# Original Article

# Tri-Lineage Differentiation of NTERA2 Clone D1 Cells towards Neural, Hepatic and Osteogenic Lineages *in Vitro*

(NTERA2 clone D1 cells / neurogenesis / hepatogenesis / osteogenesis/ tri-lineage differentiation)

B. SZEKY[1,2], B. MAYER[2], M. GYONGY[1], A. HAJDARA[1,2], S. BARSI[3], S. KARPATI[2], K. NEMETH[2]

[1]Roska Tamás Doctoral School of Science and Technology, Faculty of Information Technology and Bionics, Pázmány Péter Catholic University, Budapest, Hungary
[2]Stem Cell Research Laboratory, Department of Dermatology, Venereology and Dermatooncology, Faculty of Medicine, Semmelweis University, Budapest, Hungary
[3]Department of Physiology, Faculty of Medicine, Semmelweis University, Budapest, Hungary

**Abstract. Over the past decades, the *in vitro* use of pluripotent cell lines gained a crucial role in toxicology, preclinical drug testing and developmental biology. NTERA2 clone D1 cells were identified as pluripotent cells with high potential for neural differentiation. Although they are commonly used cellular sources in neuropharmacology and neurodevelopmental studies, their endodermal and mesodermal differentiation potential awaits further characterization. Here, we devised improved protocols for hepatogenic and osteogenic differentiation of NTERA2 clone D1 cells. Our *in vitro* differentiation assays showed significant up-regulation of multiple hepatogenic markers. We also observed robust mineralization and osteogenic marker expression of NTERA2 clone D1 cells upon *in vitro* osteogenic induction. These results suggest that NTERA2 clone D1 cells may be utilized as an *in vitro* model system to study various aspects of liver biology and osteogenesis. In addition, tri-lineage differentiation of NTERA2 clone D1 cells may serve as a simple experimental control system when validating pluripotency of other cell types.**

Received June 24, 2021. Accepted November 24, 2021.

This work was supported by the Hungarian National Research, Development and Innovation Office (Grant NN 114460 to SK). The research has been supported by the ÚNKP-19-3 New National Excellence Programme of the Ministry of Human Capacities, Hungary (Grant ÚNKP-19-3-III-PPKE-30). The research project has been partially supported by the European Union, co-financed by the European Social Fund (EFOP-3.6.3-VEKOP-16-2017-00002).

Corresponding author: Balázs Széky, Roska Tamás Doctoral School of Science and Technology, Faculty of Information Technology and Bionics, Pázmány Péter Catholic University, 1083, Práter utca 50/a, Budapest, Hungary. Phone: (+36)709 428 821; e-mail: szekyb@gmail.com

Abbreviations: ARS – Alizarin Red S, bFGF/FGF2 – basic fibroblast growth factor/fibroblast growth factor 2, BDNF – brain-derived neurotrophic factor, BMP4 – bone morphogenic protein 4, EGF – epidermal growth factor, FGF4 – fibroblast growth factor 4, HDM – hepatic differentiation medium, hESc/mESc – human/murine embryonic stem cell, HGF- hepatocyte growth factor, ICC – immunocytochemistry, MUSE – multilineage-differentiating stress-enduring cell, NES – nestin, OSM – oncostatin M, PBS – phosphate-buffered saline, SSEA3 – stage-specific embryonic antigen 3.

## Introduction

Pluripotent stem cells are widely used in developmental biological research, disease modelling (Rowe and Daley, 2019; Nam et al., 2020) and chemical hazard assessment (Hurtado-Gonzalez et al., 2018; Luz and Tokar, 2018; Prince et al., 2019). Assaying pluripotency markers and tri-lineage differentiation is a common experimental endpoint to analyse the differentiation potential of a sorted cell population or a given cell line under the effect of different culture conditions, toxins or genetic perturbations. Thus, reproducible protocols and well-established model systems for studying *in vitro* differentiation are highly required.

Human or murine embryonic stem cell lines (hESc, mESc) and induced pluripotent stem cells (iPSC) are commonly used pluripotent stem cell sources due to their high capacity for self-renewal and their ability to generate ectodermal, endodermal and mesodermal lineage cells upon induction of differentiation. On the other hand, the maintenance of these cell lines is expensive and complicated because of the requirement for extracellular matrix coating, use of a feeder cell layer, growth factors, and the permanent need to remove spontaneously differentiating colonies from the rest of the culture. Complicated culturing protocols can be circumvented by the use of neoplasm-derived cell lines, which are easily expandable and inducible into more differentiated phenotypes (Kim et al., 2018).

NTERA2 clone D1 is a human embryonic teratocarcinoma-derived cell line established from a lung metastasis of a 22-year-old patient's teratocarcinoma (Pleasure and Lee, 1993). Besides expressing pluripotency markers Oct3/4, SOX2, NANOG and TRA-1-60 (Pal and Ravindran, 2006), NTERA2 clone D1 cells share characteristics of committed neural progenitor cells such as expression of MAP1b, NCAM, N-cadherin, NF-L and NF-M. Furthermore, upon exposure to retinoic acid, NTERA2 clone D1 cells differentiate into mature, βIII-tubulin positive neurons with branching neurites and synaptic marker synapsin (Ahmed et al., 2019).

Compared to iPSCs and ESC lines, NTERA2 clone D1 cells do not require extracellular matrix or feeder cell-coated surfaces for their adherence, survival and proliferation. In advance to their plastic adherence, NTERA2 clone D1 cells can be grown in growth factor-free medium supplemented with foetal bovine serum. Plastic adherence and neural progenitor characteristics make NTERA2 clone D1 cells a favourable model system for neurotoxicity studies (Taylor et al., 2019) and neural (Ahmed et al., 2019; Eskandrian et al., 2019; Heebkaew et al., 2019) – especially dopaminergic neural – development (Schwartz et al., 2005; Boroujeni et al., 2019).

The utility of pluripotent NTERA2 clone D1 cells for modelling endodermal and mesodermal lineage cell functions remains less characterized. Previously published methods achieved only partial induction of certain endodermal markers in NTERA2 clone D1 cells (Pal and Ravindran, 2006), and their ability to generate mineralized tissue also requires further experimentation. Given the emerging need for stem cell-based model systems mimicking liver and bone physiology, we set out to examine the hepatogenic and osteogenic differential potential of NTERA2 clone D1 cells using improved experimental protocols.

## Material and Methods

### *Cell culture*

60,000/cm$^2$ NTERA2 clone D1 cells (Cat. No.: 01071221, Sigma Aldrich, St. Louis, MO) were seeded into cell culture flasks and assay plates. Cells were kept in DMEM low-glucose medium containing 20% foetal bovine serum (FBS, Corning, Tewksburry, MA), 1% penicillin/streptomycin and 1% GlutaMax (Gibco, Gaithersburg, MD).

We used the A172 glioma (Cat. No.: CRL-1620) and Hep3B hepatocarcinoma (HB-8064) (both purchased from ATCC, Manassas, VA) cell lines as positive controls for RT-PCR and immunocytochemistry (Fig. 4A-B), respectively. These cell lines were cultured and propagated as suggested by ATCC.

