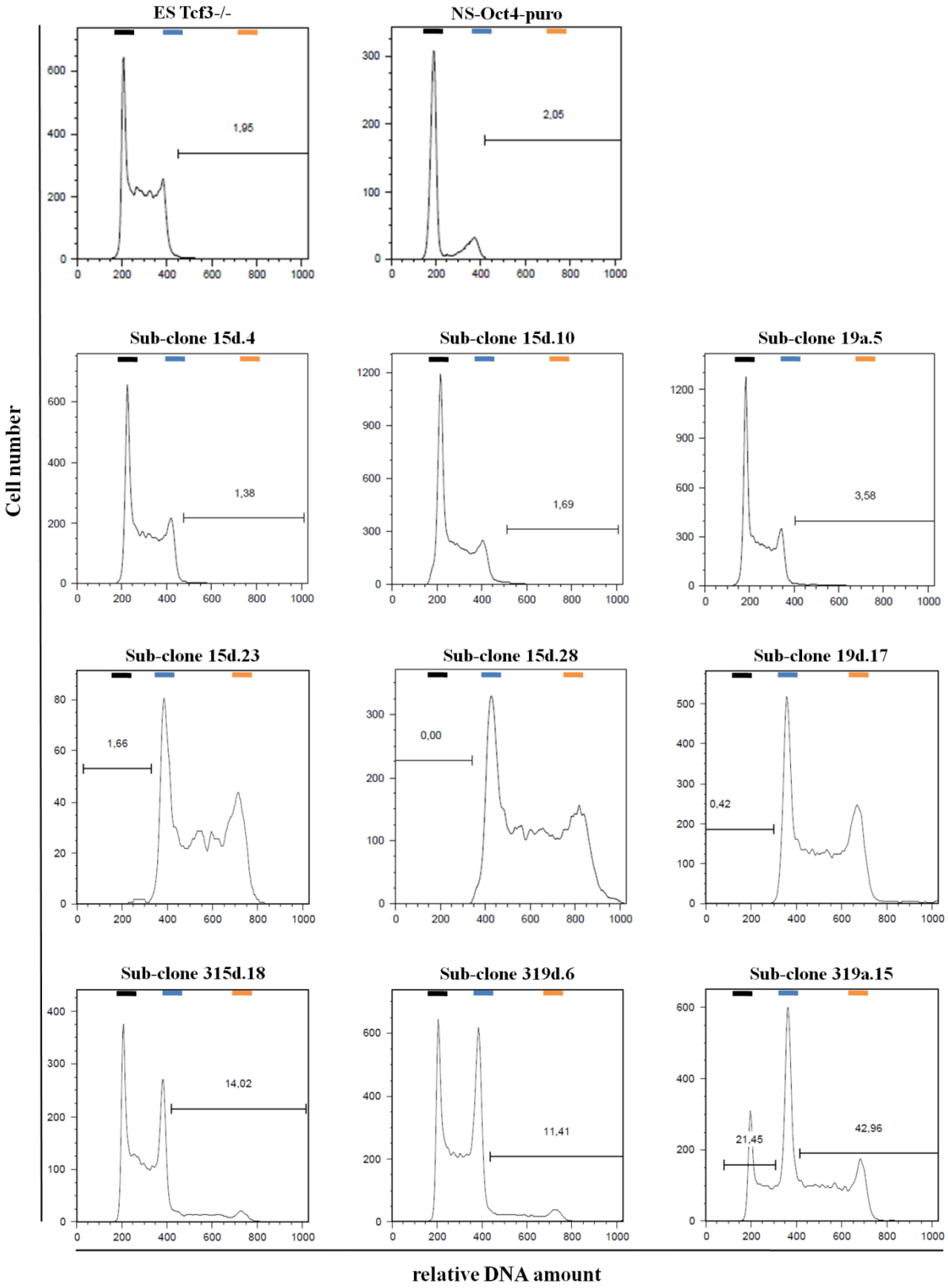
ANNEX V. Sorting of individual cells and sub-clone analysis

