Functional fluorescent materials and migration dynamics of neural progenitor cells

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NEURAL PROGENITOR CELLS CHARACTERIZATION AND LABELLING

In this chapter, we introduce the murine neural progenitor cells line C17.2 and confirm its multipotency via differentiation into a mixed population of neurons and astrocytes. Further, several fluorescent labels are explored as well as the transduction with fluorescent reporters with the focus to label cell nucleus and body. The genetically modified cell line is used for time-lapse imaging and data analysis in chapter 5.

4.1. INTRODUCTION

Neural progenitor cells (NPCs) are multipotent stem cells of the central nervous system (CNS) which reside in specific niches in adults and embryos [1]. They have the capacity for continuous self-renewal and can differentiate into the three major cell lineages of the nervous system: neurons, astrocytes, and oligodendrocytes [2]. This is crucial for embryonic as well as adult neurogenesis. Additionally, NPCs secrete neurogenic factors which contribute to a healthy neural microenvironment [3]. Transplantation studies of NPCs into injured or degenerating CNS are promising and show high potential for the treatment of neurodegenerative disorders like Parkinson's, Alzheimer's, ALS, or stroke/ischemia [4, 5]. Since the discovery of neural progenitor cells, research effort has been focused on increasing our understanding of these cells [6]. A crucial breakthrough was the establishment of the first protocols in the 1990's, enabling isolation of NPCs from rodent brains and their culture under laboratory conditions [7].

Meanwhile, primary NPCs have been successfully isolated not only from rodents but also from human foetal brains [8, 9]. As extracted primary cells resemble the phenotypic profile of original tissue the closest, they remained to the present a prominent source to obtain NPCs for research purposes. However, primary cells have only a limited lifespan in culture. The common reason is the genetical predetermination of somatic cells to undergo growth arrest or cell death after a finite number of cell divisions. This finite number is explained by the Hayflick limit stating that upon each cell division the telomeres, which protect chromosome stability, shorten, and mitosis stops completely when telomeres reach a critical length [10]. Despite the multipotent nature of NPCs, which suggests continuous proliferation capacity *in vivo*, *in vitro* isolated primary NPCs also undergo cell death and show phenotypical or genotypical modifications after several subcultures. Next to telomere shortening, this can be due to the artificial culture environment which leads to cell stress and subsequently to an accumulation of abnormalities and senescence-associated changes. Additionally, isolated NPCs are of polyclonal origin and thus exhibit various degrees of potency and commitment. In culture, this results in a variation of properties and population heterogeneity. The limited stability of primary NPCs in culture generates a recurrent need for newly isolated samples from rodents or (human) foetal tissue.

To circumvent this problem and maintain cells indefinitely in culture it is possible to genetically modify them and thus generate an immortal cell line. Immortalization is essentially a partial deregulation of the cell cycle resulting in continuous proliferation of cells. This can be achieved through a variety of chemical or viral methods. A widely used technique is inserting v-myc into the cellular genome using a replication-defective, infective retroviral vector. Myc is a family of transcription factors that control many cellular processes. It regulates the cell cycle by stimulating proliferation and stemness, and repressing differentiation [11]. Villa *et al.* (2000) compared several immortalizing genes in their capability to produce a stable human NPC line (hNS1 and hNS2, formerly HNSC.100) and found that v-myc was the most effective option [12]. The authors suggest that v-myc preserves the telomeres due to expression of high telomerase activity, avoiding accumulation of chromosomal abnormalities and aging-associated modifications upon cell division. Next to hNS1, more neural progenitor lines have been established using v-myc including C17.2, HC2S2, and MAH cells [13–15]. The stable, clonal

NPC lines have been successfully employed for neural transplantation studies [16–18].

The genetic modifications that cells undergo to become immortal can result in loss of their natural characteristics, and thus not resemble their *in vivo* tissue of origin sufficiently. The closest approach to the tissue *in vivo* is using pluripotent stem cells. Pluripotent stem cells have a higher potential than multipotent stem cells, as they can differentiate into any cell type. However, these cells are usually more laborious to culture and their differentiation is a complex process which can be challenging to implement in the laboratory for the first time. Additionally, differentiation to neurons and glia cells can take up to several weeks, thus lengthening the timeframe of every experiment. For this reason immortalized cell lines are used for convenience as they are generally easy to handle and widely recognized and have been employed in this study.

The focus of this study was to set up experiments and gain initial data into basic NPC migration and behaviour. For this, the immortalized murine cell line C17.2 was selected. This is a multipotent cell line which can differentiate into a mixed culture of neurons and glia cell within 7 days [19]. In their progenitor state, the cells grow adherent to substrate allowing to seed them homogeneously distributed onto various surfaces. Culture requirements are relatively simple and include the widely used Dulbecco's Modified Eagle's Media (DMEM) mixtures, fetal calf serum (FCS), and two neurotrophic factors (NGF and BDNF) [19]. This multipotent cell line was isolated from the cerebellum of a neonatal mouse and immortalized using a v-myc transfection [15]. C17.2 neural progenitors are used as a model system and have been employed in numerous studies including CNS toxicity [20, 21], differentiation research [22], and neural regeneration [23, 24]. In the latter, these cells integrated into the diseased or injured CNS of animals and underwent neural differentiation. Although, as mentioned above, immortalized cell lines can diverge from their *in vivo* counterparts, they have many advantages, are widely published, and thus act as a practical model system.

In this chapter, the C17.2 cell line is characterized in culture and a dual-transduced monoclonal cell line is established exhibiting a nuclear and an actin label.