### *In vitro neural differentiation*

For *in vitro* neural differentiation, we used a slightly modified version of the protocol by Wakao et al. (2011). Briefly, NTERA2 clone D1 cells were plated into wells of a 6-well ultra-low attachment plate in Neurobasal medium (Gibco) supplied with B-27 (Gibco), 30 ng/ml epidermal growth factor (EGF; Peprotech, London, UK), 30 ng/ml basic fibroblast growth factor (bFGF, FGF2) (Peprotech), 1% GlutaMax (Gibco) and 1% penicillin/streptomycin (Gibco). Medium was changed every third day until day 7, when spheroids were harvested and seeded into poly-lysine-coated dishes (100 µg/ml). Neural induction was initiated with α-MEM supplied with 2% FBS, 1% penicillin/streptomycin, 1% GlutaMax, 25 ng/ml bFGF and 25 ng/ml brain-derived neurotrophic factor (BDNF; Peprotech, Rocky Hill, NJ).

### *In vitro hepatic differentiation*

We differentiated hepatocytes from NTERA2 clone D1 cells utilizing a modified protocol previously published by Mallanna and Duncan (2013). NTERA2 clone D1 cells were seeded into plates coated with 5 µg/cm$^2$ bovine collagen I (Gibco) in DMEM supplied with 10% FBS, 1% penicillin/streptomycin, 1% GlutaMax, 1× insulin-transferrin-selenium solution (Gibco), 0.6 mg/ml nicotinamide, and 10 nM dexamethasone (from Sigma-Aldrich, St. Louis, MO) (referred to here as hepatic differentiation medium (HDM)).

Between the onset of differentiation and day 2, cells were kept in HDM containing 100 ng/ml activin A, 10 ng/ml BMP4 and 50 ng/ml FGF4. On day 2, medium was changed to HDM supplied with 100 ng/ ml activin A until day 5. Between day 5 and 10, hepatic differentiation was stimulated by HDM supplied with 20 ng/ml BMP4 and 50 ng/ml FGF4, and media were changed every 2 days. From day 10 to day 15, HDM containing 100 ng/ml HGF was added to the cells every other day. From day 15 to day 20, the cells were kept in HDM supplied with 20 ng/ml oncostatin M.

### *In vitro osteogenic differentiation and Alizarin Red S staining*

For osteogenic differentiation, cells were seeded in 6-well tissue culture treated plates, and treated with DMEM containing 20% FBS, 1% penicillin/streptomycin, 1% GlutaMax, 10 nM dexamethasone, 100 µM ascorbic acid, and 2 mM β-glycerophosphate for 21 days. To assess mineralization, cells were fixed in 4% paraformaldehyde for 15 min, and stained with Alizarin Red S (ARS) for 45 min. Excess dye was removed by washing the cells four times with double-distilled water. ARS bound to calcium phosphate complexes were extracted from the samples with 10% acetic acid and quantified by measuring $OD_{405}$ values. Concentration standards were prepared as double dilutions between 8 mM and 31.5 µM solutions of ARS.

### *qRT-PCR measurements*

We performed cell lysis and RNA extraction using the RNeasy mini-kit by Qiagen (Hilden, Germany). For cDNA synthesis, we reverse-transcribed 1 microgram

*Table 1: TaqMan Probes Used for qPCR Measurements*

| Assay ID | Gene | Assay design | Amplicon length (bp) |
|---|---|---|---|
| **Pluripotency** | | | |
| Hs01053049_s1 | *SOX2* | Within a single exon | 91 |
| Hs00999634_gH | *POU5F1* | Spans exons | 64 |
| Hs02387400_g1 | *NANOG* | Spans exons | 109 |
| **Osteocyte** | | | |
| Hs01029144_m1 | *ALPL* | Spans exons | 79 |
| Hs01587814_g1 | *BGLAP* | Spans exons | 138 |
| Hs01096681_m1 | *TGM2* | Spans exons | 129 |
| **Neuron** | | | |
| Hs04187831_g1 | *NES* | Spans exons | 58 |
| Hs00258900_m1 | *MAP2* | Spans exons | 98 |
| Hs00157360_m1 | *ENO2* | Spans exons | 77 |
| **Hepatocyte** | | | |
| Hs00173490_m1 | *AFP* | Spans exons | 82 |
| Hs00559840_m1 | *CK7* | Spans exons | 95 |
| Mm01601704_g1 | *CK18* | Spans exons | 62 |
| Hs00609411_m1 | *ALB* | Spans exons | 104 |
| **House-keeping** | | | |
| Hs99999905_m1 | *GAPDH* | Amplicon spans exons/ probe does not span exons | 122 |

RNA by using the MMLV reverse-transcriptase enzyme provided by Promega™. We measured qPCR in a Roche Lightcycler® 480 thermal-cycler. FAM-MGB-conjugated TaqMan probes (by Thermo Fisher Scientific, Waltham, Massachusetts, MA) used are listed in Table 1 below. We used *GAPDH* as a house-keeping control.

## *Flow cytometry*

Two hundred thousand cells were washed in phosphate-buffered saline (PBS), then resuspended in PBS containing 2% FBS (referred to as FC buffer). After centrifugation, cells were incubated with Fc receptor blocker solution (TruStain FcX Fc receptor blocker by BD Pharmingen™, San Diego, CA) at room temperature for 10 min. After blocking, cells were stained with biotin-conjugated, human anti-mouse SSEA3 antibody (eBioScience, San Diego) for 30 min. Cells were washed in FC buffer followed by incubation with PE-conjugated streptavidin for 30 min. Then, the cells were washed in FC buffer and stained with 7-aminoactinomycin D (7AAD, provided by Thermo Fisher Scientific™, Waltham, MA) to exclude dead cells from the measurements.

## *Immunocytochemistry (ICC)*

To stain differentiation markers and pluripotency markers, cells were fixed with 4% paraformaldehyde for 15 minutes, then washed with PBS. Fixed cells were incubated overnight with the antibodies listed in Table 2. For secondary staining, we used goat anti-mouse Cy3-conjugated antibody (purchased from Sigma-Aldrich), or donkey anti-rabbit Cy3-conjugated secondary antibody (Sigma-Aldrich). Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI).

