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Forced *LMX1A* expression induces dorsal neural fates and disrupts patterning of human embryonic stem cells into ventral midbrain dopaminergic neurons

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SUMMARY

The differentiation of human pluripotent stem cells into ventral mesencephalic dopaminergic (DA) fate is relevant for the treatment of Parkinson's disease. Shortcuts to obtaining DA cells through direct reprogramming often include forced expression of the transcription factor *LMX1A*. Although reprogramming with *LMX1A* can generate tyrosine hydroxylase (TH)-positive cells, their regional identity remains elusive. Using an *in vitro* model of early human neural tube patterning, we report that forced *LMX1A* expression induced a ventral-to-dorsal fate shift along the entire neuroaxis with the emergence of roof plate fates despite the presence of ventralizing molecules. The *LMX1A*-expressing progenitors gave rise to grafts containing roof plate-derived choroid plexus cysts as well as ectopically induced TH-positive neurons of a forebrain identity. Early activation of *LMX1A* prior to floor plate specification was necessary for the dorsalizing effect. Our work suggests using caution in employing *LMX1A* for the induction of DA fate, as this factor may generate roof plate rather than midbrain fates.

INTRODUCTION

The ventral midbrain (vMB) dopaminergic (DA) neuron lineage has attracted much attention in the last decades, due to its association with Parkinson's disease, which is hallmarked by neurodegeneration of the DA midbrain nucleus termed *substantia nigra*. Extensive molecular characterization of the progenitor population located in the ventral floor plate of the developing neural tube has led to the discovery of numerous genes implicated in the patterning, specification, and maturation of DA neurons (Blaess and Ang, 2015). Out of a multitude of transcription factors expressed locally and involved in the regionalization of the vMB domain, the LIM-homeodomain *LMX1A* has been identified as a determinant factor for the specification of the DA progenitors and neurons (Andersson et al., 2006; Doucet-Beaupre et al., 2015). Although the *Lmx1a* mouse mutant Dreher displays a mild reduction of the vMB DA neuronal population, compensated by the paralog *Lmx1b* (Yan et al., 2011), ectopic expression of *Lmx1a* can drive the formation DA neurons in the ventral neural tube, but not in dorsal neural tube, of mouse and chicken embryos (Andersson et al., 2006; Lin et al., 2009; Nakatani et al., 2010). Moreover, forced expression of *Lmx1a* in mouse embryonic stem cells (mESCs) (Andersson et al., 2006; Friling et al., 2009) and mouse fetal fibroblasts, together with other neuronal reprogramming factors (Caiazzo et al., 2011; Dell'Anno et al., 2014; Kim et al., 2011), can *in vitro* induce the formation of cells expressing tyrosine hydroxylase (TH), the rate-limiting enzyme in DA production. Altogether, these observations have supported several reprogramming studies using *LMX1A* to induce DA progenitors or neurons from human cells *in vitro* (Caiazzo et al., 2011; Friling et al., 2009; Pfisterer et al., 2011; Rivetti di Val Cervo et al., 2017). However, attempts to apply *LMX1A* and other factors for obtaining DA neurons *in vivo*—through either direct *in vivo* reprogramming or transplantation of induced neurons (iNs)—have generated disappointing results with only very few TH^+ neurons obtained (Pereira et al., 2014; Torper et al., 2013). Thus, the regional neural identity resulting from forced expression of *LMX1A* in human cells remains elusive.

In this study, we applied a human embryonic stem cell (hESC)-based *in vitro* model of early human neural

