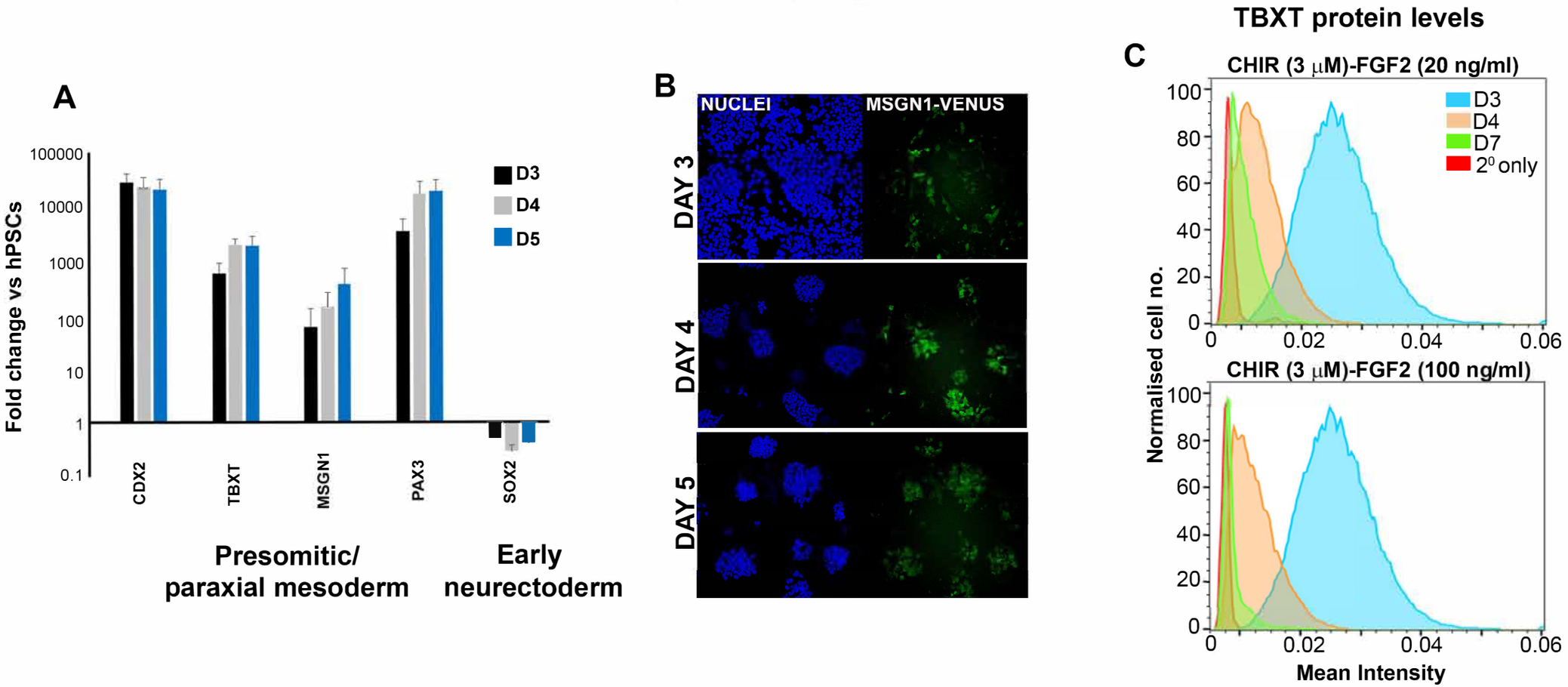

Figure S1. (A) qPCR expression analysis of indicated markers at different time points during the differentiation of hPSC-derived NMP-like cells in the presence of 8 μM CHIR and 40 ng/ml FGF2 as described in Fig. 1A. Error bars=S.D. **(B)** Fluorescence analysis of the expression of MSNG1-VENUS at indicated time points during the differentiation of hPSC-derived NMP-like cells (D3) cells in the presence of 8 μM CHIR and 40 ng/ml FGF2 as described in Fig. 1A. Cells were derived from a MSGN1-VENUS reporter hPSC line (Frith et al., 2018). **(C)** Mean fluorescence intensity of TBXT protein expression in NMP-like (D3) cells and their derivatives during differentiation in the presence of 3μM CHIR and 20/100 ng/ml FGF2 as described in Fig. 1A.

