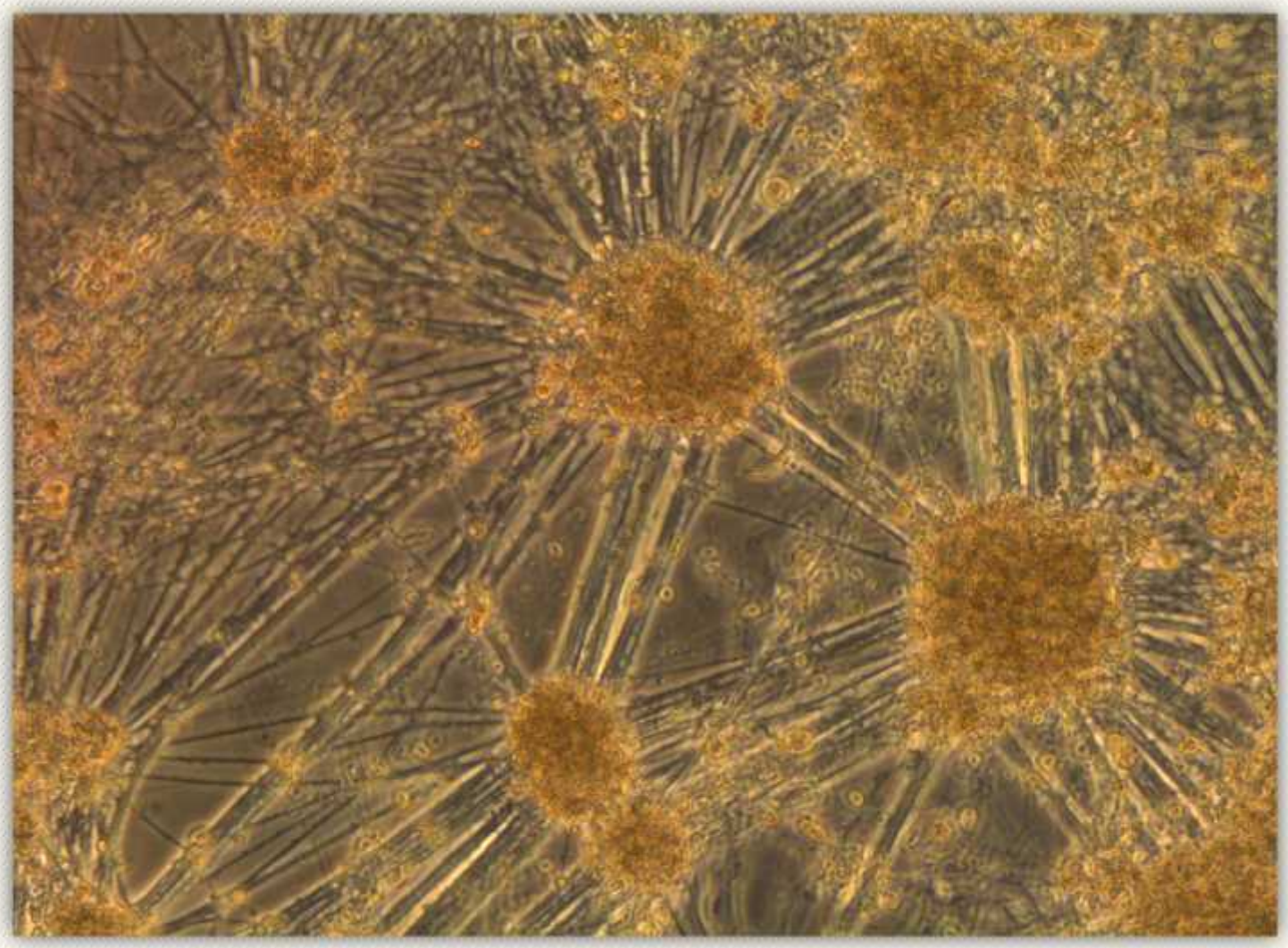

André Filipe Santos Sousa

Generation and characterization of functional sensory neurons from hESCs and hiPSCs

Tese de Mestrado em Biologia Celular e Molecular

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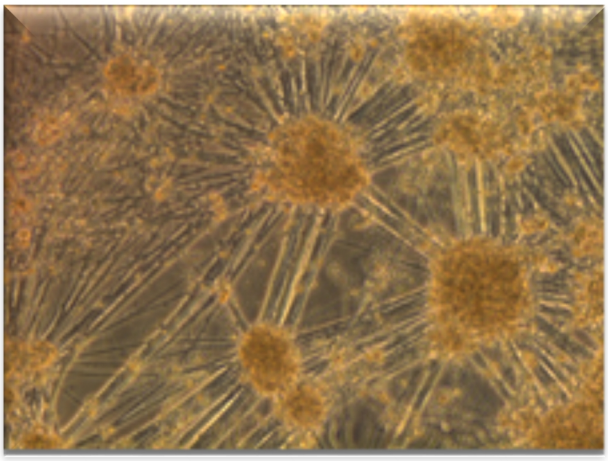
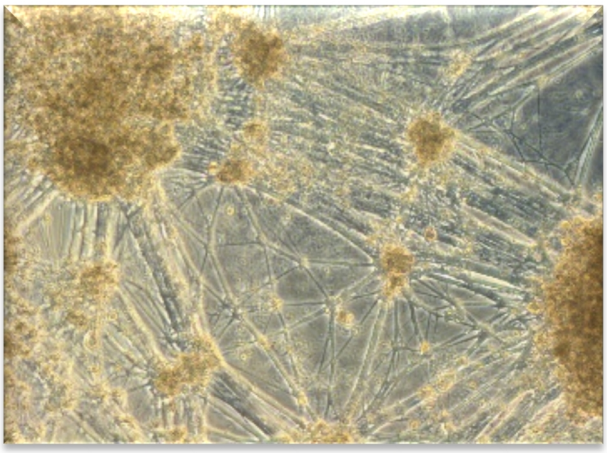


Master's Thesis nominated to obtain the academic degree in Cellular and Molecular Biology, Life Sciences Department, University of Coimbra by André Sousa.

Supervisor: Carlos Duarte



THIS PROJECT WAS PERFORMED IN THE STEM CELL INSTITUTE OF KU LEUVEN, BELGIUM, DEPARTMENT OF DEVELOPMENT AND REGENERATION IN THE LABORATORY OF CATHERINE VERFAILLIE AND UNDER SCIENTIFIC GUIDANCE OF BELA SCHMIDT.

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"Eu tentei 99 vezes e falhei, mas na centésima tentativa eu consegui, nunca desista dos seus objetivos mesmo que esses pareçam impossíveis, a próxima tentativa pode ser a vitoriosa."

Albert Einstein

ABSTRACT

The lack of access to the neuronal tissue, limited our understanding about its development and physiology of pain in humans. Therefore, the generation of functional human sensory neurons for disease modelling, drug screening and clinical applications is an urgent and unmet need. Nociceptors are the sensory neurons related to sense the pain, characterized by the presence of transient receptor potential channels which have sensory functions linked to transduction of noxious stimuli as well as signalling within the pain system. In this thesis, we first characterized the nociceptors generated from induced pluripotent stem cell with a protocol developed on the laboratory, comparing it to a protocol described by Young *et al.,* 2014. We used techniques as real-time reverse transcription-polymerase chain reaction and staining to check the neuronal phenotyping of the cells generated, as well as calcium imaging as a functional assay, to test about the functionality of transient receptor potential (TRP) channels, in order to characterize the neurons generated. We showed that only after 50 days of differentiation with both protocols we can have functional mature sensory neurons, with transient receptor potential cation channel sub family V, member 1 and transient receptor potential cation channel subfamily A, member 1 being activated. Moreover, the best results were achieved with the adapted protocol. Next, we focused on different approaches to improve the differentiation protocol. Changing cell line and replating dilution, did not enhance the protocol. Hence, we next hypothesized that current hurdles to speed up differentiation might be overcome by overexpression of key transcription factors involved in the sensory neurons differentiation: PR domain 12, brain-specific homeobox 3A, insulin gene enhancer and kruppel-link zinc finger transcription factor. A technique used on the laboratory, recombinase-mediated cassette exchange, was employed to generate two cell lines, one overexpressing PRDM12 and the other overexpressing ISL1-BRN3A-KLF7. We proved that in the new cell lines, the transcription factors were being correctly overexpressed, and collectively our results demonstrate that overexpressing PRDM12 improves the homogeneity of the cells generated. Further studies are required to evidence the impact of this new cell lines on the upgrading of the differentiation protocol. This work will open new opportunities for investigating in vitro disease modelling and evaluation of pharmacological responds to pain research.

Key words: *iPSC, differentiation, sensory neurons, nociceptors, pain, TRP channels, RMCE, overexpression, transcription factors*

RESUMO

A falta de acesso ao tecido neuronal limita o nosso conhecimento acerca do seu desenvolvimento e da fisiologia da dor nos humanos. Portanto, a geração de neurónios sensoriais humanos funcionais de forma à criação de modelos de doenças relacionadas com a dor, triagem de drogas e aplicações clinicas é uma necessidade urgente e não atendida. Os *nociceptors* são os neurónios sensoriais responsáveis por receber o estímulo da dor, caracterizados pela presença dos *TRP channels* que têm funções sensoriais na medida em que estão ligados à transdução de estímulos nocivos assim como à sinalização dentro do sistema da dor. Nesta tese, primeiramente caracterizamos os *nociceptors* gerados a partir de células estaminais recorrendo a um protocolo desenvolvido no nosso laboratório, comparando-o a um protocolo descrito por Young *et al.,* 2014. Foram usadas técnicas como *qRT-PCR* e *staining,* de forma a conferirmos o fenótipo neuronal das células geradas, assim como *calcium imaging* como um teste funcional acerca da atividade dos *TRP channels* com o objetivo de caracterizarmos os neurónios gerados. Aqui, demonstramos que apenas após 50 dias de diferenciação com ambos protocolos conseguimos obter neurónios sensoriais maduros funcionais, com a ativação de *TRPV1* e *TRPA1*. Ainda, o protocolo adaptado no laboratório teve melhores resultados. De seguida, focamo-nos nas várias formas de melhorarmos o protocolo de diferenciação. Mudar a linha celular usada e a diluição na fase de *replating* não aperfeiçoou o protocolo. Sendo assim, supomos que as barreiras em conseguir uma diferenciação mais rápida podiam ser ultrapassadas pelo aumento de expressão de fatores de transcrição envolvidas na diferenciação de neurónios sensoriais: *PRDM12*, *BRN3A*, *ISL1* e *KLF7*. Uma técnica usada no laboratório, *RMCE*, foi usada de forma a gerar duas linhas celulares, uma com o aumento de expressão de *PRDM12*, e a outra com o aumento de expressão de *ISL1, BRN3A* e *KLF7*. Neste trabalho provamos que as novas linhas celulares estavam de facto com a expressão aumentada dos fatores de transcrição inseridos, e os nossos resultados demonstram que o aumento de expressão de *PRDM12* contribuiu para o melhoramento da homogeneidade das células geradas. Futuros estudos têm de ser feitos de forma a evidenciar o impacto destas duas novas linhas celulares no aprimoramento do protocolo de diferenciação. Este trabalho vai abrir novas oportunidades para a investigação *in vitro* de modelos de doenças e a avaliação de respostas farmacológicas na investigação da dor.

Palavras-chave: *células estaminais, diferenciação, neurónios sensoriais, nociceptors, dor, TRP channels, RMCE, aumento de expressão, fatores de transcrição.*

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LIST OF ABBREVIATIONS

AAVS1	Adeno-associated virus integration site 1
BDNF	Brain-derived neurotrophic factor
BMP	Bone morphogenetic protein
BRN3A	Brain-specific homeobox 3A
CRISPR	Clustered regularly interspace short palindromic repeats
DRG	Dorsal Root Ganglia
DSB	Double Strand Break
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
Flp	Flippase
FRT	Flippase-recombinase target
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDNF	Glial-derived neurotrophic factor
HDR	Homology-directed repair
hESC	Human embryonic stem cell
hiPSc	Human induced pluripotent stem cell
hPSC	Human pluripotent stem cell
HYG-tk	Hygromycin phosphotransferase – Herpes simplex virus type 1 thymidine kinase gene
IDT	Integrated DNA Technologies
ISL1	Insulin gene enhancer
iPSC	Induced pluripotent stem cell
KLF4	Kruppel-link factor 4
KLF7	Kruppel-link zinc finger transcription factor
MCL	Mother Cell Line
mRNA	Messenger Ribonucleic Acid
NCC	Neural Crest Cell
NEB	New England Biolabs

NHEJ Non-homologous end joining

NGF Nerve growth factor

NGN1 Neurogenin 1

NGN2 Neurogenin 2

NSAIDs Nonsteroidal anti-inflammatory drugs

NT3 Neurotrophin 3

OCT4 Octamer binding transcription factor-4

PCR Polymerase Chain Reaction

PDGF Platelet-derived growth factor

PFA Paraformaldehyde

PPP1R12C Protein phosphatase 1, regulatory (inhibitor) subunit 12C

PRDM12 PR Domain 12

PS Pregnenolone sulfate

qRT-PCR Real-time reverse transcription-polymerase chain reaction

RMCE Recombinase-mediated cassette exchange

RNA Ribonucleic Acid

ROCK-inhibitor Rho-associated coiled-coil containing protein kinase inhibitor

RUNX1 Runt-related transcription factor 1

SOX2 Sex determining region Y-box 2

TAC Tachykinin Precursor 1

TALE Transcription activator-like effectors

TALEN Transcription activator-like effector nuclease

TF Transcription Factors

TGF-B Transforming growth factor beta

TRP Transient receptor potential

TRPA1 Transient receptor potential cation channel, subfamily A, member 1

TRPV1 Transient receptor potential cation channel subfamily V member 1

TRPM3 Transient receptor potential cation channel subfamily M member 3

TRPM8 Transient receptor potential cation channel subfamily M member 8

TrkA Tropomyosin receptor kinase A

TrkB Tropomyosin receptor kinase B

TrkC Tropomyosin receptor kinase C

VEGF Vascular endothelial growth factor

ZFN Zinc finger nuclease

1 INTRODUCTION

1.1 IPS CELLS

Stem cells are unspecialised cells, with the capacity for self-renewal and differentiation into the all types of cells (Stadtfeld *et al.,* 2010). These cells were discovered in 1961, by Till and McCulloch, and since then they have been receiving a huge focus from the scientific community (Maximow, 1909; Till and McCulloch, 1961). Stem cells can be categorized in embryonic stem cells (ECS), mesenchymal stem cells (MCS), adult stem cells (ADS) and induced pluripotent stem cells (iPSC). ESCs are derived from the inner cell mass and demonstrate excellent pluripotency, however their use has some ethical issues, once the destruction of the blastocyst is necessary to obtain it (Evans, *et al.,* 1981). The first MSCs were discovered in the bone marrow and were shown to be capable of making bone, cartilage and fat cells. They are obtained from these tissues, thus free from ethical concerns. However, their use is limited by low cell numbers and a reduced pluripotency. ASCs are tissue-specific cells, and they don't seem to self-renew in culture as easily as embryonic stem cells do (Thomson *et al.,* 1998). The major breakthrough came in 2006 when Takahashi and Yamanaka opened a completely new venue in stem cell research by generating stem cells (iPSC) that were having properties relating to ESCs (Takahashi and Yamanaka, 2006). The discovery of iPSCs dramatically altered the previous dogma of cellular differentiation as a unidirectional, nonrevertible developmental process, resulting in a paradigm shift in the field of developmental biology (Diecke *et al.*, 2014). iPSCs were generated from fibroblasts by using a combination of 4 reprogramming factors, including octamer binding transcription factor-4 (OCT4), sex determining region Y-box 2 (SOX2), kruppel like factor-4 (KLF4), and c-Myc and were demonstrated both self-renewing and differentiating into all cell types like ESCs, and thus, making iPSCs an attractive cell source for translational and regenerative medicine applications, and could be used as an alternative for ESCs in various researches. Indeed, ethical concerns, limited availability, and possible immunogenicity are the main disadvantages of ESCs over iPSCs (Dieck *et al.*, 2014; Singh *et al.,* 2015). Since their discovery, iPSCs have opened new fields of investigation not only in cellular reprogramming itself but also in creating human models for disease modelling and drug target discovery (Soares *et al.*, 2014). Furthermore, and possibly the most promising application of iPSCs is in the field of regenerative medicine, through the regeneration of tissue-specific cells for the transplantation to patients of various injuries or degenerative diseases (Singh *et al.*, 2015).

1.1.1. GENERATION OF IPSC

In 2006, Takahashi and Yamanaka demonstrated that terminally-differentiated somatic cells (fibroblasts) can be reverted into a cell like ESC by overexpressing transcription factors that regulate the maintenance of ESC pluripotency. These induced pluripotent stem cells are characterized by expression of typical pluripotency markers OCT4, SOX2, KLF4 and c-Myc (Zhou, *et al.,* 2009). OCT4 is a transcription factor associated with the maintenance of the pluripotency of ESCs, and it creates a heterodimer with SOX2 in ESCs, so that SOX2 binds to chromatin neighbouring to the OCT4 binding sites, and thus being able to preserve the developmental potential of stem cells (Niwa *et al.,*2000; Chambers and Tomlinson, 2009; Avilion *et al.,* 2003). In

turn, KLF4 plays an important role in regulating processes like differentiation, development, proliferation, apoptosis and maintenance of normal tissue homeostasis and c-Myc is a protein and is a part of the process of cell growth, cell proliferation, apoptosis and cellular metabolism (Shi, 2013; Boxer and Dang, 2001).

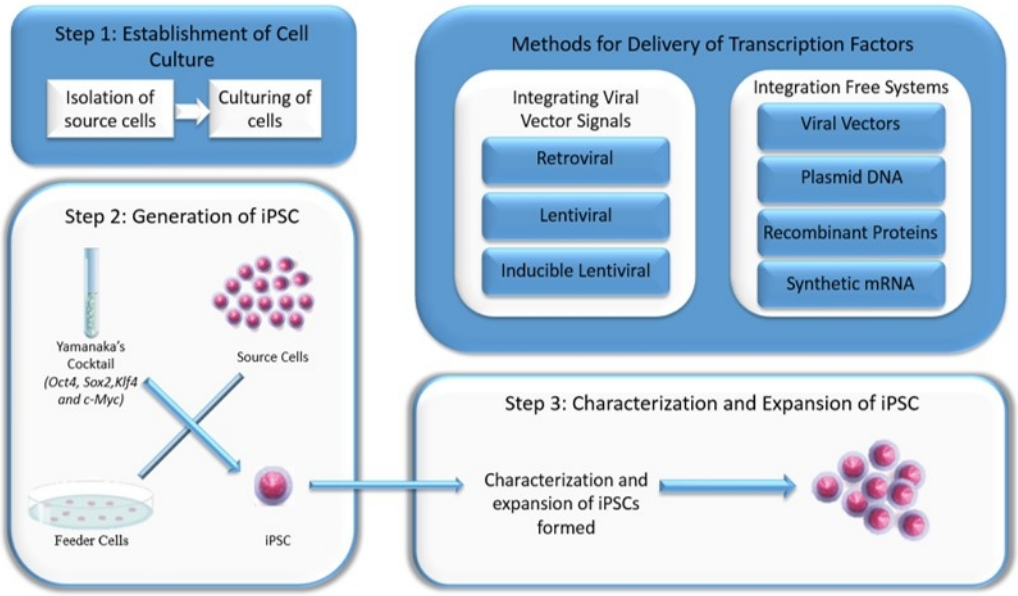


Figure 1. Overview of the methodology for the generation of iPSCs.

iPSCs can be generated by introducing this factors into somatic cells. Various sources of cells have been used to generate it, like fibroblasts (Takahashi *et al.,* 2007), cord blood, peripheral blood, bone marrow (Singh *et al.,* 2015), and even a few viable epithelial cells from urinal track (Zhou *et al.,* 2008). The process starts with the establishment of initial cell culture, followed by the induction of iPSCs and then the characterization and expansion (figure 1). After the source cells being isolated and cultured, the reprogramming factors are inserted into those cultured cells. Some methods introduce the transgenes on viral vectors, resulting in their integration into the genome of the cells. However, this method has the risk of insertional mutagenesis (Gonzalez *et al.,* 2011). Several non-integrating reprogramming techniques have thus been developed to circumvent the risk of spontaneous tumor formation and to improve the quality of the generated iPSCs (Stadtfeld *et al.,* 2008). Then, the most common approaches now used are non-integrating and rely on adenoviral (Stadtfeld *et al.,* 2008), Sendai virus (Fusaki *et al.,* 2009), or episomal plasmid vectors (Yu *et al.,* 2009). Other non-integrating methods use synthetic RNAs (Warren *et al.,* 2009) and protein-based reprogramming (Kim *et al.*, 2009). These methods vary in efficiency, complexity, labour required and compatibility with different somatic cell types. The most preferred method is the episomal plasmids, followed by Sendai virus and modified ribonucleic acids (RNAs), and even this ones has some limitations: episomal plasmids have lower reprogramming efficiencies, Sendai viruses require higher biosafety and are relatively costly and messenger RNA reprogramming is labor intensive (Soares *et al.*, 2014).

After the transfection, the cells are incubated on feeder layers under appropriate conditions, and the generation of iPSCs occurs after the expression of the reprogramming factors. The originated colonies can be characterized by different morphological and

physiochemical methods. Reprogrammed colonies are tightly packed, sharp edged, flat and mitotically very active. However, it is hard to characterize these cells only based on the morphology. So, iPSCs can be defined on the basis of the expression of transcription factors and cell surface proteins (SSEA-4, alkaline phosphatase) (Singh *et al.*, 2015).

1.1.2. DIFFERENTIATION INTO SENSORY NEURONS

The lack of access to sensory neurons has limited researcher's understanding about the development and the physiology of pain in humans (Young *et al.*, 2014). Indeed, obtaining and manipulating sensory neurons is a tough job, so traditional methods for studying sensory neurons have largely relied on animal models (Scholz and Wolf, 2002; Rubin, 2008). However, human mechanisms of pain and itch perception can differ from those of rodents, thus it is necessary to produce functionally responsive, human sensory neurons in sufficient number for studying the aspects of pain, itch and other disorders that affect the sensory neurons (Blanchard *et al.,* 2015). The sensory neurons generated *in vitro* from pluripotent cells are suitable models for pain and sensory disease modelling, pharmacological assays, drug screening, especially *in vitro* screening for the next generation of analgesic drugs (Young *et al.,* 2014).

The potential of iPSCs to differentiate *in vitro* into neural precursor cells and neurons has been reported (Yuan *et al.,* 2013). It was demonstrated that iPSCs could generate astrocytes, motor neurons, sensory neurons and oligodendrocytes (Wang and Doering, 2012). Chambers and his team, in 2009, proved that iPSCs efficiently induces formation of neural cells with a help of fibroblast growth factor-2 (FGF-2), Rho-associated coiled-coil containing protein kinase inhibitor (ROCK-inhibitor), transforming growth factor beta (TGF-B) inhibitor and Noggin (Chambers *et al.,* 2009). Three years later, the same author described a protocol that originate sensory neurons from iPSCs in just 10 days of differentiation (Chambers *et al.,* 2012). Usually, the *in vitro* derivation of postmitotic neurons from Human pluripotent stem cells hPSCs requires extended culture periods, during 30 days or more (Zhang, 2009; Elkabetz *et al.,* 2008). He showed that with the addition of a combination of 3 small molecules (CHIR99021, DAPT, SU5402) to the standard protocol used by him back on 2009, it enables the acceleration of the differentiation and fate specification toward a sensory phenotype. SU5402 is a potent inhibitor of vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF) tyrosine kinase signaling12, CHIR99021 can act as a WNT agonist by selectively inhibiting GSK-3β stabilizing βcatenin13, and DAPT is a γ-secretase inhibitor blocks Notch signalling (Chambers *et al.,* 2012).

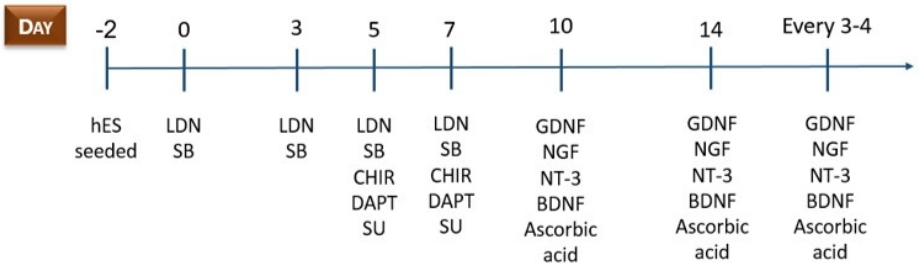


Figure 2. Protocol of sensory neuron differentiation. Day 0-5 – addition of the molecules LDN and SB to drive anterior neuroectoderm specification. Day 5-10 - nociceptor induction, by adding CHIR, DAPT, and SU together with LDN and SB. From day 10 on, it is the maturation step and there's the addition of GDNF, NGF, NT3, BDNF and ascorbic acid.

