Universitat de Girona
Facultat de Ciències

Characterization of *NF1 (+/+)*, *(+/-)*, and *(-/-)* iPSC lines generated through genetic editing using CRISPR/Cas9

Judit Farrés Casas
juditfarres66@gmail.com
Grau en Biologia

Tutor: Oriol Vidal Fàbrega
Correu electrònic: oriol.vidal@udg.edu

Co-tutor: Meritxell Carrió Llach
Correu electrònic: mcarriol@igtp.cat
Empresa/Institució: Institut d'Investigació Germans Trias i Pujol (IGTP)

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List of Abbreviations

aNF	Atypical neurofibroma
BDM-NC	Basal differentiation medium-Neural crest medium
cNF	Cutaneous neurofibroma
CRISPR-Cas9	Clustered regularly interspaced short palindromic repeats associated with nuclease 9
crRNA	CRISPR RNA
DMEM	Dulbecco's Modified Eagle Medium
ESC	Embryonic stem cells
FiPS	Fibroblasts-derived induced pluripotent stem cell
GAP	GTPase activating protein
GTPase	Guanosine triphosphatase
InDels	Insertions or deletions
iPSC	Induced pluripotent stem cell
LOH	Loss of Heterozygosity
MAPK	Mitogen-Activated Protein Kinase
MPNST	Malignant peripheral nerve sheath tumor
NC	Neural crest
NF	Neurofibroma
NF1	Neurofibromatosis Type 1
PAM	Protospacer adjacent motif
pNF	Plexiform neurofibroma
QOL	Quality of life
RNP	Ribonucleoprotein complex
SC	Schwann cell
sgRNA	Single-chain guide RNA
tracrRNA	Trans-activating crRNA

RESUM

L'ús de cèl·lules mare embrionàries derivades d'oòcits i embrions ha estat motiu de discussió ètica. Per això, la reprogramació de cèl·lules somàtiques per produir cèl·lules mare pluripotents induïdes (iPSCs) és una alternativa per evitar la problemàtica i una oportunitat per desenvolupar noves estratègies terapèutiques i models de malaltia.

En els darrers anys, els investigadors del grup de 'Càncer Hereditari' de 'l'Institut de Recerca Germans Trias i Pujol (IGTP)' han fet ús de la tecnologia de les iPSCs per estudiar la Neurofibromatosi de tipus 1, una malaltia genètica autosòmica dominant causada per mutacions al gen *NF1* amb una predisposició a desenvolupar tumors associats al sistema nerviós perifèric anomenats neurofibromes.

Al laboratori de l'Eduard Serra, han desenvolupat un model cel·lular d'iPSC-*NF1* mitjançant la reprogramació de cèl·lules amb una o les dues còpies del gen *NF1* mutades*, NF1 (+/-)* i *(-/-),* a partir de neurofibromes plexiformes, generant diverses línies d'iPSC amb diferents genotips. Aquestes línies són capaces de capturar l'estat genòmic dels tumors originals i de mantenir les característiques de les cèl·lules mare, ja que es poden diferenciar cap a cèl·lules mare de la cresta neural i més enllà cap a cèl·lules de Schwann, el principal tipus cel·lular dels tumors. A més, quan les cèl·lules de Schwann *NF1 (-/-)* s'injecten al nervi ciàtic de ratolins atímics, es formen neurofibromes. Addicionalment, també s'ha utilitzat la tecnologia de CRISPR/Cas9 per editar genèticament l'exó 2 del gen *NF1* en línies control d'iPSC *NF1(+/+)*, amb l'objectiu de generar mutacions en un o els dos al·lels del gen *NF1* per obtenir clons addicionals amb els genotips *NF1 (+/-)* i *(-/-)*.

En aquest projecte, s'han identificat tres clons diferents d'aquest experiment d'edició: un d'ells porta una mutació només en un al·lel del gen *NF1* (*NF1(+/-)*); un altre que porta mutacions en els dos al·lels (*NF1(-/-)*); i el tercer sense cap mutació (*NF1 (+/+)*) com a línia de control. Aquests clons s'han caracteritzat per confirmar l'estat genètic del gen *NF1* i avaluar les capacitats biològiques de les cèl·lules mare.
Les mutacions a l'exó 2 del gen *NF1* es van confirmar determinant la seqüència de cada clon; la capacitat de pluripotència *in vitro* es va avaluar mitjançant l'anàlisi de l'expressió de marcadors específics; i la capacitat de diferenciar-se en cèl·lules del llinatge de NC es va demostrar mitjançant l'anàlisi de l'expressió de factors de transcripció i marcadors de membrana específics del llinatge.

Aquestes línies cel·lulars seran útils per ampliar el nombre de línies d'iPSC-*NF1* al laboratori per generar models cel·lulars per la NF1 amb l'objectiu d'utilitzar-les com a models preclínics per provar noves estratègies terapèutiques.

RESUMEN

El uso de líneas de células madre pluripotentes derivadas de ovocitos y embriones ha sido motivo de discusión ética. Por eso, la reprogramación de células somáticas para producir células madre pluripotentes inducidas (iPSCs) es una alternativa para evitar la problemática y una oportunidad para desarrollar nuevas estrategias terapéuticas y modelos de enfermedad.

En los últimos años, los investigadores del grupo de 'Cáncer Hereditario' del 'Instituto de Investigación Germans Trias y Pujol (IGTP)' han utilizado la tecnología de las iPSCs para estudiar la Neurofibromatosis de tipo 1, una enfermedad genética autosómica dominante causada por mutaciones en el gen *NF1* capaz de desarrollar tumores asociados al sistema nervioso periférico llamados neurofibromas.

En el laboratorio de Eduard Serra, han desarrollado un modelo celular de iPSC-*NF1* mediante la reprogramación de células *NF1 (+/-)* y *(-/-)* de neurofibromas plexiformes, generando diferentes líneas de iPSC *NF1 (+/-)* y *(-/-)*. Estas líneas celulares de iPSC son capaces de capturar el estado genómico de los tumores originales y mantener las características de las células madre, ya que se pueden diferenciar hacia células madre de la cresta neural y más allá hacia células de Schwann, el principal tipo celular de los tumores. Además, cuando las células de Schwann *NF1 (-/-)* se inyectan en el nervio ciático de ratones atímicos, se forman neurofibromas. Adicionalmente, se ha utilizado la tecnología de CRISPR/Cas9 para editar genéticamente el exón 2 del gen *NF1* en líneas control de iPSC *NF1(+/+)*, con el objetivo de generar mutaciones en uno o ambos alelos de *NF1* para obtener clones adicionales con los genotipos *NF1 (+/-)* y *(-/-)*.

En este proyecto, se han identificado tres clones distintos de este experimento de edición: uno de ellos lleva una mutación solo en un alelo del gen *NF1 (NF1(+/-)*); otro que lleva mutaciones en ambos alelos (*NF1(-/-)*); y el tercero sin mutación alguna (*NF1 (+/+)*) como línea de control. Estos clones se han caracterizado por confirmar el estado genético del gen *NF1* y evaluar las capacidades de las células madre.
Las mutaciones en el exón 2 del gen *NF1* se confirmaron determinando la secuencia de cada clon; la capacidad de pluripotencia *in vitro* se evaluó mediante el análisis de la expresión de marcadores específicos; y la capacidad de diferenciarse en células del linaje de NC se demostró mediante el análisis de la expresión de factores de transcripción y marcadores de membrana específicos del linaje.

Estas líneas celulares serán útiles para ampliar el número de líneas de iPSC-*NF1* en el laboratorio para crear modelos de células *NF1* con el objetivo de utilizarlas como modelos preclínicos para probar nuevas estrategias terapéuticas.

ABSTRACT

The use of pluripotent stem cell lines from oocytes and embryos has been a cause of ethical discussion. For that reason, reprogramming somatic cells to produce induced pluripotent stem cells (iPSCs) has been an alternative to avoid this issue and an opportunity to develop new therapeutic strategies and disease models.

In the last few years, researchers of the 'Hereditary Cancer' group in the 'Germans Trias i Pujol Research Institute (IGTP)' have been using this iPSC technology to study Neurofibromatosis type 1, an autosomal dominant genetic condition caused by mutations in the *NF1* gene and capable of developing tumors associated with the peripheral nervous system called neurofibromas.