*Table 2. Primary Antibodies Used for ICC*

| Manufacturer/Cat. No. | Target | Host/Clonality | Specificity | Isotype | Dilution |
|---|---|---|---|---|---|
| **Pluripotency** | | | | | |
| Santa Cruz/Sc-5279 | Oct 3/4 | Mouse, Monoclonal (C10) | Mouse, Rat, Human | IgG2b | 1 : 50 |
| Merck Millipore/AB5603 | Sox2 | Rabbit, Polyclonal | Human, Mouse | | 1 : 50 |
| Merck Millipore/AB9220 | NANOG | Rabbit, Polyclonal | Human, Mouse | | 1 : 50 |
| Abcam/Ab16288 | TRA-1-60 | Human, Mouse Monoclonal (clone TRA-1-60) | Human | IgGM | 1 : 100 |
| **Neuron** | | | | | |
| Merck Millipore/MAB5326 | Nestin | Mouse, Monoclonal (clone 10C2) | Human | IgG1 | 1 : 00 |
| Merck Millipore/AB5622 | MAP2 | Rabbit, Polyclonal | Human, Mouse, Rat | | 1 : 100 |
| **Hepatocyte** | | | | | |
| Santa Cruz/sc-271605 | Albumin | Mouse, Monoclonal (clone F-10) | Mouse, Rat, Human | IgG1 | 1 : 50 |
| R&D/MAB1368 | Alpha-fetoprotein | Mouse, Monoclonal (clone # 189502) | Human, Mouse | IgG1 | 1 : 50 |
| **Secondary Ab** | | | | | |
| Sigma Aldrich/C2181 | Anti-Mouse F(ab')2 fragment-Cy3 | Sheep, Polyclonal | Mouse | IgG | 1 : 200 |
| Sigma Aldrich/C2306 | Anti-Rabbit F(ab')2 fragment-Cy3 | Sheep, Polyclonal | Rabbit | IgG | 1 : 100 |

### *Statistical analyses*

Statistical analysis was carried out using Graphpad Prism version 7 (Graphpad Software, San Diego, CA). All data are represented with mean + SEM. RT-PCR results were analysed by two-way ANOVA with Bonferroni's post hoc test. A value of $P < 0.05$ was considered as statistically significant. ARS staining results were analysed by Student's *t*-test with $P < 0.05$ considered statistically significant.

## Results

First, we validated the presence of pluripotency markers in cultured NTERA2 clone D1 cells. As shown by immunocytochemistry and qPCR, NTERA2 clone D1 cells expressed OCT3/4, SOX2, TRA-1-60 (Fig. 1B-C). The presence of stage-specific embryonic antigen 3 (SSEA3), a surface marker of embryonic stem cells, was also confirmed in NTERA2 clone D1 cells by flow cytometry (Fig. 1A).

Next, we examined the neural differentiation potential of NTERA2 clone D1 cells. For neural differentiation, NTERA2 clone D1 cells were grown as cellular spheroids in neural induction media containing EGF and bFGF, growth factors known to promote neural stem cell proliferation (Zhao et al., 2019). After seven days of neural induction, spheroids were plated into poly-lysine-coated plates. EGF and FGF2 were removed from the culture and brain-derived neurotrophic factor, an astrocyte-derived growth hormone was added to the culture to promote nerve cell maturation. NTERA2 clone D1-derived spheroids attached to poly-lysine and acquired neural stem cell-like morphology on day 21. Furthermore, we detected up-regulation of *NES* (nestin), *ENO2* (enolase-2 or neuron-specific enolase) and *MAP2* (microtubule-associated protein 2) by qRT-PCR (Fig. 2A), and up-regulation of MAP2 and nestin by ICC (Fig. 2B and C). During the process of differentiation, down-regulation of pluripotency marker *POU5F1* was also confirmed (Fig. 2A).

After the validation of NTERA2 clone D1 cell pluripotent phenotype and well-known ectodermal (neural) differentiation potential, we wanted to investigate whether NTERA2 clone D1 cells are able to differentiate into endodermal and mesodermal lineages. Endodermal differentiation potential was studied by inducing liver-specific differentiation. To recapitulate *in vivo*

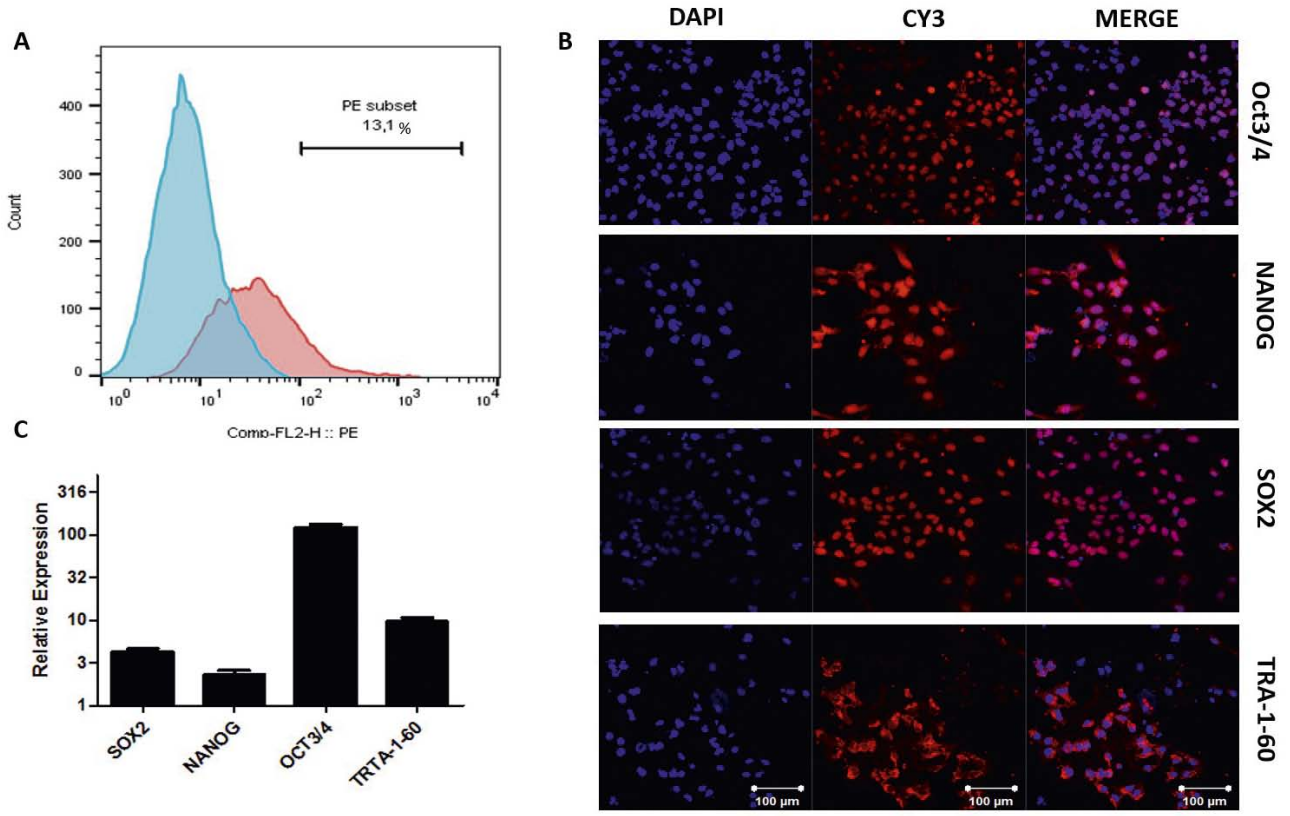



*Fig. 1.* Pluripotency markers expressed by NTERA2 clone D1 cells. (**A**) Expression of SSEA3 confirmed by flow cytometry. Blue histogram: unstained control (cells incubated with streptavidin-PE, but without biotinylated anti-mouse SSEA3 antibody), red histogram: NTERA2 clone D1 cells stained with biotin-conjugated anti-mouse SSEA3 antibody and streptavidin-PE. (**B**) Expression of OCT 3/4, NANOG, SOX2 and TRA-1-60 was detected by immunocytochemistry (scale bar: 100 µm) and (**C**) qRT-PCR (expression values relative to GAPDH expression). Values are means ± SEM from N = 4 experiments.