1.2. Sensory Neurons

The nervous system consists of many different types of neuron, among them are the sensory ones. These neurons enable us to sense touch, warm, cold, pain, limb movements and limb spatial position. The sensory neurons derive from neural crest cells, upon specific inductive signals. The multipotent neural crest cells can generate other types of cells of peripheric nervous system, like sympathetic and enteric neurons (Lallemend and Ernfors, 2012). Two factors, WNT1 and WNT3A, are essential in directing neural crest cells towards the sensory lineage (Levanon *et al.,* 2002; Nakamura *et al.,* 2008). Indeed, in mice and cultured neural crest cells (NCCs) with disrupted β-catenin, the intracellular mediator of the canonical WNT signalling pathway, multipotent NCCs fail to differentiate into sensory neurons (Inoue *et al.,* 2007; Averil *et al.,* 1995).

Different types of sensory neurons are specialized for the different perceptual modalities. Each functional type of sensory neuron has unique molecular characteristics, contains unique sets of ion channels and responds to unique sets of stimuli. However, all sensory neurons express BRN3A and ISL1, genes that are related with sensory specification and largely with the commitment of the NCCs to sensory neuronal fate (Marmigère and Ernfors, 2007). There are three major subsets of sensory neurons including proprioceptors, mechanoceptors, and nociceptors distinguished by the specific expression pattern of neurotrophic receptor and the transcription factor associated (Chambers et al., 2012). These receptors are crucial for peripheral innervation of the appropriate targets, cell survival and expression of several ion channels, defining the functional characteristics of the different types of sensory neuron. The nociceptors are neurons with small diameter related to sense the pain and are characterized by the presence of Tropomyosin receptor kinase A (TrkA). In turn, the larger diameter low threshold mechanoreceptive neurons conveying mechanical sensations possess the Tropomyosin receptor kinase B/C (TrkB/TrkC) receptors, whereas the proprioceptive neurons only has the TrkC receptors, and are related to sense limb movement and position (Marmigère and Ernfors, 2007) (table 1).

Table 1. Differences between the three main types of sensory neurons. Nociceptors express RUNX1 and TrkA receptor, while Mechanoreceptors and Proprioceptors express RUNX3 and TrkC.

	Transcription Factor	Receptor	Function
Nociceptor	RUNX1	TrkA	Pain sensation
Mechanoreceptors	RUNX3	TrkB/TrkC	Mechanical sensations (ex. touch)
Proprioceptors	RUNX3	TrkC	Limb movement and position

There are some transcription factors that have key roles in the diversification of sensory neurons, like the runt-related transcription factor (RUNX) family (36, 61-68). RUNX1 is important for diversifying the early small diameter TrkA population, producing the nociceptive neurons. In turn, RUNX3 is confined to all TrkC neurons (Levanon *et al.,* 2002).

1.2.1. PAIN CHANNELS IN SENSORY NEURONS

Pain sensation protects your body against imminent threat. In response to pain, electrical signals for conducting the nociceptive neural signals through axons are generated. Then, these action potentials are conveyed to specific areas in the spinal cord and in the brain, and the body makes an effective response (Lee *et al.,* 2005).

Sensory neurons in the dorsal root ganglia (DRG) are functionally, anatomically, and neurochemically diverse. Approximately three-quarters of DRG neurons are nociceptors, the ones related to sense the pain and most are excited by noxious heat or cold and chemical irritants (Meyer *et al.,* 2005). It is the differential expression of ion channels that lend neurons their unique functional attributes (Julius and Basbaum, 2001). The main channels responsible for inward membrane currents in nociceptors are voltage-activated sodium and calcium channels, while outward current is carried mainly by potassium ions. In addition there are others channels related to pain sensation, like TRP channels. This ones are associated with many sensory functions and linked to transduction of noxious stimuli as well as signalling within the pain system (Sexton et al., 2014). Here in this thesis we used TRP channels as the marker to assess about the functionality of the sensory neurons generated. Our lab has a protocol with the laboratory of Prof. Voets, being in this lab possible to perform functional tests (calcium imaging) in which the addition of specific TRP channels agonists into the cells, allow us to see whether the neurons are responding and what channels are active in a specific moment. TRP channels have a restricted expression pattern among sensory neurons involved in nociception (Clapham, 2003; Jordt *et al.,* 2003). TRPV1 is the best characterized TRP channel, and its expression is often considered a marker for nociceptors (Sexton *et al.,* 2003). TRPV1 is sensitive to noxius heat and acid (Caterina and Juluis, 2001), and is activated by capsaicin. Capsaicin is a main ingredient of chilli peppers and causes burning pain and irritation when stimulates the nociceptors (Lee *et al.,* 2005). There are other TRP channels that are activated in the pain response, like TRPA1, transient receptor potential cation channel subfamily M member 8 (TRPM8), transient receptor potential cation channel subfamily M, member 3 (TRPM3) and transient receptor potential cation channel subfamily V, member 4 (TRPV4). TRPA1 is activated by several pungent chemicals, including cinnamaldehyde and mustard oil (Story *et al.,* 2003; Bautista *et al.*, 2006). TRPM8 is activated by cold stimuli, menthol, and other cooling compounds (McKemy *et al.*, 2002; Peier *et al.*, 2002). In turn, TRPM3 and TRPV4 are activated by noxius heat (Sexton *et al.,* 2014). When these channels are activated, the sensory neurons are depolarized, which in turn triggers action potentials. Nociceptors normally activate only following intense, potentially damaging stimuli to provide a protective warning of imminent tissue injury (Wainger *et al.,* 2015).

These channels are good molecular target for developing antagonists as analgesics. A better understanding of the underlying mechanisms of pain could lead to the development of more effective analgesics. Indeed, existing analgesics, centred on opioids and nonsteroidal anti-inflammatory drugs (NSAIDs), are limited in their efficacy and are frequently associated with undesirable side effects and can induce dependence. Thus, it is very important to characterize

molecular properties of this channels to make better pain treating drugs (Lee *et al.,* 2005; Sexton *et al.,* 2014).

1.3. TRANSCRIPTIONAL NETWORKS REGULATING SENSORY NEURONS DIFFERENTIATION AND MATURATION

1.3.1 NEURAL CREST INDUCTION AND MIGRATION

The first step towards sensory neurons differentiation is neural crest cells induction and migration. Under the influence of bone morphogenic protein (BMP) and WNT, cells of the dorsal neural tube undergo an epithelial-to-mesenchymal transition into NCCs. During migration, two transcription factors neurogenin 1 (NGN1) and neurogenin 2 (NGN2) play an important role in neurogenesis: NGN2 initiates a first wave of neurogenesis, while NGN1 initiates a second wave of neurogenesis in SOX+10 multipotent cells (Marmigère and Ernfors, 2007; Lallemend and Ernfors, 2012). The first wave give arise to mechanoreceptive and proprioceptive neuronal subtypes (TrkB/TrkC), while the wave initiated by NGN1 produces small TrkA as well as TrkB and TrkC. Two studies made in mice allow this conclusion. Indeed, two different models were made, one with NGN1 mutant and another with the NGN2 mutated. In the NGN2 mutant mice, NGN1 was sufficient to allow large TrkB/TrkC neurons, in turn NGN1 mutant mice, NGN2 was unable to compensate for the loss of the NGN1, once mutant mice lack small TrkA neurons (Fode *et al.,* 1999).

1.3.2 SENSORY NEURON SUBTYPE DIVERSIFICATION

The diversification into the different neuronal types involves transcriptional activities inducing and maintaining expression of the growth factors receptors (Lallemend and Ernfors, 2012). Indeed, in a knock-in animal, replacement of TrkA by TrkC led to a change of the nociceptors to proprioceptive neurons (Moqrich *et al.,* 2004). Then, the activation of Trk receptors are important to turning neuronal progenitors into specific subclasses of sensory neuron. The best characterized is the TrkA promoter, and transcription factors like BRN3A, ISL1, KLF7 and RUNX1 can drive its activation and maintains expression (Marmigère and Ernfors, 2007).

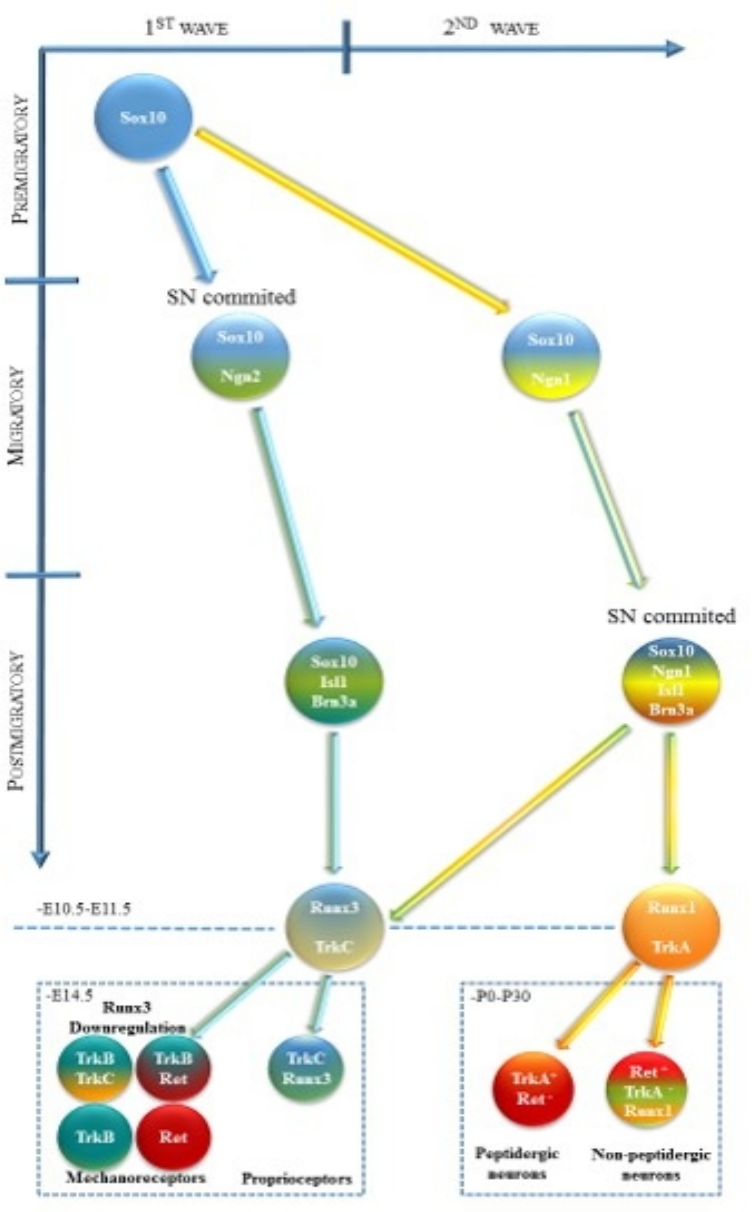


Figure 3. Overview of the differentiation pathway

Expression of BRN3A and ISL1 occurs in all sensory neurons, and coincides with sensory specification and with the commitment of the neural crest cells to a neuronal fate (McEvilly *et al.,* 1996). Indeed, studies involving deletion of BRN3A results in the death of all subtypes of sensory neurons, showing the importance of this transcription factor to keep cell survival (Xiang *et al.,* 1996). BRN3A regulates TrkA expression directly by binding to two binding sites in the TrkA promoter (Lei *et al.,* 2003). The Kruppel-link zinc finger transcription factor KLF7 cooperates with BRN3A to activate TrkA expression, and bind itself to TrkA promoter. In mice, a loss of function of KLF7, leads to a reduction in TrkA but not in TrkB or TrkC expression, highlighting the importance of this transcription factor in TrkA expression (Marmigère and Ernfors, 2007).

RUNX1 and RUNX3 are differentially expressed in sensory neurons. RUNX1 is found in small TrkA neurons while RUNX3 is present in TrkC neurons (Levanon *et al.,* 2001). RUNX3 is first detected at E10.5 in DRG and cranial ganglia and is cofined to all TrkC neurons at E12.5. RUNX3–/– mice have a loss of TrkC expression, and consequently a loss of propioceptive neurons (Levanon *et al.,* 2002). Evidences on chick demonstrate that the blocking of the activity of both leads to loss of TrkA, TrkB and TrkC neurons (Marmigere *et al.,* 2006), determining the importance of the RUNX1 to activate TrkA and the importance of RUNX3 to establish an early TrkC population, from which mechanical and proprioceptive (TrkB/TrkC) neurons arise (Marmigère and Ernfors, 2007). Interestingly, RUNX1 acts as a gene activator but also as a repressor at later embryonic stages (Chen, et al., 2006). At earlier stages, RUNX1 is necessary for the emergence of the TrkA population, while during postnatal development RUNX1 exerts a transcriptional repression of genes associated with the peptidergic subclass of nociceptors. The

peptidergic subclass of nociceptors maintain TrkA expression, extinguish RUNX1 expression and starts to express substance P (gene tachykinin precursor - TAC). For non-peptidergic ones, which mostly bind isolectin B4, the receptor Ret replaces TrkA in a process dependent of the expression of Runx1 (Wainger, 2015). Peptidergic nociceptors (TrkA+) respond to nerve growth factor (NGF), while nonpeptidergic nociceptors (Ret+) are sensitive to glial-derived neurotrophic factor (GDNF) (Chen *et al.,* 2006).

In support of this, mice lacking RUNX1 showed expression of TrkA in non-peptidergic neurons not used to it, and the forced expression of RUNX1 lead to a complete extinction of TrkA (Kramer *et al.,* 2006). RUNX1 mutants exhibit a delayed reaction to a noxious heat stimulus and cold stimulus, as well as deficits in responses to neuropathic pain. Indeed, TRPV1 that also serves as the receptor for capsaicin, lost their high-level expression in this mutants. As contrast, there was no change in the sensitivity to threshold mechanical stimulus. Taking together these results, RUNX1 activity is important in sensory neurons for the detection of acute thermal and neuropathic pain, but not mechanical stimuli (Chen *et al.,* 2006).

1.3.3 PRMD12 AS A KEY REGULATOR IN SENSORY NEURONS PATHWAY?

A new finding made by Chen and his team in 2015, highlighted the importance of PRDM12 in developing sensory neurons in humans. They analysed 11 families with congenital insensitivity to pain, and interestingly they found that all of them share mutations in PRDM12. PRDM12 is member of a family of epigenetic regulators that control neural specification and neurogenesis in vertebrates, having a SET domain that is known to methylate histone lysines (Hohenauer and Moore, 2012).

PRMDM12 is essential for the sensing of pain in humans, as pathogenic mutations cause a congenital loss of pain perception. Indeed, their clinical and histological findings suggest that mutations in this gene cause defects in the development of the sensory neurons that are destined to become nociceptors, the ones responsible to sense pain. Taken together these findings show an essential function of PRDM12 during nociceptor neurogenesis, suggesting PRDM12 as a key regulator of sensory neurogenesis (Chen, 2015).

1.4. GENERATION OF SENSORY NEURONS BY OVEREXPRESSION OF SPECIFIC TRANSCRIPTION FACTORS

Reprogramming cells and forcing differentiation into a specific neuronal subtype can generate good models for disease modelling. Wainger and his team in 2015, generated nociceptors from fibroblasts through overexpression of specific transcription factors. From an initial set of twelve factors (ASCL1, DRGX, EBF1, ETV1, ISL2, KLF7, MYT1L, NGN1, PKNOX2, BRN3A, RUNX1 and TLX3), they found that the expression of five was sufficient to generate functional mouse nociceptor neurons (ASCL1, MYT1L, KLF7, ISL2, NGN1). Both ASCL1 and MYT1L have roles in neuronal lineage reprogramming also (Vierbuchen *et al.,* 2010). In a comparison between adult mouse nociceptors, and the ones generated from the fibroblasts, the reprogrammed neurons produced functional TRPV1, TRPA1 and TRPM8 in similar relative percentages to those found in adult mouse nociceptors. The induced neurons also modelled inflammatory peripheral sensitization. Reprogrammed nociceptors can be useful as an *in vitro* model for pain, as the pain-sensitization process mimicked by the induced nociceptors contributes to the pathological transition to chronic pain (Wainger *et al.,* 2015). Gene therapy

based on the correction or disruption of disease-relevant genes is foreseen as the future of regenerative medicine (Cai and Yang, 2014).

1.5. GENOME ENGINEERING TOOLS

Genome engineering gives the ability to modify mammalian cells, introducing efficiently a variety of genetic alterations, ranging from single nucleotide modifications to whole gene addition or deletion (Rajat and Musunuru, 2014). Genome-edited differentiated stem cells allow a better genomic understanding of diseases for which there is no treatment, and this new tool has a great promise for acceleration of therapeutic translation (Lin *et al.,* 2016). Disease modelling by using a combination of human stem cells and genome engineering offer the opportunity to study the contribution of individual genetic variabilities and therefore, develop precise treatments (Yuda, 2015).

The classical method for gene modification is homologous recombination. This technique has been used to create knockout or knock in in mice, but has the disadvantage of taking more than a year to generate a genetically modified mouse (Rajat and Musunuru, 2014). Alternative approaches to knock down gene expression like RNAi have been used prior to genome editing. However, the effect is usually incomplete and the ablation of gene is time limited (Lin and Musunuru, 2016). These shortcomings promoted the development of genetic engineering techniques based on nucleases. The first described genome editing technique was by using ZFNs, which comprises a nuclease class that binds the sequence target via a chimeric enzyme consisting of target-specific zinc-finger binding domains coupled to a nuclease domain of the bacterial restriction enzyme FokI. Each zinc finger domain is able to recognize a 3-4 base pair in the DNA sequence, and tandem domains can bind to an extended nucleotide sequence (usually 9 bp to 18 bp) in order to create specificity inside the genome. ZFNs are constructed to recognize and bind two sequences flanking the site. Once bound, the Fok1 nuclease domains of the ZFN pair dimerize and generate a double-strand break, that are then repaired by the cell through non-homologous end joining (NHEJ) or homology-directed repair (HDR). Although ZFN technology offers advantages over RNAi, there are some limitations associated, like the limitations on the target-site selection, once ZFNs do not facilitate targeting sequences that are guanine-poor (Lin and Musunuru, 2016). In hPSCs ZFN has been reported to mediate efficient disruption and insertion of genes (Li et al., 2014).

Later on, TALENs were developed. TALENs are chimeric enzymes with the same FokI domain fused to a DNA-binding domain derived from tandem repeats of transcription activator-like effectors (TALEs) with 10 to 30 repeats that bind and recognize extended DNA sequences. Each amino acid recognizes one base pair, evidencing this technique as an improvement over ZFNs (Lin and Musunuru, 2016).

More recently, in 2013, the CRISPR/Cas9 system, was first applied in mammalian cells. This system uses a combination of a Cas9 protein with a guide RNA (two sepate RNAs or a single chimeric RNA), that creates a DSB within a genomic target site with 20 bp sequence, due to the endonuclease activity of Cas9. CRISPR/Cas9 can be adapted to target any genomic sequence by changing the 20 bp sequence of the guided RNA without re-engineering Cas9. This capacity is a significant advantage over ZFN and TALEN, due to the ability of generate a large set of vectors to target numerous sites, or even using multiple guide RNAs to target multiple sites at the same time in the same cell (Rajat and Musunuru, 2014). Studies done in hPSCs, showed that

CRISPR/Cas9 is more efficient than TALENs, however the precision is lower due to the use of only one guide RNA which caused more off target effects (Ding *et al.,* 2013).

1.5.1. Recombinase-Mediated Cassette Exchange (RMCE) in the AAVS1 locus

Despite these advances, there is still a need to develop tools that allow rapid and efficiently genetic modifications. RMCE technology is a good tool to have a fast generation of transgenic hPSC lines. Recombinase-Mediated Cassette Exchange (RMCE) in the AAVS1 locus is a technology that is been used in the lab, and has allowed the generation of transgenic hPSC lines by FLPe mediated RMCE in 15 days with 100% targeting efficiency and without random integrations (Ordovas *et al.,* 2015).

The adeno-associated virus integration site 1 (AAVS1) locus is located within the protein phosphatase 1, regulatory (inhibitor) subunit 12 C (PPP1R12C) gene in the chromosome 19. This site serves as a specific integration locus for AAV serotype 2 (AAV2), a non-pathogenic human parvovirus (Ramachandra et al., 2011). AAVS1 is considered a "safe harbour" for addition of a transgene into the human genome, once is characterized as a transcription-competent environment with open chromatin structure that has native insulators to avoid transgene silence. Moreover, the disruption of the PPP1R12C gene causes no adverse to the cell, and the transcriptional competence of a transgene cassette inserted in the site remains across diverse cell types (Ogata *et al.,* 2003). This way, this locus has been targeted in hPSCs for gene downregulation as well as inducible or constitutive gene expression (Tiyaboonchai *et al.,* 2014).

1.5.1.1. Generation of hPSC master cell lines suitable for FLPe RMCE in the AAVS1 locus

People on the lab generated hPSC master lines containing FRT sites between the first and second exon of the AAVS1 locus. The first step is the nucleofection with ZFNs, where a selection cassette flanked with FRT sites and containing homology regions for the AAVS1 is knocked in into hPSCs. There will be a DNA break in the AAVS1 locus which is recognized by the selection cassette due to the homology with the flanking regions and the cassette will be inserted by homology-directed repair. The cassette also contains a CAGGS promoter driving GFP and herpes simplex virus type 1 thymidine kinase gene (HYG-tk).

1.5.1.2. Cassette exchange

RMCE site-specific recombinases are able to rearrange DNA fragments by recombining specific DNA sequences, the recognition sites. Flippase (Flp) recognize the Flp-recombinase target (FRT) and it is able to switch the sequences (cassette) present in a donor plasmid that is flanked by the FRTs into a genomic locus (Ramachandra *et al.,* 2011).

Once master cell lines are nucleofected with a FRT-flanked donor cassette plasmid containing a promoter, a resistance gene to puromycin, and also a plasmid containing Flp-RNA, colonies with the inserted cassette will be selected with progressively increasing concentrations of puromycin, to select for cells with the correct integration of the donor plasmid, combined with negative selection with FIAU, to select for exchange events resulting in loss of the HSV-tk gene (Ordovas *et al.,* 2015). The promoter is inducible by doxycycline, which allow us to start and stop overexpressing the transcriptions factors inserted into the donor plasmid by adding (or not) doxycyclin.

One of our goals in this project is to generate cell lines overexpressing key transcription factors involved in the sensory neurons differentiation, in order to have a faster and more homogenous differentiation into nociceptors.

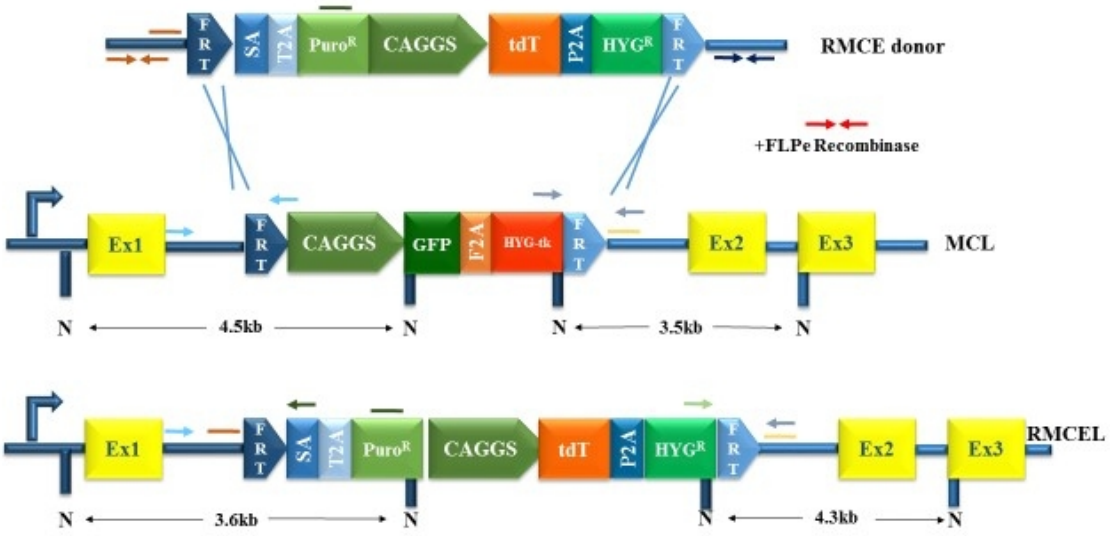


Figure 4. Ilustration of the RMCE mediated cassete exchange process. The first step is the nucleofection with ZFNs, where a selection cassette is knocked in into hPSC, giving rise to the Mother Cell Line (MCL). Next the MCL is nucleofected with a FRT-flanked donor cassette plasmid (pZ M2rtTA_CAGG TetON-3xFLAG-tdT v2 in this thesis) and also a plasmid containing Flp-RNA. Then, the flippase switch the information present in the RMCE donor into the MCL, making the RMCE line (RMCEL).