4.2. RESULTS AND DISCUSSION

4.2.1. CHARACTERIZATION OF C17.2 CELLS

C17.2 NPCs were purchased from Sigma (♯07062902) and cultured as advised by the provider in serum-containing media (details see Materials). The cells were maintained in culture for up to 25 passages. Under these conditions the progenitor state of the cells remained stable: no spontaneous neural differentiation or other changes were observed. In their undifferentiated state, C17.2 cells showed a homogeneous, elongated morphology with multiple processes (Figure 4.1 a).

In contrast to the by Sigma advised surface coating with Poly-L-Lysine (PLL), the cells were grown on laminin-coated dishes. Initially, cells were cultured on PLL - coated dishes but showed stress signs and exhibited higher death rates. Laminin was considered as an alternative coating as this extracellular matrix-protein is widely employed for neural cultures. A comparison by growing the cells on laminin and PLL for 20 passages showed a healthier cell appearance on laminin-coated surfaces with lower death num-

bers and no signs of any morphological changes (data not shown). Thus, laminin was selected for the daily culture protocol. To confirm the progeny state of the cells and to verify its preservation on laminin, an immunofluorescence targeting the neuroepithelial stem cell protein (nestin) was performed. Nestin is a cytoskeletal intermediate filament which is found during early development of the CNS and is characteristic for neural progenitor cells. It is related to essential functions like proliferation, differentiation, and migration [25]. As expected, cultured C17.2 cells showed a high expression of nestin [19] on both surface coatings (Figure 4.1 c, e).

C17.2 are a particularly interesting cell line due to their multipotent capacity and were shown to produce a mixture of neurons and astrocytes [19]. To confirm their ability for neural maturation, cells were cultured in differentiation media following the protocol established by Lundqvist *et al.* (2013). After 7 days, C17.2 produced as expected a mixed neural population (Figure 4.1 b). Morphological analysis showed typical neuronal phenotypes with small, spherical bodies and variable branches of neurites building a complex network (Figure 4.1 b, inset). Additionally, among the neurons, more flattened cells were found resembling astrocytes (Figure 4.1 c; inset, white arrow) [19]. An immunofluorescence analysis confirmed protein expression of the neuronal marker βIII-tubulin (Figure 4.1 d) [26], as well as the presence of the glial fibrillary acidic protein (GFAP) (Figure 4.1 f) [27].

In summary, cultured C17.2 cells were confirmed to be in their immature state and differentiated as expected into a neural population.

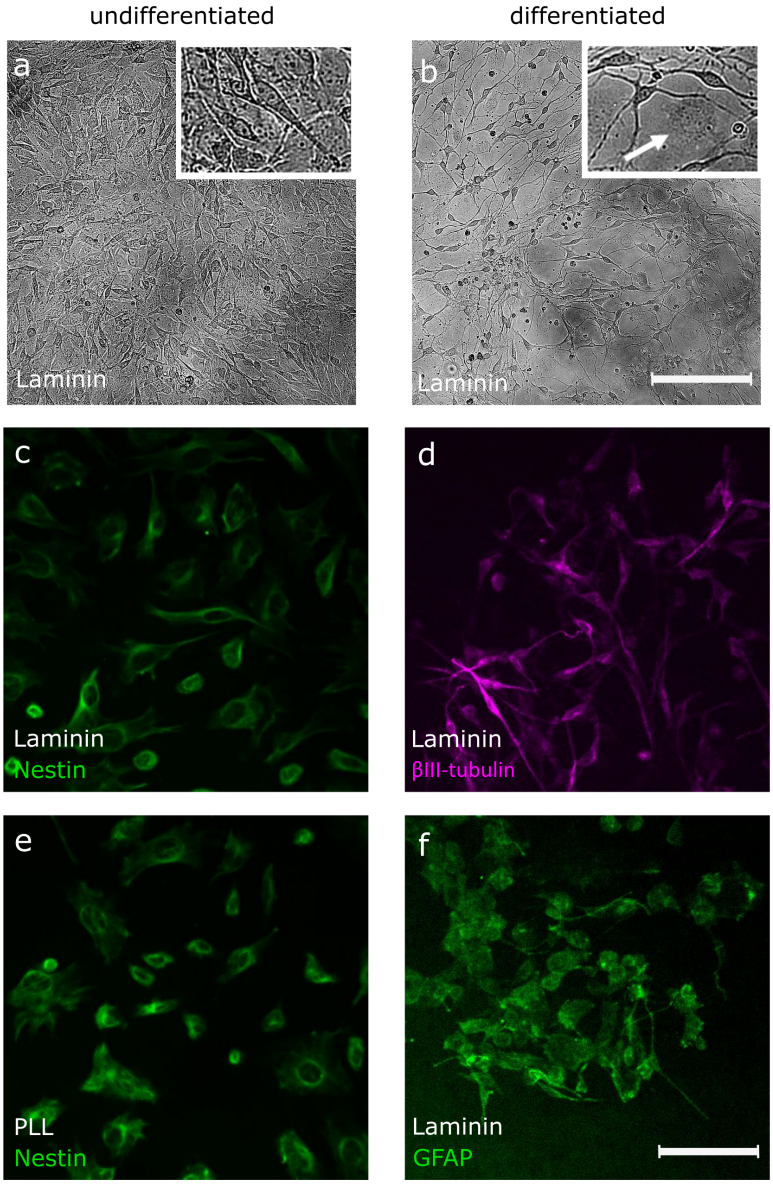

Figure 4.1: C17.2 NSCs in their immature and differentiated state. C17.2 NSCs in their immature and differentiated state. Immature C17.2 cells show an elongated cell body with processes (a) and express the neural progenitor marker nestin when cultured on laminin (c) and PLL-covered (e) surfaces. Differentiated C17.2 cells yield a neural network (b) with neurons showing small cell bodies and expressing the neuronal marker βIII-tubulin (d). Additionally, astrocytes showing a flat morphology (inset, white arrow) express the glial marker GFAP (e). The scale bar is 200 μm for (a-b) and 100 μm for (c-f).