In Eduard Serra's lab, they have developed an *NF1* iPSC-based cellular model by reprogramming *NF1 (+/-)* and *(-/-)* cells from plexifrom neurofibromas, generating *NF1 (+/-)* and *(-/-)* different iPSC lines. These iPSC cell lines were capable of capturing the genomic status of the original tumors and maintaining the characteristics of stem cells, as they were able to differentiate toward neural crest stem cells and further toward Schwann cells, the main cell type of the tumors. Moreover, when Schwann cells *NF1 (-/-)* are injected into the sciatic nerve of a nude mice, neurofibromas are formed. In addition, they have also used the technology of CRISPR/Cas9 to genetically edit exon 2 of the *NF1* in *NF1(+/+)* control iPSC lines, with the aim of generating mutations in one or both *NF1* alleles to obtain extra clones with the genotypes *NF1 (+/-)* and *(-/-)*.

In this project, we have identified three different clones from this editing experiment: one of them carrying a mutation only in one allele of the *NF1* gene (*NF1(+/-)*); another one carrying mutations in both alleles (*NF1(-/-)*); and the third one without any mutation (*NF1 (+/+)*) as a control line. These clones have been characterized to confirm their *NF1* genetic status and evaluate their stem cell abilities.
Mutations in exon 2 of the *NF1* gene were confirmed by determining the sequence of each clone; *in vitro* pluripotency capacity was tested by the analysis of the expression of specific markers; and the ability to differentiate into cells of the NC lineage was demonstrated by analyzing the expression of transcription factors and membrane markers specific to the lineage.

These newly characterized cell lines will be useful to expand the number of *NF1* iPSC line in the lab to create *NF1* cell models and establish non-perishable cell lines to test new therapeutic strategies before preclinical *in vivo* models.

REFLECTION ON ETHICS

Embryonic stem cells (ESC) are presented as a tool of biological interest, but they are derived from the inner cell mass of the 5- to 7-day blastocyst, a contentious ethical and political finding that is avoided by iPSC technology (Lo and Parham, 2009).

As a matter of religious faith, there are those who consider that human life begins at conception, so an embryo has interests and rights that must be respected (President's Council on Bioethics (US), 2004), while others defend that a blastocyst is not equivalent to a fetus because it has no chance of thriving in a uterus (EuroStemCell, 2016). In 2006, Takahashi and Yamanaka were capable of reprogramming somatic cells to produce iPSCs, which preserve the same characteristics as ESC.

For that reason, this technique allows the investigation of cellular, molecular, and genetic systems without generating moral arguments over the beginnings of life.

REFLECTION ON SUSTAINABILITY

The laboratory produces a large quantity of daily residues that fall into different categories of environmental impact. In the IGTP cell culture rooms, we work with materials of biological origin, which are recycled in three different containers:

Sharp objects are disposed of in puncture-proof containers with lids. Liquid biosanitary waste is collected and neutralized with bleach, while the rest of the material is deposited in black containers. All containers remain with the lid closed in their location and are not filled to more than 80% of their capacity, at which time they are sealed, removed, and replaced.

In this way, good recycling of the waste is ensured by minimizing the risks and impact on the environment.

REFLECTION ON GENDER PERSPECTIVE

Science is usually associated with a male activity due to the existence of roles and stereotypes imposed by society and the lack of reference women.

Although women represent 41% of research employees in Spain (Unidad de Mujeres y Ciencia, 2021), they face different gender biases, worse assessments, and less funding compared to their male counterparts. Besides, those who decide to become mothers find it difficult to reconcile family life with their research career (Dinu, 2021).

Although talent has no gender, the existence of some handicaps for women makes it complicated for them to develop their qualities at senior stages, a fact that should be considered and compensated in some way (Dinu, 2021).

INTRODUCTION

1.1. Neurofibromatosis type 1

1.1.1. The disease

Neurofibromatosis type 1 (NF1) is a tumor predisposition genetic disease caused by the inheritance of a pathogenic variant in the *NF1* tumor suppressor gene, where almost 50% of cases are familial and 50% are due to de *novo* mutations (DeBella, Szudek, and Friedman, 2000).

NF1 affects ~ 1 in 3,000 people worldwide (Riccardi, 1992; Kallionpää RA et al. 2018), and their penetrance is nearly 100% at 8 years of age. Even so, it represents one of the most frequently diagnosed cancer predisposition disorders involving the nervous system (Cimino and Gutmann, 2018).

Importantly, NF1 is a disorder with a high degree of clinical heterogeneity since multiple tissues can be involved and patients can show high phenotypic variability. The main implicated tissues are the skin, bones, and the central and peripheral nervous systems (Cimino and Gutmann, 2018). Cells of the lineage of the embryonic neural crest are implicated in the development of all these tissues and are mainly involved in this condition.

One of the hallmarks of the disease is the development of tumors from the peripheral nervous system called neurofibromas.

1.1.2. Diagnosis and clinical manifestations

This disease is mainly characterized by multiple café-au-lait macules (CALMs), skinfold freckling (more correctly termed lentiginous macules since they occur in non-sun-exposed areas), iris Lisch nodules, and tumors of the nervous system (Legius et al., 2021). Additional features include skeletal abnormalities, tibial dysplasia, optic pathway gliomas, and behavioral deficits such as attention deficit hyperactivity disorder (Gutmann et al., 2017).

Diagnosis criteria were established in 1987 by the NIH (National Institutes of Health) Consensus Development Conference on Neurofibromatosis and have been updated lately (Legius et al., 2021). The agreed diagnosis is based on clinical assessment, and two or more of the features in **Table 1** are required.

Table 1. **NIH revised diagnostic criteria for Neurofibromatosis type 1 (NF1)** (Legius et al., 2021).

- Six or more café-au-lait macules > 5 mm in greatest diameter in prepubertal individuals and > 15 mm in greatest diameter in postpubertal individuals
- Freckling in the axillary or inguinal region
- Two or more neurofibromas of any type or one plexiform neurofibroma
- Optic pathway glioma
- Two or more iris hamartomas (Lisch nodules)
- A distinctive osseous lesion such as sphenoid dysplasia, anterolateral bowing of the tibia, or pseudarthrosis of a long bone
- A heterozygous pathogenic *NF1* variant with a variant allele fraction of 50% in apparently normal tissue such as white blood cells

1.1.3. *NF1* Gene and protein

The *NF1* gene, located at chromosome 17q11.2 (Wallace, 1990; Viskochil, 1990), is one of the largest genes in the human genome, with 60 exons and extended over 280 kb of genomic DNA (Li, 1995). It is considered a classical tumor suppressor gene with an important role in the downregulation of Ras protein in the MAPK/ERK signaling pathway, which regulates cellular proliferation and differentiation (Rasmussen et al., 2000).

NF1 encodes for neurofibromin, a protein highly expressed in neurons, astrocytes, oligodendrocytes, and Schwann cells (SCs) that has an estimated molecular mass of 327 kDa and consists of 2818 amino acids (Cawthon, 1991). Neurofibromin possesses multiple functions, including the regulation of cyclic AMP levels and microtubule binding. Even though, the best characterized function is the Ras GTPase activating protein (GAP) activity, by promoting the hydrolysis of Ras-bound GTP to GDP, thus transitioning Ras to its inactive state (Korfhage and Lombard, 2019).

1.1.4. Tumors associated with NF1

1.1.4.1. Type of tumors

NF1 predisposes patients to develop benign nerve sheath tumors termed neurofibromas (NFs).

NFs appear when the *NF1* gene is completely inactivated in a cell from the SC lineage, following the two-hit model proposed by Knudson et al. (1971) (**Figure 1**). The ‘first hit’ in the *NF1* gene is inherited or acquired as a germline pathogenic variant. The ‘second hit’ disrupts the remaining wild-type allele of the *NF1* gene of a cell from the SC lineage and results from a somatic event. In 25% of cases, the somatic mutation is caused by

somatic rearrangements, deletions, and somatic recombination, generating loss of heterozygosity (LOH) (Serra et al., 2001).

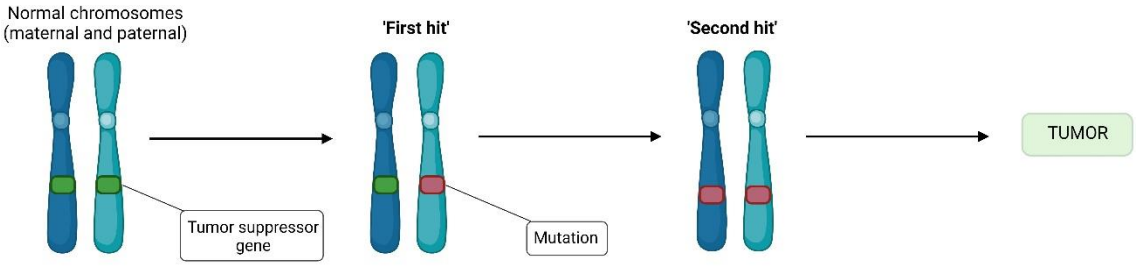


Figure 1. Representation of the "two-hit" Knudson hypothesis.
The chromosomes, one maternal and the other paternal, are represented in blue, and the normal allele of the tumor suppressor gene is represented by the green line. The 'first hit' is due to a germline mutation (in red), and the 'second hit' occurs from a somatic mutation that leads to tumor formation.