1.6. OBJECTIVES

The generation of human sensory neurons from iPSc is a good tool to study the biology of pain. Indeed, their study has been reliant on the use of rodent models due to the lack of accessibility of human nociceptive neurons. Therefore there is the need to use stem cells and differentiate them towards sensory neurons. Young et al., 2014 have reported a protocol that generate functional sensory neurons in 50 days. Here on this thesis, we took advantage of this protocol and our aims were [1] differentiate hiPSc into sensory neurons using the normal protocol described and an adapted one, to check about the morphology, gene expression and functionality of the sensory neurons generated [2] trying to improve protocol using a different cell line and doing replating differences [3] using RMCE to overexpress key transcription factors involved in the differentiation of sensory neurons and [4] improvement of the differentiation protocol by using the two cell lines generated from RMCE, one overexpressing PRDM12 and the other overexpressing ISL1-BRN3A-KLF7.

2 EXPERIMENTAL PROCEDURES

2.1. CELL CULTURE

2.1.1 STEM CELL LINE MAINTENANCE

The stem cell lines used were epithelial-1 normal human iPSC line (female) purchased from Sigma (IPSC0028) and the ChiPSC6B normal human iPSC line (male) purchased from Cellartis-Takara (Y11032) (Cellectis). We had to culture them in order to expand them to be able to start differentiation. These cells were kept undifferentiated in 10cm dish, in Essential 8™ (E8) Medium (Gibco®) with geltrex coating (3,5 mL per dish). When cells become confluent, they were passaged in a ratio according to cell confluence and experimental requirements, using 3 mL of EDTA and incubate them 3 minutes at 37ºC. Cells were manipulated in a vertical laminar flow bench, and incubated at 37ºC and 5% CO2 in a cell incubator.

2.1.2 STEM CELL LINE DIFFERENTIATION

When these cells were confluent, we applied splitting acutase step to make single cells to start differentiation. 5 mL of acutase (Sigma-Aldrich) plus 5 minutes at 37ºC were used to achieve it. Cell lines were differentiated in Corning® Costar® flat bottom (Sigma-Aldrich) 24 well plate, before coated with matrigel BD Matrigel™ (250 ul). Then, mTeSR medium™ (Stem Cell Technologies) was added until we get total confluence of cells, which took approximately 3 days. Once cells are confluent, we can start differentiation (day 0).

2.1.2.1 NOCICEPTOR INDUCTION

In this thesis, in order to generate nociceptors, we used a protocol described by Young et al., 2014 (figure 5) and we compared to an adapted protocol (figure 6) using different medium (B27+N2 vs KOES) and also half concentration of the molecules between day 5-10. We used half concentration of this molecules in the adapted protocol, because full concentration was toxic for the cells, and they started to detach around day 8.

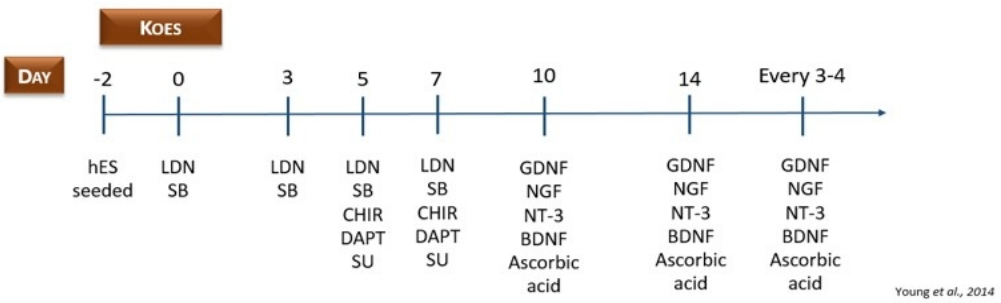


Figure 5. Protocol described by Young et al., 2014 to generate nociceptors from iPSC

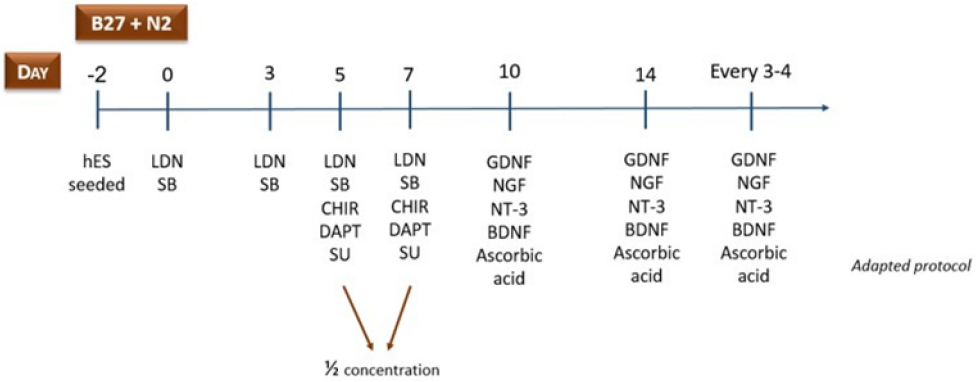


Figure 6. Adapted protocol to generate nociceptors from iPSC

From day 0 until day 10 we need to change medium every day. From day 10 on, it's medium change 3 times a week. Cell lines overexpressing transcription factors followed the same protocol, but with the addition of doxycyclin (2 µL/mL) from day 7 on. In table 2, the differentiation protocol is depicted, and the mediums are described in table 3.

Table 2. Differentiation protocol

Day 0-5 (Neuroectoderm specification)

- Addition of the molecules LDN193189 (1 µmol/l) and SB-431542 (10 µmol/l) to both medium

Day 5-10 (Nociceptor indution)

- In KOES medium, CHIR99021, DAPT, SU5402 and SB were added at 10 µmol/l and LDN at 1 µmol/l.
- In B27 + N2 medium, those molecules were added at half concentration.

From day 10 (Maturation)

- Maturation medium:

- DMEM/F12 144 mL
- FBS 10% 16 mL
- BDNF, GDNF, NGF and NT3 (each 10 ng/ml)
- Ascorbic acid (200 µmol/l)

Table 3. Description of the KOES and B27+N2 medium

KOES	B27 + N2
- KO DMEM 281,75 mL - KO SERUM 49,61 mL - NEAA 1x 3,3075 mL - GLUTAMAX 1x 3,3075 mL - Penstrep 3,3075 mL - B mercapto 0,01 mmol/l 66,15 µL	- DMEM/F12 159 mL - N2 1,67 mL - Insuline 83 µL - L-glutamine 1,67 mL - NEAA 1,67 mL - B mercapto 299 µL - Penstrep 1,67 mL - Neurobasal medium 162 mL - B27 3,3 mL

2.1.2.2. – REPLATING

On day 6, we do a replating with 1/2 dilution, otherwise cells detach due to the high density. First, we coated with matrigel BD Matrigel™ (250 mL) a new Corning® Costar® flat bottom (Sigma-Aldrich) 24 well-plate, and then we add 1 mL of acutase to our cells and wait 6 min at 37ºC. Afterwards, we add 250 µL to each well of new medium together with rock inhibitor (Sigma-Aldrich) and finally, add 250 µL from cell suspension to each well.

2.1.2.3. DIFFERENTIATION HUMAN EMBRYONIC STEM CELL (H9) OVEREXPRESSING TRANSCRIPTION FACTORS

The cell line used to insert the donor plasmid was a human embryonic stem cell (H9), because in the lab there were already H9 **(WA09) (WiCell Research Institute)** lines with the insertion of the cassette.

2.2 STAINING

The fixation was done with 4 % paraformaldehyde (PFA) solution, under laminar flow hood. The PFA was then drained and wells were washed by PBS, 3 times for five minutes and after last wash PBS was left in the wells and the plates were stored in +4 °C. To stain, wells were treated with a solution containing 10% normal donkey serum, 0.1% Triton-X 100, 1% bovine serum albumin in PBS, incubated overnight at 4ºC, together with primary antibody anti-BRN3A (Chemicon®), 1:80 diluted. In the day after, wells were washed 3 times with PBS Triton-x 0,1%. Then, secondary antibody was applied. The used secondary antibody was Alexa 488 donkey anti-rabbit. It was used in 1:500 dilution and one hour incubation. Then wells were washed first three times with PBS for 5 minutes and then two times with phosphate buffer without saline for 5 minutes. Lastly wells were aspired dry and VECTASHIELD® (Vector Laboboratories, USA) with DAPI dye for nuclei staining and cell mounting was applied with a cover slip on top. After staining the plates were stored at +4 °C.

2.3 Gene expression and quantification

2.3.1 RNA extraction and cDNA synthesis

At different time points during cell differentiation, cells were lysed by adding 350 ul of Lysis Buffer (Sigma-Aldrich) with 1% β-mercaptoethanol and collected cell lysates were stored at -80ºC. RNA was extracted from lysed cells with the commercially available kit GenElute™ Mammalian Total RNA Miniprep Kit (Sigma-Aldrich). Afterwards, RNA concentration was measured at 260 nm and 280 nm with NanoDrop (Thermo Scientific™). cDNA was generated using the SuperScript™ III First-Strand Synthesis Supermix for qRT-PCR (Invitrogen™) following the manufacturer's protocol, and using 1 ng of RNA. Then, cDNA samples were diluted 1:10 and stored at -20ºC or used to qRT-PCR.

2.3.2 Real-Time Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

qRT-PCR was performed in ViiA™ 7 Real Time PCR System (Applied Biosystems®). For that, 5 µL of Platinum® SYBR® Green (Invitrogen™) were mixed with 1 µL of 2.5 µM forward and reverse primers (all primers described on table 4), together with 3 µL of H20 plus 2 µL cDNA. Then, 10 µL were added to each well. All the work was done under UV2 PCR Workstation (UVP). Every sample and gene was measured in triplicates, and mean CT was obtained. Then, outliers were discarded and ΔCT values were obtained by normalizing with the housekeeping gene Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Data analysis and graphs were performed with LibreOfficeCalculator.

Table 4. Primers list used (from IDT)

Primer	Sequence
RUNX1	5'-TCATCTAGTTTCTGCCGATGTC-3' 5'-TCTGACCATCACTGTCTTCAC-3'
PRDM12	5'-GAGTTTCCGTACCACACCAG-3' 5'-AACGAACAGGAGCAGAACC-3'
TRPV4	5'-GTCCTCATTGCACACCTTCA-3' 5'-TCTACTTGCTCTTCATGATCGG-3'
TRPV1	5'-GGGACCAGGGCAAAGTTC-3' 5'-GGCATCATCAACGAAGACC-3'
TRPM8	5'-TGTCGCAGGACAGACGTAT-3' 5'-CCAGATCAACCAAAGTGAGAA-3'
TRPM3	5'-CATTCCTTGGTCATCAGGTGTA-3' 5'-CATCAGCAAACACACTCAACTC-3'
TRPA1	5'-GAAACCAAAGTGGCAGCTTC-3' 5'-GACATTGCTGAGGTCCAGAA-3'
ISL1	5'-GTACAACCACCATTTCACTG-3' 5'-CCCGTACAACCTGATATAATCTC-3'
TAC1	5'-CGGACCAGTAATTCAGATCATCA-3' 5'-GAGGAACCAGAGAAACTCAGC-3'
BRN3A	5'-AGCAGCGTCTCGTCCAG-3' 5'-CTCACTTTGCCATGCATCC-3'

KLF7	5'-GCGTTTCCTTTAGACACTAGC-3' 5'-CCCTTCAATGCAACCACTG-3'
KLF7-NT*	5'-CAGCTCCGTAAAGGTGAGAAG-3' 5'-ACCTGTGTGTTTCCTGTAGTG-3
ISL1-NT*	5'-GCCTTGCAGAGTGACATAGAT-3' 5'-CTGGAAGTTGAGAGGACATTGA-3'

*NT – New transcripts

2.4 CLONING

2.4.1 DESIGN OF PLASMIDS

We constructed two plasmids in silico, one containing PRDM12 and the other containing ISL1-BRN3A-KLF7 with the help of a plasmid editor (ApE).

2.4.2 BACTERIAL TRANSFORMATION

50 µL of competent Escherichia coli (New England Biolabs) were added to 4 ng of plasmid DNA. After 30 minutes on ice, a heat shock at 42ºC for 30 seconds was given. Then, 2 minutes again on ice. After it, 950 µL of SOC outgrowth medium (New England BioLabs) were added and bacteria were incubated for 60 minutes at 37ºC and 250 rpm in an Innova® 40 incubator shaker (New Brunswick Scientific). Finally, bacteria were spread on LB agar plates containing ampicillin (100 µg/ml) and incubated overnight at 37ºC in a Heraeus incubator (Thermo Scientific).

2.4.3 VECTOR LINEARIZED

In order to linearize our vector, we did a digestion with appropriate restriction enzymes (AflII and MluI), all from Fermentas. To do it, we add 3 µL of Buffer O, 2 µL AflII, 4 µL MluI, together with 4 µg of DNA, in a total reaction of 20 µL. The reaction was incubated at 37ºC for 2h30m, followed by enzyme inactivation at 80ºC for 20 min in SimpliAmp™ Thermal Cycler (Applied Biosystems®). Digestion products were run in a 0,8% agarose gel electrophoresis and then the correct band was extracted and purified by PureLink® Quick Gel Extraction Kit (Invitrogen™). Then, plasmid DNA was isolated with Purelink™ Quick Plasmid Miniprep Kit (Invitrogen™).

2.4.4 AGAROSE GEL ELECTROPHORESIS

Agarose gel electrophoresis was performed to confirm the size of DNA fragments. 0,8% Agarose (Sigma-Aldrich) in 1x Tris-AcetateEDTA (TAE) Buffer (from Ultrapure™ 10X TAE Buffer, Invitrogen™) gels were prepared and SYBR Safe DNA Gel Stain (Invitrogen™) was added (9µl). Once solidified, gels were placed in a running chamber with 1x TAE Buffer. Then, 9 µL of digested and undigested plasmid were added with 10x Blue Gel Loading dye (New England Biolabs) (1µL) and further loaded into the gel. Besides, 10 µL of 1 kb Plus DNA Ladder (Invitrogen™) were loaded into the gel. Gels were run by using Bio-Rad Power PAC 300 (Bio-Rad), which was set at 135V and 400 mA for 45 minutes. Gels were imaged with Molecular Imager® Gel Doc™ XR+ System (Bio-Rad) and Image Lab™ Software (Bio-Rad).

2.4.5 INSERT FRAGMENTS

Insert fragments were ordered as gBlocks from IDT. Silent mutations had to be made to lower the GC content and complexity, otherwise the gBlocks could not be synthesized.

Table 5. Sequences ordered as gBlock from IDT.

PRDM12	gcagagctcgtttagtgaaccgtcagatcgcttaagGCCACCATGATGGGCTCCGTGCTCCCGGCTGAG GCCCTGGTGCTCAAGACCGGGCTGAAGGCGCCGGGACTGGCGCTGGCCGAGGTTATCAC CTCCGACATCCTGCACAGCTTCCTGTACGGCCGCTGGCGCAACGTGCTCGGGGAGCAGCTC TTCGAGGACAAGAGCCACCACGCCAGCCCCAAGACAGCCTTCACCGCCGAGGTGCTGGCG CAGTCCTTCTCCGGCGAAGTGCAGAAGCTGTCCAGCCTGGTGCTGCCTGCGGAGGTGATC ATCGCTCAGAGCTCCATCCCTGGCGAGGGCCTCGGCATCTTCTCCAAGACGTGGATCAAGG CGGGAACCGAGATGGGCCCCTTCACCGGCCGCGTGATCGCCCCGGAGCACGTGGACATCT GCAAGAACAACAACCTCATGTGGGAGGTGTTCAATGAGGATGGCACGGTGCGCTACTTCA TCGATGCCAGCCAGGAGGACCACCGGAGCTGGATGACCTACATCAAGTGTGCACGTAACG AACAGGAGCAGAACCTGGAGGTGGTCCAGATCGGCACCAGCATCTTCTACAAGGCCATTG AGATGATCCCACCTGACCAGGAACTGCTGGTGTGGTACGGAAACTCACACAACACCTTCCT GGGGATCCCAGGTGTGCCCGGGCTAGAGGAGGACCAGAAAAAGAACAAGCATGAGGACT TCCACCCGGCGGACTCGGCGGCTGGCCCCGCGGGCCGCATGCGATGCGTCATCTGCCACC GCGGCTTCAACTCGCGCAGCAACCTGCGCTCGCACATGCGCATCCACACGCTGGACAAGCC CTTCGTGTGCCGCTTCTGCAACCGCCGCTTCAGCCAGTCGTCCACGCTGCGCAACCACGTG CGCCTGCACACGGGCGAGCGCCCCTACAAGTGCCAGGTGTGCCAGAGCGCCTACTCGCAG CTGGCCGGCCTGCGCGCCCACCAGAAGAGCGCGCGGCACCGGCCGCCCAGCACCGCGCT GCAGGCACACTCGCCCGCGCTGCCCGCCCCGCACGCGCACGCGCCCGCGCTCGCCGCCGC CGCCGCCGCCGCCGCCGCCGCCGCGCACCACCTGCCGGCCATGGTGCTGTGAACGCG TGGGGGAGGCTAACTGAAACACGGAAGGAGA
KLF7	gcagagctcgtttagtgaaccgtcagatcgcttaagGCCACCATGGACGTGTTGGCTAGTTATAGTATA TTCCAGGAGCTACAACTTGTCCACGACACCGGCTACTTCTCAGCTTTACCATCCCTGGAGGA GACCTGGCAGCAGACATGCCTTGAATTGGAACGCTACCTACAGACGGAGCCCCGGAGGAT CTCAGAGACCTTTGGTGAGGACTTGGACTGTTTCCTCCACGCTTCCCCTCCCCCGTGCATTG AGGAAAGCTTCCGTCGCTTAGACCCCCTGCTGCTCCCCGTGGAAGCGGCCATCTGTGAGAA GAGCTCGGCAGTGGACATCTTGCTCTCTCGGGACAAGTTGCTATCTGAGACCTGCCTCAGC CTCCAGCCGGCCAGCTCTTCTCTAGACAGCTACACAGCCGTCAACCAGGCCCAGCTCAACG CAGTGACCTCATTAACGCCCCCATCGTCCCCTGAGCTCAGCCGCCATCTGGTCAAAACCTCA CAAACTCTCTCTGCCGTGGATGGCACGGTGACGTTGAAACTGGTGGCCAAGAAGGCTGCT CTCAGCTCCGTAAAGGTGAGAAGCCTTATAAGTGCTCATGGGAGGGATGTGAGTGGCGTT TTGCACGAAGCGATGAGCTCACGAGGCACTACAGGAAACACACAGGTGCAAAGCCCTTCA AATGCAACCACTGCGACAGGTGTTTTTCCAGGTCTGACCATCTTGCCCTCCACAGATATCgg tggtggctcgggggggaggagcgacaaacttta
BRN3A	gacgttgaggagaatcccggaccaggattcACCGGTATGTCCATGAACAGCAAGCAGCCTCACTTTG CCATGCATCCCACCCTCCCTGAGCACAAGTACCCGTCGCTGCACTCCAGCTCCGAGGCCAT CCGGCGGGCCTGCCTGCCCACGCCGCCGCTGCAGAGCAACCTCTTCGCCAGCCTGGACGA GACGCTGCTGGCGCGGGCCGAGGCGCTGGCGGCCGTGGACATCGCCGTGTCCCAGGGCA AGAGCCATCCTTTCAAGCCGGACGCCACGTACCACACGATGAACAGCGTGCCGTGCACGT CCACTTCCACGGTGCCTCTGGCGCACCACCACCACCACCACCACCACCACCAGGCGCTCGA ACCCGGCGATCTGCTGGACCACATCTCCTCGCCGTCGCTCGCGCTCATGGCCGGCGCGGGC GGCGCGGGCGCGGCGGCCGGCGGCGGCGGCGCCCACGACGGCCCGGGGGGCGGTGGC GGCCCGGGCGGCGGCGGCCCGGGCGGCGGCCCCGGGGGAGGCGGCGGTGGCGGC

	CCGGGGGGCGGCGGCGGCGGCCCGGGCGGCGGGCTCCTGGGCGGCTCCGCGCACCCTCA CCCGCATATGCACAGCCTGGGCCACCTGTCGCACCCCGCGGCGGCGGCCGCCATGAACAT GCCGTCCGGGCTGCCGCACCCCGGGCTGGTGGCGGCGGCGGCGCACCACGGCGCGGCAG CGGCAGCGGCGGCGGCGGCGGCCGGGCAGGTGGCAGCGGCATCGGCGGCGGCGGCCGT GGTGGGCGCAGCGGGCCTGGCGTCCATCTGCGACTCGGACACGGACCCGCGCGAGCTCG AGGCGTTCGCGGAGCGCTTCAAGCAGCGGCGCATCAAGCTGGGCGTGACGCAGGCCGAC GTGGGCTCGGCGCTGGCCAACCTCAAGATCCCGGGCGTGGGCTCACTCAGCCAGAGCACC ATCTGCAGGTTCGAGTCGCTCACGCTCTCGCACAACAACATGATCGCGCTCAAGCCCATCC TGCAGGCGTGGCTCGAGGAGGCCGAGGGCGCCCAGCGCGAGAAAATGAACAAGCCTGA GCTCTTCAACGGCGGCGAGAAGAAGCGCAAGCGGACTTCCATCGCCGCGCCCGAGAAGC GCTCCCTCGAGGCCTACTTCGCCGTGCAGCCCCGGCCCTCGTCCGAGAAGATCGCCGCCAT CGCCGAGAAACTGGACCTCAAAAAGAACGTGGTGCGGGTGTGGTTTTGCAACCAGAGACA GAAGCAGAAGCGGATGAAATTCTCTGCCACTTACATCGATggtggtggctcgggggggaggagcgac aaacttta
ISL1	gacgttgaggagaatcccggaccaggattcGTTAACATGGGAGACATGGGAGATCCACCAAAAAAA AAACGTCTGATTTCCCTATGTGTTGGTTGCGGCAATCAGATTCACGATCAGTATATTCTGAG GGTTTCTCCGGATTTGGAATGGCATGCGGCATGTTTGAAATGTGCGGAGTGTAATCAGTAT TTGGACGAGAGCTGTACATGCTTTGTTAGGGATGGGAAAACCTACTGTAAAAGAGATTAT ATCAGGTTGTACGGGATCAAATGCGCCAAGTGCAGCATCGGCTTCAGCAAGAACGACTTC GTGATGCGTGCCCGCTCCAAGGTGTATCACATCGAGTGTTTCCGCTGTGTGGCCTGCAGCC GCCAGCTCATCCCTGGGGACGAATTTGCGCTTCGGGAGGACGGTCTCTTCTGCCGAGCAG ACCACGATGTGGTGGAGAGGGCCAGTCTAGGCGCTGGCGACCCGCTCAGTCCCCTGCATC CAGCGCGGCCACTGCAAATGGCAGCGGAGCCCATCTCCGCCAGGCAGCCAGCCCTGCGGC CCCACGTCCACAAGCAGCCGGAGAAGACCACCCGCGTGCGGACTGTGCTGAACGAGAAG CAGCTGCACACCTTGCGGACCTGCTACGCCGCAAACCCGCGGCCAGATGCGCTCATGAAG GAGCAACTGGTAGAGATGACGGGCCTCAGTCCCCGTGTGATCCGGGTCTGGTTTCAAAAC AAGCGGTGCAAGGACAAGAAGCGAAGCATCATGATGAAGCAACTCCAGCAGCAGCAGCC CAATGACAAAACTAATATCCAGGGGATGACAGGAACTCCCATGGTGGCTGCCAGTCCAGA GAGACACGACGGTGGCTTACAGGCTAACCCAGTGGAAGTACAAAGTTACCAGCCACCTTG GAAAGTACTGAGCGACTTCGCCTTGCAGAGTGACATAGATCAGCCTGCTTTTCAGCAACTG GTCAATTTTTCAGAAGGAGGACCGGGCTCTAATTCCACTGGCAGTGAAGTAGCATCAATGT CCTCTCAACTTCCAGATACACCTAACAGCATGGTAGCCAGTCCTATTGAGGCATGAACGCG TGGGGGAGGCTAACTGAAACACGGAAGGAGA

2.4.6 GIBSON ASSEMBLY

In order to assemble TF over-expression Flip-in plasmids we used NEBBuilder Kit (New England Biolabs). To do that, we add 0,5 pmole of each TF into 10 µL of NEBBuilder Master Mix, in a total reaction of 20 µl. Reaction was incubated at 50ºC for 1h. Afterwards we use 2 and 5 µL of each assembly reaction to perform a similar transformation as described before (2.4.2), and then plasmid DNA was isolated again with Purelink™ Quick Plasmid Miniprep Kit (Invitrogen™).

2.4.7 CLONES SCREENING

We had to check if the plasmids were correctly assembled, so we set up some digestions with appropriate restriction enzymes. Samples were run in a 0,8% gel as described before (2.4.4), and the clones with the best band pattern were chosen to get plasmid DNA using Purelink™ HiPure Plasmid Filter Maxiprep Kit (Invitrogen™).

2.4.8 SELECTION

The first step in the process is the hPSC transfection by nucleofection. Approximately $2x10^6$ cells were used in the Nucleofector 2b Device (Amaxa) and program F16 (feeder-free), together with 2,5 µg of the flp plasmid plus 12 µg of donor plasmid.