4.2.2. FLUORESCENCE LABELS EXAMINATION

The objective of the study was to analyse NPC migration and behaviour by acquiring time-lapse imaging data using a fluorescence confocal microscope. Thus, fluorescent labels of cell nuclei and cell bodies were required. Ideally, the label should yield a stable signal over at least 24 h of data acquisition, a sufficiently high intensity for a clear signal at a 20x magnification (lens used here for tracking analysis), not interfere with the cellular functions and preferably absorbing at longer wavelengths. Exposing living cells to laser pulses of high intensity light can have a phototoxic effect, and particularly near UV range light can induce DNA damage. Several commercially available products have been assessed as possible fluorescent markers and are listed below. Hoechst 33342, FM4-64, Fluorescein diacetate (FDA), NeuroFluor™ CDr3, NucSpot® Live 650, were available in stock and thus tested for their usability. Whereas BioTracker, CellTracker, and SiR Actin labels were purchased with consideration to the above-mentioned requirements.

Hoechst 33342 (ThermoFisher) is a well-known and popular nuclear dye for living cells. Testing it at concentrations 2-5 μM produced the best results at 5 μM (Figure 4.2 a). Although the nuclei labelling was successful, the signal intensity was not very high and variable among cells. Additionally, the clear disadvantage of Hoechst is its absorption at 405 nm. As mentioned before, blue light is damaging to living cells compared to longer wavelengths, which becomes particularly problematic during frequent and long-term exposure of time-lapse imaging.

BioTracker 488 Green Nuclear Dye (Merck) was tried as an alternative nuclear label. This label is advertised as having higher photostability than Hoechst 33342 and has the advantage of absorbing in the green spectra which is moderately less toxic to living cells. Following the manufacturer's protocol, the labelling failed as the dye adsorbed onto the laminin-coated surface on which C17.2 cells were grown (Figure 4.2 b). The approach to label the cells in suspension before seeding was tested but yielded no nuclear signal (data not shown). In contrast, in parallel tested breast cancer cell line MDA-MB-231 did show nuclear staining (not shown). Lastly, NucSpot® Live 650, a nuclear dye advertised for long-term live cell imaging, was tested but yielded no fluorescence. Thus, all tested nuclear labels were discarded as an option for C17.2 tracking.

In parallel, several markers to visualize the cellular body were tested. Fluorescein diacetate (FDA) is commonly used as a viability probe as it fluoresces upon intracellular enzymatic activity. It absorbs in the green spectra and yields a cytoplasmic staining. Concentration of 50 μg/ml generated strongly labelled cells but impacted their health and lead to plasma membrane blebbing - a characteristic of injured cells - and cell death (data not shown). Lower tested concentrations did not produce a sufficiently strong signal. Additionally, upon laser exposure the dye bleached visibly within seconds.

NeuroFluor™ CDr3, a red fluorescent dye selectively labelling neural progenitor cells, was also tested. The staining was successful visualizing the cell cytoplasm (Figure 4.2 d). However, the label was not distributed homogeneously throughout the cell, thus making it difficult to distinguish the cell contours. Furthermore, time-lapse imaging resulted in fast bleaching of the dye within 2 h.