There are different tumors associated with NF1, many of them implicating the peripheral nervous system: cutaneous neurofibromas (cNFs), plexiform neurofibromas (pNFs), and malignant peripheral nerve sheath tumors (MPNSTs).

cNFs are benign skin tumors that typically appear during the stage of puberty and affect almost more than 95% of adults with NF1 (Wolkenstein et al., 2001). cNFs may be very variable in number, ranging from tens to thousands, and may be greatly disfiguring, causing a significant impact on the quality of life (QOL) of the patients. The only actual treatment for them is surgery removal.

On the other hand, pNFs are congenital benign tumors that arise in deeper nerves and can become large. They may compress other structures and cause disfigurement, pain, and different neurologic symptoms (Prada et al., 2012). The only currently approved treatment for inoperable pNFs in children is Selumetinib, an inhibitor of MEK. In contrast to cNFs, these tumors are capable of progressing towards a malignant tumor termed MPNST. In the path to malignancy, there is an intermediate pre-malignant state termed atypical neurofibroma (aNF), which involves the additional loss of the CDKN2A/B locus (Beert et al., 2011; Higham et al., 2018; Carrió et al., 2018; Magallón-Lorenz et al., 2021).

MPNSTs are an important factor in the morbidity and mortality of the disease, where NF1 patients have an 8–15% lifetime risk of developing them (Evans et al., 2002; Uusitalo, 2016). These malignant tumors are typical of being hyperploid and present highly disarranged genomes, and recent studies have identified recurrent mutations in the genes EED and SUZ12, key components of the polycomb repressive complex 2 (PRC2) (Lee et al., 2014).

1.1.4.2. Cellular composition of neurofibromas

NFs are composed of different cell types, mainly SCs and endoneurial fibroblasts, as well as perineurial cells and infiltrating immune cells, all embedded in an abundant collagen-rich extracellular matrix (Krone et al., 1983; Peltonen et al., 1988).

In a nonpathological state, a single peripheral nerve fiber is associated with myelinating or non-myelinating SC. Several nerve fibers and associated SCs are clustered into a nerve bundle (or fascicle), and each fascicle is surrounded by concentric layers of perineurial cells. Multiple nerve fascicles, bound by loose connective tissue, constitute an individual nerve.

These tumors are composed of the same cell types present in nerves, but in a complete disorganized state and exhibiting an increased number, particularly SCs, that are found dissociated from nerves. It is also observed that the perineurium is disrupted (Cichowski and Jacks, 2001; **Figure 2**).

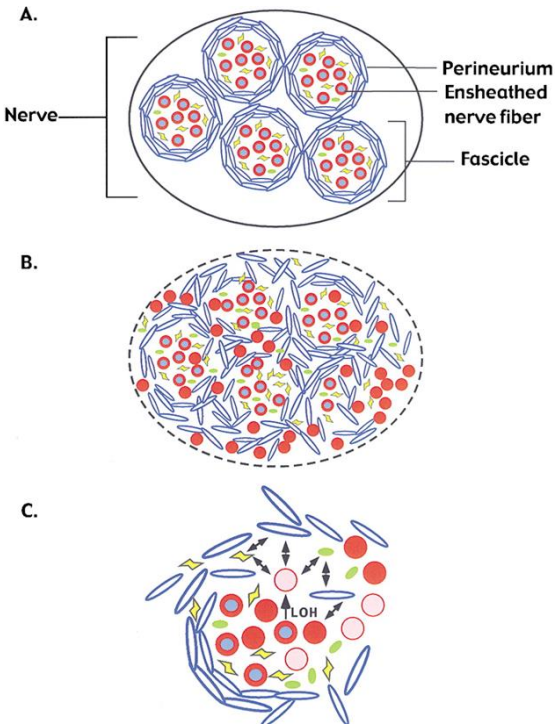


Figure 2. Normal Peripheral Nerve and Model of Neurofibroma Development. (A) Peripheral nerves are comprised of multiple cell types; neurons (blue), Schwann cells (red), perineurial cells (gray), fibroblasts (yellow), and occasional mast cells (green). (B) Neurofibromas exhibit an increased number of all cell types. In addition, Schwann cells are often dissociated from neurons, and the perineurium is disrupted. (C) Neurofibroma development is initiated by mutation or loss of the inherited wild-type *NF1* allele in Schwann cells (pink cells are *NF1(-/-).* This triggers tumor development by initiating a cascade of changes in other cell types as a result of their interdependency and/or haploinsufficiency. Extracted from Cichowski and Jack, 2001.

1.2. iPSC

1.2.1. Reprogramming of somatic cells

Stem cells are undifferentiated cells that are present in the embryonic, fetal, and adult stages of life and give rise to differentiated cells that are the building blocks of tissue and organs. This cell type is majorly characterized by its capacity for self-renewal, its ability to extensively proliferate, and its potency to differentiate into different cell types (De Los Angeles, 2015).

Their use in experimentation in the fields of regenerative therapies and personalized medicine has opened a controversial debate about life limits, so the use of induced pluripotent stem cell (iPSC) technology has transformed biological research for its applications in the development of disease modeling and drug screening.

In 2006, Kazotushi Takashi and Shinya Yamanaka demonstrated the induction of pluripotent stem cells from somatic cells by introducing four factors, OCT3/4, SOX2, C-MYC, and KLF4, under embryonic stem cell (ESC) culture conditions. These cells, designated iPSCs, exhibit the morphology and growth properties of ESCs and express their marker genes. Their pluripotency capacity can be tested by specific expression markers such as intracellular proteins like OCT4, SOX2, NANOG, and LIN28, as well as surface markers like SSEA3, SSEA4, TRA-1-60, TRA-1-81, and TRA2-49 (Cevallos et al., 2021).

iPSC technology has been used to reprogram tumor cells. In 2019, Carrió et al. reprogrammed, using these transcription factors, pNF-derived primary cells *NF1 (-/-)* and *(+/-)* into iPSCs. These iPSCs were capable of capturing the genetic status and tumorigenic properties of *NF1* pNFs and were able to differentiate toward NC stem cells and further toward SCs. iPSC-derived *NF1 (-/-)* SCs exhibited a continuous high proliferation rate, poor myelination ability, and a tendency to form 3D spheres that express the same markers as their PNF-derived primary SC. Moreover, when they were engrafted in the sciatic nerve of nude mice, they generated neurofibroma-like tumors, indicating they constitute a good pre-clinical *NF1* model (Mazuelas et al., 2022).

1.2.2. Genetic edition using CRISPR/Cas9

Clustered regularly interspaced short palindromic repeats associated with nuclease 9 (CRISPR/Cas9) is a system of gene targeting that takes advantage of the characteristics of the ancient adaptative immune system found in archaea and eubacteria (Sontheimer and Barrangou, 2015).

This DNA editing tool is based on a chimera with a single guide RNA (sgRNA) formed by a CRISPR RNA (crRNA), a 20-nucleotide sequence homologous to the target genomic region, and a trans-activating CRISPR RNA (tracrRNA), which folds into a hairpin structure required for Cas9 protein interaction. Additionally, a PAM region (5'-NGG/NCC-3') must be present in the target sequence for Cas9 recognition and cleavage to occur (Gasiunas et al., 2012; Ran et al., 2013). As a result of the complex interaction with the Cas9 protein, double strand breaks (DSBs) are then induced, and the damage is corrected by the NHEJ or HDR DNA repair systems using random insertions or deletions (InDels) or precise corrections, respectively (Wyman and Kanaar, 2006; **Figure 3**).

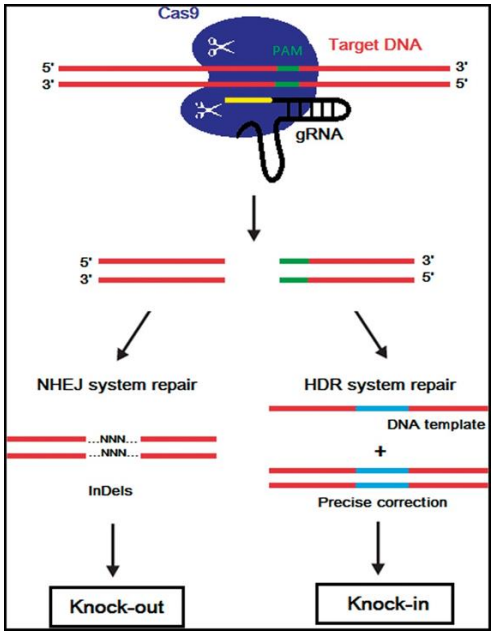


Figure 3. Mechanism of action of CRISPR/Cas9. The gRNA interacts with Cas9, which causes a DNA DSB in the specific genomic region. Subsequently, the systems of DNA repair mend the damage. Extracted from D'Agostino and D'Aniellov, 2017.