Once the cassette is inserted we wanted to see which the colonies have the inserted cassette (from donor vector) correctly. For that, we add hESC medium with 100 ng/mL of puromycin, and we observe cell growth so that death and growth are balanced. When growth rate overtakes cell death, increase puromycin concentration in blocks of 25-50 ng/mL up to a maximum of 250 ng/mL. Keep puromycin selection 5-7 days. Three-four days after starting puromycin selection, around day 6 post-transfection, start selection with 0.5 µM FIAU. Change media daily and maintain FIAU for no more than 7 continuous days. This selection allow us to select for cells with the correct integration of the donor plasmid.

2.4.9 FLOW CYTOMETRY

We analyzed whether the cells had the donor vector correctly inserted in BD FACSCanto™ (BD Biosciences) using FACSDiva software (BD Biosciences). Green fluorescence was detected with 502 LP and 530/30 filters.

2.5. CALCIUM IMAGING

For [Ca2+] measurements, cells were incubated with 2 µM Fura-2AM for >20min at 37 °C in culture medium. [Ca2+] cyt was measured by monitoring the fluorescence ratio (F340/F380). Signals were corrected for background. The standard imaging solution contained (in mM): 138 NaCl, 5.4 KCl, 2 CaCl2, 2 MgCl2, 10 glucose, 10 HEPES, (ph7.4 with NaOH).

Agonists are:

Menthol (100µM); PS (40µM); MO (100µM); Capsaicin (1µM); High Potassium (50mM)

2.6. CELL IMAGING

Differentiating cells were observed under Axiovert 40C (Zeiss) inverted microscope for morphological evaluation. Cell fluorescence was observed under Axiovert 40 CFL (Zeiss) inverted microscope and pictures were taken with AxioCam MRc5 and AxioVision Vs40 v4.8.2 (Zeiss).

2.6. STATISTICS

Statistical tests were performed in n=3 experiments.

3 RESULTS

3. RESULTS

3.1. CHARACTERIZATION OF SENSORY NEURONS DIFFERENTIATED FROM IPSC (CELLECTIS) WITH KOES MEDIUM AND B27+N2 MEDIUM

Previous studies done by Young *et al.,* 2014, have reported the differentiation of sensory neurons from hiPSC. Then, we first characterized the sensory neurons originated by this protocol (figure 7) compared to the ones originated from an adapted protocol that we developed (figure 8), in order to identify which one is the best to be used as a differentiation protocol.

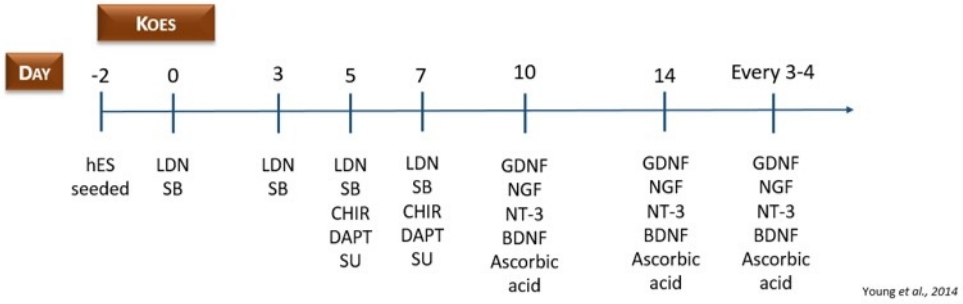


Figure 7. Protocol described by Young et al., 2014, with KOES medium.

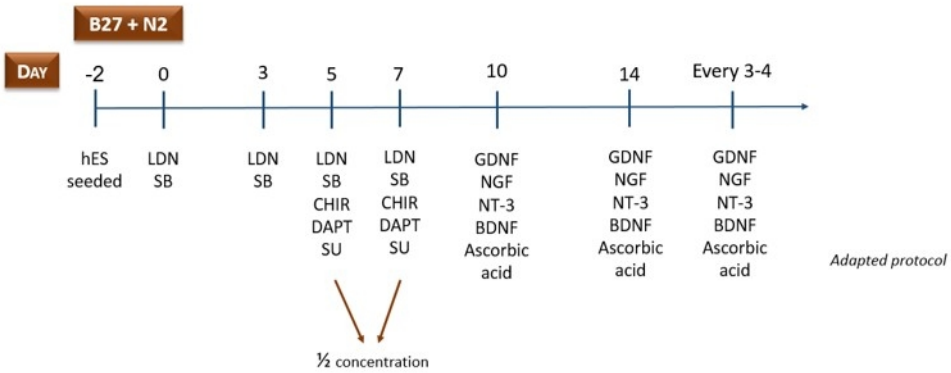


Figure 8. Adapted protocol with a different medium (B27 + N2) and half concentration of the molecules between day 5-10.

3.1.1. MORPHOLOGY AND GENE EXPRESSION ANALYSIS OF TRANSCRIPTION FACTORS INVOLVED IN NOCICEPTORS DIFFERENTIATION

iPSC cellectis were differentiated into sensory neurons as detailed in table 6 and 7. Cells were harvested at days 0, 15, 30, 50, and RNA was analysed by qRT-PCR.

Morphologically, sensory neurons in both protocols seem similar. They all started from the same point (figure 9A), total confluent, and then on day 15 (figure 9B and 9E), they already look like sensory neurons, with clump of cells and the presence of neurites. Then, in both cases, until day 50, they just lose density and the neurites become better defined, connecting the clump of cells. Changes in gene expression of markers specific for the different stages of differentiation protocol were also observed together with the morphological changes. In both

cases, ISL1 and BRN3A, the markers of sensory neuron stage, go up on day 15 (figure 10A) and then stay stable. In turn, the marker for nociceptor stage RUNX1 only go up on day 30, and then the expression decrease on day 50. TAC1, the other nociceptor marker, already increases expression on day 15, but on day 30 there is a big increase on the expression of this gene, which stays constant until day 50 (figure 10B).

Regarding the expression of TRP channels, TRPA1 and TRPV1, the most predominant TRP channels in sensory neurons from human DRG (as we can see by the lower ΔCT), are only upregulated and similar to levels expected in normal sensory neurons around day 50 in both protocol (Figure 10C). TRPM3 and TRPM8 in both cases peak early, at day 15, and then TRPM3 expression reduces while TRPM8 stay stable. In turn, TRPV4 only peak at day 50, reaching similar levels to what is observed in sensory neurons.

Then, and taking together the results, during the differentiation protocol, around day 15 we already have sensory neurons , but only after 30 days we have nociceptor phenotype. Due to the increase of the TRP channels expression on day 50, probably we only have functional nociceptors after 50 days of differentiation.

Both protocol showed similar qRT-PCR results, however in the adapted protocol with B27+N2 medium the expression of most of the markers are closer to what is expected to see in normal sensory neurons from human DRG, so we decided to continue experiences using this protocol. Hence, on day 50 of the B27+N2 differentiation protocol, we did staining to assess about the homogeneity of the plate (figure 11). We can see that around 70% of cells are expressing BRN3A, a general marker for sensory neurons, meaning that a huge amount of cells generated are sensory neurons.

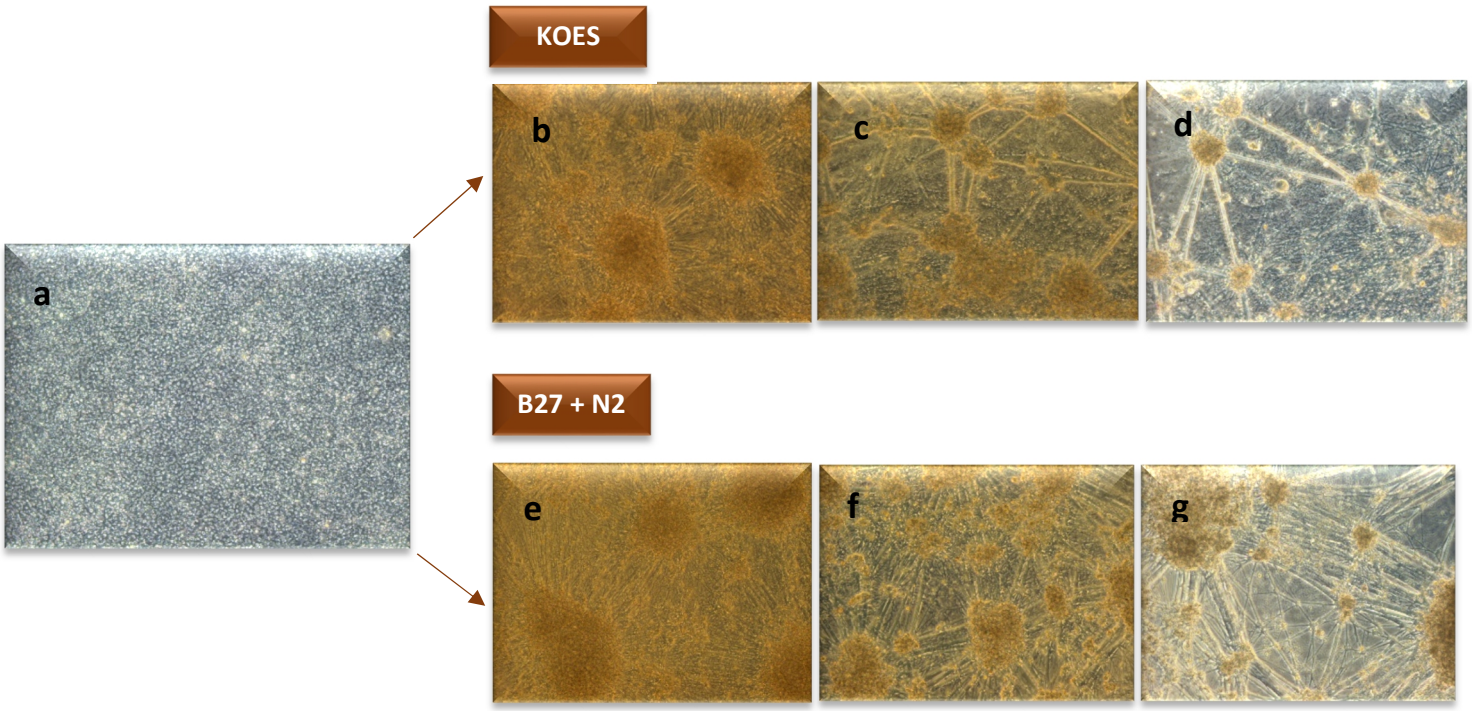
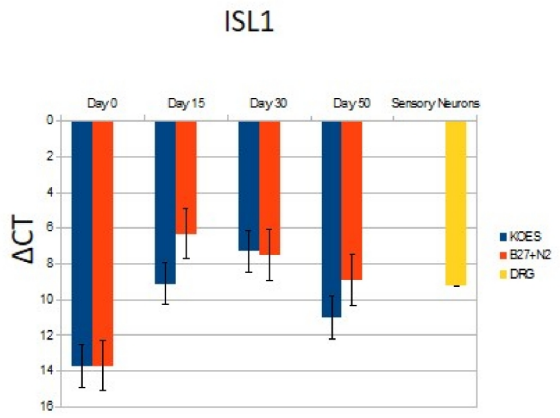
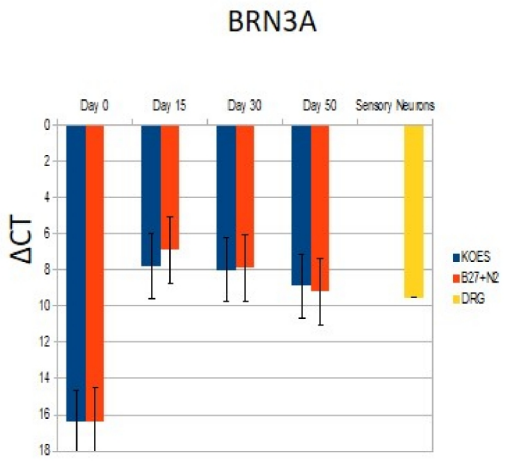
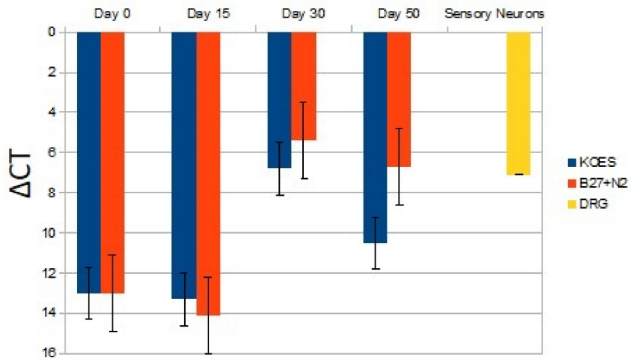


Figure 9. Cellectis cells were differentiated into sensory neurons following the protocol described in figure 7 and 8. Cell morphology of cells on day 0 (A), KOES day 15 (B), day 30 (C), day 50 (D), B27+N2 day 15 (E), day 30 (F), day 50 (G) was assessed throughout the protocol under the inverted microscope.

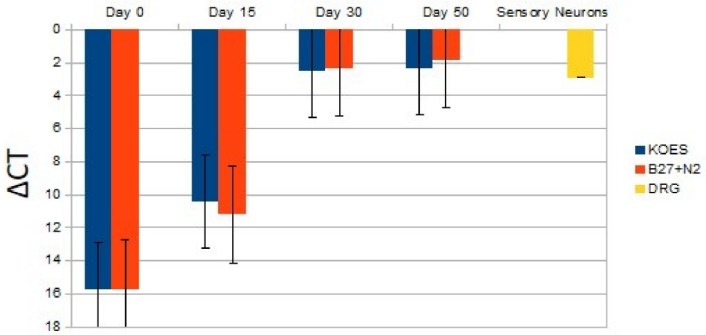

ISL1
Day 0
Day 15
Day 30
Day 50
Sensory Neurons
ΔCT
KOES
B27+N2
DRG

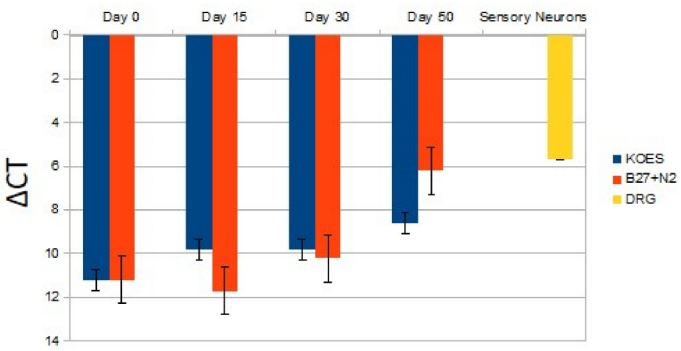
A


BRN3A
Day 0
Day 15
Day 30
Day 50
Sensory Neurons
ΔCT
KOES
B27+N2
DRG

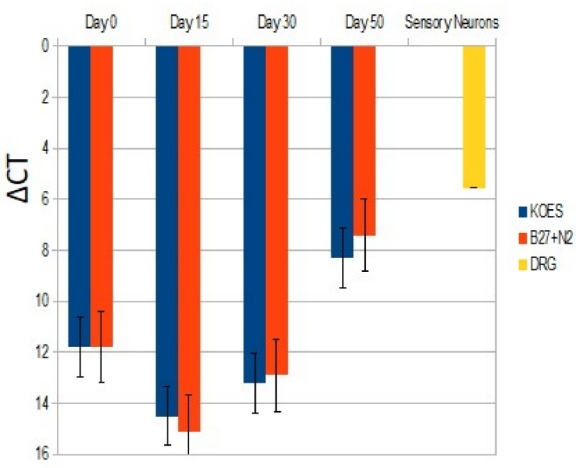

RUNX1
Day 0
Day 15
Day 30
Day 50
Sensory Neurons
ΔCT
KOES
B27+N2
DRG

B


TAC1
Day 0
Day 15
Day 30
Day 50
Sensory Neurons
ΔCT
KOES
B27+N2
DRG


TRVP1
Day 0
Day 15
Day 30
Day 50
Sensory Neurons
ΔCT
KOES
B27+N2
DRG

C


TRPA1
Day 0
Day 15
Day 30
Day 50
Sensory Neurons
ΔCT
KOES
B27+N2
DRG

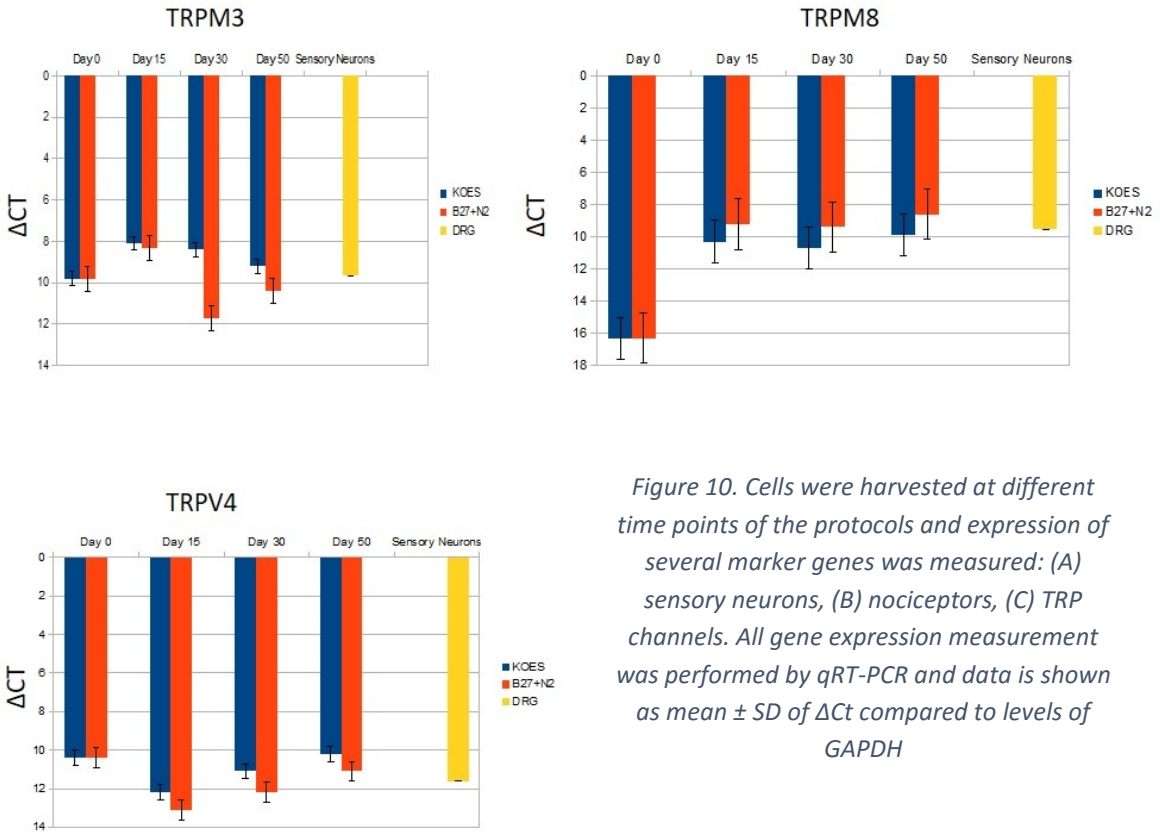


Figure 10. Cells were harvested at different time points of the protocols and expression of several marker genes was measured: (A) sensory neurons, (B) nociceptors, (C) TRP channels. All gene expression measurement was performed by qRT-PCR and data is shown as mean ± SD of ΔCt compared to levels of GAPDH

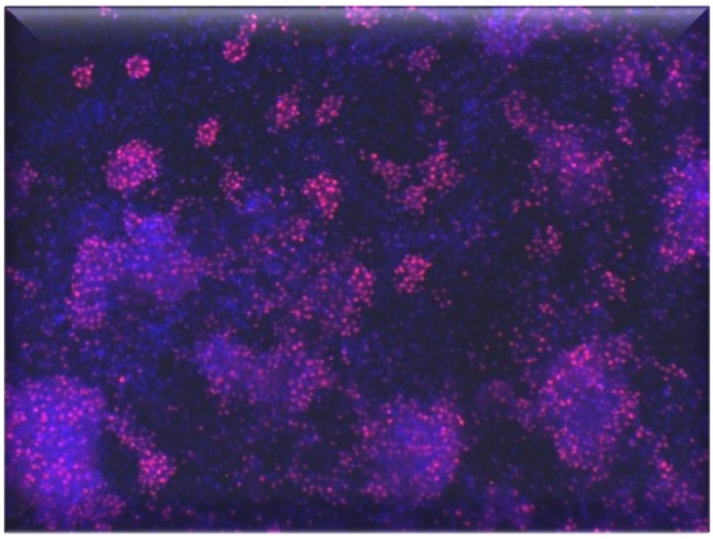


Figure 11. Sensory neurons originated from the adapted protocol were fixed at day 50 and then stained for antibodies: DAPI (blue) and anti-BRN3A (red).

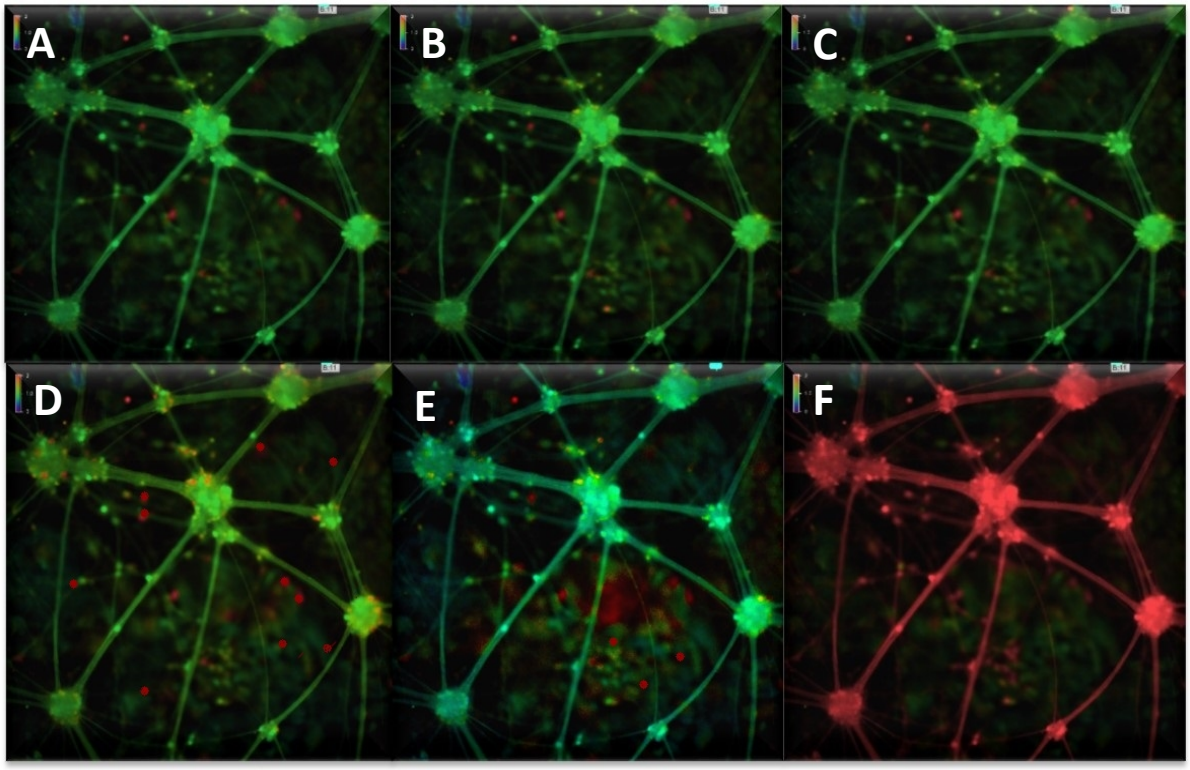
3.1.2. FUNCTIONAL TESTS (CALCIUM IMAGING)

Nociceptive neurons play an essential role in pain sensation by transmitting painful stimuli to the central nervous system. Thus, it's important to check if the nociceptors generated are able to respond to agonists of the TRP channels (pain channels), to see if they are functional. We did this experiments together with the lab of Prof. Voets, and we first measured nociceptors from both protocols at day 50.

3.1.2.1. DAY 50

In qRT-PCR, we saw that only after 50 days the main TRP channels (TRPV1 and TRPA1) were upregulated, so we start measure on day 50. Gene expression data suggested that TRPA1 and TRPV1 were the most expressed TRP channels in nociceptors originated from both protocol. Functional tests are in accordance with this. When we have a red dot (but only a red dot that is not present in the control before experiment), means that in that spot the TRP channel is activated (influx of calcium) by the agonist. In both cases, we can see more red dots after the addition of capsaicin (TRPV1 agonist), and mustard oil (TRPA1 agonist), with the best response being caused by capsaicin. TRPM3 and TRPM8 agonist caused almost no response. After the addition of high potassium, we can observe that all the cells responded, so all the cells were alive. Therefore, this results pointed the functionality of the nociceptors generated from both protocols after 50 days of differentiation (having TRPV1 and TRPA1 activated).