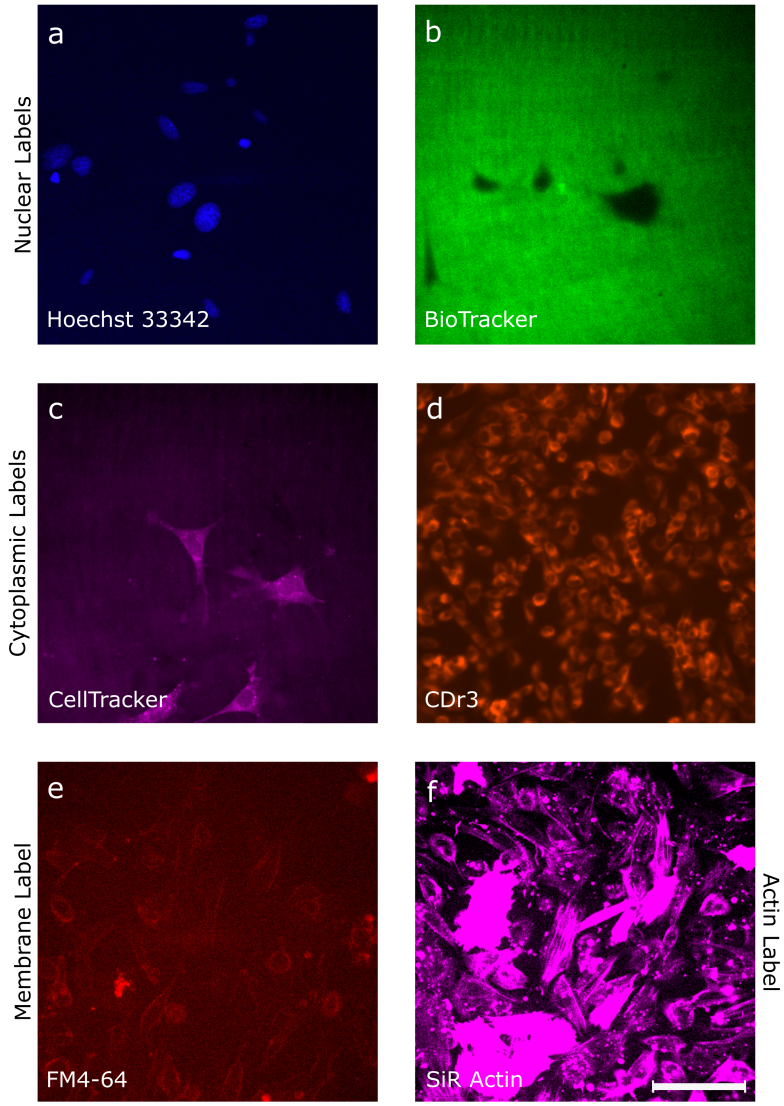

Figure 4.2: Testing of various fluorescence labels with C17.2 cells. Testing of various fluorescence labels with C17.2 cells. The nuclear label Hoechst 33342 produced a clear but partially weak signal (a), whereas BioTracker adsorbed to the laminin surface and failed to label cell nuclei (b). The cytoplasmic label CellTracker produced an insufficiently strong fluorescence with background noise (c), whereby the fluorescence of neural progenitor label CDr3 was not homogeneously distributed throughout the cell body (d). FM4-64 produced only a weak staining of the cell membrane (e). SiR Actin produced heterogeneous fluorescence intensity among cells with insufficiently clear actin label and dye clumping (f). Scale bar for (a-f) is 100 μm.

Next, CellTracker™ Deep Red Dye (ThermoFisher) is advertised as a multigenerational tracking dye with retention times above 72 h and non-toxic to cells. Along with its absorption maxima at 630 nm, it made an interesting candidate for planned experiments. However, the produced signal was very low with some accumulation of the dye around the nucleus and partial adsorption to the substrate (Figure 4.2 c). Testing different concentrations and incubation times, as well as labelling the cells in suspension before seeding did not change the outcome.

FM™ 4-64 Dye (ThermoFisher) was considered as an alternative as it is visualizing cell contours although it has a broad absorption spectrum between 300 - 600nm (maximum at 515nm) and short retention times of only several hours. It is a lipophilic dye staining cellular membrane and commonly used to study endocytic pathways. But this dye did not produce a sufficiently strong signal at 20x magnification and thus was also discarded as a label.

The optimal way to visualize the cell body for the experiments was considered to be a label of the cytoskeleton protein actin. However, availability of actin labels for living cells is still limited due to its dynamic nature that makes it highly challenging to produce a label which does not interfere with its polymerization. SiR actin (tebu-bio) is based on the fluorescent silicon rhodamine (SiR) and is advertised as showing high labelling specificity of F-actin in live cells with low background. With its absorption maxima at 652 nm it was a promising candidate. The manufacturer advises to use concentrations below 100 nM for time-lapse imaging to avoid interference of the probe with actin. Labelling produced a highly heterogeneous signal among individual cells as well as within the cell. Figure 4.2 f shows several over-exposed cells while other exhibit a weak signal. Also, the actin visualization within the cell was suboptimal with localized higher and lower signal intensity. Different concentrations and incubation times have been tested, but none yield a satisfactory result (data not shown). Thus, also this label was discarded as an alternative.

In summary, several commercially available fluorescence markers have been tested on C17.2 but none produced an effective labelling. Although fluorescent labels have revolutionized cell research, their success still faces challenges. A label needs to be cell-membrane permeant, specifically bind to the region of interest, show only fluorescence when specifically bound, not impair cell function, have a sufficient retention time within the cell, exhibit high fluorescence intensity, be photostable, and have a homogeneous distribution among and within cells. Generally, the response to exogenously introduced chemicals and proteins can vary among different cell types and even cell cultures established in different laboratories, thus complicating their application. Another reason for the failed fluorescence dye labelling could be high activity of transmembrane efflux pumps. Nestin-positive neural stem cells were found to express transmembrane ABC-transporters [28], which actively pump out toxins and other molecules from the cell interior. Additionally, C17.2 NSCs were found to be more sensitive to neurotoxins compared to other neural cell lines. This information coupled with our observations suggests that C17.2 cells can be challenging to label using fluorophore dyes and thus require other labelling methods.

4.2.3. DUAL-TRANSDUCTION WITH NUCLEAR AND ACTIN LABEL

An attractive alternative to using fluorescent dyes is to introduce genes coding for fluorescent proteins into the cellular genome. A transfection (non-viral) or transduction (viral) can also lead to impairment of cellular functions as it integrates exogeneous genes randomly into the cellular genome and thus can disrupt DNA transcription. The clear advantage of this method is the generation of cells which continuously express fluorescent molecules bound to the protein of interest, thus circumventing many complications which fluorescent dyes show. As non-viral transfection is known to be inefficient in neural progenitor cells and can be toxic [29, 30], the commercially available BacMam 2.0 transduction system was tested on C17.2 cells. BacMam uses a modified insect virus (baculovirus) which is advertised to be ready-to-use and produce a transient protein expression for up to 5 days. The systems CellLight™ Nucleus-RFP and CellLight™ Actin-GFP were tested with different protocol parameters, however no labelling signal was achieved (data not shown).

As published data showed successful transduction of C17.2 cell using a lentiviral vector [31, 32], this method was selected to eventually obtain a stable cell line with bright and homogeneous nuclei and actin labels. Lentiviral transduction demands higher safety measures and GMO-approval, as well as several weeks of time to establish a transduced cell line. Thus, this method was only selected after the possibility of using fluorescence dyes was ruled out.