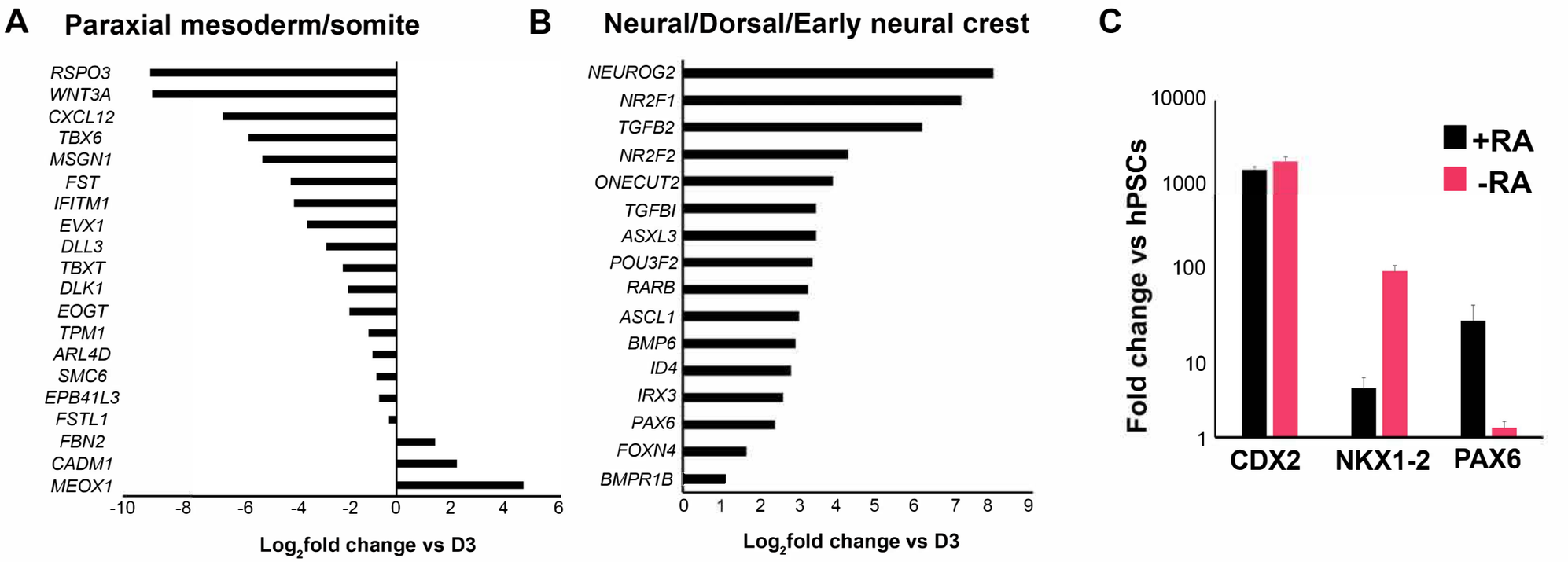

Figure S2. (A) Log fold induction of representative paraxial mesoderm/somite-associated markers in D7 WNT-FGF treated cultures compared to D3 NMP-like cells. Selection of transcripts was based on Diaz-Cuadros et al. 2020. **(B)** Log fold induction of representative neural/dorsal/BMP/TGFβ-associated markers in D7 WNT-FGF treated cultures compared to D3 NMP-like cells. **(C)** qPCR expression analysis of indicated spinal cord progenitor/neural markers in D7 early neurectoderm cultures induced by WNT and FGF in the presence (+RA) and absence (-RA) of RA. Error bars=S.D (n=3).