V.A)



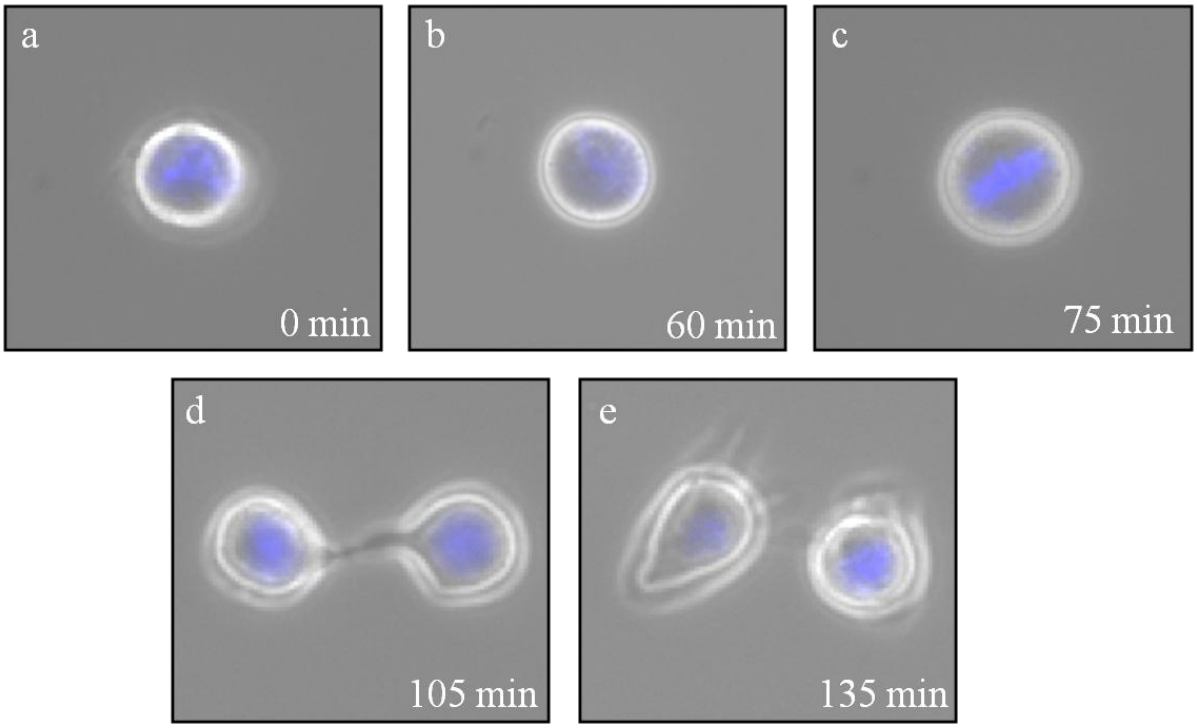
Gating strategy for the sorting of individual single cells: cells were sorted for the right size and granularity by Forward Scatter (FSC) and Side Scatter (SSC) channels, respectively; doublets were excluded in order to exclusively keep the single cells; finally dead cells were excluded on the basis of Propidium Iodide (PI) incorporation. Each single cell (blue dots) was sorted to a single well.

V.B)



DNA ploidy shown by cell cycle analysis of representative sub-clonal lines obtained from single sorted cells. Horizontal bars indicate the amount of DNA that corresponds to 2n (black), 4n (blue) and 8n (red) ploidies. Percentages of the marked regions are shown in the plots.

ANNEX VI. Bipolar mitosis of a tetraploid fusion-derived hybrid.



Live-imaging pictures of a single mononucleated (a) tetraploid hybrid cell in different mitotic stages. The cell begins the DNA condensation (a and b) and rapidly enters in metaphase (c). Then the chromosomes are distributed in two independent cells (d and e). Cell structures were tracked with DIC and DNA was stained with Hoechst (pseudocolored blue). Time of picture capture is indicated. (400x magnification)