Previous work from the Hereditary Cancer lab at IGTP has used this technique, using ribonucleotide complexes (RNP) to edit the *NF1* gene in a control cell line, iPSC-*NF1(+/+)*, to obtain mutated clones with *NF1 (+/-)* and *(-/-)* genotypes (I. Uriarte TFM, 2019). The engineered sgRNA was site-specific for the target region of exon 2 of the *NF1* gene, and some of the modified knockout cells with target mutations in the gene were characterized to determine the type of mutation and its length, and their genotype.

1.2.3. Differentiation towards Neural Crest Cells

NC cells constitute a population of embryonic multipotent cells that emerge from the neural tube, migrate to diverse locations, and change into lineage-restricted precursors. During embryonic development, NC cells give rise to diverse derivatives, including mesenchymal, neuronal, glial, secretory, and pigmented cells (Simões-Costa and Bronner, 2015), so that NC cells are sometimes referred to as the 'fourth germ layer' (Hall, 2000).

After the closure of the neural tube, the last stage of neurulation, NC cells leave the neural tube via an epithelial-to-mesenchymal transition (EMT), which results in their transformation into migratory cells (Bronner and LeDouarin, 2012). Later, they reach their target tissues through specific migration pathways and differentiate towards some mechanisms of specification (Simões-Costa and Bronner, 2015). NC induction is mediated by four major developmental pathways: Wnt, FGF, BMP, and Notch signaling (Prasad et al., 2019). Wnt signaling is crucial for important aspects of NC induction, i.e., formation of the neural plate border and subsequent activation of neural crest-specifying transcription factors (Ji et al., 2019). FGF signaling has been especially implicated in the early phases of neural crest specification and BMP, appears to be critical after initial neural crest induction, and requires an intermediate activity level mediated by BMP antagonizing signals (Anderson et al., 2006; Tribulo et al., 2003; Marchant et al., 1998). Finally, Notch signaling is relevant in neural crest induction, migration, differentiation, and inhibition of neurogenesis (Noisa et al., 2014)**.** Some specific markers of the NC lineage are used to identify such transcription factors as SOX9, SOX10, and TFAP2A, as well as cell membrane markers such as p75 and HNK1 (Mazuelas et al., 2020).

Some protocols have been described for human embryonic stem cells (hESCs) and iPSCs to be propagated *in vitro* to generate NC cells. Menendez et al. (2013) and Lee et al. (2007) described an efficient differentiation protocol that employs a chemically defined medium to activate Wnt signaling while inhibiting Activin/Nodal/transforming growth factor β signaling. Then, these derived-NC cells can be differentiated and directed toward peripheral nervous system lineages (peripheral neurons and SCs) and mesenchymal lineages (smooth muscle, adipogenic, osteogenic, and chondrogenic cells) (Lee et al., 2007).

OBJETIVES

As was explained before, previous work in the lab was able to generate clones of iPSCs with genotypes *NF1 (+/-)* and *(-/-)* by editing the *NF1* gene by CRISPR/Cas9 technique to establish cell lines for further studies.

Not all the clones obtained were characterized. For that, the purpose of this study was to characterize one *NF1 (+/-)* and one *NF1 (-/-)* iPSC clone and confirm their mutations on the *NF1* gene, as well as test their pluripotency capacity and differentiation potential towards the NC lineage.

For that, the objectives of this project are:

a) To confirm *NF1* mutations to validate the genotype of the NF1 edited iPSC lines.
b) To evaluate the pluripotency capacity of the edited *NF1* iPSCs.
c) To differentiate the edited *NF1* iPSC lines toward the NC lineage.
d) To evaluate the differentiation potential towards the NC lineage of the edited *NF1* iPSCs.

METHODS

2.1. Cell culture

2.1.1. iPSCs samples

The iPSC lines used were derived from foreskin fibroblasts and generated at the Center for Regenerative Medicine, Barcelona (CMRB), and were named FiPS. This line was edited in exon 2 of the *NF1* gene using CRISPR/Cas9 technology (I. Uriarte TFM, 2019).

The three different clones from this edition were called: G3, B8, and F1.

2.1.2. Coatings and media

For the culture and maintenance of iPSC and NC cells, it is required to use plates coated with extracellular matrix. Matrigel is an extracellular matrix composed mainly of laminin (59%) and collagen VI (31%), and was used 1:20 (for iPSC) or 1:40 (for NC) Matrigel dilutions prepared in cold DMEM:F12 (1:1) media without supplements.

To coat the plates, Matrigel was added to 6-well plates and incubated for 45 minutes at room temperature (RT). After that, plates were washed with DMEM:F12, and 1.5 mL of iPSC or NC media was added.

iPSC cells were grown in mTeSR media, a feeder-free maintenance complex medium for human ES and iPSC (STEMCELL Technologies). NC cells were grown in BDM-NC media (**Table 2**), a specific media containing compounds that will direct NC differentiation.

Table 2. Components of BDM-NC media

BASAL DIFFERENTIATION MEDIA (BDM)	CATALOG NUMBER	MEDIA CONCENTRATION
DMEM:F12	Gibco 2196035/21765029	
Bovine Serum Albumin	Sigma 05470-1G	5 mg/mL
Penicillin/Streptomycin (10000units/ml:10000µg/ml)	Gibco 15140122	1X
Glutamax-I 200mM	Gibco 35050038	1X
MUM NEAA	Gibco 11140-035	1X
Trace Elements A	Corningn 25-021-Cl	1X
Trace Elements B	Corningn 25-022-Cl	1X
Trace Elements C	Corningn 25-023-Cl	1X
2-bmercaptoethanol	Gibco 313505-010	90 µM
Apo-Transferrin human	Sigma T2036	10 µg/mL
L-ascorbate	Sigma A4034	50 µg/mL
NC MEDIUM		
BDM		
Recombinant human Heregulin beta-1	Peprotech 100-03	10 ng/mL
Recombinant human IGF-I LR3	Peprotech 100-11R3	200 ng/mL
Recombinant human FGF basic	Peprotech 100-18C	8 ng/mL
CHIR99021	STEMCELL Technologies 72054	2 µM
SB431542	STEMCELL Technologies 72234	20 µ

2.1.3. Thawing and freezing iPSC and NC

For thawing the cells, the cryovial was quickly defrosted at 37°C, and its content was transferred into a tube with 15 mL of DMEM:F12 (1:1). Cells were centrifugated at 1200 rpm and plated on coated-Matrigel 1:20 DMEM:F12 (1:1) with mTeSR or BDM-NC medium.

Besides, cells from the three clones of iPSC and NC were cryopreserved as backups during their amplification. For freezing, the cells were washed with PBS, detached with accutase, collected with DMEM:F12 (1:1) media, centrifuged, and resuspended with 400 uL of freezing media for each cryovial. The cells must be rapidly placed on ice since the freezing medium is toxic at RT because it contains DMSO, a reagent that prevents, by osmosis, cells from popping when frozen.

2.1.4. iPSC culture

iPSCs are delicate cells that need some special care in culture; for that, all procedures must be done rapidly. They are grown in compact multicellular colonies with defined borders in Matrigel-coated plates 1:20 DMEM:F12 (1:1) and mTeSR medium.

iPSCs need to be observed daily to check their morphology, the presence of spontaneously differentiated cells, the size of colonies, and their compaction. When colonies are expanded over the plate and confluence, they are ready to split. The ideal ratio for passaging iPSCs is 1:2. Cells are washed with PBS, detached with accutase, collected with DMEM:F12 (1:1) media, and plated in mTeSR medium.

mTESR medium must be replaced every day, and cells may require time to adapt after thawing and splitting. Cells are incubated at 37°C in a humidified CO_2 incubator.

2.1.5. Differentiation iPSC-NC

Following the lab protocol, adapted from Menéndez et al. (2013), $9x10^4$ iPSC cells/cm^2 were plated on 6-well 1:20 diluted Matrigel-coated plates in mTeSR medium. For that, cell counting was performed using a Neubauer chamber, even though high clamp compaction made the measurement difficult.