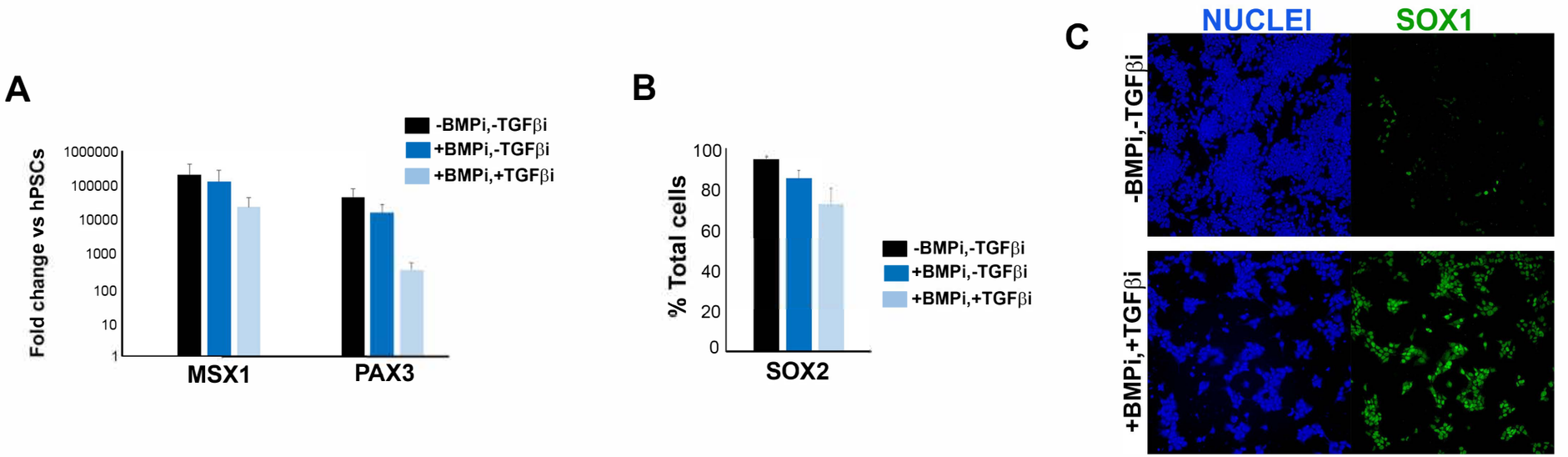

Figure S3. (A) qPCR expression analysis of indicated dorsal markers in the absence (black bars) and presence of BMP only (dark blue bars) or TGFβ and BMP (light blue bars) inhibitors (i) at day 8 during the differentiation of NMP-like cells to MNs as described in Fig. 3A. Error bars=S.D. (n=3). **(B)** Quantification of cells marked by expression of SOX2 at D8 during the differentiation of hPSCs in the absence (black bars) and presence of BMP only (dark blue bars) or TGFβ and BMP (light blue bars) inhibitors toward MNs as described in Fig. 3A and following immunofluorescence and image analysis. The data in the graph were obtained after scoring 4-5 random fields. Error bars = S.D. **(C)** Immunofluorescence analysis of the expression of SOX1 in D8 cultures following differentiation of hPSCs in the absence and combined presence of TGFβ and BMP inhibitors. Results were obtained using a hiPS (SFCi55-ZsGr) cell line.