For the nuclear label, the commercial product IncuCyte® NucLight™ Red Lentivirus Reagent (EF-1 Alpha, Puro) from Essen BioScience was purchased. It is advertised to produce a homogeneous expression of a nuclear-restricted red fluorescent protein, mKate2. The result was a 100% transduction efficiency showing a uniform and strong fluorescence signal of mKate2 (see signal distribution in Figure 4.3 c). For the dual label, the nuclear-labelled cells were transduced with an in-house lentivirus reagent by Dr. Sylvia de Dévédec (LACDR), expressing GFP lifeact. Lifeact is a marker visualizing the cytoskeletal element F – actin, without interfering with its dynamics [33]. The result was a high-efficiency transduction but with a heterogenous signal intensity (Figure 4.2 a, c). Furthermore, as both vectors incorporated a puromycin resistance for selection, many cells showed either the nuclear or the actin label (Figure 4.2 a).

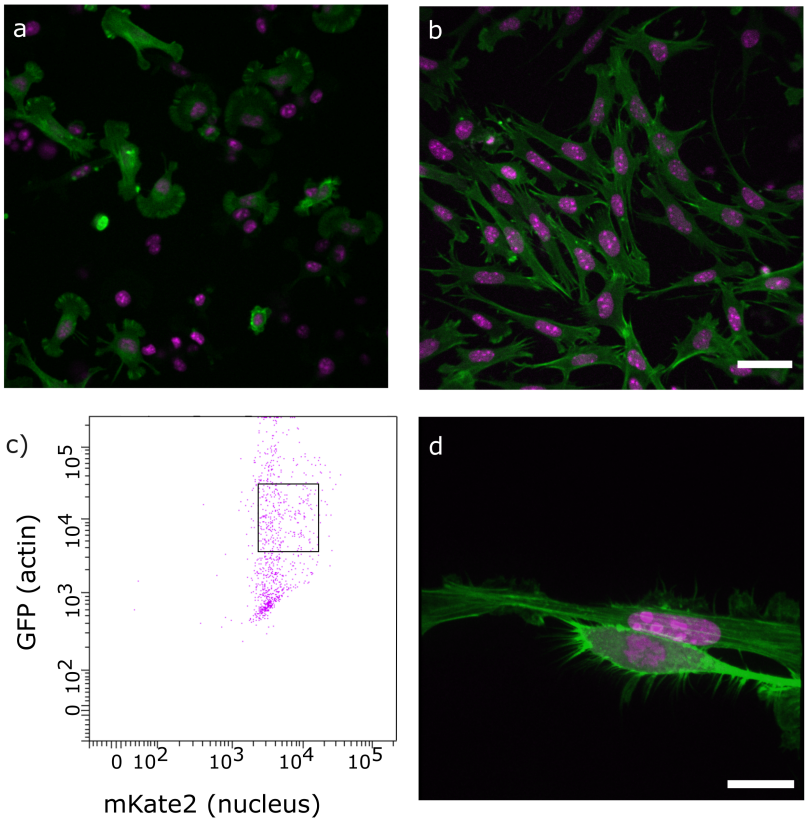

Figure 4.3: Dual-transduced nuclear- and actin-labelled C17.2 cells. Dual-transduced nuclear- and actin-labelled C17.2 cells. Dual-transduction produced a heterogeneous intensity of GFP-lifeact among cells with homogeneous fluorescence of mKate2-nucleus label, whereby several cells exhibited only one of the two labels (a). FACS data confirmed the presence of broad GFP fluorescence intensity and narrow mKate2 fluorescence intensity distribution (c); each point equals one cell. Black square shows the fluorescence parameters of the cell population selected for sorting. After FACS sorting and clone selection, 100 % of the cell population expressed double labels for actin and nuclei with homogeneous and strong fluorescence among (b) and within cells (d).

To obtain a pure culture of dual-labelled cells showing a homogeneously high fluorescence signal, Fluorescence-Activated Cell Sorting (FACS) was performed. Hereby, the fluorescence signal for a sample of the cell population is measured and cells expressing the desired signal intensity can be selected for further culture. The rest will be discarded. Figure 4.2 c shows the homogeneous signal intensity for mKate2 (nucleus) and the broadly heterogeneous signal distribution for GFAP (actin). The black square represents the parameter window for signal intensity based on which individual cells were selected for further culture. To establish a monoclonal cell line exhibiting the same location of gene insertion, FACS equipment was programmed to select single cells and direct

them into single wells of a 96-well plate with a feeder-layer. Feeders are growth-arrested cells which condition the media and thus enable an individual cell to survive and proliferate. A cell in isolation without conditioned media by its neighbours or feeder-cells would undergo growth arrest or cell death. The individual C17.2 cells were observed in culture over the following weeks. As the location and number of the inserted genes is not controlled, many monoclonal cell populations exhibited failed proliferation or changed morphology. Eventually one clone was selected based on its normal morphology and proliferation characteristics, as well as homogeneous and strong fluorescence signal of both labels (Figure 4.2 b). A close-up of two cells supports the homogeneous distribution of the nucleus and actin-bound fluorophores (Figure 4.2 d). This transduced monoclonal cell line was expanded and used for experiments shown in this thesis.

4.2.4. CONCLUSIONS

This chapter introduced the multipotent neural progenitor cell line C17.2 and confirmed its immature state as well as its capacity for neural differentiation. Additionally, a range of commercial fluorescent dyes has been tested to find a suitable nuclear and cell body label for fluorescence time-lapse imaging, but none produced a sufficient result. Lastly, viral transduction has been successfully employed to establish a monoclonal cell line with a homogeneous and strong fluorescence signal within the nucleus and actin of C17.2 neural progenitors. Although cell morphology and proliferation suggested no visible interference with cellular function, viral transduction can negatively affect neural differentiation [29] and should still be tested for.

