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Directed Expression of Gata2, Mash1, and Foxa2 Synergize to Induce the Serotonergic Neuron Phenotype During In Vitro Differentiation of Embryonic Stem Cells

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Key Words. Embryonic stem cells • Neural differentiation • Serotonergic neurons • Tissue-specific expression • Lineage commitment • Neural development

ABSTRACT

Investigation of serotonergic neuronal activity and its relationship to disease has been limited by a lack of physiologically relevant in vitro cell models. Serotonergic neurons derived from embryonic stem cells (ESCs) could provide a platform for such studies and provide models for use in drug discovery. Here, we report enhancement of serotonergic differentiation using a genetic approach. Expression of *Gata2* increased the yield of serotonergic neurons. Enhancement was only achieved when *Gata2* was expressed under the control of the tissue-specific promoter of the transcription factor *Nkx6.1*. High levels of *Gata2* expression in ESCs compromised pluripotency and induced non-neuronal differentiation. Combined directed expression of *Gata2*, proneural gene *Mash1*, and forkhead transcription factor *Foxa2* further enhanced serotonergic neural differentiation, resulting in a 10-fold increase in serotonin content. These neurons were also capable of depolarization (KCl, 30 mM)-induced elevations of intracellular Ca^{2+}. The presence of sonic hedgehog during differentiation produced a further modest increase in numbers (1.5-fold). Transgene expression did not influence the number of tyrosine hydroxylase positive neurons in the cultures after 20 days, implying that *Gata2*, *Mash1*, and *Foxa2* modulate in vitro differentiation at a time beyond the decision-point for dopaminergic or nondopaminergic commitment. This study demonstrates that the directed expression of specific transcription factors enhances serotonergic neuron differentiation in vitro and highlights the importance of transgene expression at the right stage of ESC differentiation to effect the generation of a desired neural subtype. STEM CELLS *2011;29:928–939*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

5-Hydroxytryptamine (5-HT or serotonin) neurons develop in mice between embryonic day E10.75 and E12 from the ventral most neuroepithelial domain of the hindbrain, immediately after it has produced brachiomotor and visceromotor (BM/VM) neural precursors [1, 2]. This complex of cells is subdivided into rostral (r1–r3) and caudal (r5–r7) groups by a region that lacks any 5-HT neurons (r4). In r4, the generation of facial brachial motor neurons carries on until E12.5 and no serotonergic differentiation occurs [2], whereas in ventral r1, where the first 5-HT neurons appear, no BM/VM neuronal differentiation takes place at all [2]. Three major growth factors, sonic hedgehog (Shh) and fibroblast growth factors (FGF) 4 and 8, have been associated with the emergence of 5-HT neurons. In particular, Shh is obligatory for the patterning of all central nervous system 5-HT neurons in vivo [3]. Some 5-HT subpopulations also display a requirement for FGF family members. FGF8 secreted by the midbrain-hindbrain organizer (MHO) supports the induction of rostral 5-HT neurons but not of 5-HT neurons located more caudally [4]. Furthermore, it is assumed that FGF4, secreted from the primitive streak, prepatterns neural progenitors caudally of the MHO so that exposure to Shh and FGF8 leads to the induction of 5-HT neurons.

Our understanding of the genetic cascades that regulate the differentiation of 5-HT neurons in vivo is growing but still incomplete (Fig. 1). During development, the homeodomain transcription factors *Nkx2.2* and *Nkx6.1* are among the earliest genes transiently expressed in all future 5-HT neurons [2, 5]. The classical proneural gene *Mash1* is expressed in Nkx positive precursors, although in r2–r3 and r5–r7 its proneural activity is suppressed by *Phox2b* [6]. Early expression of the forkhead transcription factor *Foxa2* in r1 prevents expression of *Phox2b* and motor neuron differentiation, while delayed expression of *Foxa2* in r2–r3 and r5–r7 down regulates *Phox2b* expression and allows the switch from motor neuron to 5-HT neuron differentiation [7]. Postmitotically, the differentiation of all 5-HT neurons is marked by the expression of zinc finger (*Gata2* and *Gata3*), homeodomain (*Lmx1b*), and Ets domain (*Pet1*) transcription factors [6].

Author contributions: C.M.N.: collection and assembly of data, manuscript writing; J.M.H. and C.W.P.: conception and design, manuscript writing.