The following day, the medium was replaced with BDM-NC media to start NC differentiation.

2.1.6. NC culture

In contrast to iPSCs, NC cells present a stellate morphology and are grown as single cells in Matrigel-coated 1:40 DMEM:F12 (1:1) plates in BDM-NC medium.

BDM-NC medium should be changed every 1-2 days, and cells should be ideally split in 1:3 and 1:6 dilutions every 3-5 days when they reach 90% confluency, using the same protocol as iPSCs.

2.2. DNA extraction, spectrophotometer, PCR, and Agarose gel

Genomic DNA was extracted from iPSC and NC cells using the Maxwell 16 cell LEV DNA Purification Kit (Promega), an instrument to extract DNA from cells grown either in suspension or attached to plates, following its protocol. DNA was quantified by a Nanodrop 1000 (Thermo Scientific) spectrophotometer.

Exon 2 of the *NF1* gene was amplified by PCR using *NF1_gDNA_exon2_F2* and *NF1_gDNA_exon2_R2* primers (**Table 3**) under the conditions described in **Table 4**. Each PCR reaction had the following components: 5 uL of Buffer Promega 5x, 1.5 uL of MgCl 25 mM, 0.5 uL of dNTPs 10 mM, 1 uL of each primer 10 nM, 0.15 uL of GoTaq 5U/mL Promega, 50 ng of DNA, and sterile miliQ H_2O water until a final volume of 25 uL.

Table 3. Primers information.

Primer name	DNA binding region	Product size (bp)	Sequence 5'-3'
*NF1*_gDNA_exon2_F2	60912…60932	394	GAAAATCGGAGTTTGAGATGC
*NF1*_gDNA_exon2_R2	61283…61305		CCACAGAAAATCACTTTCCATAC

Table 4. **PCR Conditions for exon 2 of the *NF1* gene.**

95°C	2'
95°C	30''
Ta°C	30''
72°C	30''
72°C	7'
72°C	7'
30 cycles	
Ta°C: 58	

H_2O was used for negative control and the FiPS cell line, the non-mutated parental cell line with the genotype *NF1 (+/+)*, for positive control.
DNA amplification was confirmed by electrophoresis on a 1.5% agarose gel.

2.3. Sanger sequencing

PCR products were treated with Exosap (4:1) to purify them, incubating at 37ºC for 30 minutes and at 80ºC for 15 minutes. Then, they were mixed with H_2O and the *NF1* forward primer, *NF1_gDNA_exon2_F2*. Samples were sent for sequencing to the Stab vida company, and results were analyzed using the CLC Genomics Workbench 11 software.

The same procedure was repeated with the *NF1* reverse primer, *NF1_gDNA_exon2_R2*.

2.4. Immunocytochemistry

iPSCs or NC cells were grown in Matrigel-coated 8-well chamber slide and fixed in 4% paraformaldehyde in PBS for 15 minutes at RT. Then, cells were permeabilized with PBS-Triton 0.1%, blocked in PBS-FBS 10% for 10 minutes, washed with PBS-FBS 1%, and incubated with OCT3/4 (1:200) (mouse primary antibody) in PBS-FBS 1% overnight at 4°C. After washing three times with PBS-FBS 1% for 10 minutes each, mAF488 (1:1000) (mouse secondary antibody) in PBS-FBS 10% was incubated for 1 hour at RT. Cells were again washed three times with PBS-FBS 1% and nuclei were stained with 1:1000 DAPI in PBS for 10 minutes. Later, samples were washed with PBS and mounted with one drop of Vectashield on a coverslip, and the borders were secured with nail polish. The slide can be stored at 4°C for approximately a week. Cells were observed using a LEICA DMIL6000 microscope, and images were captured using LASX software.

The same procedure was performed with NC cells, using SOX9 (1:100) (primary mouse antibody) and SOX10 (1:400) (primary rabbit antibody), and mAF488 (1:1000) and mAF568 (1:1000) (mouse secondary antibodies).

2.5. Flow cytometry

Following Serra Lab's established protocol, 800.000 cells per well were plated in Matrigel-coated 6-well plates, and two wells of each clone were detached with accutase and collected in 1 mL of wash buffer (PBS + 1% BSA at 4°C). At this point, cells were divided in three parts: 0.5 mL of cell suspension was treated with primary and secondary antibodies, and the rest volume was kept for controls; 0.3 mL of the mix was treated only with secondary antibodies, and 0.2 mL of the mix was used for the control of single cells.

Cells in PBS+1%BSA were centrifuged at 300 g for 2 minutes at 4°C, media was aspirated, and the cells were washed again with 0.3 mL of wash buffer. Cells were centrifugated again and resuspended in 200 uL of wash buffer containing the primary p75 antibody (mouse primary antibody) diluted 1:1000 and incubated for 30 minutes.

Then, 0.3 mL of wash buffer was added, cells were centrifuged, media was aspired, wash buffer was added, and cells were centrifuged again in the same conditions described before. Cells were incubated in 200 uL of wash buffer containing mAF488 (1:1000) (mouse secondary antibody) and incubated for 30 minutes on ice.

The same procedure was performed with Hnk1 (1:1000) (mouse primary antibody) and mAF647 (1:1000) (mouse secondary antibody).

Flow cytometry was performed using CANTO and BD FACSDiva 6.2 software.

RESULTS

3.1. *NF1* genetic analysis of *NF1 (+/+)*, *(+/-),* and *(-/-)* iPSC lines using molecular biology techniques and Sanger sequencing

The edited *NF1* iPSC clones, G3, B8, and F1, were thawed and maintained in culture until their amplification with mTeSR media, a specific medium for the growth of human ES and iPSC. The DNA of each cell line was extracted to amplify exon 2 of the *NF1* gene, the edited target region, by PCR using specific primers. Electrophoresis in an agarose gel at 1.5% was performed to confirm PCR amplification and the presence of the mutated region.

Figure 4A shows a schematic representation of the edited NF1 region in exon 2 and the primers used to amplify the region.

The results of the agarose gel are displayed in **Figure 4B**, which confirms amplification of exon 2 in the three iPSC lines (G3, B8, and F1) and in the FiPS *NF1 (+/+)* control line. The absence of a band for H_2O, the negative control, confirms that the samples are free of DNA cross-contamination.

Next, the PCR products were purified and prepared for Sanger sequencing. By using CLC software, the sequences were assembled with a *NF1* gene reference gDNA sequence, and exon 2 was compared to this sequence to identify the mutation type and its size in each edited clone.

The FiPS sequence, the control cell line, and the three edited *NF1* clones sequences are all displayed in **Figure 4C**.

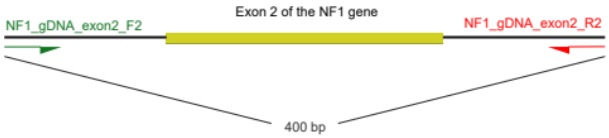
The results show that the FiPS sequence of the non-mutated cell line *NF1 (+/+)* matches the reference sequence for both alleles.

Also demonstrates the mutation that the iPSC G3 clone carries. The CLC Genomics Workbench 11 program is not capable of analyzing large alterations, so the sequences were manually aligned and compared. This allowed us to identify the cell line as a *(+/-) NF1* heterozygote with an eleven-nucleotide loss in one of the *NF1* alleles.

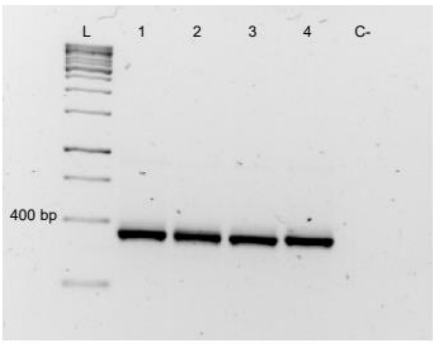
The iPSC B8 has a deletion in four nucleotides of both NF1 alleles, so it is a negative homozygote for the *NF1* gene.

Finally, since the genotype of the iPSC F1 is identical to the reference sequence *NF1 (+/+)*, CRISPR/Cas9 has not altered it.