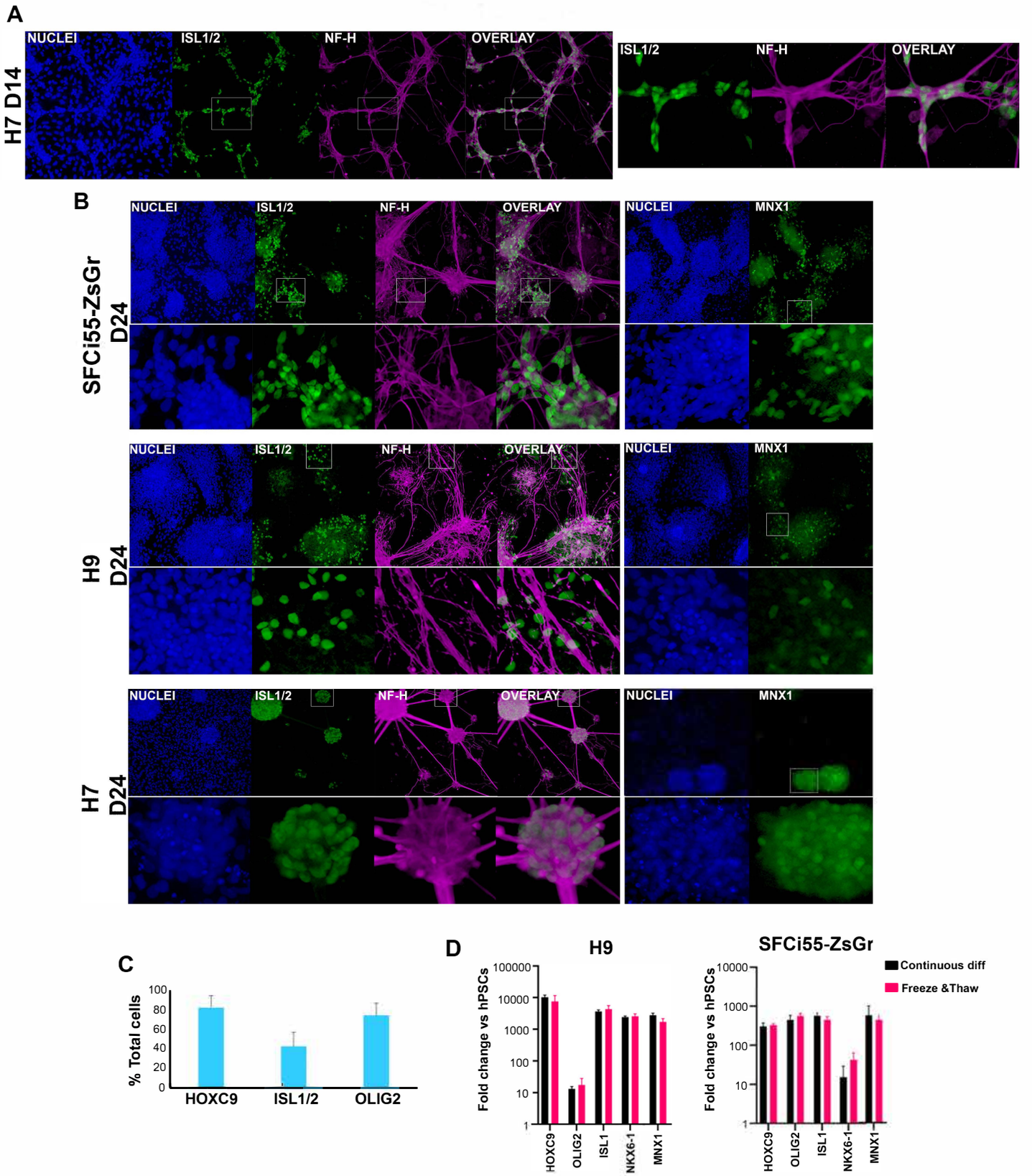

Figure S4. (A) Immunofluorescence analysis of the expression of the MN marker ISL1/2 together with the neuronal marker Neurofilament Heavy Chain (NF-H) in day 14 cultures differentiated from H7 hES cells in the presence of BMP/TGFβ inhibitors as described in Fig. 3A. Magnified regions corresponding to the insets are also shown. **(B)** Immunofluorescence analysis of the expression of ISL1/2 and NF-H in day 24 cultures differentiated from indicated hPSC lines in the presence of BMP/TGFβ inhibitors as described in Fig. 3A. Magnified regions corresponding to the insets are also shown. **(C)** Quantification of cells marked by expression of MN-associated proteins at D14 after differentiation of indicated MIFF1 hiPSCs in the presence of BMP/TGFβ inhibitors as described in Fig. 3A, and following immunofluorescence and image analysis. The data in the graph were obtained after scoring five random fields per experiment (two independent replicates). Error bars = S.D. **(D)** qPCR expression analysis of indicated thoracic, MN progenitor and MN markers at day 24 of differentiation of a hES (H9) and hiPS (SFCi55-ZsGr) cell line following freezing and thawing of cultures at D14 vs continuous differentiation controls. Error bars=S.D. (n=3).