KOES DAY 50



B27 + N2 DAY 50

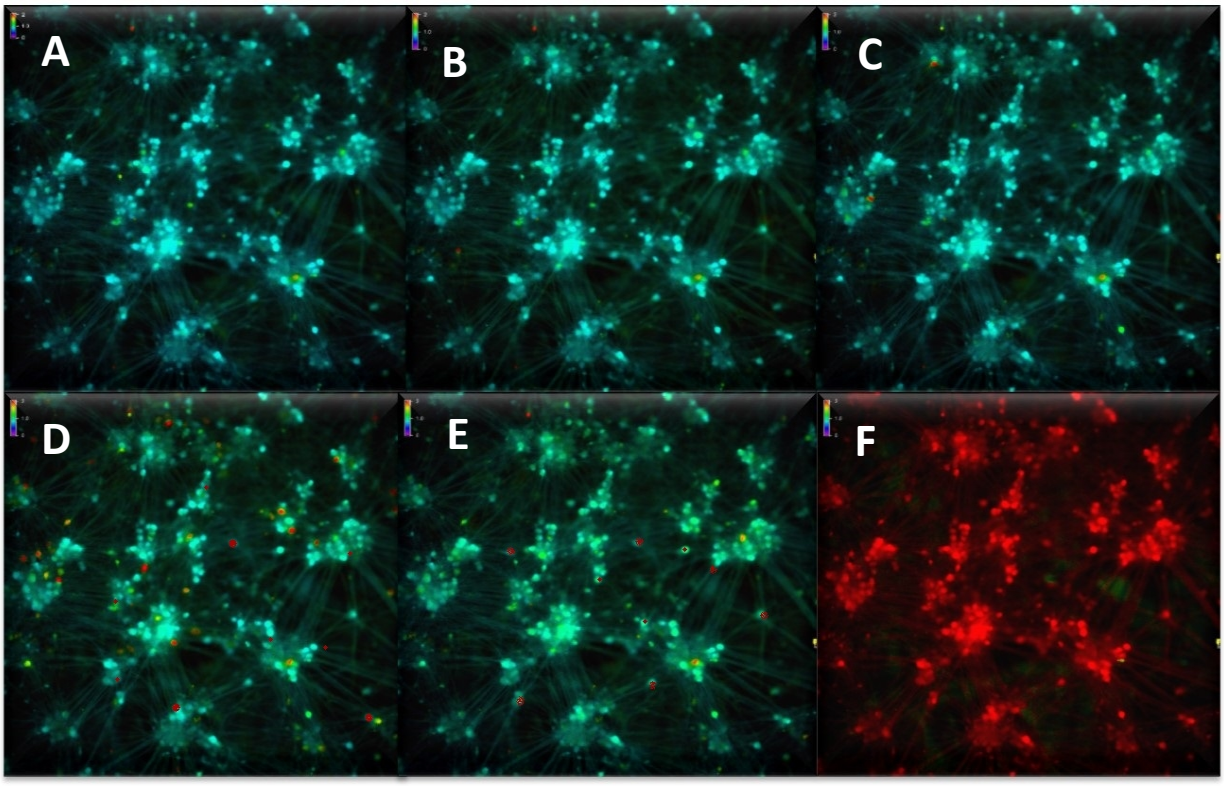


Figure 12. Calcium imaging. Sensory neurons generated from both protocols were measured for TRP channels agonists at day 50: A. Fura-2 ratio picture before experiment; B. After PS [40µM] application (TRPM3 agonist); C. After Menthol [100µM] application (TRPM8 agonist); D. After Capsaicin [1µM] application (TRPV1 agonist); E. After mustard oil [100µM] application (TRPA1 agonist); F. After high potassium [50mM] (depolarizes all neurons).

3.1.2.2. DAY 15 AND DAY 30

Next, we decided to see if the TRP channels were already activated in earlier stages. For that, we repeated the experience, this time only with B27+N2 protocol, because this one had better qRT-PCR results, and we measured at day 15 and day 30.

Interestingly, after the addition of PS (TRPM3 agonist), we can observe a huge response (figure 13), meaning that TRPM3 was already activated at day 15. Indeed, this TRP channel peaked expression at day 15, and then it decreases, so functional tests are according with qRT-PCR results. At this time point, no more responses were recorded after the addition of the same agonists as before (supplementary data). At day 30, there were no relevant response to any agonists (supplementary data). In the table 6 it is resumed the activation of TRP channels during differentiation protocol.

Table 6. Summary of the functionality tests

DAY 15	DAY 30	DAY 50
TRPM3 ACTIVATED	**NO ONE**	**MAINLY TRPV1 AND TRPA1 ACTIVATED**

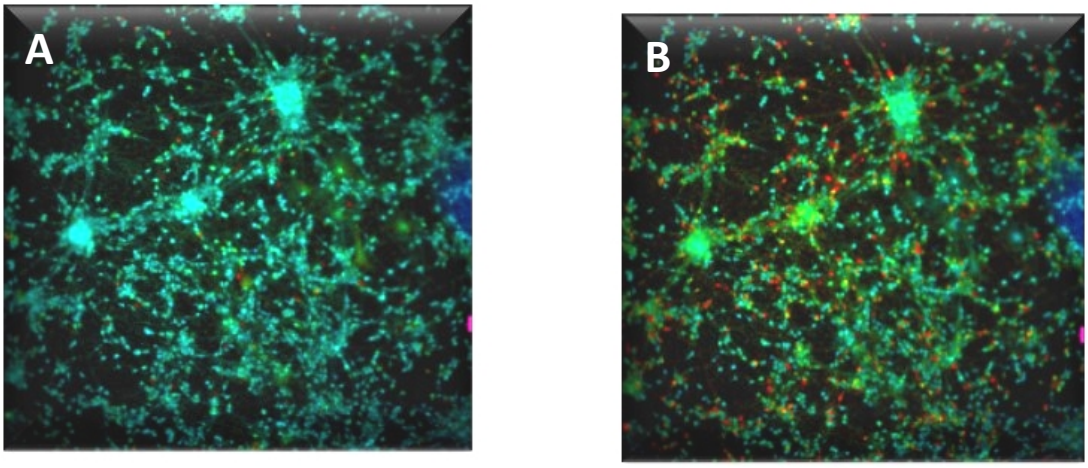


Figure 13. Calcium imaging. Sensory neurons generated from adapted protocol were measured for TRP channels agonists at day 15 A. Fura-2 ratio picture before experiment; B. After PS [40μM] application (TRPM3 agonist)

3.2. REFINEMENT OF SENSORY NEURONS DIFFERENTIATION PROTOCOL

After having characterized the sensory neurons generated from the differentiation protocol, we noticed that only after 50 days we have fully mature sensory neurons. Our next point was trying to improve this by making some changes to the protocol used.

3.2.1. REPLATING DIFFERENCES DIDN'T IMPROVE DIFFERENTIATION

The first change we did was in the replating step (figure 14). Normally, on day 6, we do a replating with ½ dilution. To do the functional tests, the less density it has, the better the results. For that, we tested whether doing a replating with a dilution ¼ and 1/6 we could improve differentiation. Globally, there was not seen a significant difference (p >0,05) between dilutions (see supplementary data). On day 50 (figure 15), in the ½ and ¼ dilution there is no difference.

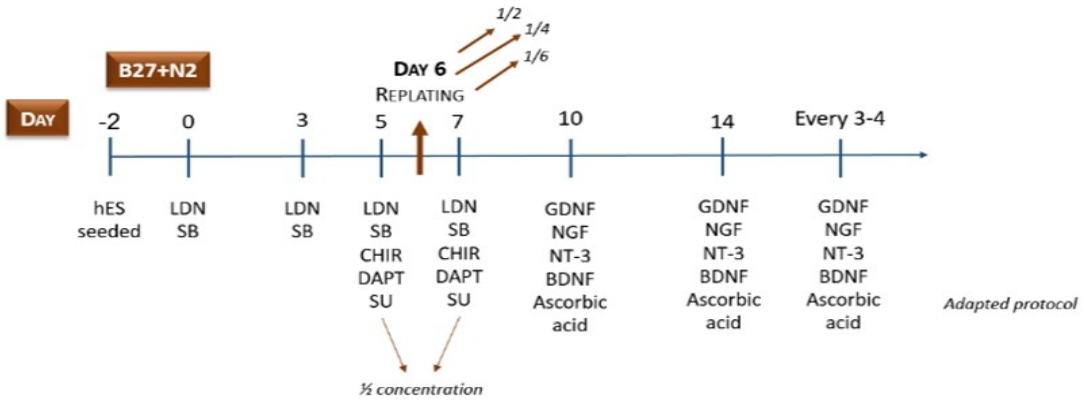
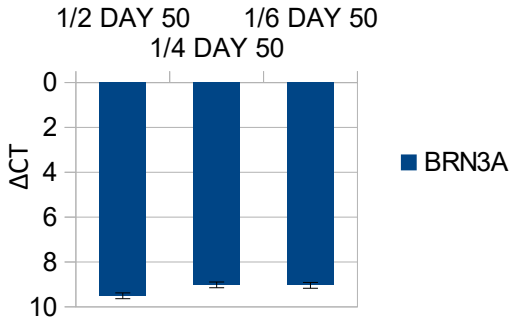
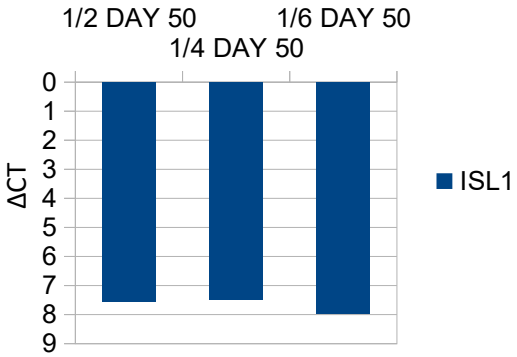
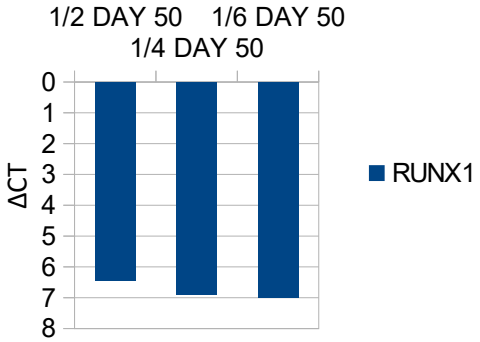


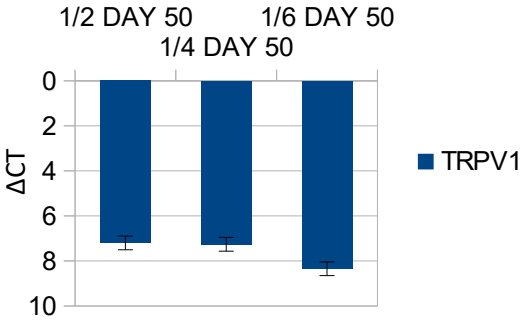
Figure 14. Adapted protocol used with differences on the replating step

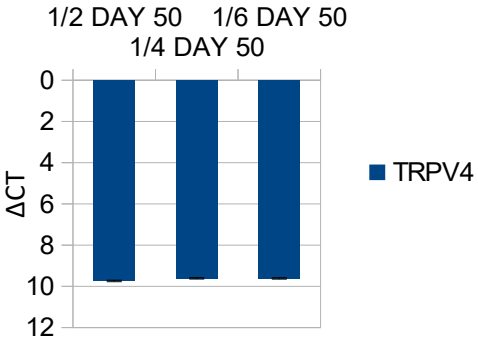
However, in the 1/6 dilution there's a significant difference (less expression) on the TAC1 and TRPA1. Then, doing this replating differences didn't improve differentiation, so we continued using ½ dilution. We didn't make the functional tests, because the cells diluted ¼ and 1/6 weren't looking as good as the ones diluted ½.

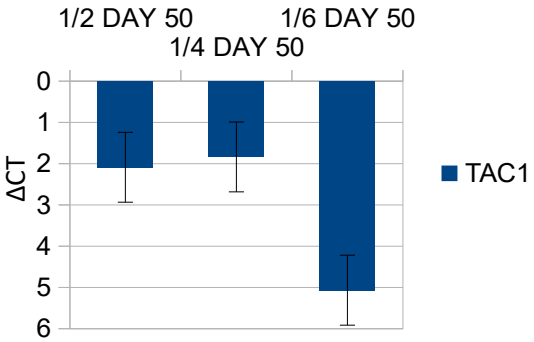












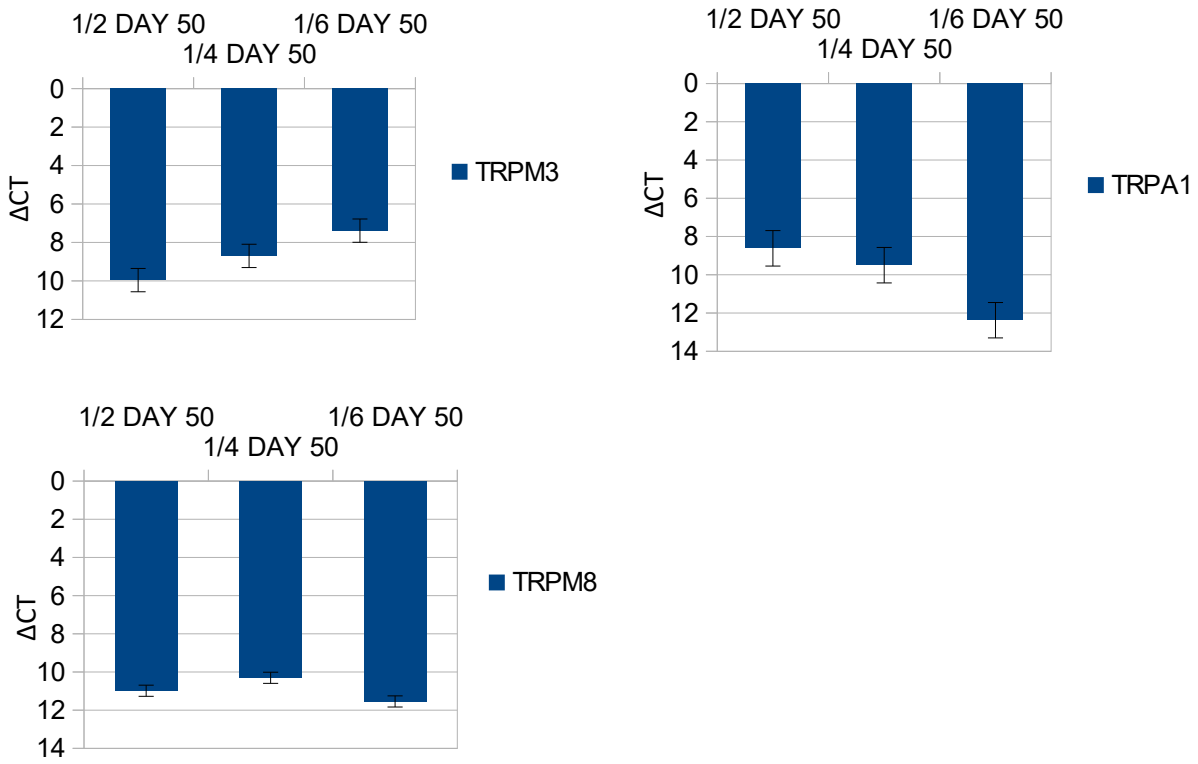


Figure 15. Cells were harvested at day 50 of the protocol and expression of several marker genes was measured. All gene expression measurement was performed by qRT-PCR and data is shown as mean ± SD of ΔCt compared to levels of GAPDH

3.2.2. DIFFERENTIATION IS NOT ENHANCED BY USING DIFFERENT CELL LINE (SIGMA 0028)

Next, we wanted to see if the protocol is reproducible in a different cell line, and if we could have better results. For that we used the same adapted protocol in the sigma 0028 cell line.

Morphologically, we have the same pattern as observed in the cellectis line: day 0 (figure 16A), cells total confluent, on day 15 (figure 16B) already the presence of the neurites and clump of cells, and then on (figure 16C and D) is just a question of losing density and the neurites becoming mature, connecting the clump of cells. In terms of gene expression the markers for sensory neurons had similar results: ISL1 and BRN3A go up on day 15 and stay stable (figure 17A). On the other hand, about markers for nociceptors, there were small differences: RUNX1 were already upregulated in day 15, and the increase between day 15 and 30 in TAC1 were lower comparing to cellectis (figure 17B). Regarding the expression of the TRP channels (figure 17C), there are significant differences in two: TRPV1 and TRPV4. TRPV1 is more expressed in nociceptors generated from cellectis, and TRPV4 is more expressed in nociceptors generated from sigma 0028.

With this results, we could see that the protocol is reproducible in different cell lines, although slightly differences are noticed. The values on the gene expression obtained from the

cellectis are closer to what we expect to see on sensory neurons from human DRG, so using different cell line did not enhance the protocol differentiation.

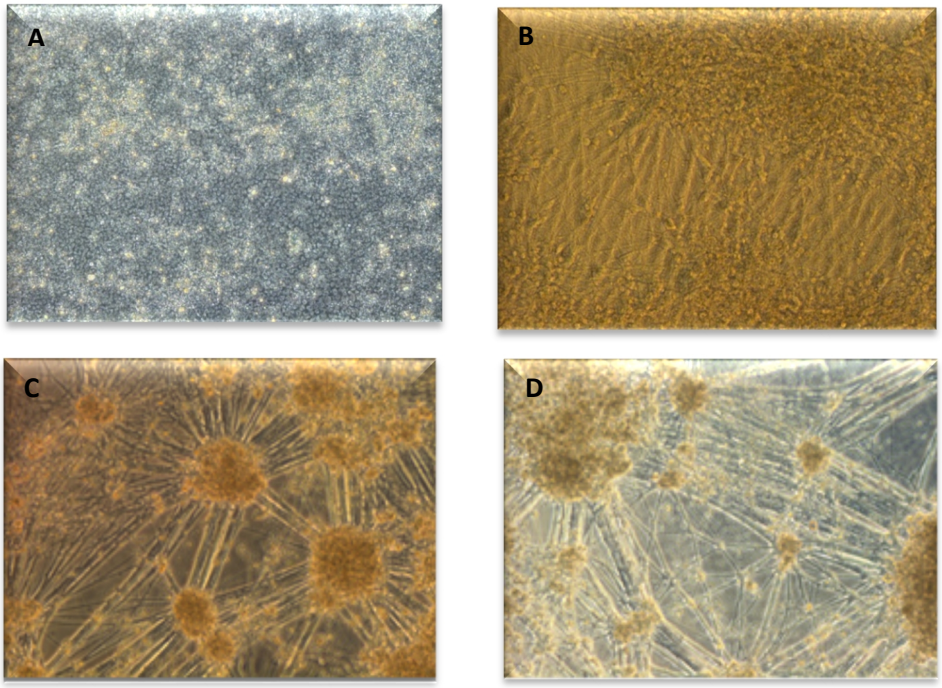
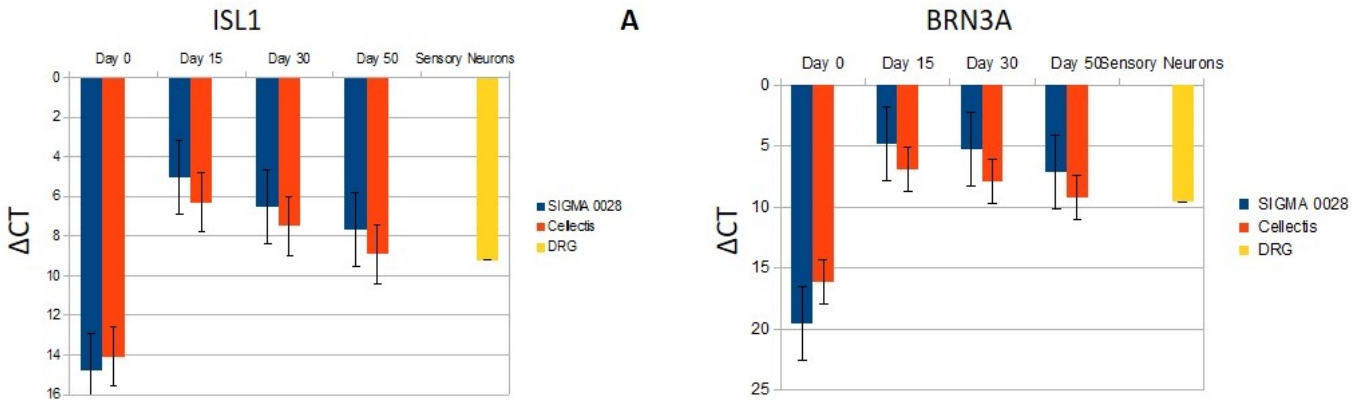
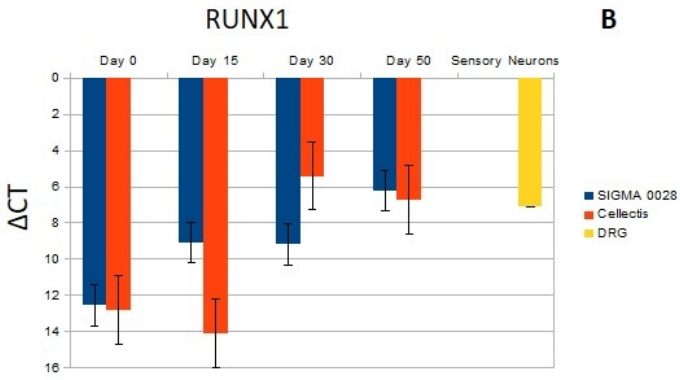
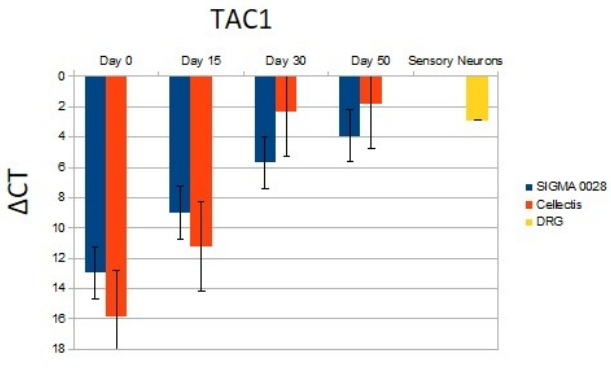


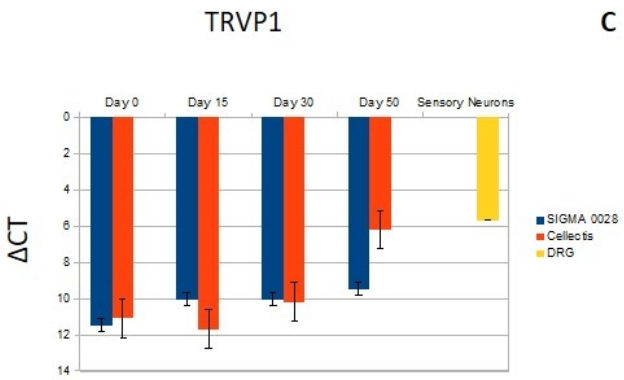
Figure 16. Sigma cells were differentiated into sensory neurons following the adapted protocol. Cell morphology of cells on day 0 (A), day 15 (B), day 30 (C), day 50 (D), was assessed throughout the protocol under the inverted microscope.