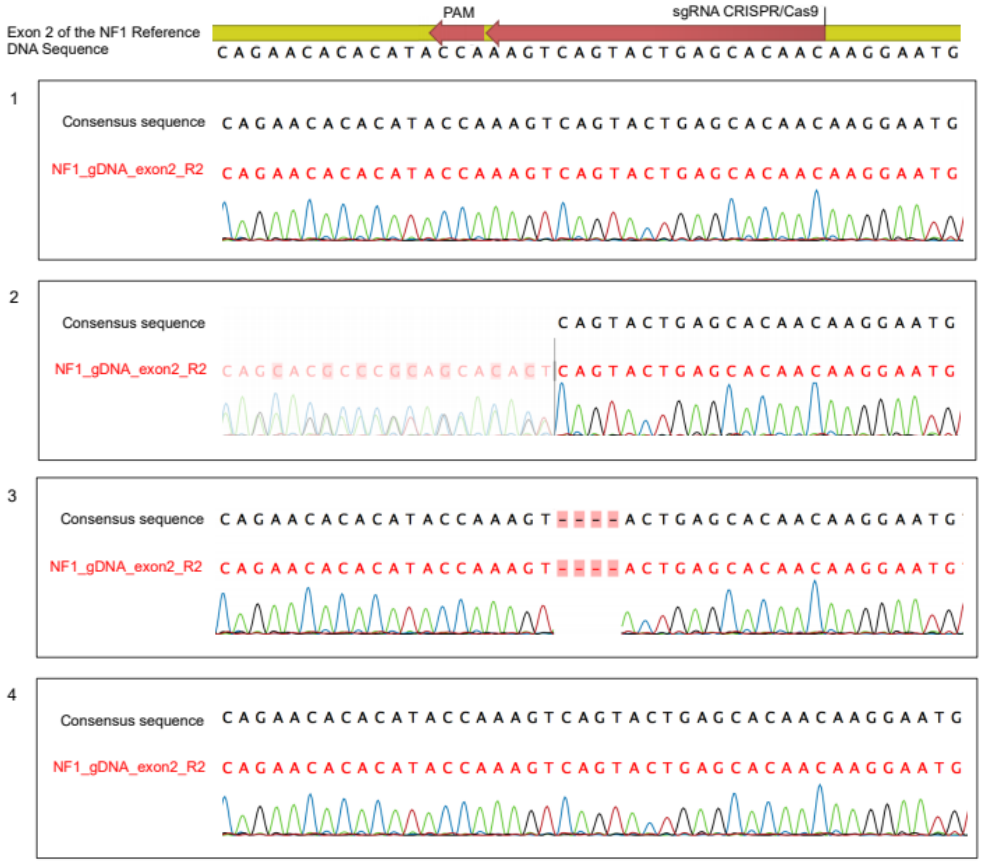
A)



B)



C)



D)

Clone	Alleles	gDNA mutation	cDNA mutation	Protein
2	Alelle 1	g.66037_66047del	c.90_101del	p.H31_Qfs*2
3	Alelle 1	g.66048_66051del	c.102_105del	p.S35_Lfs*7
	Alelle 2	g.66048_66051del	c.102_105del	p.S35_Lfs*7

Figure 4. Confirmation of the iPSC mutations in exon 2 of the *NF1* gene.

(A) Representation of exon 2 of the *NF1* gene with the forward and reverse primers of exon 2 of the *NF1* gene and its product size (400 bp).

(B) Agarose gel showing amplification of exon 2 of the *NF1* gene in the iPSC clones. L: ladder; 1: FiPS, control *NF1 (+/+)* cell line; 2: G3 clone; 3: B8 clone; 4: F1 clone; C-: negative control, H_2O.

(C) Sanger sequence analysis of the *NF1* edited region in the iPSC clones from PCR products with the reverse primer of exon 2 of the *NF1* gene. The first sequence is the reference *NF1* gDNA with the PAM sequence and the sgRNA. The next sequences are the control line and the three iPSC clones. 1: FiPS, control *NF1 (+/+)* cell line; 2: G3 clone; 3: B8 clone; 4: F1 clone.

(D) Table of the mutations in the edited iPSC clones. 2: G3 clone; 3: B8 clone.

Figure 4D shows the mutations identified in the alleles of the edited clones. In the case of the B8 clone, which has the genotype *NF1 (-/-)*, it was discovered that it carries the same deletion for both alleles of exon 2 of the NF1 gene.

These results demonstrate that the CRISPR/Cas9 system was able to edit some clones and produce various mutations in either one or both NF1 alleles, and three different iPSC genotypes were discovered: *NF1 (+/+)*, *NF1 (+/-),* and *NF1 (-/-).*

3.2. Evaluation of the pluripotency capacity by immunocytochemistry

iPSCs were maintained in culture for 18 days, and after performing 4-5 passages for the amplification of *NF1 (+/+), NF1 (+/-),* and *NF1 (-/-)* cells, the pluripotency capacity of the iPSC clones was tested by analyzing the expression of the OCT3/4 transcription factor by immunocytochemistry. Cells in culture were fixed in an 8-well chamber slide, incubated with the OCT3/4 antibody, and incubated with DAPI to stain nuclei. **Figure 5** displays the captured images of the three iPSC clones.

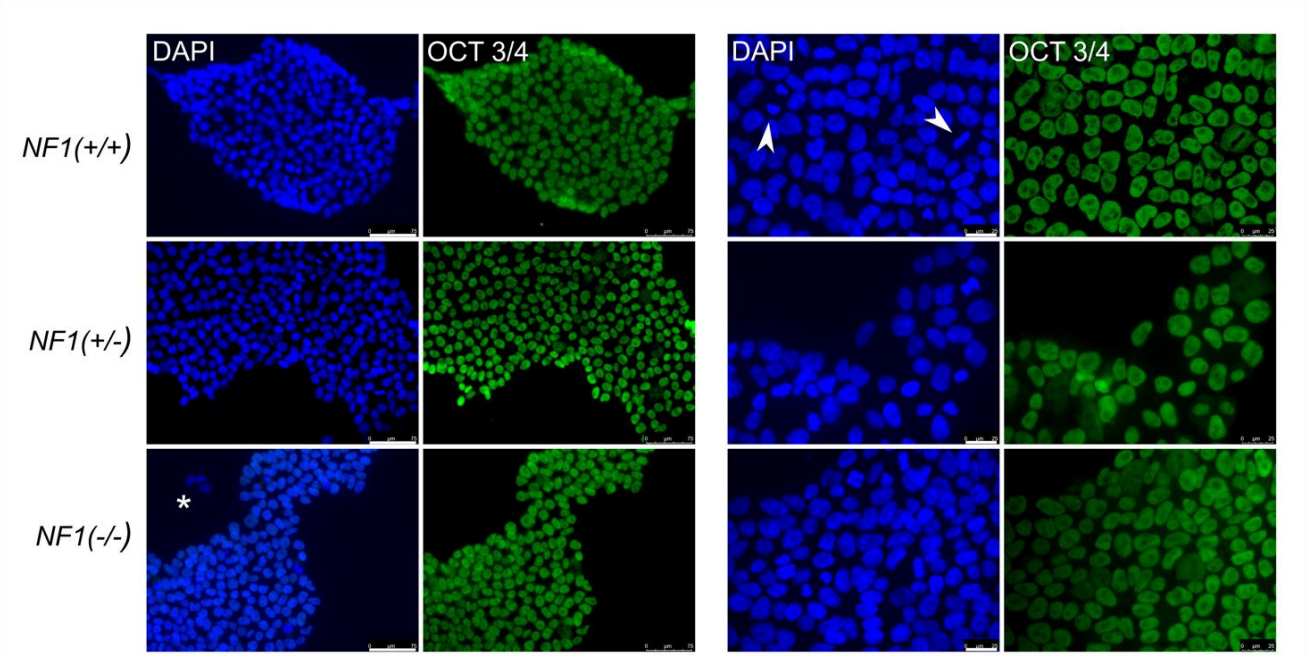


Figure 5. Analysis of a pluripotency marker in iPSC *NF1 (+/+)* F1, iPSC *NF1 (+/-)* G3, and iPSC *NF1 (-/-)* B8. Immunocytochemistry was performed with the OCT3/4 marker (in green). Nuclei were stained with DAPI (in blue). Scale bars: 75um (left panel) and 25um (right panel). The arrowhead points to a mitotic cell; the asterisk shows a group of cells negative for OCT3/4.

As is observed in **Figure 5,** OCT3/4 was expressed in all the nuclei of *NF1 (+/+)*, *NF1 (+/-),* and *NF1 (-/-)* cells, so the three iPSC clones have the same capacity to manifest pluripotency ability, and no differences are seen between the *NF1* genotypes of the cell lines. It is worth mentioning that the colonies observed showed an elevated presence of mitosis due to their self-renewal capacity (see arrowhead). Besides, some spontaneous differentiation was detected in single cells and small clumps of the *NF1 (-/-)* iPSC culture, as they do not express the pluripotency marker OCT3/4 (see asterisk).

These results confirmed that all iPSCs are able to maintain stem cell characteristics in culture, such as pluripotency and the ability to extensively proliferate, regardless of their *NF1* genotype.