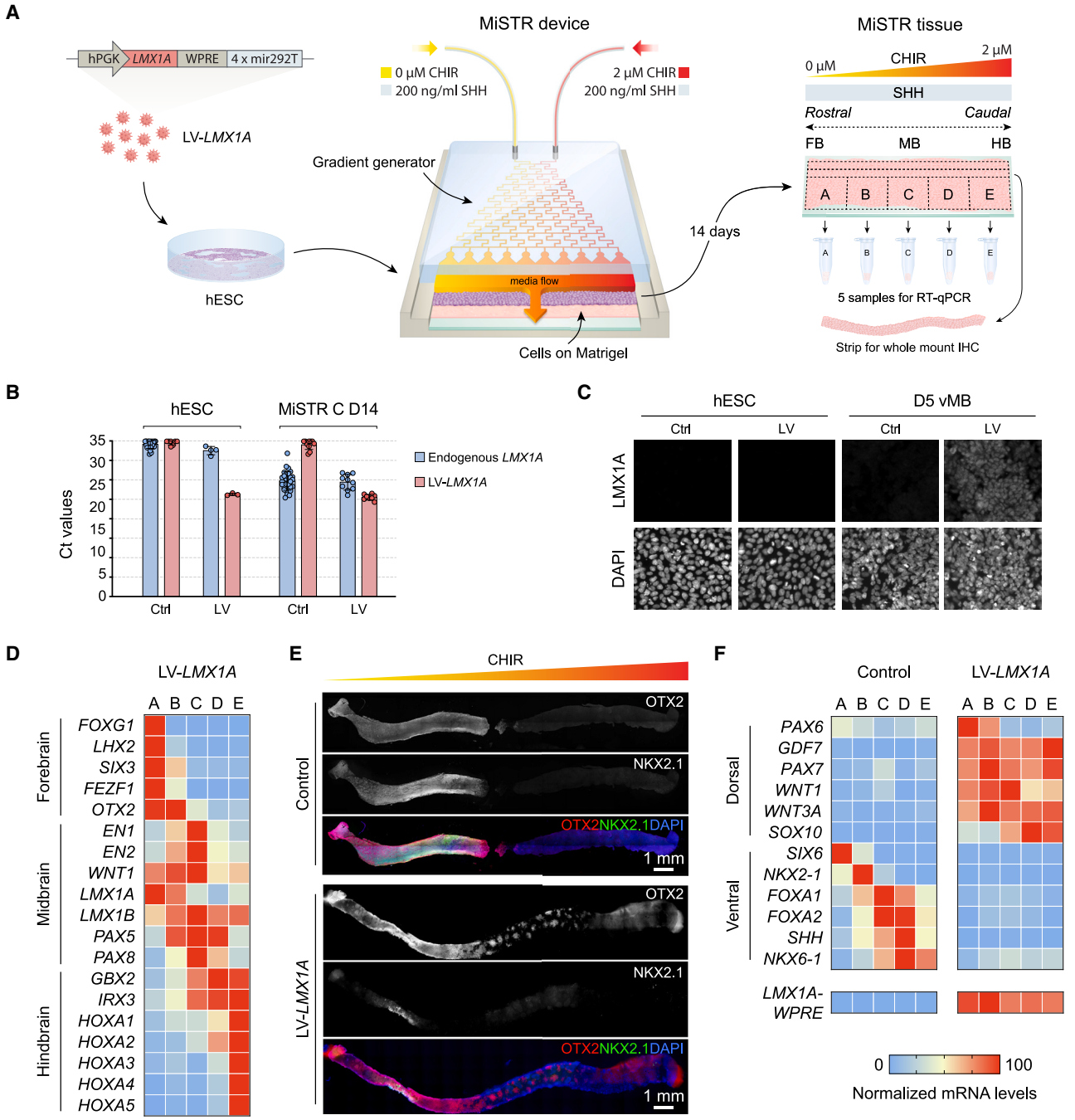

Figure 1. Forced lentiviral expression of *LMX1A* in a stem cell-based *in vitro* model of ventral neural tube patterning induces dorsal identity

(A) A lentiviral construct with *LMX1A* under control of 4x mir292 target sequences was applied to hESCs (referred to as LV-*LMX1A*). The LV-*LMX1A*-expressing hESCs were seeded into the MiSTR model of human rostro-caudal patterning of the neural tube. After 14 days, the tissue was cut into 5 regional portions along the rostro-caudal gradient for RT-qPCR analysis, or a longitudinal strip was taken for immunofluorescence.

(B) Cycle threshold values (Ct) from RT-qPCR analysis using primers specific for endogenous *LMX1A* (*LMX1A*-UTR) or for lentiviral *LMX1A* (*LMX1A*-WPRE) expression. Results are displayed for hESCs and MiSTR day-14 samples (section C) with and without LV-*LMX1A* expression. hESC data are from at least 3 independent experiments while MiSTR data are from at least 7 independent experiments.

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patterning (Rifes et al., 2020) to investigate the outcome of forced *LMX1A* expression along the entire rostro-caudal neuraxis. We found that despite the presence of ventralizing factors, forced *LMX1A* expression caused a global ventral-to-dorsal fate shift of neural progenitors, regardless of their rostro-caudal regionality. We further confirmed the dorsalizing effect of *LMX1A* through transplantation studies and found that *LMX1A* induced the formation of choroid plexus cysts as well as ectopic TH expression in forebrain cells, while abrogating the formation of DA neurons from midbrain cells.