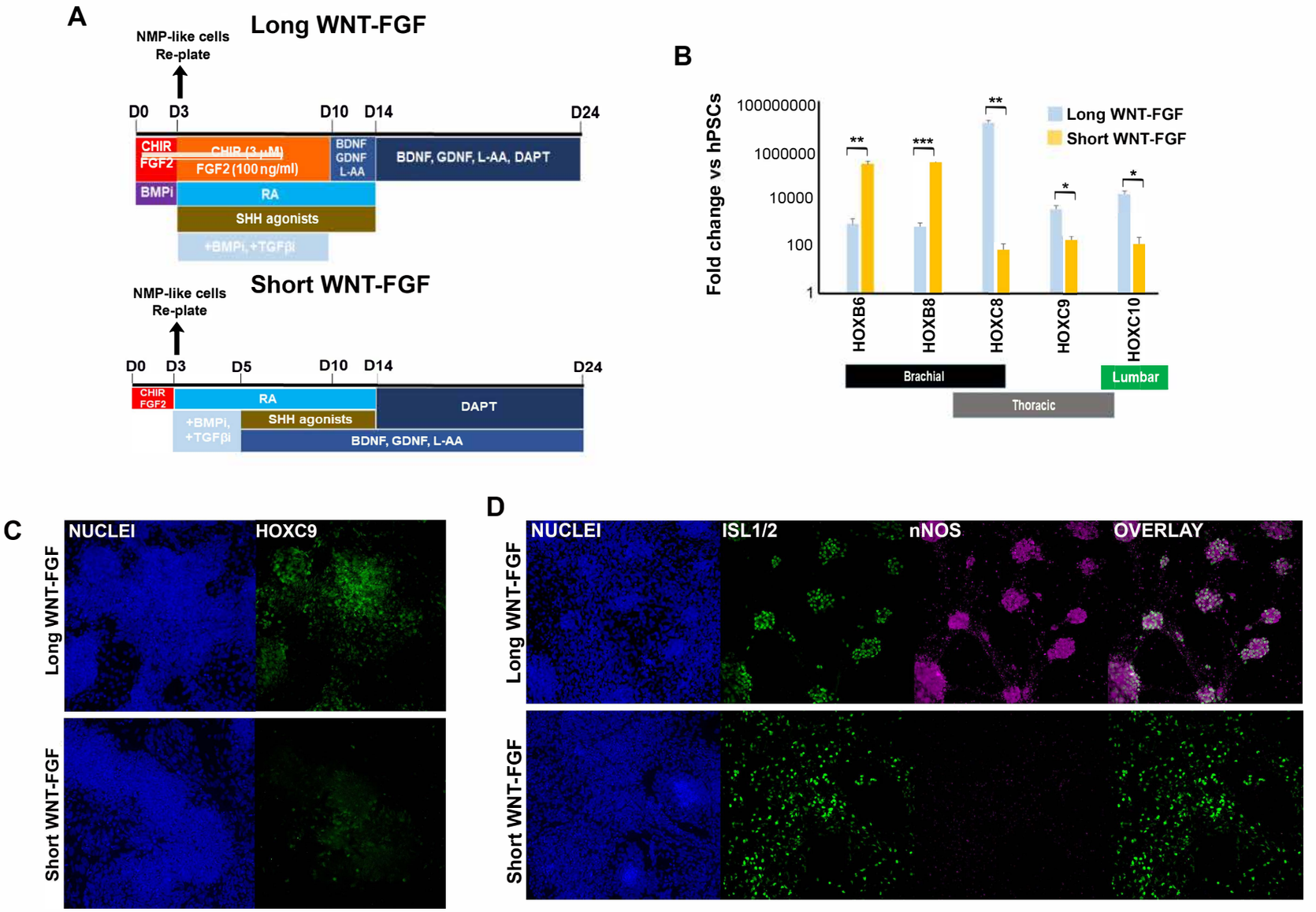

Figure S5. (A) Diagrams comparing the culture conditions employed in our protocol ("Long WNT-FGF") to those in conventional NMP-based approaches ("Short WNT-FGF") aiming to generate posterior spinal cord MNs from hPSCs. **(B)** qPCR expression analysis of indicated HOX transcripts (bottom) in day 24 MN cultures generated in parallel from hiPS cells (MIFF1) using the two protocols depicted in (A). Error bars=S.D. (n=3). *P<0.05, **P<0.005, ***P<0.0005 (Paired t-test). **(C)** Immunofluorescence analysis of the expression of HOXC9 in D24 MN cultures generated from hiPS cells (SFCi55-ZsGr) using the indicated protocols depicted in (A). **(D)** Immunofluorescence analysis of the expression of the PGC/MN markers nNOS and ISL1 in day 24 MN cultures generated from H7 hES cells using the two protocols depicted in (A).