4.3. MATERIALS AND METHODS

4.3.1. CELL CULTURE

C17.2 cells (Merck, 07062902) were maintained in plastic p60 (9cm^2)) plastic culture dishes in 5 mL Dulbecco's Modified Eagle Medium with phenol red (DMEM; Sigma), supplemented with 2 mM Glutamine (Gibco), 10% fetal calf serum (FCS; Biowest), 100 U/mL penicillin and streptomycin (P/S; Duchefa) under standard culturing conditions (humidified, 37 °C atmosphere containing 5.0% CO2). Culture dishes were pre-coated with 1-2 μg/mL laminin (Merck, L2020) in DMEM for at least 1 h in the incubator at 37 °C. For poly-l-lysine (PLL) coating, dish was covered with 10 μg/ml PLL (Sigma, P6282) in sterile distilled water for 5 min at room temperature. Then the solution was removed, and the dish dried with an open lid in the sterile hood for at least 1 h. Cells were split every 3-4 days when reached confluency of 80 - 90% and seeded 1 - 2 x 105 cells per p60 dish. Cells were cultured for up to 25 passages.

4.3.2. CELL DIFFERENTIATION

For neural differentiation, cells were seeded on laminin-coated dishes with 5 x 103 cells per cm2 in complete growth medium. On the next day, cells were washed twice with Phosphate Buffered Saline (PBS; Merck) and differentiation media added. Differentiation media consisted of DMEM : F12 (ThermoFisher) with N2 supplement (Invitrogen,

17502-048), 10 ng/ml brain-derived neurotrophic factor (BDNF; R&D systems, 248-BDB) and 10 ng/ml nerve growth factor (NGF; Merck, N6009). Media was refreshed every second day. Protocol is based on publication by Lundqvist *et al.* (2013) [19].

4.3.3. IMMUNOFLUORESCENCE (IF)

Cells were cultured on laminin-coated 8-chambered microscopy slides (ibidi, 80821). For this, uncoated (hydrophobic) μ-Slide 8 Well (ibidi, 80821) were exposed to UV for 30 min and incubated with laminin as described for regular cell culture above. For IF, cells were washed 2x with PBS and fixed for 10 min using 4% paraformaldehyde (PFA; Gibco). After 2x washing steps with PBS, cells were permeabilized with 0.1% TritonX for 5 min. Washed twice with PBS and incubated for 1 h in blocking buffer (1% bovine serum albumin (BSA; Gibco) and 0.3% Triton X (xxx) in PBS). Primary antibodies were diluted in blocking buffer as followed: Nestin (BioLegend, 839801) 1:1000, βIII-tubulin (BioLegend, MMS-435P) 1:1000, GFAP (Merck, HPA056030) 1:500. Cells were incubated with primary antibody solution overnight at 4 °C and washed 3x with PBS. Secondary antibodies were diluted in blocking buffer as followed: Alexa 488 goat anti-rabbit (ThermoFisher, ♯A-11034) 1:500, Alexa 568 anti-mouse (Abcam, ab175701) 1:500. Cells were incubated with secondary antibodies for 1 h at room temperature in the dark. After three washing steps with PBS, the cells were ready to be imaged.

4.3.4. FLUORESCENCE DYE LABELLING

For all labelling tests, cells were grown on laminin-coated 35 mm μ-Dishes (ibidi, 81151). For this, uncoated μ-Dishes were exposed to UV for 30 min and incubated with laminin as described for regular cell culture above. All protocols were repeated at least twice. All incubation steps were performed at 37°C in the dark.

Hoechst 33342 (ThermoFisher, 62249)
Cells were washed twice with PBS and incubated with 5 μM Hoechst 33342 in complete media for 30 min in the incubator. Then, cells were washed again twice with PBS and covered with complete media.

BioTracker™ Nuclear Dye (Merck, SCT120)
Cells media was removed, cells covered with BioTracker solution (diluted 1:1000 and 1:500 in culture medium) and incubated for 10 - 30 min at 37°C and imaged. Alternatively, trypsinized cells were incubated in a falcon tube with the BioTracker solution for 20 min, centrifuged, resuspended in fresh media, and seeded onto a laminin-coated dish.

NucSpot® Live 650 (Biotium, 40082)
Cell media was removed, cells covered with NucSpot solution (diluted 1:1000 and 1:500 in culture medium) and incubated for 10 - 30 min at 37°C and imaged. Alternatively, trypsinized cells were incubated in a falcon tube with the NucSpot solution for 20 min, centrifuged, resuspended in fresh media, and seeded onto a laminin-coated dish.

CellTracker™ Deep Red Dye (ThermoFisher, C34565)
Cells were washed with PBS and incubated with CellTracker solution (250 nM, 1 μM, 5μM, and 25 μM in serum-free culture medium) for 15-45 min. Then staining solution was removed and cells covered in complete culture medium. Alternatively, trypsinized cells were incubated in a falcon tube with the CellTracker solution for 30 min, centrifuged, resuspended in fresh media, and seeded onto a laminin-coated dish.

Fluorescein Diacetate (FDA; Merck, F7378)
Cells were washed with PBS and incubated with 20 μg/ml and 50 μg/ml FDA solution. Cells were ready to be imaged almost immediately.

FM4-64 (ThermoFisher, T13320)
FM™ 4-64 Dye (N-(3-Triethylammoniumpropyl)-4-(6-(4-(Diethylamino) Phenyl) Hexatrienyl) Pyridinium Dibromide). Cells were washed with PBS and incubated for 10 min in FM4-64 staining solution (2 μg/ml and 5 μg/ml in cell medium).