RESULTS

Lentiviral expression of *LMX1A* in an *in vitro* model of early human neural patterning

We have demonstrated previously that neural differentiation of a layer of hESCs under a microfluidic-controlled gradient of the small-molecule WNT activator CHIR99021 (0–2 μM) recapitulates the earliest steps of rostro-caudal neural tube patterning *in vitro* and generates a coherent neural tissue ranging from forebrain to hindbrain (Rifes et al., 2020). In this model, termed MiSTR (microfluidic stem cell regionalization), the global addition of sonic hedgehog (SHH) agonists (SHH and purmorphamine) results in an hESC-derived neural tissue with a ventral identity, generating a rostro-caudal neuraxis at the level of the floor plate (Rifes et al., 2020). To investigate the patterning effect of forced *LMX1A* expression along the entire ventral human neuroaxis, we delivered a lentiviral construct of *LMX1A* (LV-*LMX1A*) to hESCs and subjected these cells to MiSTR differentiation (Figure 1A). The LV-*LMX1A* construct applied contained a target sequence for the pluripotency-specific microRNA mir292 within the 3′-UTR. This construct has previously been shown to efficiently silence transgenic protein translation in pluripotent cells and to activate expression only 3–5 days after initiation of differentiation (Sachdeva et al., 2010). Indeed, while expression of the LV-*LMX1A* mRNA was detected by quantitative reverse-transcription PCR (RT-qPCR) in both hESCs and differentiated day-14 cells (Figure 1B), we confirmed that LMX1A was detected at protein level only in differentiated cells but not in hESCs (Figure 1C). Furthermore, we demonstrated that the human phosphoglycerate kinase (hPGK) promoter induced LV-*LMX1A* expression at physiological levels comparable to endogenous *LMX1A* levels in vMB cells (Figure 1B). Surprisingly, we found through OTX2 immunolabelling and RT-qPCR analysis that ventralized MiSTR tissue originating from hESCs expressing LV-*LMX1A* did not induce a global midbrain signature but instead maintained the overall rostro-caudal patterning from forebrain to hindbrain (Figures 1D and 1E), similar to what has previously been demonstrated for wild-type hESCs (Rifes et al., 2020). However, when assessing the dorsoventral identity of the tissues, it was revealed that LV-*LMX1A* expression resulted in a strongly reduced expression of ventral markers such as *NKX2-1*, *SIX6*, *FOXA2*, and *SHH* (Figures 1E and 1F). Concomitantly, there was an induction of dorsal neural tube and roof plate markers (e.g., *GDF7* and *WNT3A*), which were absent in the ventralized control tissue without LV-*LMX1A* (Figure 1F). Our MiSTR results indicate that, despite the presence of ventralizing molecules, forced expression of *LMX1A* did not induce global vMB fates but in contrast abrogated ventralization and provoked a ventral-to-dorsal fate shift along the entire rostro-caudal neuraxis in human neural tissue.

Grafts from LV-*LMX1A*-expressing cells confirm a ventral-to-dorsal fate shift

To validate whether the observed ventral-to-dorsal fate shift observed in the MISTR model also held true upon *in vivo* transplantation, we patterned individual cultures of hESCs (control and LV-*LMX1A* cells) toward either ventral forebrain (vFB) and vMB or ventral hindbrain (vHB) fates (Figure 2A; Figure S1), using a previously published protocol (Kirkeby et al., 2012). These cells were then transplanted into the striatum of adult rats which had been unilaterally lesioned with 6-hydroxydopamine (6-OHDA) to eliminate endogenous DA neurons, thereby enabling better visualization of graft-derived DA neurons (Figure 2B). The transplanted cells from all 6 groups survived implantation into the rat brain and gave rise to human neuron-containing grafts after 6 weeks (Figure 2C). Interestingly, we observed the formation of large cystic structures in grafts of the LV-*LMX1A* group (5 out of 9 animals), which were not present in the control grafts (0 out of

(C) Immunolabeling for LMX1A displays immunoreactive expression only in LV-*LMX1A* cells at day 5 of differentiation indicating the protein is expressed only once cells exit pluripotency. No expression was detected in hESCs nor differentiated control cells.
(D) Normalized expression of forebrain, midbrain, and hindbrain markers (RT-qPCR) along the A-E regions of ventralized LV-*LMX1A* MiSTR tissue at day 14. $n = 7$ tissues from independent experiments.
(E) Whole-mount MiSTR tissue immunolabelling for the forebrain/midbrain marker OTX2 and ventral marker NKX2-1 in control and LV-*LMX1A* tissues. Note that Matrigel-derived autofluorescence is noticeable as a diffuse red haze on the OTX2-negative side.
(F) Normalized RT-qPCR expression values show induction of dorsal markers in LV-*LMX1A* MiSTR tissues compared to control tissue. $n = 10$ (control tissues), $n = 7$ (LV-*LMX1A*) from independent experiments.