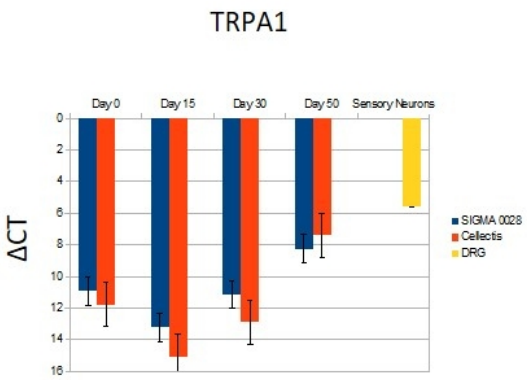

RUNX1
Day 0
Day 15
Day 30
Day 50
Sensory Neurons
ΔCT
SIGMA 0028
Cellectis
DRG

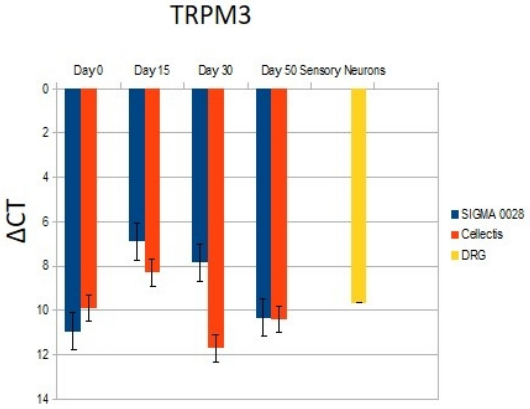
B

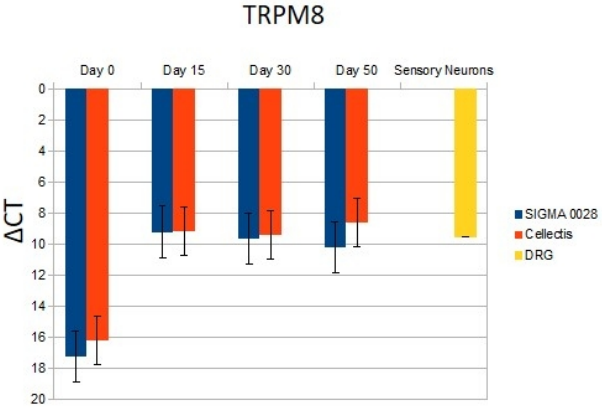

TAC1
Day 0
Day 15
Day 30
Day 50
Sensory Neurons
ΔCT
SIGMA 0028
Cellectis
DRG


TRVP1
Day 0
Day 15
Day 30
Day 50
Sensory Neurons
ΔCT
SIGMA 0028
Cellectis
DRG

C


TRPA1
Day 0
Day 15
Day 30
Day 50
Sensory Neurons
ΔCT
SIGMA 0028
Cellectis
DRG


TRPM3
Day 0
Day 15
Day 30
Day 50
Sensory Neurons
ΔCT
SIGMA 0028
Cellectis
DRG


TRPM8
Day 0
Day 15
Day 30
Day 50
Sensory Neurons
ΔCT
SIGMA 0028
Cellectis
DRG

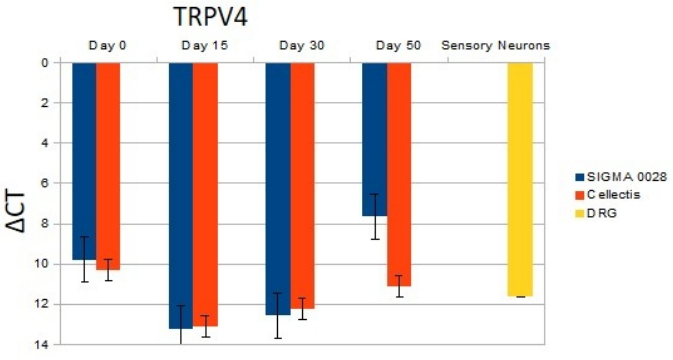


Figure 17. Cells were harvested at different time points of the protocols and expression of several marker genes was measured: (A) sensory neurons, (B) nociceptors, (C) TRP channels. All gene expression measurement was performed by qRT-PCR and data is shown as mean ± SD of ΔCt compared to levels of GAPDH

3.3. IMPROVEMENT OF SENSORY NEURONS DIFFERENTIATION PROTOCOLS BY OVEREXPRESSION OF TRANSCRIPTION FACTORS

We didn't improve the differentiation protocol doing previous attempts, so we tried something different. We used a technique used on the lab (RMCE) and a mother cell line (MCL) hESC line (H9) (figure 18), suitable for FLPe RMCE in the AAVS1 locus, previously generated in the lab (see section 1.5.1.1.) to overexpress key transcription factors involved in sensory neurons differentiation. Using this line, we were able to generate transgenic hESC lines in 15 days by using positive/negative selection. Our goal is to see if we can enhance differentiation with this new cell lines.

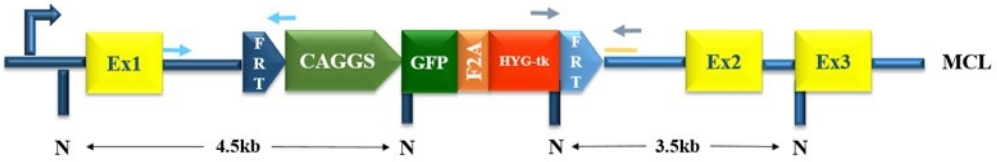


Figure 18. Mother cell line (MCL) previously established in a H9 cell line

3.3.1. EXPRESSION OF KEY TRANSCRIPTION FACTORS

We found on literature that KLF7, BRN3A and ISL1 play an important role on the differentiation towards sensory neurons. Moreover, PRDM12 is essential for the sensing of pain in humans. Therefore, the first thing we did was a short differentiation using the adapted protocol on cellectis line, to see how this genes are regulated. On figure 19, we can see that BRN3A, ISL1 and KLF7 peak at the same time (day 15), meaning that they could act together in the differentiation. In turn, PRMD12 peaked earlier, at day 9.

Knowing this, we were able to make the constructions. BRN3A, ISL1 and KLF7 can go in the same construction (figure 20), and PRDM12 was alone in a different construction (figure 21).

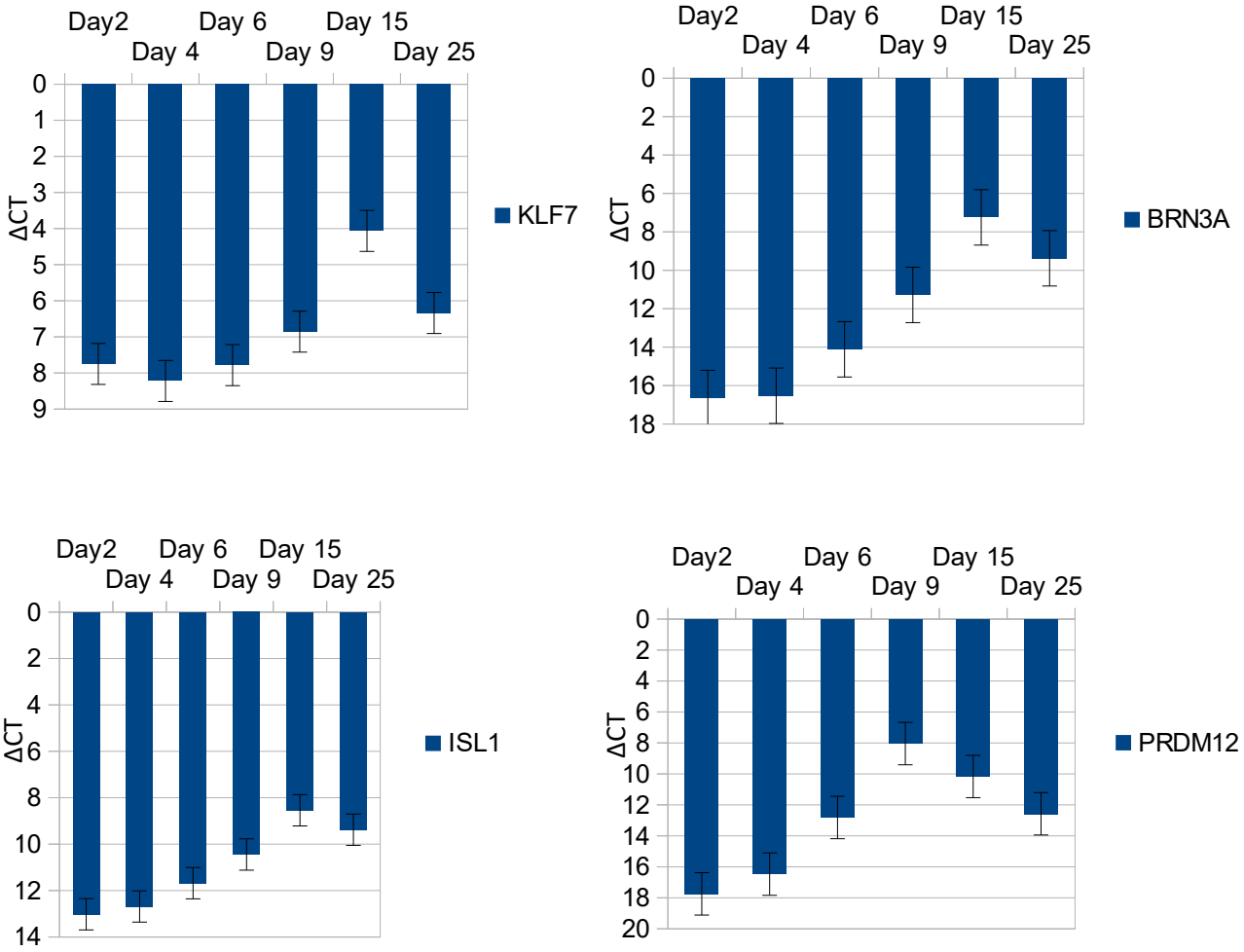


Figure 19. Cells were harvested at different time points (Day 2, 4, 6, 9, 15, 25) of the protocol and expression of KLF7, BRN3A, ISL1 and PRDM12 was measured. All gene expression measurement was performed by qRT-PCR and data is shown as mean ± SD of ΔCt compared to levels of GAPDH.

Figure 20. Donor vector containing ISL1, BRN3A and KLF7

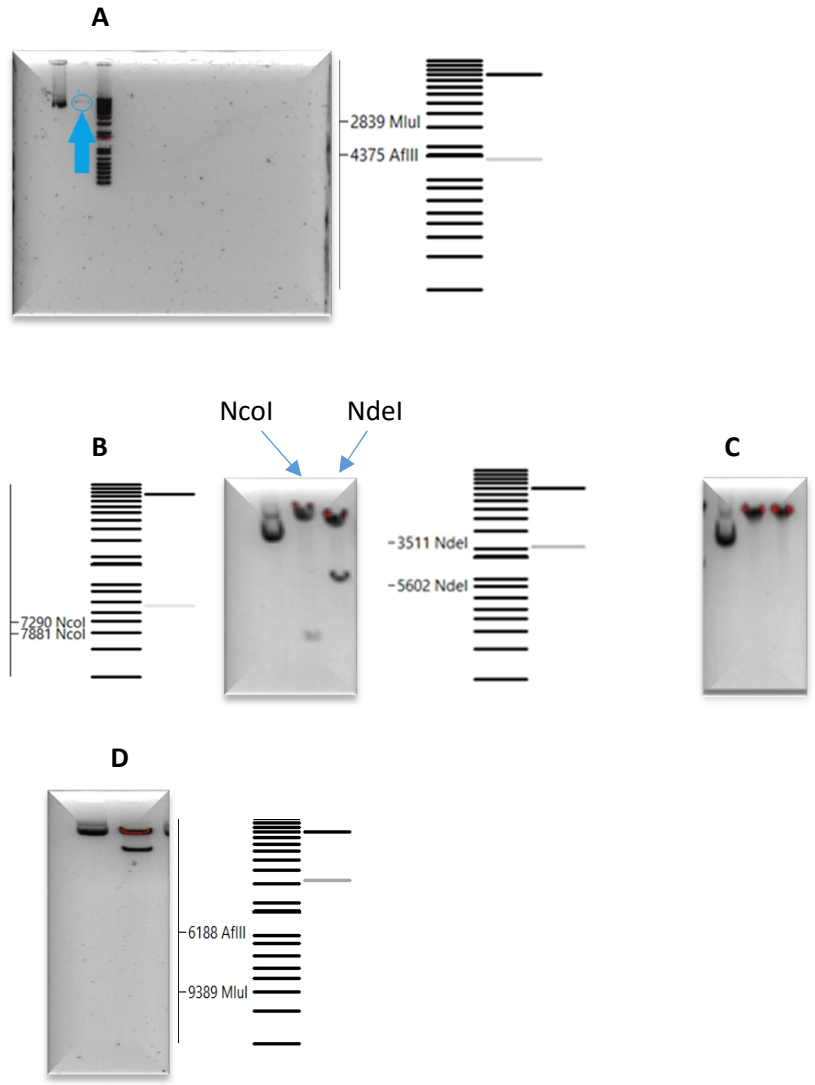
Figure 21. Donor vector containing PRDM12

3.3.2. CLONING TRANSCRIPTION FACTORS REGULATING SENSORY NEURONS DIFFERENTIATION INTO THE RMCE DONOR VECTOR

In order to insert the transcription factors into the donor vector, we needed to perform cloning.

Our first point was to linearize the vector pZ M2rtTA_CAGG TetON-3xFLAG-tdT v2 (figure 4) with appropriate enzymes, AflII and MluI. The bands match (figure 22A), so we extracted and purified the bigger band (9 kb) because it was our opened vector.

Next, we cloned TF using gibson assembly. We generated two separate RMCE donor vectors with three (BRN3A, ISL1, KLF7) and one (PRMD12) transcription factors. These were ordered as gBlock® gene fragment from Integrated DNA Technologies. After the first gibson assembly reaction we performed digestions with NcoI and NdeI to check if the assembly was done correctly. For fragment containing PRDM12 the expected bands are there (figure 22B), but for fragment containing the 3 TFs there was no cut (figure 22C). Then, we repeated the gibson assembly reaction for the fragment with 3 TFs, 1/10 diluted. After doing some digestion tests with AflII+MluI (figure 22D), Bam HI and HindIII (figure 22E), we observed the expected bands, proving that both constructs were ready to nucleotransfect.



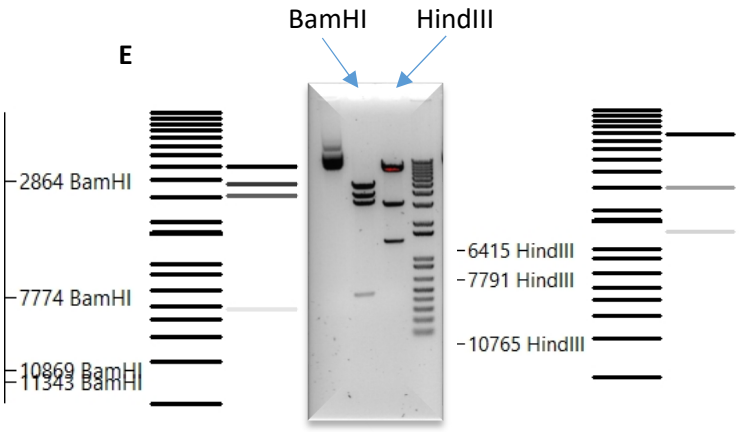
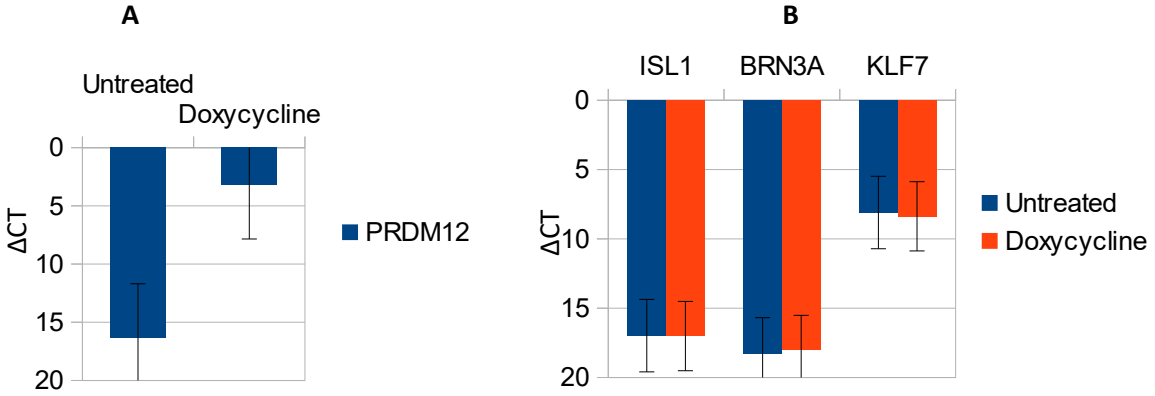


Figure 22. 0,8% agarose gel electrophoresis of digested RMCE vectors to test for correct assembly. The digestion pattern in silico is depicted on next to each gel. In all gels 1Kb DNA ladder was also loaded. In every first lane there is the uncut plasmid

3.3.3. TEST OF THE DOXYCYCLINE INDUCIBLE OVEREXPRESSION SYSTEM

After nucleotransfection, and before starting the differentiation protocol, we wanted to check if the transcription factors were correctly assembled. First, we performed a FACS, and we observed that in the mother cell line (MCL) (figure 24E) there was the expected expression of the GFP (dots on the Q4). On the other hand, in both cell lines generated (figure 24F and G), the expression of GFP was lost (dots on Q3), meaning that the flipase system worked fine.

Next, we added doxycycline to both cell lines during 1 day to see if the inducible system is working. In the cell line overexpressing PRMD12 (figure 23A), it is clear the overexpression of PRDM12 after the addition of doxycycline. In turn, in the cell line overexpressing the 3 TF (figure 23B), there was no difference in gene expression. So, we added doxycycline for 2 days in this cell line. Finally we noticed some difference (figure 23C) in gene expression, but only for BRN3A. It's supposed to overexpress all of them, and not only one, so something was wrong. Indeed, to order the fragments as gblocks we did silent mutations, due to the high amount of CG, for IDT being able to make it. Therefore, our normal primers were not able anymore to recognize the new ISL1 and KLF7 transcripts, only still recognize BRN3A. Then, and after ordering right primers, we saw that also the system overexpressing BRN3A, ISL1 and KLF7 were working (figure 23D). At this point we had all the information to start the differentiation protocol.



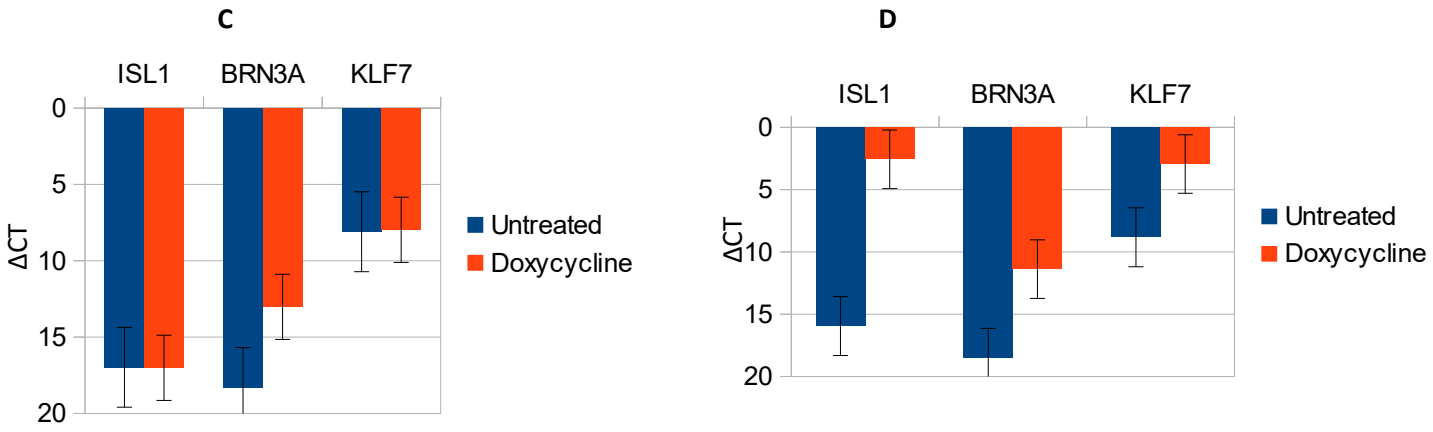


Figure 23. Cells were harvested after 1 day (A, B) and 2 days (C, D) of doxy. New transcripts from fragment 2 were measured with the normal primers (C) and right primers (D). All gene expression measurement was performed by qRT-PCR and data is shown as mean ± SD of ΔCt compared to levels of GAPDH.

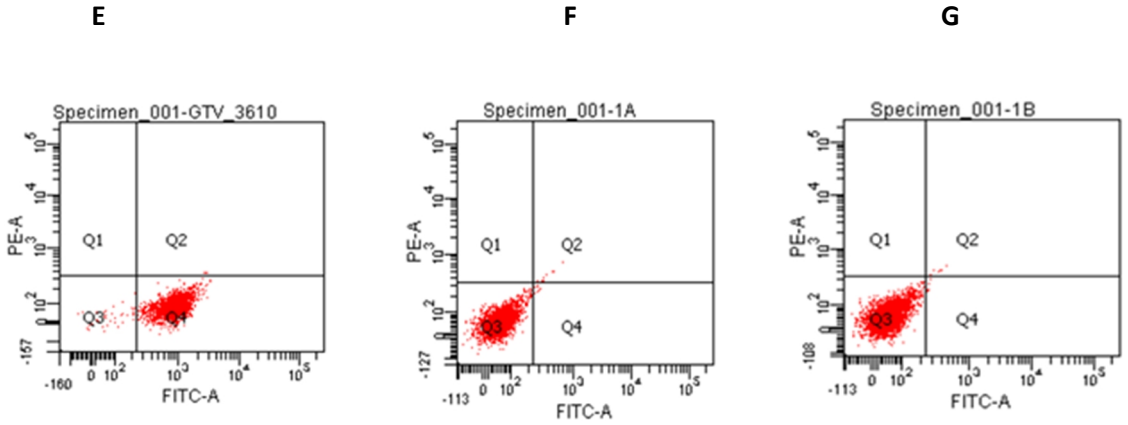


Figure 24. Quantification of GFP performed by FACS on (E) normal mother cell line and after 15 days of selection on (F) fragment PRDM12; (G) fragment ISL1-BRN3A-KLF7

3.3.4. CHARACTERIZATION OF SENSORY NEURONS DIFFERENTIATED FROM HESC (H9) OVEREXPRESSING PRMD12

Once the efficiency of the doxycycline-inducible system was confirmed, we were able to start differentiation. Unfortunately, we took some time to solve the problem on the cell line overexpressing BRN3A, ISL1 and KLF7, so I only had the time to start differentiation on the cell line overexpressing PRDM12. This cell line was differentiated into sensory neurons following protocol described in figure 8, and doxycycline was added from day 7. Cells were harvested at different time points (day 10, 19, 25 and 35) during differentiation and gene expression was analysed by qRT-PCR.

3.3.4.1. MORPHOLOGY AND GENE EXPRESSION ANALYSIS OF TRANSCRIPTION FACTORS INVOLVED IN NOCICEPTORES DIFFERENTIATION

The morphological pattern on day 15 without adding doxycycline (figure 25A) is similar to what we got before on different cell lines (figure 9B and E; figure 16B): clump of cells with already neurites present. On day 15 in the doxycycline condition (figure 25B), we can observe the same clump of cells and the neurites, however the plate looks much more homogeneous, without the presence of "unknown cells". The same is registered at day 35, in which the doxy condition (figure 25D) looks cleaner, without those "unknown cells" comparing to no doxy condition (figure 25C).

The expression of sensory neurons markers, ISL1 and BRN3A (figure 26A) are significantly increased at day 25 in the doxy condition, and then at day 35 the expression is similar between doxy and no doxy condition. In terms of the expression of the nociceptors markers, RUNX1 is lower expressed in the doxy condition at day 19 and 25 (figure 26B), but on day 35 there is a small increase on the expression of the RUNX1 in the doxy condition. In turn, TAC1 is lower expressed in the doxy condition until day 19, but from day 25 on is significantly overexpressed (figure 26C). There is no significant difference in KLF7 expression (figure 26D). PRDM12 is obviously overexpressed in the doxy condition, because this was the transcription factor inserted in the system (figure 26E).

Concerning TRP channels expression, there is no significant difference in TRPA1 (figure 26F). In turn, the expression of TRPV1 is already increased on day 19 in the doxy condition, and stays increased in day 35 (figure 26G). Interestingly, the most differences are observed in TRPM3 (figure 26H) and TRPM8 (figure 26I) expression. In both cases, from day 25 on, there is a big increase on the expression in the doxy condition, especially in TRPM3. TRPV4 expression is also increased in the doxy condition, but only on day 35 (figure 26J).