SiR Actin (Tebu-bio, SC006)
Cells were covered with SiR staining solution of 100 nM and 1 μM diluted in cell medium. Also, the addition of 10 μM verapamil (efflux pump inhibitor) was tested. After incubation of 1 h of 1 μM, and from 6 h - 12 h for 100 nM (as advised by manufacturer) cells were imaged. For better signal-to-noise ratio staining solution has been replaced with fresh cell medium.

CellLight^{TM} Nucleus-RFP and Actin-GFP, BacMam 2.0 (ThermoFisher, C10603 and C10506)
Cells were used at 30 - 50 % confluency. BacMam solution was mixed with media to 30, 40, and 50 particles per cell (PPC) and incubated with cells for 16 h.

NeuroFluor™ CDr3 (Stemcell, ♯01800)
Cells were incubated for 1.5 h with 1 - 2 μM CDr3 solution in cell medium. Staining solution was removed, cells washed twice with PBS, and fresh medium added.

4.3.5. LENTIVIRAL DUAL-TRANSDUCTION AND FACS SORTING

Cells were seeded 24 h before and reached about 30% confluency. For the initial transduction with IncuCyte® NucLight Red Lentivirus Reagent (Sartorius, 4476), six different concentrations and combinations of the lentivirus reagent (3 - 6 MOI) with and without Polybrene (4 – 8 μg/mL) were applied and incubated overnight. Then cell media was refreshed and incubated for another 24 h. For stable cell line generation, a kill curve was performed on C17.2 cells, and the optimal puromycin concentration of 100 μg/ml determined. 48 h after transduction, cells were maintained in cell medium with 100 μg/ml puromycin. Medium was refreshed every 2 -3 days until only living cells were present, which were further cultured and expanded.

For dual-transduction, NucLight Red-transduced C17.2 cells were seeded 24 h before and reached 30% confluency for transduction. Cells were transduced with GFP-lifeact by Dr. Sylvia de Dévédec (LACDR, Leiden University) at different concentrations, and selected by culturing in cell medium with 100 μg/ml puromycin.

Fluorescence activated cell sorting (FACS) was performed at the LUMC Flow cytometry Core Facility (FCF). Cell population was sorted using GFP and mKate2 fluorescence channels. Single cells were seeded into a 96-well plate containing a layer of feeder-cells. Feeder cells consisted of mouse fibroblasts (MEFs) which were treated with mitomycin to inactivate proliferation and were seeded the day before at low concentration. Sorted C17.2 cells were growth in full medium and split when reached a confluency of 80 - 90%. Cells exhibiting normal growth rate and typical C17.2 morphology, as well as stable expression of GFP (actin label) and mKate2 (nucleus label) were expanded. Other cells were discarded.

4.3.6. FLUORESCENCE MICROSCOPY

Images of live and fixed cells were acquired on a Nikon Ti Eclipse inverted microscope (Nikon Corporation, Japan) equipped with a Yokogawa 10,000 rpm spinning disc unit (Andor Technology Ltd., United Kingdom) and a stage-top miniature incubation chamber (Tokai Hit, Japan; INUG2E-TIZ) with a TIZD35 sample holder mounted on a Nikon Ti-S-ER motorized stage. The cells were imaged using a 20x (Nikon Plan Apo WD 1.0, numerical aperture (NA) 0.75), or a 60x (Nikon Oil Plan Apo λ, NA 1.4). An Agilent MLC400B monolithic laser combiner (AgilentTechnologies, Netherlands) was used for excitation at 405 nm, 488 nm, 561 nm, and 640 nm in combination with a Semrock custom-made quad-band dichroic mirror for excitation wavelengths 400 – 410, 486 – 491, 460 – 570, and 633 – 647 nm. The emission was filtered using a Semrock quad-band fluorescence filter (TR-F440-521-607-700), which has specific transmission bands at 440 ± 40 nm, 521 ± 21 nm, and 607 ± 34 nm, or otherwise a Semrock TR-F447-060 for λexc = 405 nm or a Semrock TR-F607-036 for λexc = 561 nm. All images were captured by an Andor iXon Ultra 897 High-speed EM-CCD camera. Image acquisition was automated using NisElements software (LIM, Czech Republic).

4.4. REFERENCES

[1] Julia P Andreotti et al. “Neural stem cell niche heterogeneity”. In: *Seminars in cell & developmental biology*. Vol. 95. Elsevier. 2019, pp. 42–53.

[2] Kirsten Obernier and Arturo Alvarez-Buylla. “Neural stem cells: origin, heterogeneity and regulation in the adult mammalian brain”. In: *Development* 146.4 (2019).

[3] Paul Lu et al. “Neural stem cells constitutively secrete neurotrophic factors and promote extensive host axonal growth after spinal cord injury”. In: *Experimental neurology* 181.2 (2003), pp. 115–129.

[4] Linda Ottoboni, Beatrice von Wunster, and Gianvito Martino. “Therapeutic plasticity of neural stem cells”. In: *Frontiers in neurology* 11 (2020), p. 148.

[5] Yasushi Takagi. “History of neural stem cell research and its clinical application”. In: *Neurologia medico-chirurgica* 56.3 (2016), pp. 110–124.

[6] Joshua J Breunig, Tarik F Haydar, and Pasko Rakic. “Neural stem cells: historical perspective and future prospects”. In: *Neuron* 70.4 (2011), pp. 614–625.

[7] Brent A Reynolds and Samuel Weiss. “Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system”. In: *science* 255.5052 (1992), pp. 1707–1710.

[8] Armine Darbinyan et al. “Isolation and propagation of primary human and rodent embryonic neural progenitor cells and cortical neurons”. In: *Neuronal Cell Culture*. Springer, 2013, pp. 45–54.

[9] Weixiang Guo et al. “Isolation of multipotent neural stem or progenitor cells from both the dentate gyrus and subventricular zone of a single adult mouse”. In: *Nature protocols* 7.11 (2012), pp. 2005–2012.