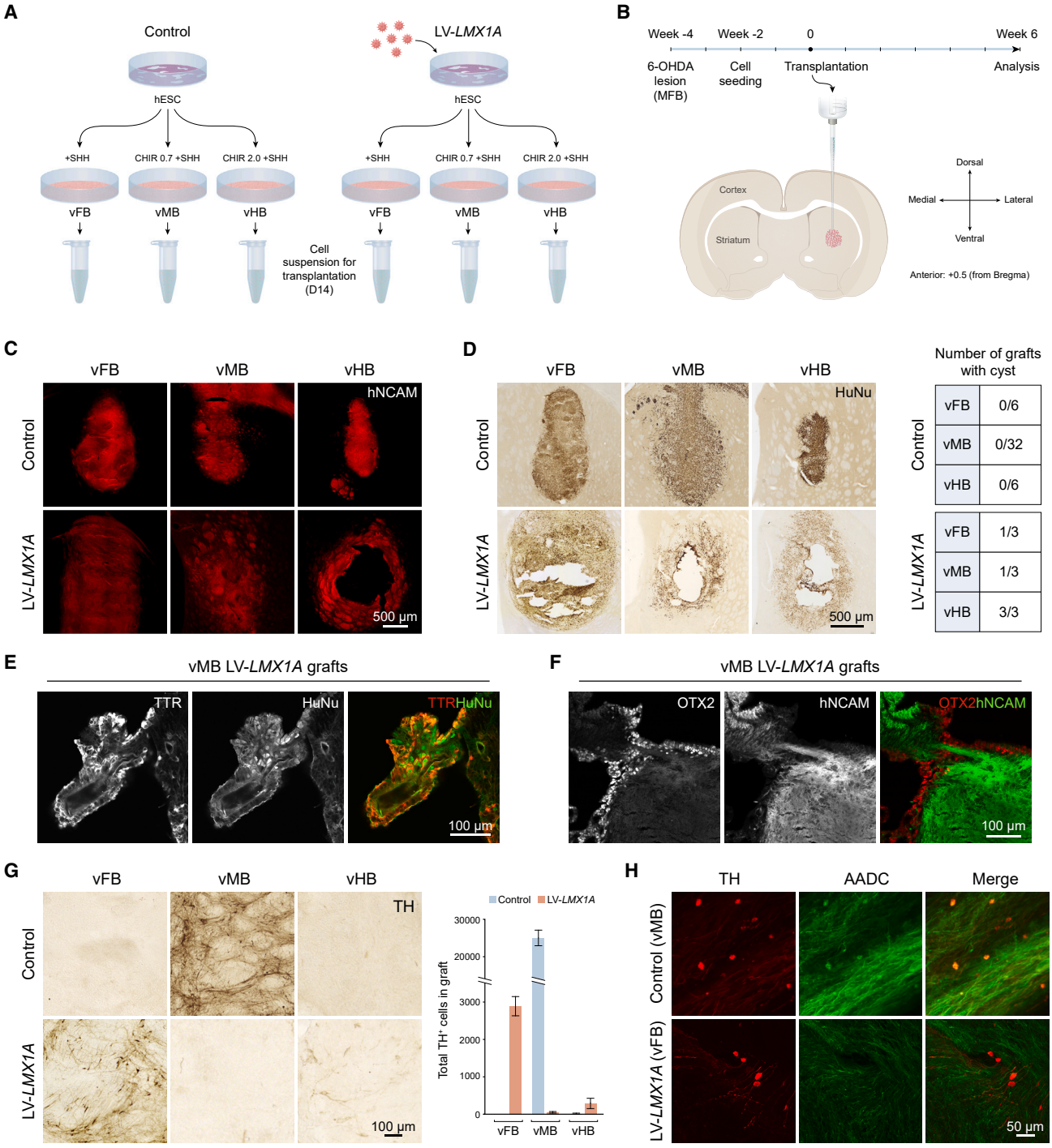

Figure 2. Ventrally differentiated LV-*LMX1A* hESCs form cystic structures with choroid plexus marker TTR upon grafting into rat brain

(A) Control hESCs and LV-*LMX1A* hESCs were patterned toward different ventral fates (forebrain, midbrain, and hindbrain; vFB, vMB, vHB, respectively) and prepared as cell suspensions for transplantation to the rat brain.

(B) Cell suspensions of patterned cells were injected into the striatum of 6-hydroxydopamine-lesioned rat brains at 4 weeks post lesion, and the grafts were analyzed 6 weeks post-transplantation.