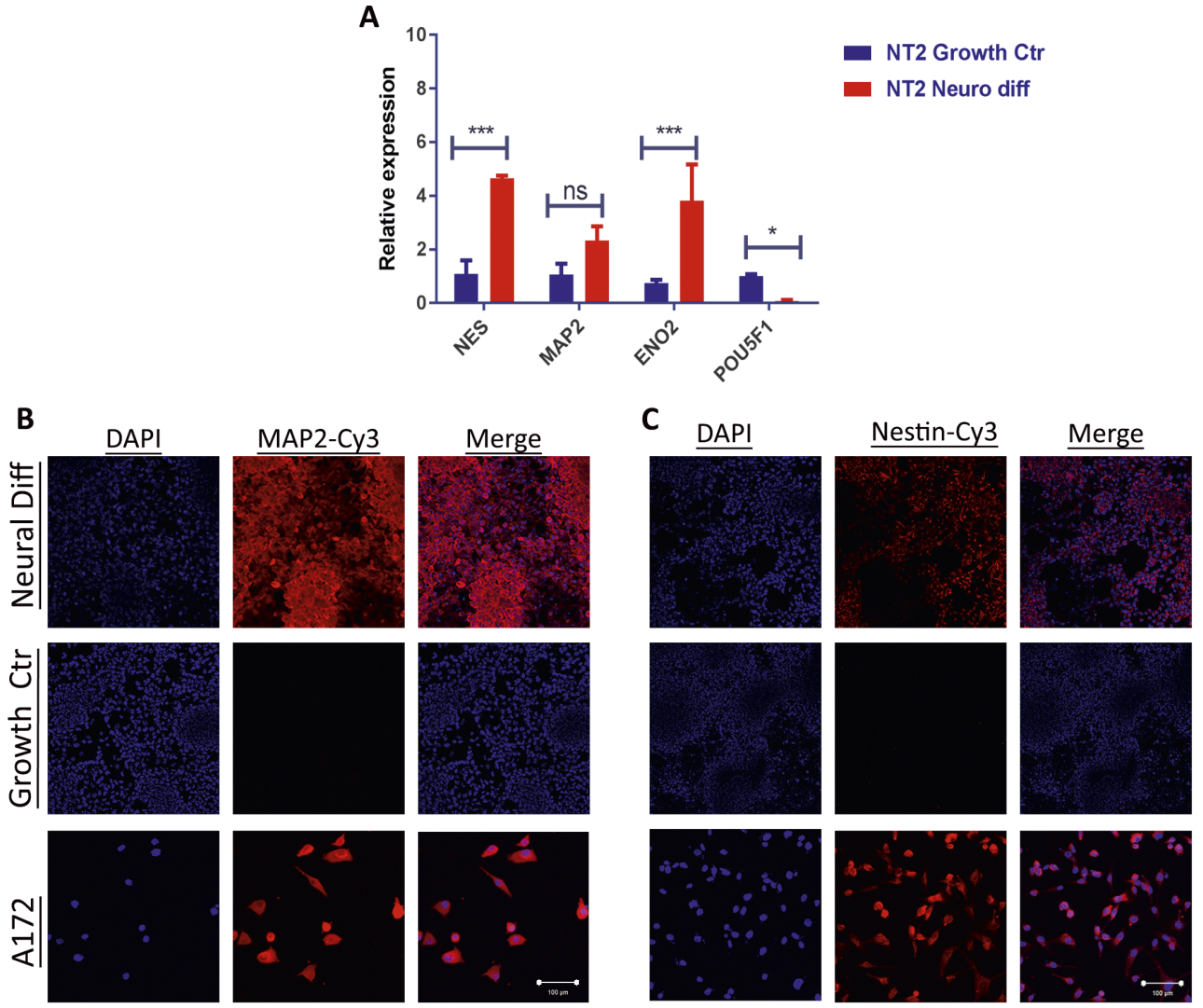



*Fig. 2*. Differentiation of NTERA2 clone D1 cells into neural-progenitor-like cells. (**A**) Neural marker (*NES, MAP2, ENO2*) and *POU5F1* expression were assessed by qRT-PCR (expression values relative to *GAPDH* expression). Values are means ± SEM from N = 4 experiments. Neural differentiation of NTERA2 clone D1 cells analysed by immunocytochemical staining of nestin. (**B**) and MAP2 (**C**) in differentiated (top panels), undifferentiated cells (middle panels) and A172 cells, which were used as a positive control (bottom panels) (scale bar: 100 μm). *** $P < 0.001$, * $P < 0.05$, ns: not significant.

hepatocyte differentiation, we plated NTERA2 clone D1 cells into collagen-coated dishes and stimulated them with activin A and BMP4 to induce endoderm specification (Fig. 3B). After five days of differentiation, we replaced activin A with FGF4 to generate hepatocyte progenitor cells. Between days 10 and 20, BMP4 was replaced by HGF and oncostatin M, growth factors promoting hepatocyte commitment and maturation (Ober et al., 2018; Myiajima et al., 2000). At the end of hepatocyte differentiation, we observed significant down-regulation of *POU5F, SOX2* and significant up-regulation of hepatic markers *AFP* (alpha-fetoprotein), *ALB* (albumin) and *CK18* (cytokeratin 18) (Fig. 4C). We also detected expression of AFP and albumin in differentiated NTERA2 clone D1 cells by ICC (Fig. 4B). Intriguingly, the expression of cholangiocyte marker cytokeratin 7 was also up-regulated, indicating a mixed culture of foetal liver- and cholangiocyte-like cells (Fig. 4C-D). We compared this method with the protocol utilized by Pal and Ravindran (2006) (Fig. 3A), who stimulated NTERA2 clone D1 cells with hepatogenic medium containing HGF, bFGF2 and oncostatin M for 20–25 days (Fig. 4D). With this method we detected only the up-regulation of *AFP* and *CK7*, slight, but not significant down-regulation of *SOX2* and significant down-regulation of *POU5F1*. In accordance with previously published results, this method induced only weak expression of hepatocyte-specific markers *(ALB)* and *CK18* (Fig. 4D). The observed differences in the results suggest that activation of TGF-β-mediated signalling