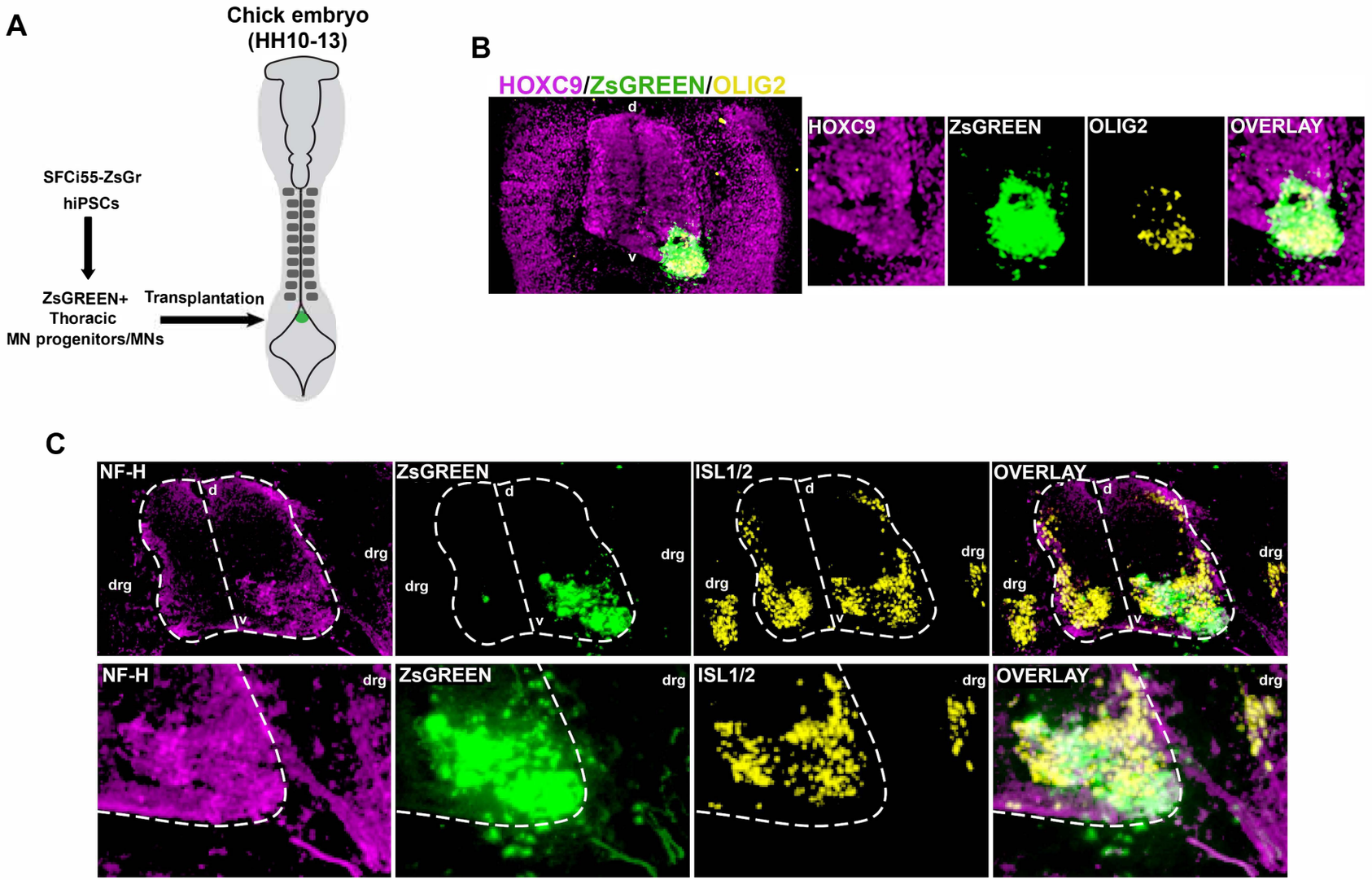

Figure S6. (A) Diagram depicting the in vitro generation of D14 posterior MN progenitors/MN from hPSCs and their transplantation into the posterior neural tube of HH10-13 chick embryos. **(B)** Immunofluorescence analysis of ZsGREEN, HOXC9 and OLIG2 expression in sections of HH26-30 chick embryos grafted with hPSC-derived ZsGREEN+ posterior D14 MN progenitor/MN cultures. Higher magnification images (right) indicate the co-expression of ZsGREEN and OLIG2 in a fraction of the grafted donor cells. Note that we employed an antibody that only detects the human version of OLIG2. D, dorsal; v, ventral. **(C)** Immunofluorescence analysis of ZsGREEN, NF-H and ISL1/2 expression marking donor human cells, neuronal projections and dorsal root ganglion (drg)/MN-containing ventral neural tube respectively, in sections of HH26-30 chick embryos grafted with hPSC-derived ZsGREEN+ posterior D14 MN progenitor/MN cultures. Higher magnification images (bottom row) indicate the co-expression of ZsGREEN and ISL1/2 in a fraction of the grafted donor cells. Dotted outlines mark the boundaries of the neural tube. D, dorsal; v, ventral.