(C) Survival and neural identity of the grafts were verified for all 6 conditions of *in vitro*-differentiated cells through immunolabeling for human neural cell adhesion molecule (hNCAM). CL, cyst lumen.

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44 animals, Figure 2D). These cysts (4 out of 5, Figure S2A) were lined with human non-neuronal cells (HuNu$^+$/hNCAM$^-$) expressing the choroid plexus markers transthyretin (TTR) and OTX2, indicating that the cysts represented ectopic hESC-derived ventricular structures (Figures 2E and 2F). The cysts were also positive for other mature choroid plexus markers such as tight junction protein Zonula occludens 1 (ZO-1), Na–K–Cl cotransporter (NKCC1), and Na/K ATPase α1 (ATP1A1) (Figure S2B). Given that choroid plexus cells are derived from dorsal roof plate structures in the developing embryo, these findings substantiated the ventral-to-dorsal fate shift observed in the LV-*LMX1A* MiSTR tissue (Figure 1F). The LV-*LMX1A*-induced generation of choroid plexus cells particularly in progenitors of hindbrain fate further explained the presence of OTX2$^+$ cell patches in the hindbrain region of the LV-*LMX1A* MiSTR tissue (Figure 1E).

We next analyzed the grafts for the presence of dopamine neurons. In line with our previous studies (Grealish et al., 2014; Kirkeby et al., 2012), when using wild-type control hESCs, we observed TH$^+$ neurons only in grafts derived from vMB-patterned progenitors, but not in vFB or vHB grafts (Figure 2G). In contrast, vMB-patterned grafts from LV-*LMX1A*-expressing cells were surprisingly devoid of TH$^+$ neurons, indicating again a loss of ventral fates induced by *LMX1A*. Interestingly, LV-*LMX1A* expression in the vFB setting induced the formation of TH$^+$ neurons (Figure 2G) that were, in contrast to transplanted hESC-derived vMB DA neurons, negative for the dopamine synthesis enzyme aromatic l-amino acid decarboxylase, AADC (Figure 2H). This indicated that the ectopically induced forebrain-derived TH$^+$ neurons were not of a bona fide vMB-dopamine-producing phenotype. In conclusion, the *in vivo* data thus further corroborated our *in vitro* MiSTR observations and demonstrated that forced expression of *LMX1A* not only failed to induce vMB fates but instead initiated a dorsal transcriptional program in the cells *in vitro* and *in vivo*.

Forced *LMX1A* expression overrides early ventralizing transcriptional program

Taking advantage of the *in vitro* MiSTR model to access events prior to the observed ventral-to-dorsal fate shift induced by LV-*LMX1A* expression, we reassessed the dynamics of the expression of ventral and dorsal markers at earlier time points in the system (day 2 and day 6 of differentiation; Figure 3A). RT-qPCR analyses revealed that a ventral transcriptional program with induction of *NKX2-1*, *NKX6-1*, *SIX6*, *FOXA1*, and *FOXA2* was initiated in both wild-type and LV-*LMX1A*-expressing MiSTR tissue at day 6 of differentiation. However, by day 14 of differentiation, these markers were almost completely lost in the LV-*LMX1A* tissues and instead replaced by the induction of dorsal roof plate markers such as *GDF7*, *SOX10*, and *WNT1/3A* (Figure 3A). Interestingly, the early expression of floor plate genes (*FOXA1/2*, *SHH*) by day 6 tended to be reduced, albeit not statistically significant, in the LV-*LMX1A* tissues (Figure 3B), indicating that forced *LMX1A* expression abrogated the positive feedback loop for ventralization between day 6 and 14. We further explored the effect of timing of forced *LMX1A* expression by applying an inducible Tet-*O*-*Lmx1a* system (Figure S3A). Through addition of doxycycline, we induced *Lmx1a* expression at day 3, 6, or 9 of differentiation and analyzed the cells at day 14 (Figure 3C). Immunocytochemistry for NKX2.1, FOXA2, and PAX6 showed that early induction of *Lmx1a* expression led to dorsalization (day 3 and to lesser extent day 6) while activation of Tet-*O*-*Lmx1a* on day 9 had only a minor effect (Figure 3D). Analysis of the cells at day 5 showed that induction of *Lmx1a* expression caused significant activation of the WNT pathway (i.e., increase in *WNT1* and *LEF1*) as well as antagonism of the SHH pathway (i.e., inhibition of *PTCH1)* already at two days after initiation of doxycycline treatment (Figure 3E). In line with this, supraphysiological activation of SHH signaling through combination of high SHH and purmorphamine was able to initiate earlier and stronger expression of FOXA2 and thereby prevented the dorsalizing effect from forced expression of *Lmx1a* starting on day 3 (Figure S3B). These data indicate that ventral and dorsal transcriptional programs are in competition and that forced *LMX1A* expression during early stages can prematurely activate strong WNT signaling, thereby overriding an emerging ventral floor plate program and inducing a ventral-to-dorsal fate shift.