3.3. Differentiation of iPSCs towards the NC lineage

iPSCs also have the potential to differentiate into different cell types, so following the lab protocol adapted from Menéndez et al. (2013), the capacity of the three iPSC clones to differentiate towards the NC lineage was tested. For the NC induction, iPSC cells were plated in mTeSR media, and the following day the media was changed to BDM-NC differentiation medium.

Figure 7 shows the evolution of the process of differentiation of the iPSC towards NC.

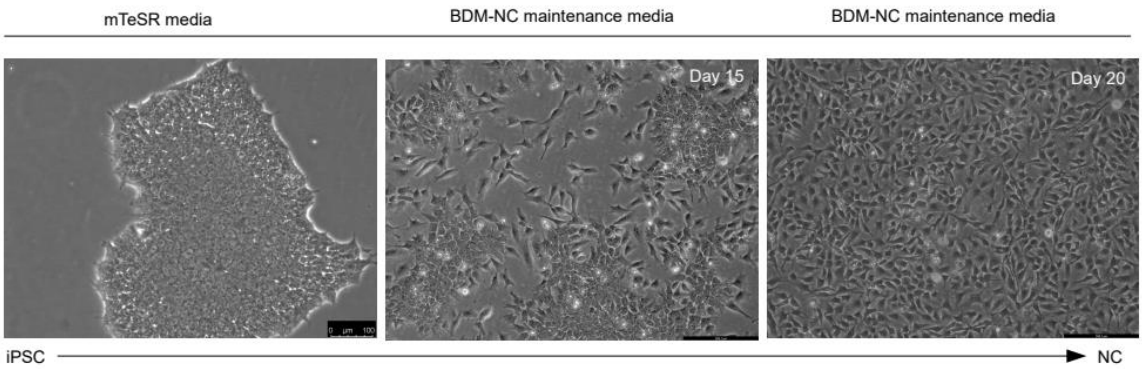


Figure 7. **Differentiation process to NC of the iPSC for 20 days.** It showed the morphology of the iPSCs, the iPSCs 15 days after NC induction, and the NC cells after 20 days of NC induction. Scale bar: 100um and 205,7um.

iPSCs cultivated in mTeSR media (left picture) showed the classical iPSC structure, where all cells were grouped in big compacted colonies with defined borders. After 15 days of NC induction (middle picture), a mixture of cell types with different morphologies were present, iPSC colonies turned smaller and started to spread out, and cells on their borders began developing into NC cells. Finally, after 20 days of NC differentiation (right picture), cells adopted a stellate morphology typical of NC cells. Either there was no presence of colonies or small clumps, and cells proliferated as single cells.

It is worth noting that during the differentiation, it was observed that the process was harder for the *NF1 (-/-)* B8 clon, compared to the *NF1 (+/+)* and *(+/-)* clones. *NF1 (-/-)* cells required more days in BDM-NC medium, so they needed more passages to turn into NC cells due to their slow growth.

Overall, these experiments demonstrate that all three clones of iPSC have the capacity to differentiate into NC cells, but the potential of the differentiation process is influenced by the *NF1* genotype.

3.4. Evaluation of the differentiation capacity of the NC lineage by flow cytometry and immunocytochemistry

The cells were completely differentiated after 20 days of NC induction. NC identity was confirmed by flow cytometry and immunocytochemistry by analyzing different specific NC markers, such as p75 and Hnk1, and SOX9 and SOX10, respectively.

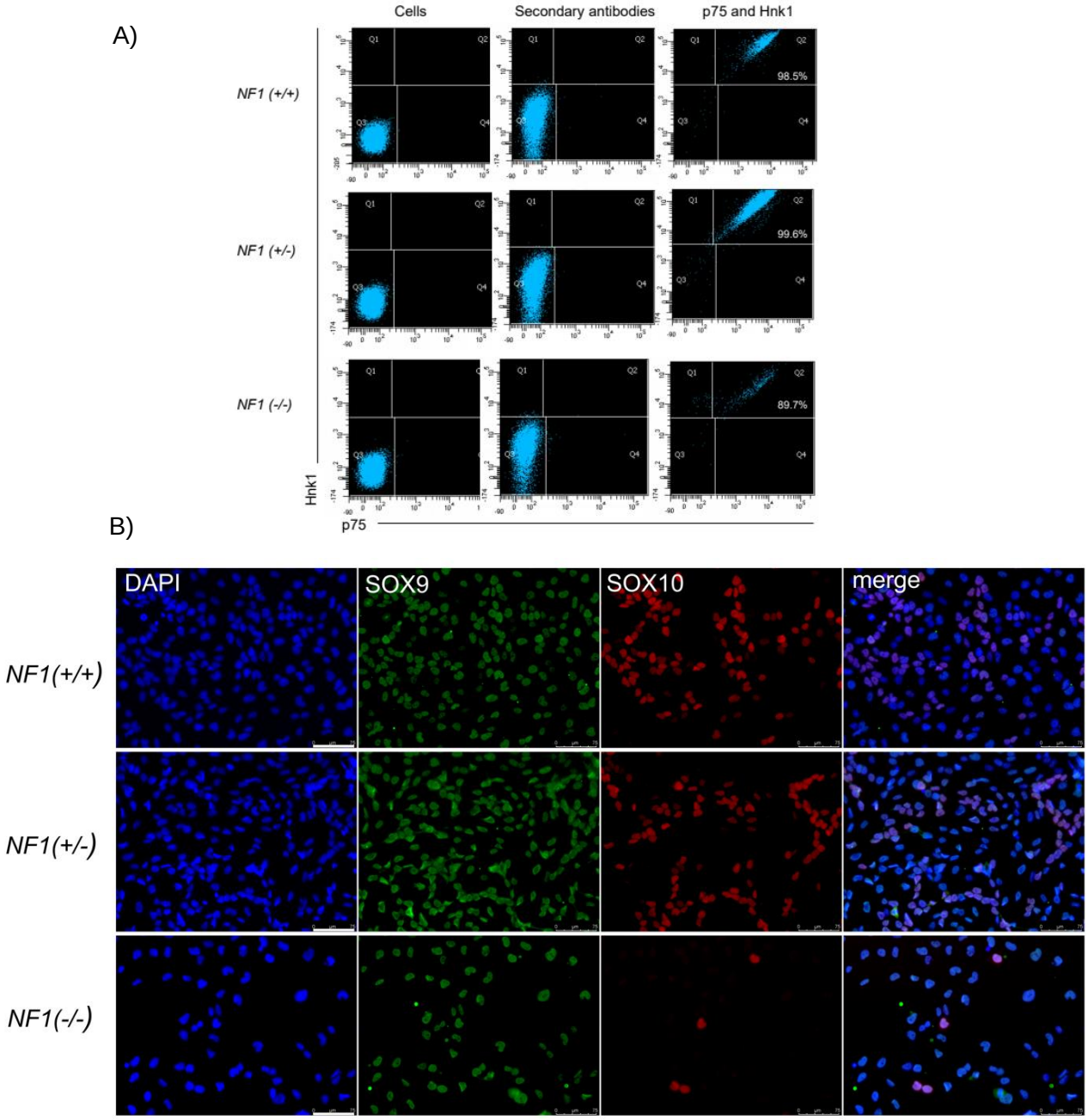


Figure 8. Analysis of NC lineage markers in NC *NF1 (+/+)* F1, NC *NF1 (+/-)* G3, and NC *NF1 (-/-)* B8 clones.

(A) Flow cytometry analysis for p75 and Hnk1. Each NC clone was evaluated using the two controls (single cells and cells incubated with secondary antibodies) and cells incubated with p75 and Hnk1. Hnk1 (Y axis) and p75 (X axis), and the percentage of double-positive cells is shown in the Q2 square. 10.000 cells were analyzed for the F1 and the G3 clones, and 2.000 cells for the B8 clone.

(B) Immunocytochemistry for Sox9 and Sox10. Performed with SOX9 (in green) and SOX10 (in red). Nuclei were stained with DAPI. Scale bar: 75um.

For flow cytometry, cells from the three clones were plated on Matrigel-coated 6-well plates, detached with accutase, and collected in wash buffer. Each sample was separated into three parts: a control part of single cells, a control with cells just exposed to secondary antibodies, and cells exposed to p75 and Hnk1 and secondary antibodies (**Figure 8A**).

Differentiation was also monitored by analyzing SOX9 and SOX10, two master transcription factors, by immunocytochemistry. Cells in culture were fixed in an 8-well chamber slide, incubated with SOX9 and SOX10 antibodies, and nuclei were stained with DAPI (**Figure 8B**).