[10] Leonard Hayflick and Paul S Moorhead. “The serial cultivation of human diploid cell strains”. In: *Experimental cell research* 25.3 (1961), pp. 585–621.

[11] Gabriel Bretones, M Dolores Delgado, and Javier León. “Myc and cell cycle control”. In: *Biochimica et Biophysica Acta (BBA)-Gene Regulatory Mechanisms* 1849.5 (2015), pp. 506–516.

[12] Ana Villa et al. “Establishment and properties of a growth factor-dependent, perpetual neural stem cell line from the human CNS”. In: *Experimental neurology* 161.1 (2000), pp. 67–84.

[13] Susan J Birren and David J Anderson. “A v-myc-immortalized sympathoadrenal progenitor cell line in which neuronal differentiation is initiated by FGF but not NGF”. In: *Neuron* 4.2 (1990), pp. 189–201.

[14] Minoru Hoshimaru et al. “Differentiation of the immortalized adult neuronal progenitor cell line HC2S2 into neurons by regulatable suppression of the v-myc oncogene”. In: *Proceedings of the National Academy of Sciences* 93.4 (1996), pp. 1518–1523.

[15] Elizabeth F Ryder, Evan Y Snyder, and Constance L Cepko. "Establishment and characterization of multipotent neural cell lines using retrovirus vector-mediated oncogene transfer". In: *Journal of neurobiology* 21.2 (1990), pp. 356–375.

[16] Alberto Martínez-Serrano and Anders Björklund. "Immortalized neural progenitor cells for CNS gene transfer and repair". In: *Trends in neurosciences* 20.11 (1997), pp. 530–538.

[17] Evan Y Snyder et al. "Multipotent neural cell lines can engraft and participate in development of mouse cerebellum". In: *Cell* 68.1 (1992), pp. 33–51.

[18] Booma D Yandava, Lori L Billinghurst, and Evan Y Snyder. ""Global" cell replacement is feasible via neural stem cell transplantation: evidence from the dysmyelinated shiverer mouse brain". In: *Proceedings of the National Academy of Sciences* 96.12 (1999), pp. 7029–7034.

[19] Jessica Lundqvist et al. "Optimisation of culture conditions for differentiation of C17. 2 neural stem cells to be used for in vitro toxicity tests". In: *Toxicology in Vitro* 27.5 (2013), pp. 1565–1569.

[20] Kristina Attoff et al. "Whole genome microarray analysis of neural progenitor C17. 2 cells during differentiation and validation of 30 neural mRNA biomarkers for estimation of developmental neurotoxicity". In: *PloS one* 12.12 (2017), e0190066.

[21] Jessica Lundqvist et al. "Altered mRNA expression and cell membrane potential in the differentiated C17. 2 cell model as indicators of acute neurotoxicity". In: *Applied In Vitro Toxicology* 3.2 (2017), pp. 154–162.

[22] Bu Wang, Sabrina Jedlicka, and Xuanhong Cheng. "Maintenance and neuronal cell differentiation of neural stem cells C17. 2 correlated to medium availability sets design criteria in microfluidic systems". In: *PLoS One* 9.10 (2014), e109815.

[23] Wei-Guo Liu et al. "Dopaminergic neuroprotection by neurturin-expressing c17. 2 neural stem cells in a rat model of Parkinson's disease". In: *Parkinsonism & related disorders* 13.2 (2007), pp. 77–88.

[24] Yajie Liang et al. "Neural progenitor cell survival in mouse brain can be improved by co-transplantation of helper cells expressing bFGF under doxycycline control". In: *Experimental neurology* 247 (2013), pp. 73–79.

[25] Aurora Bernal and Lorena Arranz. "Nestin-expressing progenitor cells: function, identity and therapeutic implications". In: *Cellular and Molecular Life Sciences* 75.12 (2018), pp. 2177–2195.

[26] AJI Roskams, X Cai, and GV Ronnett. "Expression of neuron-specific beta-III tubulin during olfactory neurogenesis in the embryonic and adult rat". In: *Neuroscience* 83.1 (1998), pp. 191–200.

[27] L_F Eng et al. "An acidic protein isolated from fibrous astrocytes". In: *Brain research* 28.2 (1971), pp. 351–354.

[28] Tingting Lin, Omedul Islam, and Klaus Heese. "ABC transporters, neural stem cells and neurogenesis–a different perspective". In: *Cell research* 16.11 (2006), pp. 857–871.

[29] RB Tinsley, J Faijerson, and PS Eriksson. “Efficient non-viral transfection of adult neural stem/progenitor cells, without affecting viability, proliferation or differentiation”. In: *The Journal of Gene Medicine: A cross-disciplinary journal for research on the science of gene transfer and its clinical applications* 8.1 (2006), pp. 72–81.

[30] You-Chan Kim et al. “Co-transfection with cDNA encoding the Bcl family of anti-apoptotic proteins improves the efficiency of transfection in primary fetal neural stem cells”. In: *Journal of neuroscience methods* 117.2 (2002), pp. 153–158.

[31] Anna Falk et al. “Gene delivery to adult neural stem cells”. In: *Experimental cell research* 279.1 (2002), pp. 34–39.

[32] Thor Ostenfeld et al. “Neurospheres modified to produce glial cell line-derived neurotrophic factor increase the survival of transplanted dopamine neurons”. In: *Journal of neuroscience research* 69.6 (2002), pp. 955–965.

[33] Julia Riedl et al. “Lifeact: a versatile marker to visualize F-actin”. In: *Nature methods* 5.7 (2008), pp. 605–607.