(D) Immunolabeling for a human-specific epitope (human nuclei, HuNu) in 6-week-old grafts from control and LV-*LMX1A* cells revealed the presence of cystic structures only in LV-*LMX1A*-derived grafts. The number of grafts containing cysts in each group was quantified relative to total number of animals with grafts in each group.

(E) HuNu$^+$ LV-*LMX1A* cells lining the cysts in vMB grafts displayed immunoreactivity for the choroid plexus marker TTR.

(F) LV-*LMX1A* cells lining the human-specific NCAM-stained grafts strongly expressed the choroid plexus marker OTX2.

(G) Immunohistochemistry reveals robust presence of TH$^+$ cells in control vMB-patterned grafts and to lesser extent in LV-*LMX1A* vFB-patterned grafts. The graph on the right displays the quantification of the total number of TH$^+$ cells in control and LV-*LMX1A* grafts. Data are presented as mean ± SEM, $n = 3$ grafted animals.

(H) Immunolabeling for the enzymes TH and AADC, which are essential for dopamine synthesis, reveals an absence of AADC in the LV-*LMX1A*-derived vFB TH$^+$ cells. In contrast, AADC is present in TH$^+$ cells of grafts derived from control hESCs differentiated toward vMB.

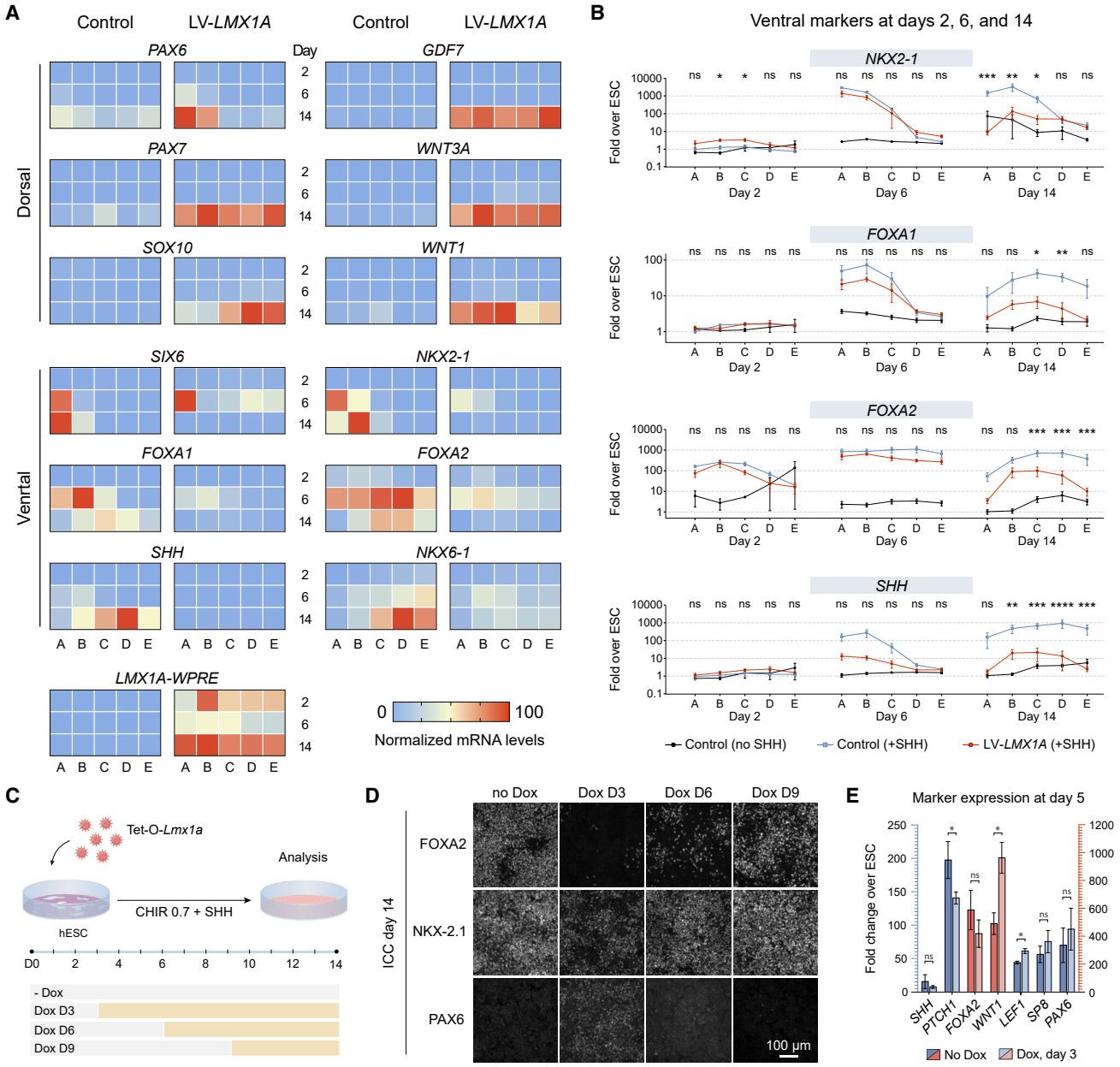

Figure 3. Temporal expression of ventral and dorsal markers in ventralized LV-*LMX1A* MiSTR tissue

(A) Normalized RT-qPCR expression values from day 2, 6, and 14 of forebrain, midbrain, and hindbrain markers along the A-E regions of ventralized MiSTR tissue displays suppression of ventral markers in LV-*LMX1A* tissues between days 6 and 14. $n = 4$ (day 2), 5 (day 6), 7 (day 14) LV-*LMX1A* MiSTR tissues from independent experiments.

(B) Non-normalized fold-change values underlying the heatmap in (A) show the transient induction of ventral markers *NKX2-1*, *FOXA1/2*, and *SHH* in ventral MiSTR differentiation, comparing control (+SHH) and LV-*LMX1A* MiSTR tissues, at 6 and 14 days. Control, non-ventralized MiSTR tissue (no SHH, black) is shown only for visual comparison. Data represented as mean ± SEM. Two-way ANOVA followed by *post hoc* Sidak test between +SHH control and LV-*LMX1A*. p-summary of comparison at each region (A-E) is shown above. $n = 4$ (day 2), 6 (day 6), 10 (day 14) control (+SHH) MiSTR tissues from independent experiments.

(C) Graphical representation of the experimental setup where *Lmx1a* expression was induced in differentiating Tet-*O*-*Lmx1a* hESCs by the addition of doxycycline from day 3, 6, or 9. Patterned cells were analyzed at day 14 and compared to control cells not treated with doxycycline.

(D) Immunolabeling of differentiated Tet-*O*-*Lmx1a* cells treated with doxycycline from day 3, 6, or 9, and an untreated control for FOXA2, NKX2.1, and PAX6 at day 14.

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DISCUSSION