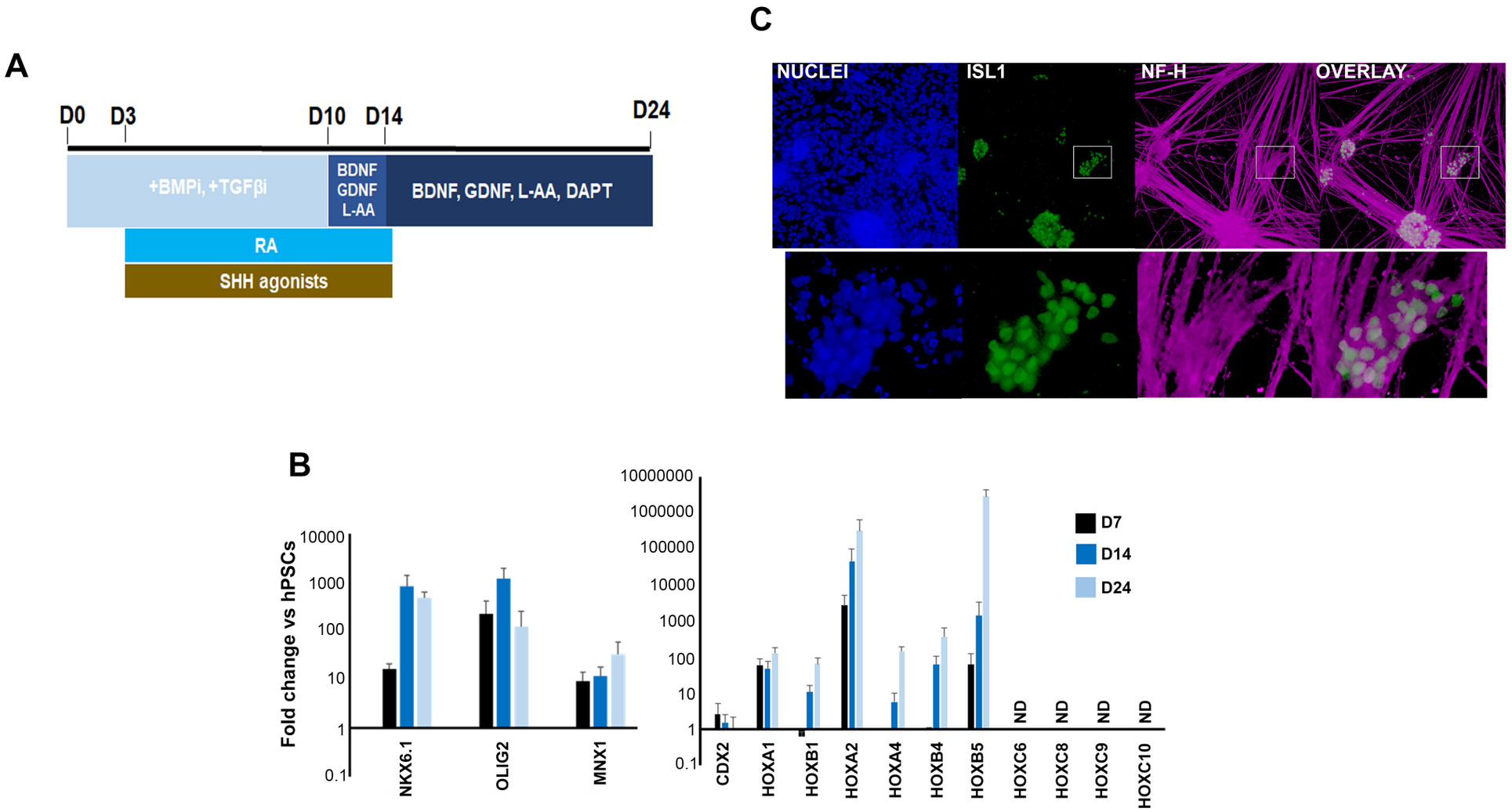

Figure S7. (A) Diagram depicting the culture conditions employed for the in vitro differentiation of hPSCs toward MNs of a hindbrain/cervical spinal cord Identity. **(B)** qPCR expression analysis of indicated MN progenitor/MN markers (left) and HOX genes/CDX2 (left) at different points during the generation of MNs from hPSCs using the culture regimen shown in A. ND, Not detected. Error bars=S.D. (n=3). **(C) I**mmunofluorescence analysis of the expression of ISL1/2 and NF-H in day 24 anterior MN cultures following differentiation of hPSCs as described in A. Magnified regions corresponding to the insets are also shown.

Table S1. Significantly up- and downregulated transcripts in day 3 NMP-like cells versus day 0 undifferentiated hPSCs, day 7 early spinal cord progenitors versus day 3 NMP-like cells and GO analysis indicating the enrichment of neural-related terms by transcripts upregulated in day 7 cultures.

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Table S2. Primer sequences

Gene	Forward	Reverse	Roche UPL Probe
GAPDH	gcccaatacgaccaaatcc	agccacatcgctcagacac	60
SOX2	ttgctgcctctttaagactagga	taagcctggggctcaaact	35
TBXT	aggtacccaaccctgagga	gcaggtgagttgtcagaataggt	23