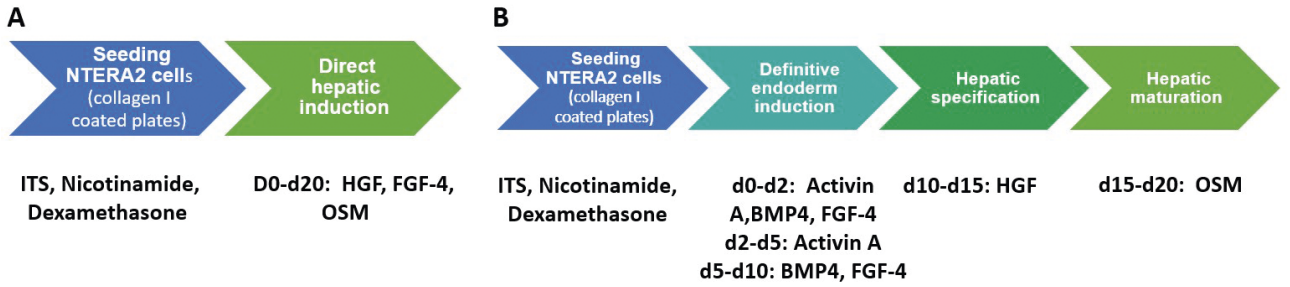



*Fig. 3*. Utilized protocols for hepatic differentiation of NTERA2 clone D1 cells. (**A**) Direct induction of hepatic differentiation by HGF, FGF4 and oncostatin M, according to the method published by Pal and Ravindran (2006). (**B**) Sequential hepatic induction with stimulation of definitive endoderm layer formation (activin A, BMP4, FGF4), hepatic specification (HGF) and hepatic maturation (OSM).

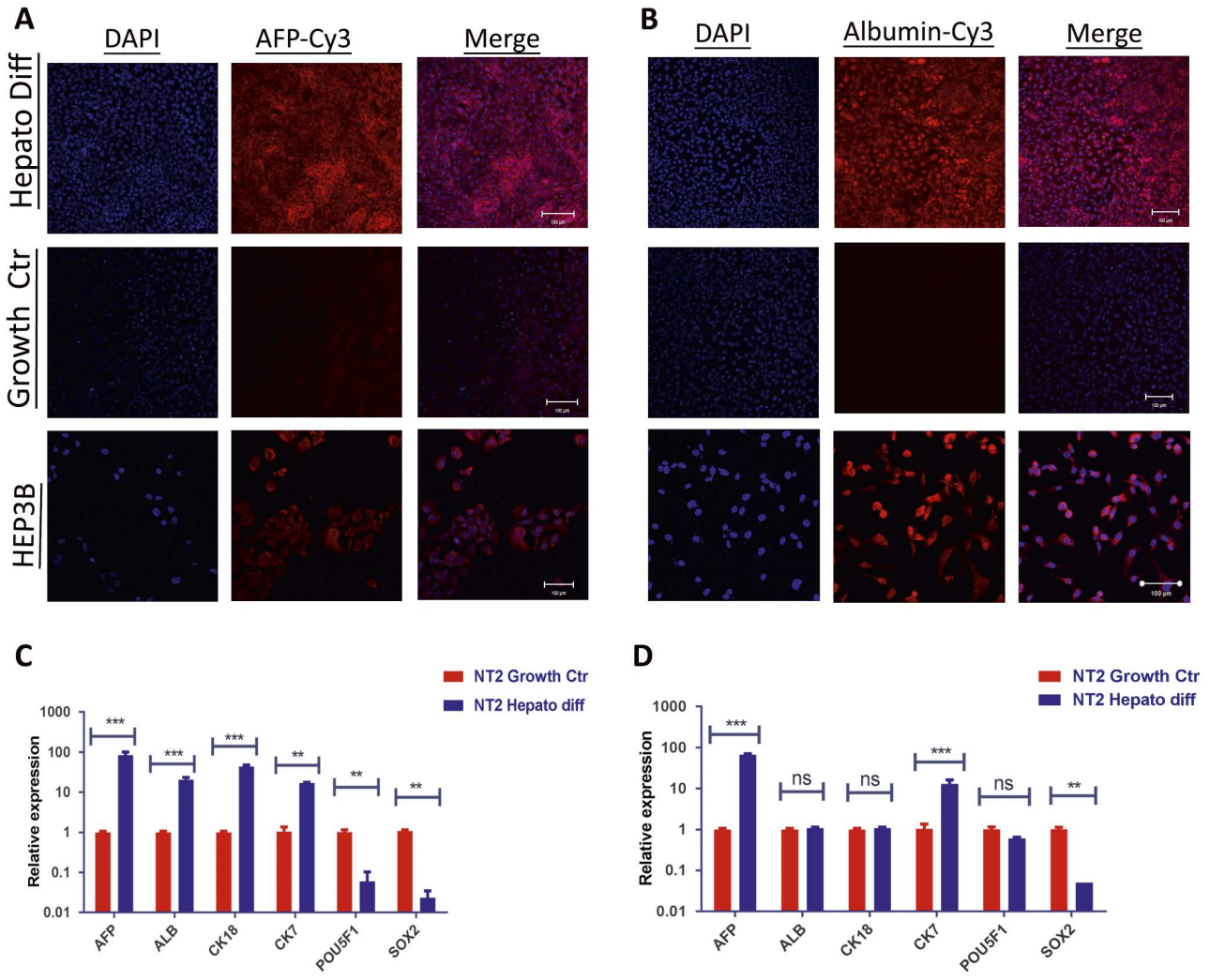



*Fig. 4.* Differentiation of NTERA2 clone D1 cells into hepatocytes. (**A**) Expression of hepatic markers α-fetoprotein (AFP) and albumin was analysed in undifferentiated NTERA2 clone D1 cells, (**B**) NTERA2 clone D1 cells with hepatocyte induction (scale bar: 100 µm). (**C**) Expression of *ALB, AFP, CK7, CK18* and pluripotency markers (*POU5F1, SOX2, NANOG*) in undifferentiated NTERA2 clone D1 cells and NTERA2 clone D1-derived hepatic cells during sequential hepatic induction or (**D**) by the protocol used by Pal and Ravindran (2006), which includes stimulation with HGF, bFGF and oncostatin M (expression values relative to *GAPDH* expression). Values are means ± SEM from N = 4 experiments. *** $P < 0.001$, ** $P < 0.01$, ns: not significant.

and concomitant endoderm specification are indispensable for hepatocyte differentiation of NTERA2 clone D1 cells.

Next, the mesodermal differentiation potential of NTREA2 cells was studied via induction of osteogenesis. Upon treatment of NTERA2 clone D1 cells with osteogenic induction medium containing ascorbic acid, beta-glycerophosphate and dexamethasone, significantly increased matrix mineralization was detected, as shown by increased Alizarin Red S staining (Fig. 5B-C). Furthermore, the expression of *ALPL* (alkaline phosphatase) and *BGLAP* (osteocalcin) were significantly up-regulated. Interestingly, the expression of transglutaminase 2 (*TG2,* Fig. 5A) was not changed despite being identified as a regulator of osteogenic maturation in murine cell line SAOS-2 (Yin et al., 2012). This suggests that osteogenic differentiation of NTERA2 clone D1 cells involves different transglutaminase-independent mechanisms, or transglutaminases other than transglutaminase 2.