In this study, we provide evidence that early forced *LMX1A* expression at physiological levels in differentiating hESCs can result in the generation of a non-vMB DA progenitor fate, despite the presence of ventralizing factors in the medium and a lentiviral design that avoids precocious delivery of LMX1A in the pluripotent stage. Our MiSTR data demonstrated that forced *LMX1A* expression resulted in a clear ventral-to-dorsal shift along the entire embryonic neural axis, even though the tissue displayed an earlier induction of the ventral floor plate markers *FOXA1/2* and *SHH*. Thereby, the transgenic *LMX1A* was capable of overriding the initiated floor plate program in early differentiating hESCs, while simultaneously inducing another, dorsal embryonic program. The competition between dorsal and ventral programs appeared to take place in a time window between days 3–6 of differentiation, where we also observed a strong WNT-activating effect of forced *LMX1A* expression. *WNT1* is a direct transcriptional target of *LMX1A* (Chung et al., 2009), and we speculate that this premature activation of strong WNT signaling induced by *LMX1A* could be responsible for overriding the early ventral *FOXA2*/*SHH* program.

In mouse and chick, expression of *Lmx1a* in early neural tube development has been shown to initiate in the dorsal midline, the roof plate anlage, preceding the onset of expression in the vMB (Chizhikov and Millen, 2004; Doucet-Beaupre et al., 2015; Failli et al., 2002). In line with this, the spontaneous *Lmx1a* mouse mutant *Dreher* displays a notorious absence of roof plate structures (Millonig et al., 2000), while displaying only a moderate loss of vMB TH^+ DA neurons, because of a partial compensatory effect of *Lmx1b* (Ono et al., 2007), which is expressed exclusively in the floor plate and also involved in midbrain DA neurogenesis (Smidt et al., 2000). Furthermore, forced *LMX1A* expression in embryonic neural tubes resulted in ectopic roof plate formation (Chizhikov and Millen, 2004), in line with our reported appearance of ectopic, *LMX1A*-induced cysts lined with choroid plexus-like cells in the vMB-patterned grafts. Moreover, lentiviral expression of *Lmx1a* in mESC-derived neural progenitors revealed a positive regulatory feedback loop with *Wnt1* (Chung et al., 2009), which we also observed in our MiSTR tissue. However, *Wnt1* is also expressed early in the roof plate (Parr et al., 1993), and, thus, the authors report that only co-delivery of *Lmx1a*, *Foxa2*, and *Otx2* results in TH/AADC double-positive DA neurons (Chung et al., 2009). Therefore, the ventral-to-dorsal shift observed in our data may reflect the execution of the embryonic *LMX1A* program as a dorsal roof plate inducer, probably before the establishment of a full floor plate identity.