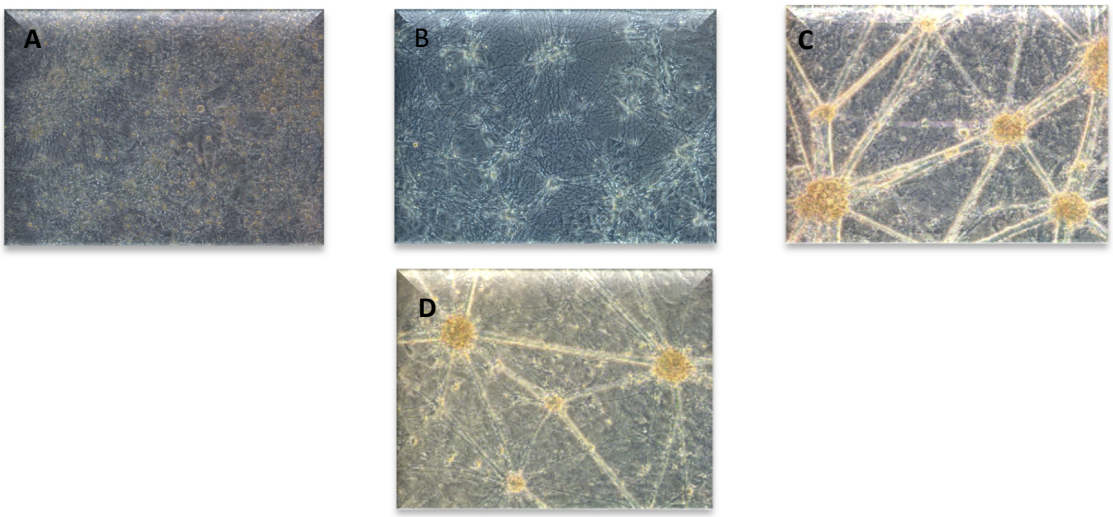
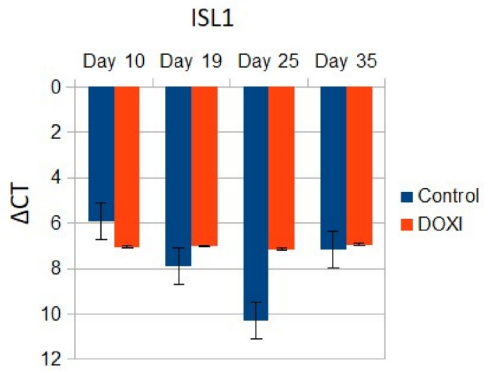
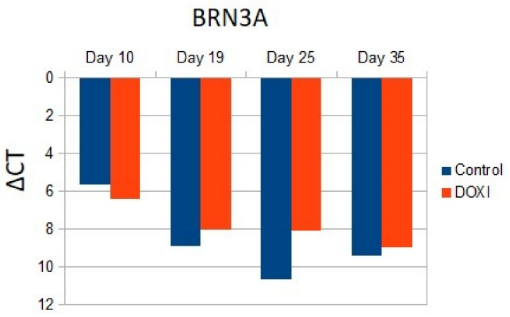
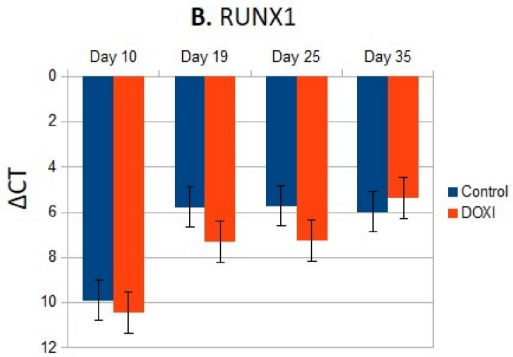


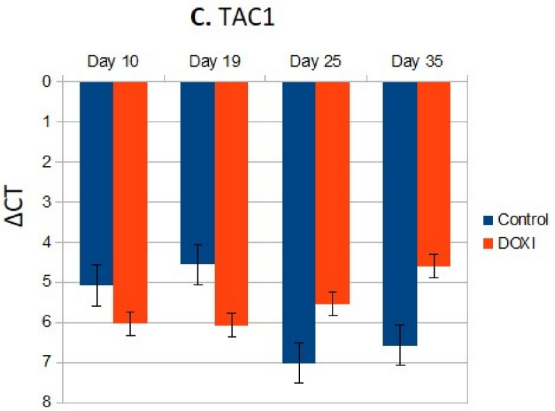
Figure 25. H9 cells overexpressing PRDM12 were differentiated into sensory neurons following the protocol described in figure 8. Cell morphology of cells on day 15 (A), 35 (C) in a no doxy condition, and cells on day 15 (B), and day 35(D) in a doxy condition, was assessed throughout the protocol under the inverted microscope.

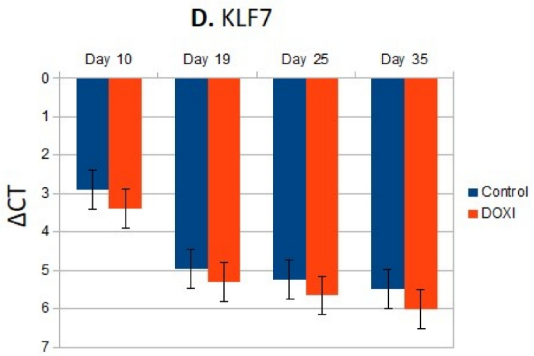

ISL1
Day 10
Day 19
Day 25
Day 35
ΔCT
0
2
4
6
8
10
12
Control
DOXI

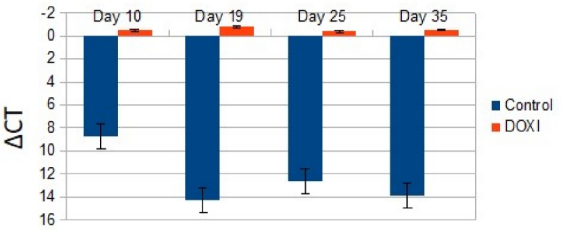
A

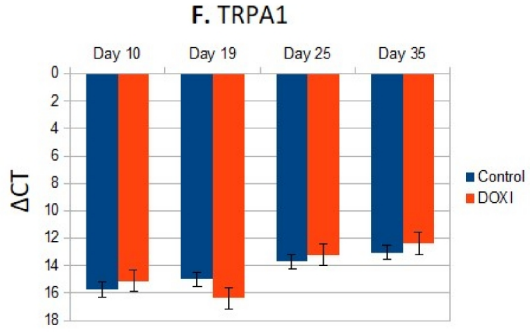

BRN3A
Day 10
Day 19
Day 25
Day 35
ΔCT
0
2
4
6
8
10
12
Control
DOXI

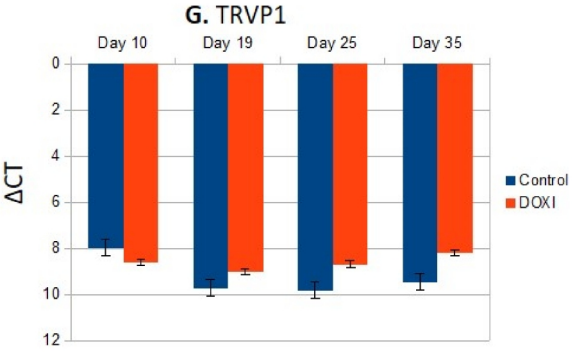

B. RUNX1
Day 10
Day 19
Day 25
Day 35
ΔCT
0
2
4
6
8
10
12
Control
DOXI


C. TAC1
Day 10
Day 19
Day 25
Day 35
ΔCT
0
1
2
3
4
5
6
7
8
Control
DOXI


D. KLF7
Day 10
Day 19
Day 25
Day 35
ΔCT
0
1
2
3
4
5
6
7
Control
DOXI


E. PRMD12
Day 10
Day 19
Day 25
Day 35
ΔCT
-2
0
2
4
6
8
10
12
14
16
Control
DOXI


F. TRPA1
Day 10
Day 19
Day 25
Day 35
ΔCT
0
2
4
6
8
10
12
14
16
18
Control
DOXI


G. TRVP1
Day 10
Day 19
Day 25
Day 35
ΔCT
0
2
4
6
8
10
12
Control
DOXI

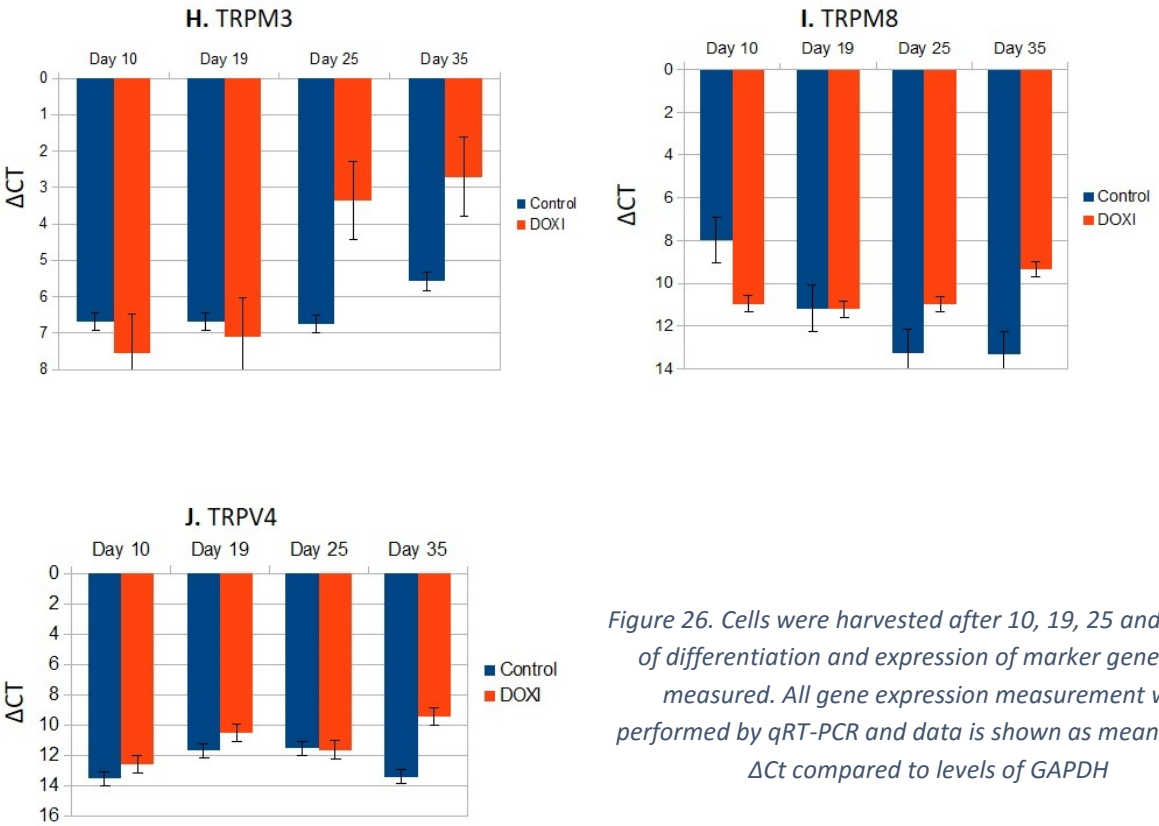
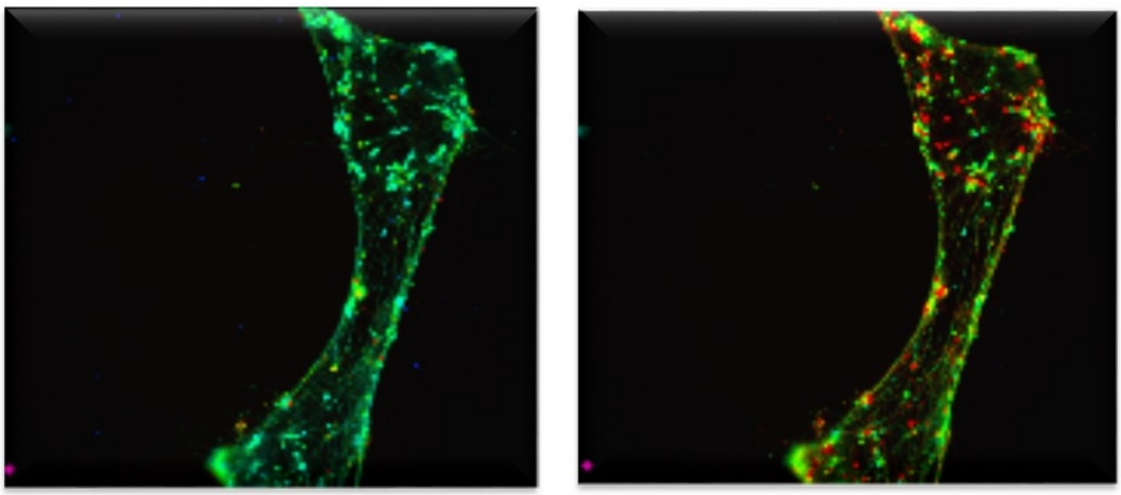


Figure 26. Cells were harvested after 10, 19, 25 and 35 days of differentiation and expression of marker genes was measured. All gene expression measurement was performed by qRT-PCR and data is shown as mean ± SD of ΔCt compared to levels of GAPDH

3.3.4.2. FUNCTIONAL TESTS (CALCIUM IMAGING)

Again, we wanted to check about the functionality of the sensory neurons generated. We measured the cells in doxy condition, and the cells in no doxy condition at day 15, but only for PS (TRPM3 agonist), because TRPM3 was the only TRP channel activated at day 15 on previous experiments. In the control plate (no doxy), the results were similar to what we got before (see section 3.1.2.2): at this point, there was already a big response to the TRPM3 agonist, PS (figure 27C). However, in the doxy condition, the response was lower to PS (we can see by the less red dots present). Unfortunately, it was impossible to measure cells at day 35 and day 50 due to the lack of time.

NO DOXY



DOXY

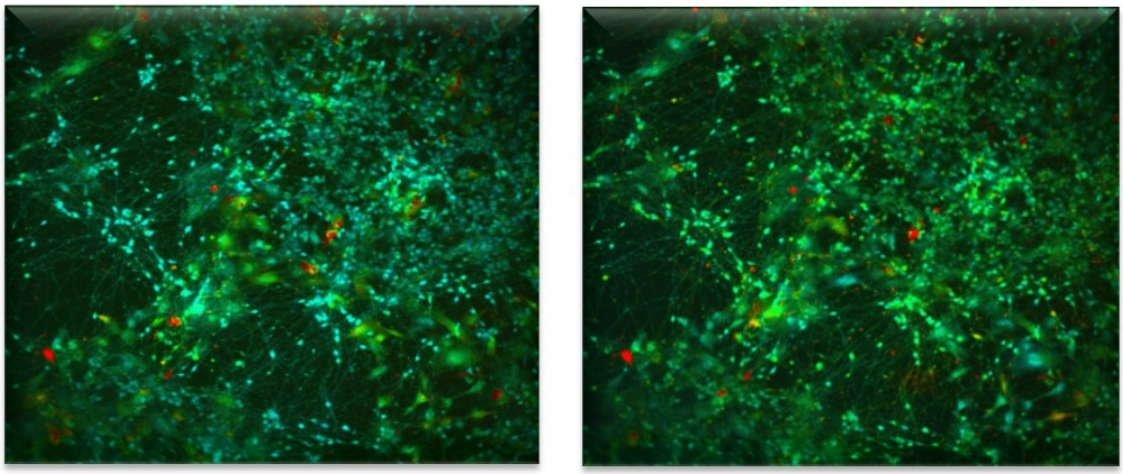


Figure 27. Calcium imaging. Sensory neurons generated from cell line overexpressing PRDM12 were measured for PS (TRPM3 agonist) at day 15 in doxy and no doxy condition A. Fura-2AM ratio picture before experiment B. Fura-2AM ratio picture before experiment C. After PS [40µM] application (TRPM3 agonist) D. After PS [40µM] application (TRPM3 agonist)

4 Discussion and Conclusions

4 DISCUSSION AND CONCLUSIONS

The generation of human sensory neurons by directed differentiation of pluripotent stem cells opens new opportunities for investigating the biology of pain. Despite the great interest in pain biology, the research in this area is mostly done on rodent cellular and animal models due to the lack of accessibility of human nociceptive neurons (Young *et al.,* 2014; Boisvert *et al.,* 2015). Over the last years, some studies have been published describing hPSCs differentiation into sensory neurons, which resulted in significant progresses such as the use of defined molecules in the differentiation protocol. In this thesis, we took advantage of an existing protocol to differentiate hPSC into sensory neurons and we changed it in order to enhance differentiation. We also took advantage of a hESC mother cell line already established in the lab to overexpress some key transcription factors involved in nociceptors differentiation.

4.1 GENE EXPRESSION AND MORPHOLOGICAL ANALYSIS OF THE IPSC DIFFERENTIATION PROTOCOLS

Young and his team in 2014, demonstrated that the protocol they use is capable to neuralize pluripotent cells, driving them to a nociceptor phenotype. Here, we tested the same protocol, and an adapted one developed on the lab with a different medium (B27+N2 instead of KOES) (see figure 7 and 8). We proved the efficiency of the protocol to generate nociceptors, and we found that both protocols are able to do it. Concerning the morphology, there was no differences between protocols, with neural colonies exhibiting neurite outgrowth. Indeed, the neurons generated via B27+N2 medium were stained for BRN3A (figure 11), confirming the sensory identity, reflecting what was observed by Young *et al.,* 2014 in his protocol using KOES medium.

The protocol used here to generate nociceptors, express sensory, nociceptors and TRP channels specific genes with a similar temporal profile between both protocols. In general, sensory markers come up at day 15, nociceptors at day 30, and TRP channels at day 50, suggesting that already on day 15 we have sensory phenotype, but the later expression of the nociceptors markers indicates the development of this specific sensory neuronal type only after 30 days of differentiation. Resembling results were expectedly obtained by Young et *al.,* 2014, in which he saw that ISL1 and BRN3A peaked expression at day 15 and then stays stable, and the expression of TAC1 and RUNX1, the nociceptors markers, only peaked at day 32. This process broadly recapitulates the *in vivo* developmental process, once BRN3A and ISL1 are expressed early in DRG neurons, coinciding with sensory specification and then maintained at constant levels throughout life. Moreover, *in vivo* RUNX1 is expressed after the activation of BRN3A and ISL1 (Marmigère and Ernfors, 2007; Lallemend and Ernfors, 2012). However, merely after 50 days of differentiation we have fully mature nociceptors as we can see by the expression of TRP channels that only reaches values similar to human DRG on day 50, specially TRPV1 and TRPA1. Indeed, TRPV1 expression is often considered a marker for nociceptors (Sexton *et al.,* 2003) and TRPA1 is co-expressed with TRPV1 (Story *et al.,* 2003), therefore expectedly, this ones are the most expressed TRP channels in the nociceptors generated.

In this work, we also assessed the genomic profile of the sensory neurons from human DRG. This information allowed us to see that TRPV1 and TRPA1 are the most expressed TRP

channels in vivo. Moreover, the mRNA expression profile of iPSC derived sensory neurons are highly similar to sensory neurons from human DRG, and this expression profile is achieved after 50 days of differentiation, highlighting these 50 days as the necessary time to have mature sensory neurons. The expression profile of the sensory neurons generated via B27+N2 medium are closer to the profile of those from human DRG than the ones originated from KOES, suggesting that B27+N2 medium has better results than KOES in differentiating iPSC into sensory neurons.

4.1.1 FUNCTIONAL TESTS

To gain insight about the functionality of the sensory neurons generated, we performed functional tests (calcium imaging) using agonists for the TRP channels (TRPV1, TRPA1, TRPM3, TRPM8). We started measuring neurons generated from both protocols at day 50. We observed that both protocols gave similar results (see section 3.1.2.1): A huge response to TRPV1 and TRPA1 agonists, especially the first, and almost no response to TRPM3 and TRPM8 agonists. This outcome proves the functionality of the sensory neurons generated after 50 days of differentiation. Indeed, TRPV1 activation was also detected by Young *et al.,* 2014 after 6 weeks in maturation medium (day 52 of differentiation). This is in accordance with the qRT-PCR results, once the most expressed TRP channels at day 50 were TRPV1 and TRPA1.

Interestingly, at day 15, we observed the early activation of TRPM3 (see section 3.1.2.2). The others TRP channels didn't have response at this time point (see supplementary data). However, at day 30, there is no response of any TRP channel, neither TRPM3 that was activated early. This early and then vanished activation of TRPM3, could suggest a role of TRPM3 in the development process. On the other hand, when the nociceptors become fully mature (after 50 days) the TRP channels activated are TRPA1 and specially TRPV1, highlighting the importance of this TRP channels in sensing pain, particularly TRPV1.

4.2 MODIFICATION OF SENSORY NEURONS DIFFERENTIATION PROTOCOL BY DOING REPLATING DIFFERENCES AND USING DIFFERENT CELL LINE

From the first results we saw that only after 50 days of differentiation the cells achieved the mature nociceptor phenotype. Then, we tried to do some changes on the protocol to see if we can speed up differentiation. In the protocol, on day 6 there's a replating step. The functional tests require plates with few density of cells. Due to that, we tried whether doing replating differences (½, ¼ and 1/6 dilution) we could see some differences on differentiation markers. We noticed there was not a significant difference between dilutions, and the only difference on day 50 was the less expression of TAC1 and TRPA1 on the 1/6 dilution. This less expression is probably caused by the less cells present on the plate. We didn't try functional tests on this, because the cells on the ¼ and 1/6 dilution were not looking as good as the ½ dilution. So, doing replating differences didn't help to speed up differentiation.

Next, we tried the differentiation protocol in a different iPSc line (sigma 0028), in order to see if the protocol is reproducible in different cell lines, and if in this new cell line we could have a faster differentiation than in the one (cellectis). In terms of morphology the cells were similar to what we got on cellectis. Also, regarding the expression of the differentiation markers, there are no big differences, and we can notice that the expression pattern of the markers is similar

between cell lines (figure 17). Indeed, in the research conducted by Young and his team in 2014, the cell line used was a hESC (Shef1), but the expression of the differentiation markers follows the same standard. This proves the reproducibility of the protocol once it is possible to be applied to different cell lines having similar results.

Concerning the expression of the TRP channels we can see differences between cellectis and sigma 0028: TRPV1 is more expressed in the nociceptors generated from cellectis line, while TRPV4 is more expressed in nociceptors generated from sigma 0028 line. Although the protocol is reproducible between cell lines, there are slightly differences associated with the different cell lines.

Comparing the expression profile of both cell lines to the sensory neurons from human DRG, we can see that using cellectis line to differentiate towards nociceptors, we have closer values to sensory neurons from DRG, meaning that the best cell line to apply the protocol is the cellectis one.

Furthermore, there were some differences in working with both cell lines. Applying the differentiation protocol to sigma line was harder than to the cellectis, with the cells detaching a lot of times and having real problems in getting attached to glass coverslips. Indeed, we did not try functional tests on sigma line, not only due to the worst qRT-PCR results, but also because the plates to functional tests (has to be on glass coverslips) were every time impossible to measure. Concluding, using a different cell line did not enhance differentiation.

4.3 RMCE-MEDIATED DOXYCYCLINE-INDUCIBLE TRANSCRIPTION FACTOR OVEREXPRESSION

Due to the unsuccessful attempts to speed up differentiation by changing replating dilution and cell line, we next studied a more direct approach by overexpressing key transcription factors.

Thus, we took advantage of the master cell line suitable for FLPe-mediated RMCE previously generated in the lab, to test whether inducible transcription factor overexpression were able to improve differentiation.

In order to check about the viability of joining transcription factors, we performed a short differentiation to get specific information about the gene expression of PRDM12, BRN3A, ISL1 and KLF7. We found that the peak of expression of BRN3A, ISL1 and KLF7 were at day 15. This result is in accordance with the research done by Young *et al.,* 2014, in which it is noticed that ISL1 and BRN3A peak expression at the same time, around day 15. In turn, PRMD12 expression reaches maximum at day 9. Indeed, in a research conducted by Chen *et al.,* in 2014, it was applied a similar protocol to differentiate iPSCs into nociceptors and they found that PRDM12 expression began to increase on day 7 and peaked at day 9, while ISL1 and BRN3A peaked around day 13, the same pattern observed in our results (see figure 19). Then, due to the early activation of PRDM12 regarding BRN3A, ISL1 and KLF7 expression, we decided to join BRN3A, ISL1 and KLF7 in the same construction and PRDM12 alone in a different construction.

We confirmed by digestion tests that our donor vectors were already done and by the outcome given by FACS that both donor vectors were correctly assembled in the locus. Next, we added doxy to see if the TF were being overexpressed. 1 day of induction was sufficient to overexpress PRDM12, while in the system containing ISL1-BRN3A-KLF7, only after two days of induction we could see some overexpression. Firstly, we only detected changes in gene expression in BRN3A, which makes no sense. Then, and after a rational thinking, we noticed that

our ISL1 and KLF7 primers were not able to recognize the new transcripts, due to the silent mutations we did. After having the right primers, we observed the overexpression of all transcription factors (figure 23D), proving the functionality of both systems generated.

4.3.1 IMPROVEMENT OF HESC DIFFERENTIATION PROTOCOL BY TRANSCRIPTION FACTOR OVEREXPRESSION

Our ultimate goal was to generate cell lines overexpressing [1] PRDM12 and [2] BRN3A, ISL1 and KLF7. We confirmed the efficiency of the doxycycline-inducible system in both cell lines generated, however due to the lack of time, I was only able to start differentiation in the cell line overexpressing PRMD12.

Morphologically, there were differences detected between the doxy and no doxy condition on day 15 and 35. Our outcome suggest that PRDM12 plays an important role in the homogeneity of the plate, with the overexpression of it being responsible in starting differentiation in a higher % of cells towards sensory phenotype, maybe due to its epigenetic function. Indeed, it's reported on the literature the importance and the role of PRDM12 in nociceptors differentiation (Chen *et al.,* 2014), having a histone-modifying activity.

In terms of gene expression, the overexpression of PRDM12 detected in all days in the doxy condition confirmed the efficiency of the system. This overexpression affects gene expression from day 25 on, with ISL1, BRN3A, TAC1, TRPV1, TRPM3 and TRPM8 expression being increased in the doxy condition. RUNX1 and TRPV4 expression were increased in the doxy condition only after 35 days of differentiation. This difference in expression could be the result of having more cells expressing this genes. About TRPA1 expression is not detected any differences, however the expression of this TRP channel is really increased from day 30 to day 50 (figure 10C), so it's impossible to take conclusions at day 35.

Interestingly, the most affected gene was TRPM3. From previous results, this TRP channel is not supposed to increase expression from day 15 to day 50, and here in the doxy condition it's clearly overexpressed from day 25 on. Moreover, the functional tests did on day 15 on doxy and no doxy condition, revealed that the cells in the no doxy condition behave like we were expecting: a huge response to PS. Surprisingly, the response of the cells in the doxy condition to PS was weaker. In previous results, we noticed that on day 15, TRPM3 was already activated and then it goes off on day 30. This could mean that our cells in the doxy condition, already on day 15, were in a further stage of differentiation, because the cells didn't react as expect to PS, resembling the behaviour on day 30.