## Discussion

Although the pluripotency and three germline commitment of NTERA2 clone D1 cells was analysed in previous studies (Pal and Ravindran, 2006), their capacity to generate endodermal and mesodermal lineage cells awaits further characterization. After the validation of the pluripotent phenotype and neural differentiation of NTERA2 clone D1 cells, we developed methods to analyse their hepatocyte and osteocyte commitment. While neural differentiation of NTERA2 clone D1 cells was addressed previously by several established protocols, only limited information is available concerning their hepatocyte and osteogenic differentiation potential. Hence, we addressed osteogenic and hepatogenic differentiation of NTERA2 clone D1 cells using our modified versions of protocols based on previously established methods to differentiate embryonic and adult pluripotent stem cells (Wakao et al., 2011; Mallana et al., 2013).

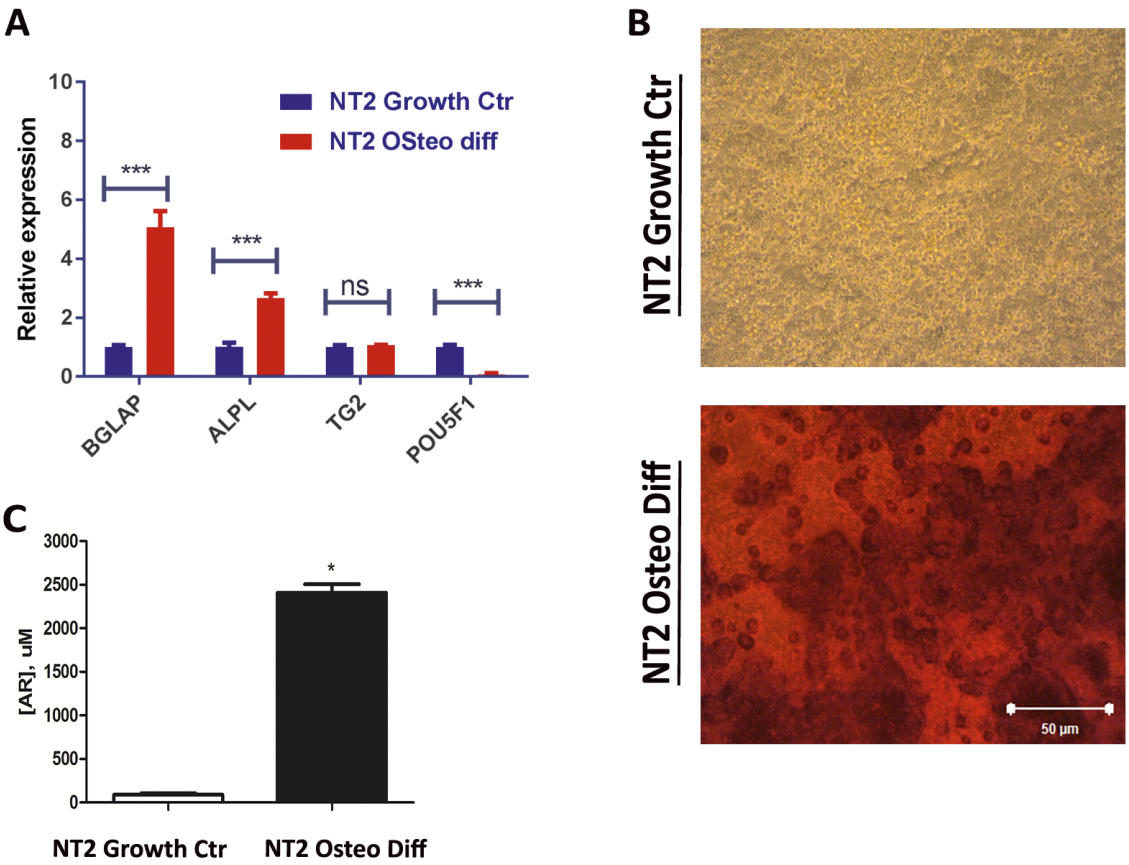



*Fig. 5.* Differentiation of NTERA2 clone D1 cells into osteocytes. (**A**) Expression of osteogenic markers *ALPL, BGLAP* and *TG2* in undifferentiated NTERA2 clone D1 cells and NTERA2 clone D1-derived osteocytes (expression values relative to *GAPDH* expression). Values are means ± SEM from N = 4 experiments. (**B**) Alizarin Red S staining of undifferentiated NTERA2 clone D1 cells (top panel) and NTERA2 clone D1-derived osteocytes (scale bar: 50 µm). (**C**) Alizarin Red S was extracted from cells and quantified by absorbance measurement. *** $P < 0.001$, * $P < 0.05$, ns: not significant

Several protocols are available for hepatocyte differentiation of mouse and human pluripotent stem cells, adult somatic stem cells or iPSC cell lines, which recapitulate the basic steps of *in vivo* hepatocyte development (Chen et al., 2012). These steps are: the endodermal commitment of stem cells, generation of hepatocyte progenitors, enrichment of immature hepatocytes, and hepatocyte maturation. Generation of a definitive endodermal layer is essential for liver, gut, and pancreatic cell development (Linneberg-Agerholm et al., 2019). Definitive endoderm formation is triggered by the activation and complex interplay of Wnt and BMP signalling pathways. Wnt and BMP signalling are both essential for endodermal lineage commitment, as inhibition of either pathway impedes liver, pancreatic, and intestinal development. Stromal cell-secreted factors, such as HGF, OSM and FGF4, are required for hepatic cell specification; acquisition of hepatocyte morphology and hepatocyte marker expression (Schmidt et al., 1995; Kang et al., 2005; Zhang et al., 2020). Besides recombinantly expressed cytokines, small molecule pathway activators or inhibitors are also frequently used for hepatocyte differentiation (Siller et al., 2015). Here, we utilized stromal cell-derived cytokines, which are well-known inducers of hepatic development (Fig. 3).

In contrast with the previously published method for NTERA2 clone D1 cell differentiation (Pal and Ravindran, 2006) (Fig. 3A), we differentiated NTERA2 clone D1 cells into hepatocytes by sequentially inducing endoderm specification, hepatocyte progenitor enrichment and commitment into foetal hepatocyte-like cells (Fig. 3B). Our approach resulted in significant up-regulation of hepatocyte markers *AFP, ALB* and *CK18* (Fig. 4 A-C), which showed only moderate up-regulation by direct hepatic induction with HGF, FGF4 and OSM (Fig. 4D). In addition, we observed up-regulation of *CK7* (cytokeratin 7) (Fig. 4C), marker preferentially expressed in cholangiocytes (Paku et al., 2005). These results highlight the importance of TGF-β-mediated signalling in hepatic differentiation and suggest a mixed hepatocyte/cholangiocyte culture obtained at the end of hepatic induction. Thus, selective enrichment of cells with pure hepatic phenotype requires more specified culture conditions or application of cell-sorting methods. Although we validated the expression of hepatocyte markers in these experiments, functional assays, such as CYP450 activity, urea and albumin secretion are required for further characterization of the NTERA2 clone D1 cell hepatic differentiation.