In summary, our data suggest caution in the use of *LMX1A* as a factor for DA reprogramming, given its capability to elicit dorsal identity as well as to generate misleading ectopic TH expression in forebrain-patterned neurons. In fact, the induction of ectopic TH expression in non-DA neurons seems to be a more general problem, as both mESCs and hESCs undergoing direct reprogramming with *Ascl1*, a non-cell-type-specific proneural transcription factor, yield a sizable fraction of TH^+ iNs (Ng et al., 2021).

Throughout embryonic development, transcription factors are essential determinants for various tissues and cell types, depending on temporal, spatial, and molecular context. In our study, we demonstrate that the use of *LMX1A* for direct reprogramming entails a risk of activating multiple different embryonic programs, some of which may yield unintended outcomes. This emphasizes the importance of thoroughly analyzing the regionalizing consequences of reprogramming factors when applying them to generate subtype-specific cells *in vitro* and *in vivo*.

EXPERIMENTAL PROCEDURES

Resources availability

Lead contact

Further information and requests for resources and reagents should be directed to the lead contact, Agnete Kirkeby (agnete.kirkeby@sund.ku.dk).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate new datasets.

hESCs

H9 hESCs were maintained on Matrigel (Corning)-coated culture dishes in StemMACS iPS-Brew XF medium (Miltenyi Biotec) and passaged with EDTA (0.5 mM) once weekly. LV-*LMX1A* hESCs were generated through transduction of H9 hESCs with a lentiviral construct containing the human open reading frame (ORF) for *LMX1A* (Pfisterer et al., 2011), which yields forced *LMX1A* expression upon exit from pluripotency (Sachdeva et al., 2010).

Statistical analysis

All RT-qPCR data (fold over H9 ESCs) were managed in Excel and statistically analyzed using GraphPad Prism 9 software. Differences were considered significant for $p < 0.05$ (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$). For analysis between A-E regions

(E) Expression of genes (RT-qPCR) in the SHH signaling pathway (*SHH*, *PTCH1*, *FOXA2*) and WNT signaling pathway (*WNT1*, *LEF1*, *SP8*, *PAX6*) at day 5 in vMB-patterned Tet-*O*-*Lmx1a* cells with or without doxycycline from day 3. Data represented as mean ± SEM. Left y-axis (blue) applies to the blue columns and the right y-axis (red) refers to the red columns. $n = 3$ independent experiments.

within one condition and time point, one-way ANOVA was performed on the original or LOG-transformed data, followed by Tukey's multiple comparison test between all regions. Each dataset was tested for its normal distribution (Shapiro-Wilk test) and equal variances (Brown-Forsythe test), and LOG transformed if needed, before ANOVA. When normal distribution or equal variance was not achieved, the non-parametric Kruskal-Wallis analysis was performed, followed by a Dunn's multiple comparison test. For analysis across MiSTR tissue regions and conditions, two-way ANOVA was performed on LOG-transformed data, followed by a *post hoc* Sidak's multiple comparison test at each A-E region between conditions. All error bars represent SEM.

Other experimental procedures

LV-*LMX1A* and control H9 cells were differentiated into an *in vitro* model of human neural development, termed MiSTR (Rifes et al., 2020), or as adherent cultures toward vFB, vMB, and vHB progenitors and grafted into the striatum of a rat model of Parkinson's disease (Kirkeby et al., 2012), as described previously. Other procedures and further details are described in the supplemental experimental procedures.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.stemcr.2024.04.010.

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AUTHOR CONTRIBUTIONS

P.R. and A.K. designed the study. P.R., J.K., J.R.C., A.S., G.S.R., A.H., D.A.W., and A.K. performed experiments. P.R., J.K., and A.K. wrote the manuscript.

DECLARATION OF INTERESTS

A.K. is the owner of Kirkeby Cell Therapy APS, performs paid consultancy to Novo Nordisk A/S, and is a co-inventor on patents WO2016162747A2/A3 and WO2019016113A1 on the generation of DA cells for treatment of PD.

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