This results suggest an important role of TRPM3 in the development process. Indeed, this TRP channel is activated early on the differentiation, and then looks no more important in mature nociceptors. Also, TRPM3 is the most affected gene in the cell line overexpressing PRMD12, highlighting the importance of this TRP channel in the differentiation.

Taking together these results, we can conclude that overexpressing PRDM12 affects the homogeneity of the plate, with more cells starting the differentiation protocol towards sensory neurons as well as gene expression from day 25 on. Moreover, it would be interesting in the following experiments, to do a staining to assess about the homogeneity in the doxy condition. The protocol is improved, however we have to check more time points (day 50) and doing more functional tests in the other time points (day 30 and day 50) to have more accurate conclusions.

Unfortunately, we don't have results on the other construction (BRNA, ISL1 and KLF7), but we expect to see a bigger improvement on the protocol, once this Tfs are directly involved in the differentiation process. Also, and once confirmed that PRDM12 overexpression affects the homogeneity, it would be worthy to join all 4 TF (PRDM12, BRN3A, ISL1 and KLF7) in a single construction.

5 CONCLUSION AND FUTURE PERSPECTIVES

5 CONCLUSION AND FUTURE PERSPECTIVES

According to its aims and experimental work, several conclusions can be taken from this thesis. First, we were able to characterize the gene expression and the morphology of functional sensory neurons generated from a protocol used by Young et al., 2014 and an adapted one, showing that the adapted one has better RT-qPCR results. Second, we demonstrated that the protocol is reproducible in different cell lines and the best replating dilution is ½. Third, RMCE mediated inducible overexpression was shown to be a valuable tool for the manipulation of stem cell differentiation due to the proved overexpression of the Tfs inserted. Finally, PRDM12 overexpression affects the homogeneity of the plate, with the biggest improvement on the gene expression being verified on TRPM3. Indeed, this thesis suggest an important role for TRPM3 in the developmental process, due to the early activation (day 15) of this TRP channel and the effect of the overexpression of PRDM12 on it. To prove this, it would be interesting to mutate this TRP channel, and see how differentiation is affected.

In the near future, we are planning to start differentiation on the cells overexpressing ISL1-BRN3A-KLF7, in order to see the impact of the overexpression of this Tfs in the differentiation process, and whether we could improve the protocol, having functional sensory neurons in less than 50 days. Moreover, we expect to be able to join the two separate constructions into a unique vector and therefore induce overexpression of the four transcription factors at the same time, having this way the ultimate construction to improve the differentiation. Finally, we can try to start (and stop) adding doxycycline at different time points during differentiation protocol, to gain information about what day is the best to start (and stop) inducing the overexpression of Tfs.

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SUPPLEMENTARY DATA

1. **FUNCTIONAL TESTS: CALCIUM IMAGING B27+N2 DAY 15**

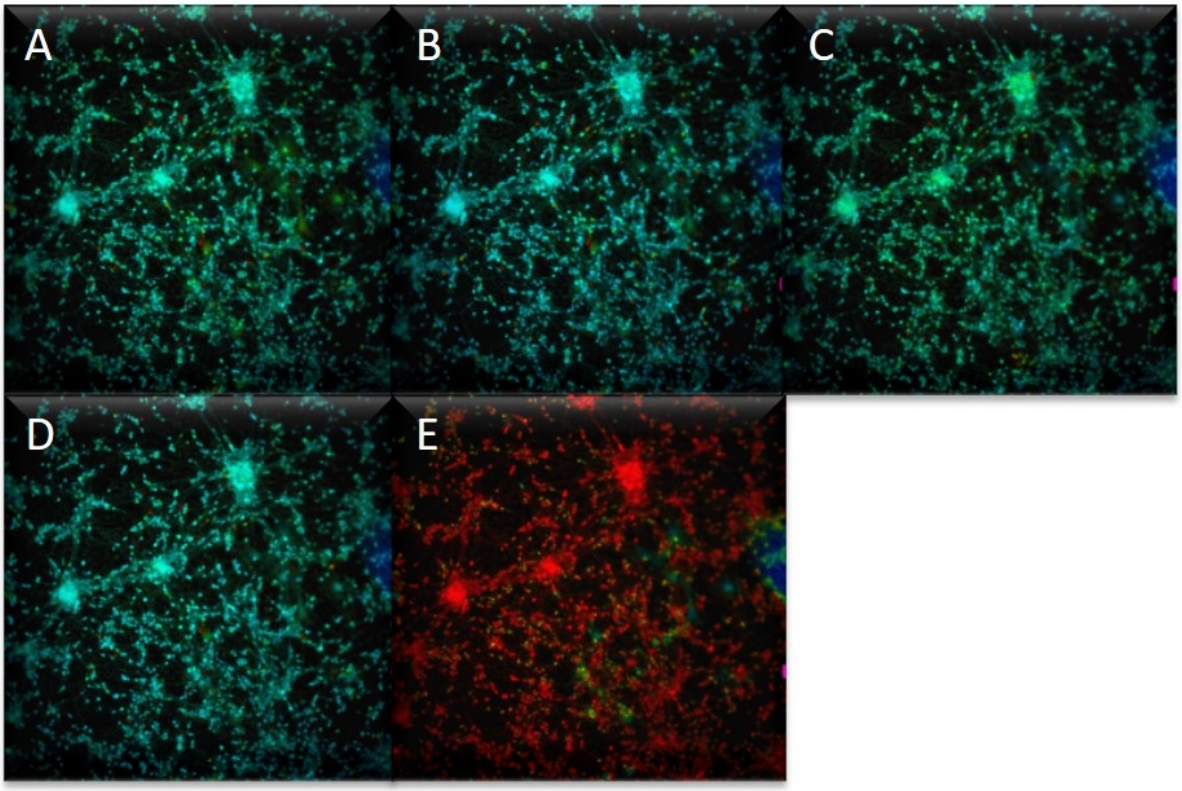


Figure S1. Calcium imaging. Sensory neurons generated from adapted protocol were measured for TRP channels agonists at day 15: A.Fura-2AM ratio picture before experiment; B.After Menthol [100µM] application (TRPM8 agonist); C.After Capsaicin [1µM] application (TRPV1 agonist); D.After mustard oil [100µM] application (TRPA1 agonist); E.After high potassium [50mM] (depolarizes all neurons).

2. FUNCTIONAL TESTS: CALCIUM IMAGING B27+N2 DAY 30

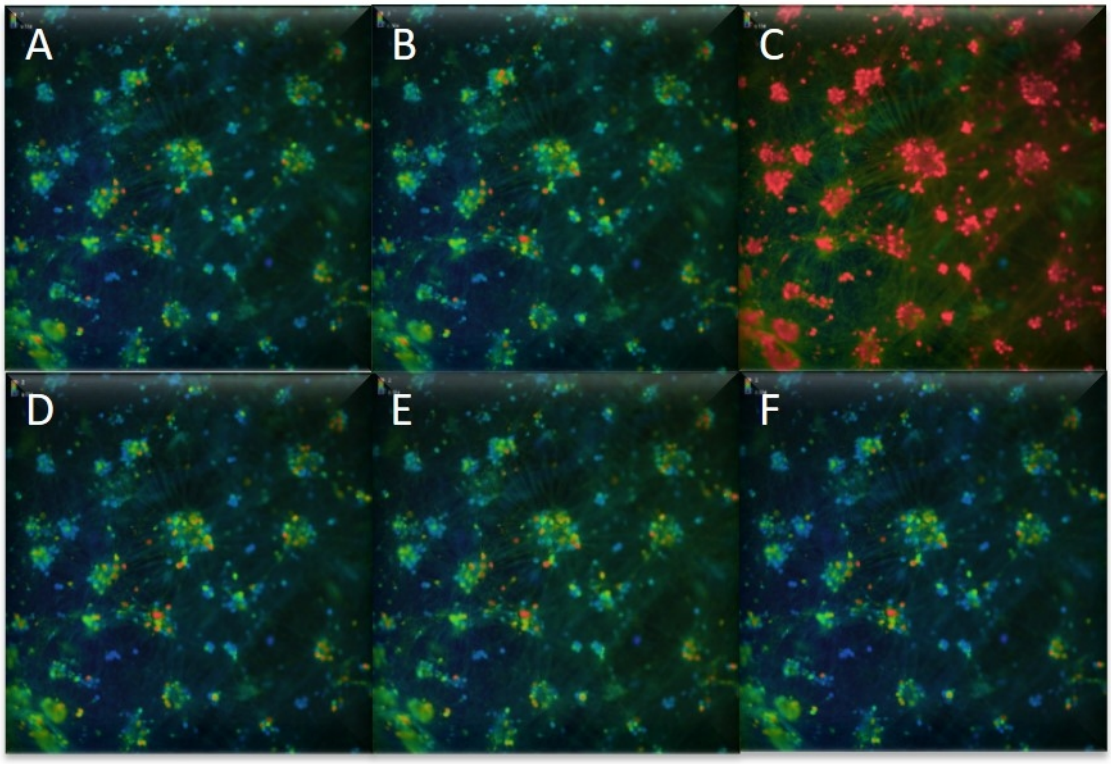
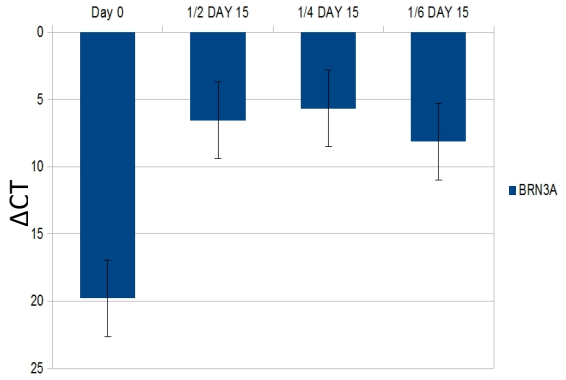
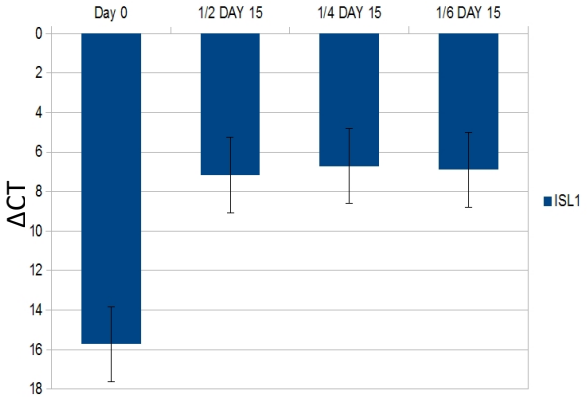
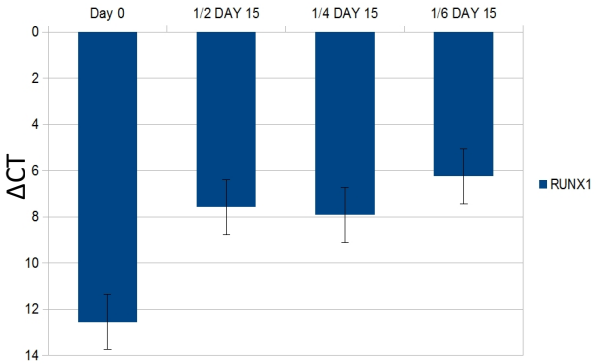


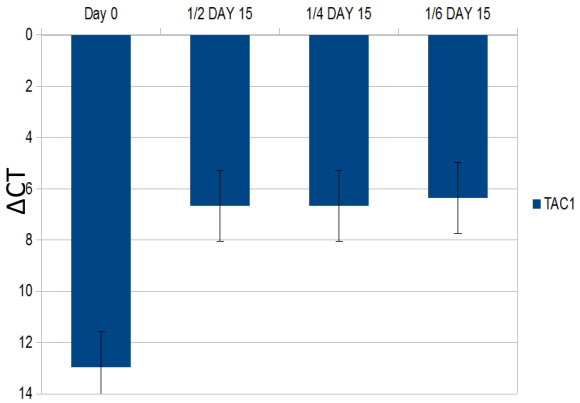
Figure S2. Calcium imaging. Sensory neurons generated from both protocols were measured for TRP channels agonists at day 30: A. Fura-2AM ratio picture before experiment; B. After PS [40µM] application (TRPM3 agonist); C. After Menthol [100µM] application (TRPM8 agonist); D. After Capsaicin [1µM] application (TRPV1 agonist); E. After mustard oil [100µM] application (TRPA1 agonist); F. After high potassium [50mM] (depolarizes all neurons).

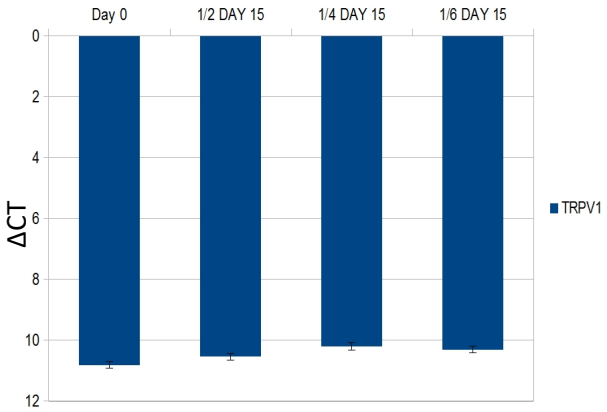
3. CELLECTIS REPLATING DIFFERENCES (DAY 15)

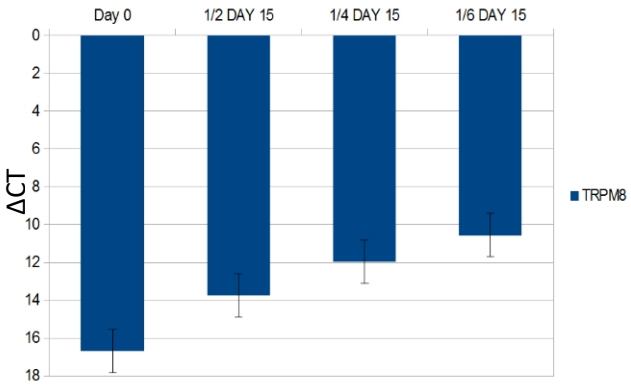


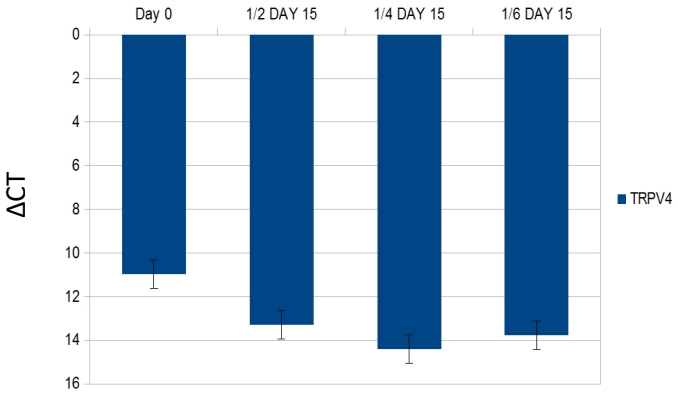


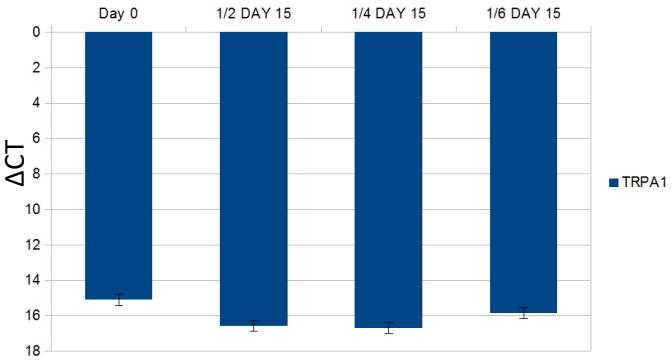












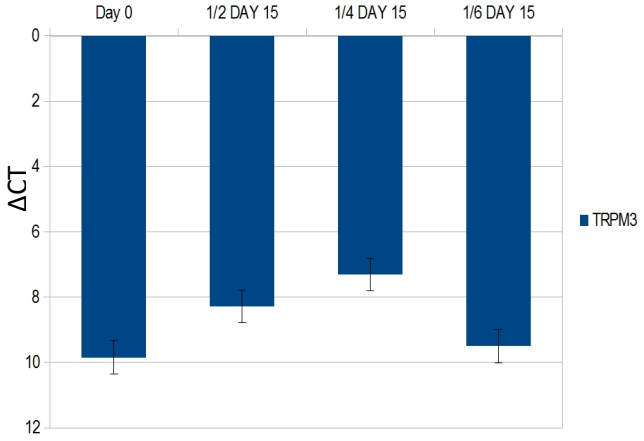
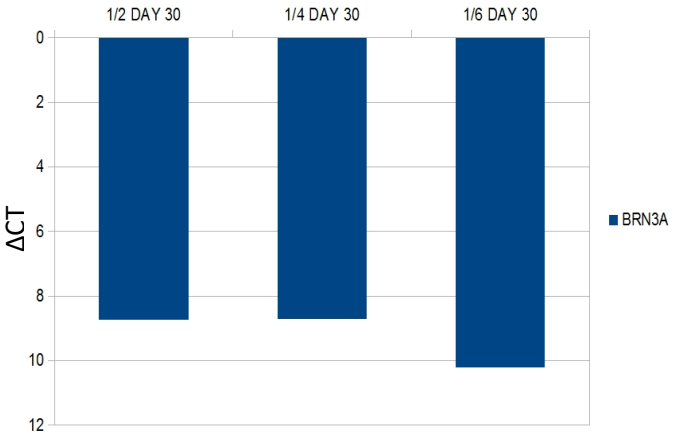
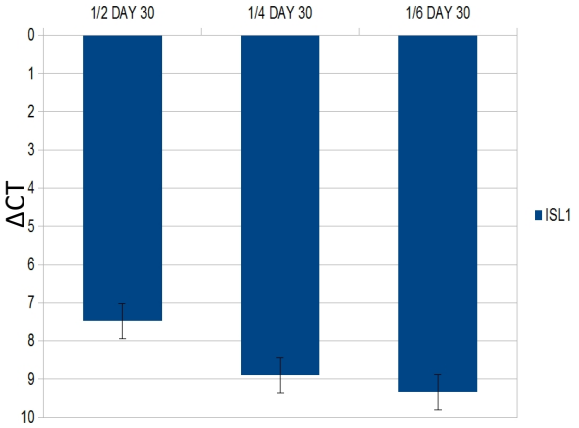
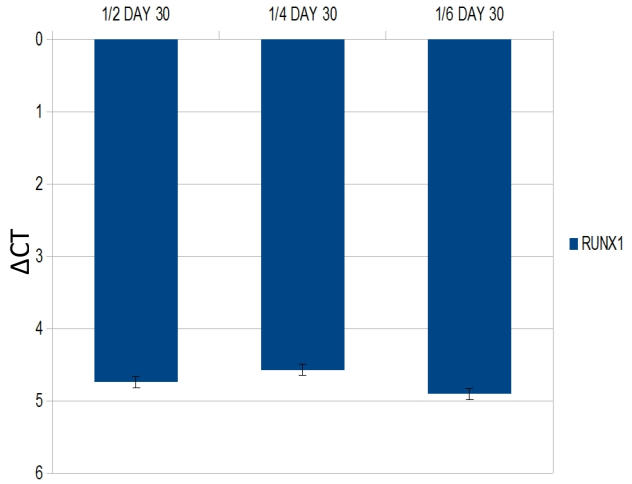


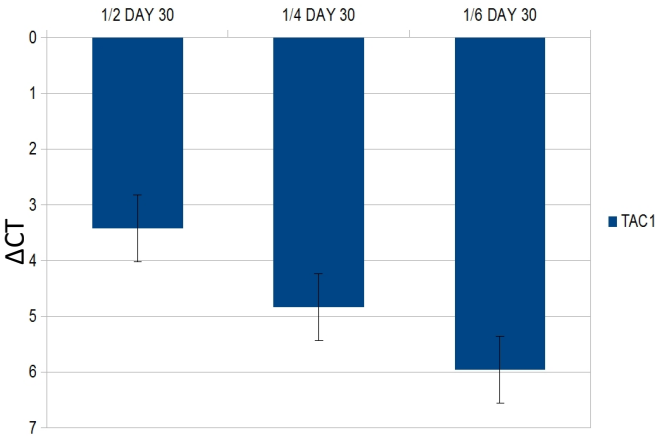
Figure S13. Cells were harvested at day 15 of the protocol and expression of several marker genes was measured. All gene expression measurement was performed by qRT-PCR and data is shown as mean ± SD of ΔCt compared to levels of GAPDH

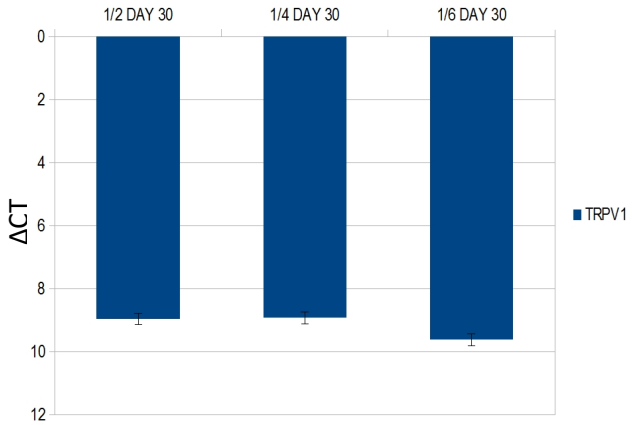
4. CELLECTIS REPLATING DIFFERENCES (DAY 30)

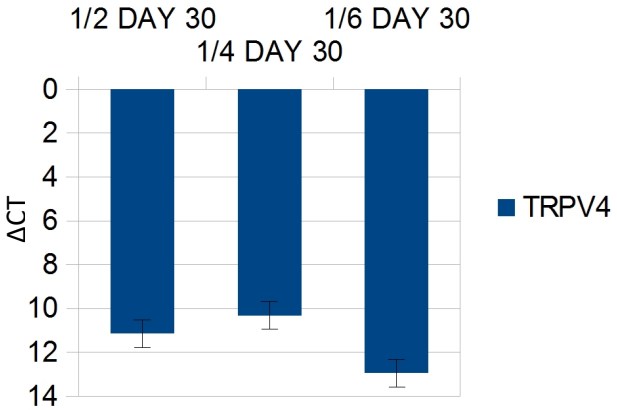


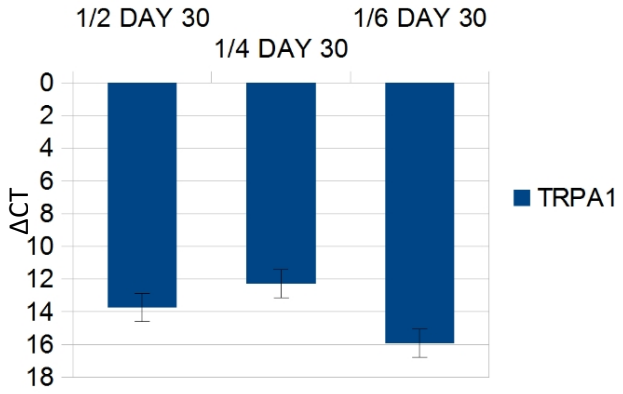


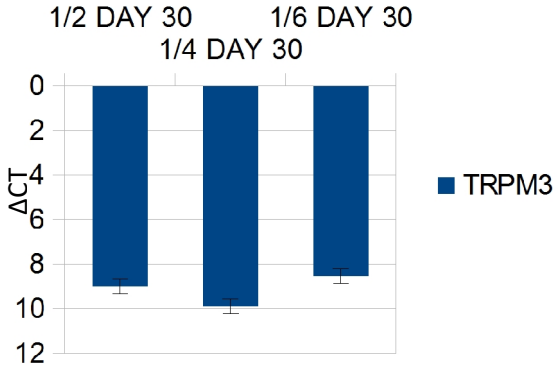












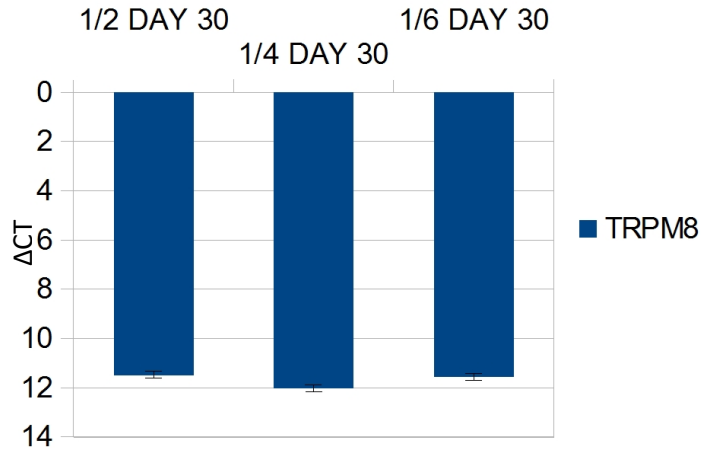


Figure S14. Cells were harvested at day 30 of the protocol and expression of several marker genes was measured. All gene expression measurement was performed by qRT-PCR and data is shown as mean ± SD of ΔCt compared to levels of GAPDH