The results of the expression of p75 and Hnk1 membrane markers by flow cytometry analysis are shown in **Figure 8A.** Both control conditions in the three cell lines (the single cells and cells treated only with mAF488 and mAF647 secondary antibodies) show non-expression of p75 and Hnk1 markers, validating the experiment.

A high proportion of *NF1 (+/+)* and *NF1 (+/-)* NC cells coexpressed p75 and Hnk1, 98.5% and 99.6%, respectively. For the *NF1 (-/-)* NC clone, only about 2,000 cells could be analyzed because of its slow proliferation, and the assay requires 10,000 cells to provide significant findings. However, within the 2,000 cells analyzed, 89.7% of them expressed both p75 and Hnk1 markers.

Figure 8B shows the expression of SOX9 and SOX10 by immunocytochemistry. The images captured displayed that all cells in the three clones are SOX9-positive, while SOX10 expression is observed at various levels depending on the clone, indicating there are variations related to *NF1* genotype.

The *NF1 (+/+)* clone was used as the control cell line, which confirms that the established protocol is valid. According to that, almost all *NF1 (+/+)* and *NF1 (+/-)* cells were double-positive for both transcription factors, and on the contrary, only a small portion of *NF1 (-/-)* cells expressed the SOX10 factor. This result may indicate that either these *NF1(-/-)* cells need more time to correctly differentiate towards NC or that *NF1* is affecting the expression levels of SOX10.

In summary, the expression of the NC stage markers analyzed (p75, Hnk1, SOX9, and SOX10) confirmed the ability of the three edited *NF1* clones to differentiate into NC cells, although *NF1 (-/-)* cells had lower levels of SOX10 than *NF1 (+/+)* and *NF1 (+/-)* clones.

3.5. Mutation confirmation of NC clones by Sanger sequencing

After the differentiation of iPSCs to NC, the presence of the target DNA, exon 2 of the *NF1* gene, was confirmed in the three cell lines in order to validate the mutation and rule out cross-contamination of cells during culture.

The DNA of the three NC clones was extracted, and a PCR was performed to amplify the target region by using specific primers. A performed agarose gel confirmed the presence of the target sequence of the three clones, and Sanger sequencing validated the genotype assigned before the differentiation.

DISCUSSION

NF1 is a rare genetic disease where peripheral nervous system is mainly implicated. Neurofibromas are benign tumors that manifest the *NF1* tumor suppressor gene, which is completely lost in progenitors of Schwann cells. The more common types of neurofibromas are cutaneous NFs, which are skin tumors that affect patients' quality of life, and plexiform NFs, which are congenital tumors that can be transformed into MPNSTs, a malignant tumor.

Previous work in Serra's lab used CRISPR/Cas9 genome editing technology, to obtain mutated clones of iPSCs in the target region of exon 2 of the *NF1* gene to establish cell lines for further *in vitro* studies.

Three of the *NF1* iPSC-edited clones, G3, F1, and B8, were selected to determine their mutations and test their stem cell abilities.

First, the three clones were characterized by confirming their *NF1* mutations. The results identified the iPSC F1 clone as a non-edited cell line having an *NF1 (+/+)* genotype, the clone iPSC G3 as an *NF1 (+/-)* carrying a deletion of eleven base pairs in one of the alleles, and the iPSC B8 clone as an *NF1 (-/-).* Surprisingly, this clone carried the same deletion of four base pairs in both alleles.

One of the important obstacles in iPSC technology is the variability between different lines caused by the genetic background. Therefore, it is crucial to create isogenic lines that differ only in the genetic mutation causing the disease. In this project, we obtained three isogenic lines, which will be very valuable for the lab.

The cells of the three clones were maintained in culture under specific conditions for iPSC amplification. All cells showed their typical iPSC compact colony morphology, regardless of *NF1* genotype. Since iPSCs are sensitive cells, every procedure was performed quickly and with careful care.

After culturing cell lines to amplify clones, pluripotency, one of the particularities of the iPSCs, was evaluated to validate that cells in culture were able to maintain their characteristics. The results showed that the three clones analyzed (*NF1 (+/+), NF1 (+/-),* and *NF1 (-/-)*) expressed OCT3/4, a pluripotency transcription factor. In the *NF1 (-/-)* clone, there were some OCT3/4 non-marked single cells, indicating there was some spontaneous differentiation in that iPSC culture. In clumps, an elevated mitosis rate was observed, another characteristic of iPSCs: self-renewal ability.

Next, iPSC differentiation ability was proved by establishing the NC differentiation procedure. To generate NCs, we used a previously described differentiation protocol that employs BDM-NC media, which contains specific factors for the activation of Wnt signaling while inhibiting Activin/Nodal/transforming growth factor β signaling (Lee et al., 2007; Menendez et al., 2013). During the iPSC-NC differentiation, it was observed that the process was harder for *NF1 (-/-)*, compared to *NF1 (+/+)* and *(+/-)* clones. This cell line develops more slowly and has a tougher time differentiating towards the NC lineage, according to previous Serra's laboratory experiments.

Culturing NC cells is more suitable and practical than iPSCs, which are grown as single cells and are characterized by their stellate morphology.

After 20 days of differentiation induction, changes in the morphology of the cells of the three different genotypes were noticed, and differentiation capacity was evaluated to confirm that all cells pertained to the NC lineage by analyzing the expression of specific lineage markers using flow cytometry and immunocytochemistry.

Flow cytometry analysis showed that approximately 95% of *NF1 (+/+), NF1 (+/-),* and *NF1 (-/-)* cells co-expressed two lineage markers, p75 and Hnk1. However, only 2.000 cells for the *NF1 (-/-)* clone could be analyzed due to its slow growth. Although this result is valid, this experiment should be performed again using more cells to confirm it.

To ensure that the differentiation process was completed, immunocytochemistry was performed using two master transcription factors for the NC lineage (SOX9 and SOX10). The results showed high levels of SOX9 expression in the three types of genotypes, while SOX10 expression was variable depending on the *NF1* genotypes. *NF1 (+/+)* and *NF1 (+/-)* cells were SOX9/SOX10 double-positive, while *NF1 (-/-)* cells expressed lower levels of the SOX10 master transcription factor. These results are in accordance with previous work from the laboratory, and they suggest *NF1* function is somehow implicated in SOX10 expression. However, other experiments would be needed to determine the exact mechanisms by which *NF1* regulates SOX10.

CONCLUSIONS

The characterization of iPSC clones with different *NF1* genotypes that demonstrate differentiation capacity towards NC will be very useful to establish non-perishable cell lines to understand the processes of the development and differentiation of NFs.

After genotyping different edited iPSC clones in the *NF1* gene, determining their *NF1* mutations, and evaluating their *in vitro* pluripotency capacity and differentiation potential towards the NC lineage, the project's conclusions are:

a) The genetic tool CRISPR/Cas9 correctly edited exon 2 of the *NF1* gene in iPSCs.

- The iPSC clone F1 has a genotype of *NF1 (+/+).*
- The iPSC clone G3 has a genotype of *NF1 (+/-)* with an eleven-nucleotide deletion in one allele.
- The iPSC clone B8 has a genotype of *NF1 (-/-)* with a deletion in four nucleotides in both alleles.
- These three iPSC lines are isogenic, and they will constitute a valuable disease model for NF1.

b) The three edited iPSC clones are able to maintain their pluripotency potential, regardless of their *NF1* genotype.

- The three iPSC clones analyzed show the typical appearance of iPSCs, so differences in *NF1* genotype do not have an effect on iPSC morphology.
- The three iPSC clones analyzed express the pluripotency marker OCT3/4.
- The three iPSC clones analyzed maintain their self-renewal potential due to their high mitosis rate.

c) The three iPSC clones have the capacity to differentiate towards the NC lineage, but *NF1* seems to affect this differentiation capacity.

- The three iPSC clones differentiated into NC cells show the typical NC appearance, so differences in *NF1* genotype do not have an effect on NC morphology.
- *NF1 (-/-)* iPSC cells present a slower differentiation capacity towards NC cells compared to *NF1 (+/+)* and *NF1 (+/-)* cells. This indicates that complete inactivation of the *NF1* gene affects the differentiation process towards the NC lineage.

- *NF1 (+/+), NF1 (+/-),* and *NF1 (-/-)* NC cells express specific NC lineage markers such as p75, Hnk1, SOX9, and SOX10, so they pertain to the NC lineage.
- *NF1 (-/-)* NC cells show lower expression of the master transcription factor SOX10 than *NF1 (+/+), NF1 (+/-),* and *NF1 (-/-)* NC cells, indicating *NF1* may influence SOX10 expression.

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