Combined treatment with ascorbic acid, β-glycerophosphate and dexamethasone induces collagen 1 synthesis, matrix mineralization and RUNX2 activation during *in vitro* osteogenesis, respectively. In a way similar to bone marrow mesenchymal stromal cells, NTERA2 clone D1 cell cultures underwent robust mineralization when treated with these osteogenic inducers for 21 days (Fig. 5B-C). Furthermore, osteogenic markers osteocalcin (*BGLAP*) and alkaline phosphatase (*ALPL*) were significantly up-regulated (Fig. 5A). These results propose NTERA2 clone D1 cells as a novel source of pluripotent stem cells for modelling osteogenesis, mineralization, and osteoporosis (Arjmand et al., 2020).

Last, but not least, this is the first time that tri-lineage differentiation towards all three germ layers is demonstrated in NTERA2 clone D1 cells by utilizing *in vitro* differentiation protocols applied to pluripotent stem cells. This highlights the potential of NTERA2 clone D1 cells to serve as a convenient source of positive control when studying pluripotency of primary cells such as induced pluripotent stem cells and multilineage-differentiation stress-enduring (MUSE) cells (Wakao et al, 2011).

### *Acknowledgements*

We thank Dr. Gabor Barna (1st Department of Pathology and Experimental Cancer Research, Semmelweis University) for his advice on flow cytometry, Mercedesz Mazan and Adrien Suba for their technical assistance in RNA isolation and qPCR measurements.

### *Author contributions*

S. K., K. N. contributed to conception, study design, supervised experimentation and manuscript preparation. S. K., B. M., A. H., S. B. contributed to all experimental work, data and statistical analysis, and interpretation of data. M. G., K. N., B. M. were responsible for overall supervision. B. S. designed and carried out experiments, drafted the manuscript, which was revised by S. K., K. N., M. G. and B. M. All authors read and approved the final manuscript.

## References

Ahmed, A., de Bock, C., Sontag, E., Hondermarck, H., Lincz, L., Thorne, R. (2019) FAT1 cadherin controls neuritogenesis during NTERA2 cell differentiation. *Biochem. Biophys. Res. Commun*. **514**, 625-631.

Arjmand, B., Sarvari, M., Alavi-Moghadam, S., Payab, M., Goodarzi, P., Gilany, K., Mehrdad, N., Larijani, B. (2020) Prospect of stem cell therapy and regenerative medicine in osteoporosis. *Front. Endocrinol. (Lausanne)* **11**, 430.

Chen, B., Wang, X., Wang, Z., Wang, Y., Chen, L., Luo, Z. (2012) Brain-derived neurotrophic factor stimulates proliferation and differentiation of neural stem cells, possibly by triggering the Wnt/β-catenin signaling pathway. *J. Neurosci. Res*. **91**, 30-41

Eskandarian Boroujeni, M., Aliaghaei, A., Maghsoudi, N., Gardaneh, M. (2019) Complementation of dopaminergic signaling by Pitx3–GDNF synergy induces dopamine secretion by multipotent NTERA2 cells. *J. Cell Biochem.* **121**, 200-212.

Heebkaew, N., Rujanapun, N., Kunhorm, P., Jaroonwitchawan, T., Chaicharoenaudomrung, N., Promjantuek, W., Noisa, P. (2019) Curcumin induces neural differentiation of human pluripotent embryonal carcinoma cells through the activation of autophagy. *Biomed. Res. Int.* 2019, **2019**, 1-12.

Hurtado-Gonzalez, P., Anderson, R., Macdonald, J., van den Driesche, S., Kilcoyne, K., Jørgensen, A., McKinnell, C.,

Macpherson, S., Sharpe, R., Mitchell, R. (2018) Effects of exposure to acetaminophen and ibuprofen on fetal germ cell development in both sexes in rodent and human using multiple experimental systems. *Environ. Health Perspect.* **126**, 047006.

Kang, X. Q., Zang, W. J., Bao, L. J., Li, D. L., Song, T. S., Xu, X. L., Yu, X. J. (2005) Fibroblast growth factor-4 and hepatocyte growth factor induce differentiation of human umbilical cord blood-derived mesenchymal stem cells into hepatocytes. *World J. Gastroenterol*. **11**, 7461.

Kim, H., Moon, S., Lee, W., Lee, H., Shivakumar, S., Lee, S., Park, B., Rho, G., Jeon, B. (2018) Inhibition of cell growth by cellular differentiation into adipocyte-like cells in dexamethasone sensitive cancer cell lines. *Anim. Cells Syst. (Seoul)* **22**, 178-188.

Linneberg-Agerholm, M., Wong, Y., Romero Herrera, J., Monteiro, R., Anderson, K., Brickman, J. (2019) Naïve human pluripotent stem cells respond to Wnt, Nodal and LIF signalling to produce expandable naïve extra-embryonic endoderm. *Development (Cambridge)* **146**, DOI: 10.1242/dev.180620.

Luz, A., Tokar, E. (2018) Pluripotent stem cells in developmental toxicity testing: a review of methodological advances. *Toxicol. Sci.* **165**, 31-39.

Mallanna, S., Duncan, S. (2013) Differentiation of hepatocytes from pluripotent stem cells. *Curr. Protoc. Stem Cell Biol.* **26**, 1G.4.1-1G.4.13

Miyajima, A., Kinoshita, T., Tanaka, M., Kamiya, A., Mukouyama, Y., Hara, T. (2000) Role of Oncostatin M in hematopoiesis and liver development. *Cytokine Growth Factor Rev.* **11**, 177-183.

Nam, K., Yi, S., Jang, H., Han, J., Lee, J. (2020) In vitro modeling for inherited neurological diseases using induced pluripotent stem cells: from 2D to organoid. *Arch. Pharm. Res.* **43**, 877-889.

Ober, E., Lemaigre, F. (2018) Development of the liver: Insights into organ and tissue morphogenesis. *J. Hepatol.* **68**, 1049-1062.

Paku, S., Dezső, K., Kopper, L., Nagy, P. (2005) Immunohistochemical analysis of cytokeratin 7 expression in resting and proliferating biliary structures of rat liver. *Hepatology* **42**, 863-870.

Pal, R., Ravindran, G. (2006) Assessment of pluripotency and multilineage differentiation potential of NTERA-2 cells as a model for studying human embryonic stem cells. *Cell Prolif.* **39**, 585-598.

Pleasure, S., Lee, V. (1993) NTera 2 cells: a human cell line which displays characteristics expected of a human committed neuronal progenitor cell. *J. Neurosci. Res.* **35**, 585-602.

Prince, L., Aschner, M., Bowman, A. (2019) Human-induced pluripotent stems cells as a model to dissect the selective neurotoxicity of methylmercury. *Biochim. Biophys. Acta Gen. Subj.* **1863**, 129300.

Rowe, R., Daley, G. (2019) Induced pluripotent stem cells in disease modelling and drug discovery. *Nature Rev Genet.* **20**, 377-388.