CDX2	atcaccatccggaggaaag	tgcggttctgaaaccagatt	34
NKX1-2	gtcgaagcggggaaagat	gatcctccgcatcctcct	78
MSGN1	agctcaggatgaggaccttg	ctggcctctctggctgtaga	87
OLIG2	agctcctcaaatcgcatcc	atagtcgtcgcagctttcg	12
MNX1	ttacctgacttatgaaacttgaaacc	cccagagacgtaagcataaacc	50
ISL1	aaacaggagctccagcaaaa	aaaggactctttcagccaagg	4
HOXA1	gacgaccgcttcctagtgg	tcccggaagtctggtaggta	9
HOXB1	ccagctaggggcttgtc	atgctgcggaggatatgg	39
HOXA2	caagaaaaccgcacttctgc	tgtgttggtgtaagcagttctca	5
HOXA4	gttgccacccaagagagaac	ccaagtagtccttctcaggtatcc	20
HOXB4	aaaccaggccccttcctac	gcacacagatattcacacatacga	45
HOXB5	aagcttcacatcagccatga	cggttgaagtggaactccttt	1
HOXB6	tggaagctgaagaagaaactgaa	gccgggtttatgatttgttg	12
HOXC6	tgaattcctacttcactaacccttc	atcataggcggtggaattga	87
HOXB8	agctcttcccctggatgc	atagggattaaataggaactccttctc	1
HOXC8	tcccagcctcatgtttcc	tgataccggctgtaagtttgc	86
HOXC9	tcctagcgtccaggtttcc	gctacagtccggcaccaa	70
HOXC10	aggagagggccaaagctg	agccaatttcctgtggtgtt	19
NKX6.1	gagatgaagaccccgctgta	gacgacgacgaggacgag	27
NKX6.2	agcacaaaccctcgaacttg	cccggattctgcaaaaatag	70
MSX1	ctcgtcaaagccgagagc	cggttcgtcttgtgtttgc	7
PAX3	attggcaatggcctctca	aggggagagcgcgtaatc	45
PAX6	gcacacacacattaacacacttg	ggtgtgtgagagcaattctcag	9
LHX3	gttcaggaggggcaggac	ctcccgtagaggccattg	12
nNOS	cagcaaagcaactgccaag	agcaccaaaaccctctgtga	61