Schmidt, C., Bladt, F., Goedecke, S., Brinkmann, V., Zschiesche, W., Sharpe, M., Gherardi, E., Birchmeler, C. (1995) Scatter factor/hepatocyte growth factor is essential for liver development. *Nature* **373**, 699-702.

Schwartz, C., Spivak, C., Baker, S., McDaniel, T., Loring, J., Nguyen, C., Chrest, F., Wersto, R., Arenas, E., Zeng, X., Freed, W., Rao, M. (2005) NTERA2: a model system to study dopaminergic differentiation of human embryonic stem cells. *Stem Cells Dev.* **14**, 517-534.

Siller, R., Greenhough, S., Naumovska, E., Sullivan, G. (2015) Small-molecule-driven hepatocyte differentiation of human pluripotent stem cells. *Stem Cell Reports* **4**, 939-952.

Taylor, M., Kan, H., Gollapudi, B., Marty, M. (2019) An in vitro developmental neurotoxicity screening assay for retinoic acid-induced neuronal differentiation using the human NT2/D1 cell line. *Neurotoxicology* **73**, 258-264.

Wakao, S., Kitada, M., Kuroda, Y., Shigemoto, T., Matsuse, D., Akashi, H., Tanimura, Y., Tsuchiyama, K., Kikuchi, T., Goda, M., Nakahata, T., Fujiyoshi, Y., Dezawa, M. (2011) Multilineage-differentiating stress-enduring (Muse) cells are a primary source of induced pluripotent stem cells in human fibroblasts. *Proc. Natl. Acad. Sci. USA* **108**, 9875-9880.

Yin X, Chen Z, Liu Z, Song C. (2012) Tissue transglutaminase (TG2) activity regulates osteoblast differentiation and mineralization in the SAOS-2 cell line. *Braz. J. Med. Biol. Res*. **45**, 693-700.

Zhang, X., Olsavszky, V., Yin, Y., Wang, B., Engleitner, T., Öllinger, R., Schledzewski, K., Koch, P., Rad, R., Schmid, R., Friess, H., Goerdt, S., Hüser, N., Géraud, C., von Figura, G., Hartmann, D. (2020) Angiocrine hepatocyte growth factor signaling controls physiological organ and body size and dynamic hepatocyte proliferation to prevent liver damage during regeneration. *Am. J. Pathol.* **190**, 358-371.

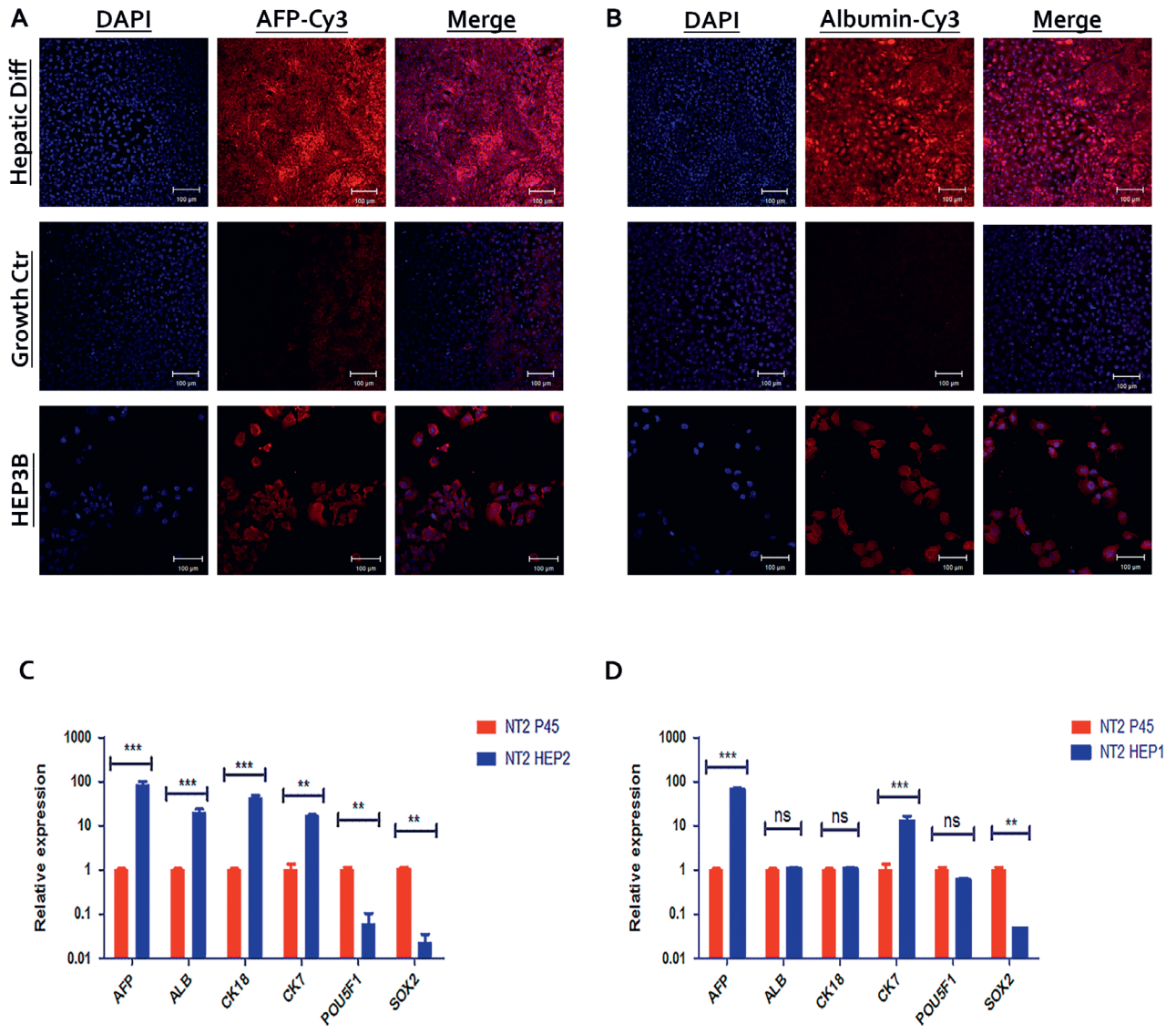
Zhao, H., Zuo, X., Ren, L., Li, Y., Tai, H., Du, J., Xie, X., Zhang, X., Han, Y., Wu, Y., Yang, C., Xu, Z., Hong, H., Li, S., Su, B. (2019) Combined use of bFGF/EGF and all-trans-retinoic acid cooperatively promotes neuronal differentiation and neurite outgrowth in neural stem cells. *Neurosci. Lett*. **690**. 61-68.

# Erratum

## Tri-Lineage Differentiation of NTERA2 Clone D1 Cells towards Neural, Hepatic and Osteogenic Lineages *in Vitro*

B. SZEKY, B. MAYER, M. GYONGY, A. HAJDARA, S. BARSI, S. KARPATI, K. NEMETH

The original article was published in Folia Biologica (Praha) Volume 67, No. 5–6 (2021), 174-182.
The images in Fig. 4 were not presented correctly.
The correct version of **Fig. 4**:




 https://doi.org/10.14712/fb2023069010040