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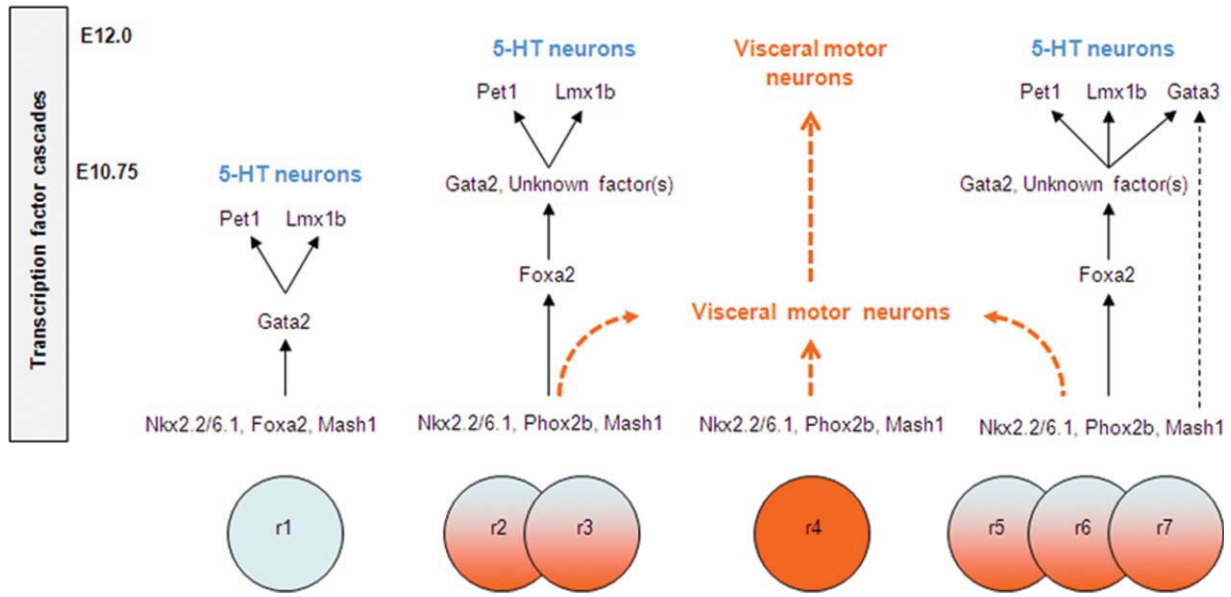

Figure 1. Overview of 5-hydroxytryptamine (5-HT) neuron differentiation. Schematic representation of the transcription factor cascades and growth factors implicated in specification of rostral (r1–r3) and caudal (r5–r7) 5-HT neuron groups (see text for more details). Abbreviations: 5-HT, 5-hydroxytryptamine; r1–r3, rostral 5-HT neuron group; r5–r7, caudal 5-HT neuron group.

Gata2, unlike *Gata3*, is required for the differentiation of all 5-HT neurons and activates expression of *Lmx1b* and *Pet1* at least in r1 [5, 6]. *Gata3* is only critical for the development of caudal groups; *Gata2* and to lesser extend *Mash1* have been implicated in its activation [5, 8].

To date, there have been very few attempts to enrich for embryonic stem cell (ESC)-derived 5-HT neurons under defined conditions, and in many cases, the differentiation of this neural phenotype has been observed as an unwanted by-product of dopaminergic differentiation protocols [9–11]. Enhancing 5-HT neuron differentiation from ESCs has been most notably achieved by treatment with growth factors [10, 12], in the presence of serum [10], or in coculture with stromal cell lines [13]. However, the variability of animal serum batches and undefined stromal cell-derived inducing activity make serum free methods, with defined media, the most attractive approach to investigate the basic mechanisms of ESC differentiation. In this study, we investigate whether the over-expression of transcription factors essential for the differentiation of 5-HT neuron in vivo increases the yield of 5-HT neurons from ESCs. Monolayer culture, producing only a few 5-HT positive cells in the absence of genetic modifications, was employed as a model for neuronal cell differentiation under chemically defined conditions [14]. After initial experiments with lentiviral constructs constitutively expressing a gene of interest, the use of transient tissue-specific promoters was explored for transgene expression at a particular stage of the differentiation process (directed expression). Using this approach, we show that expression of *Gata2* significantly enhanced 5-HT neuron differentiation. In addition, the combined directed expression of *Gata2*, *Mash1*, and *Foxa2* produced a further 10-fold increase in 5-HT neuron differentiation.

Materials and Methods

Neural Differentiation

Mouse ESCs (mESCs) (E14-tg2a) were maintained in Glasgow minimum essential medium supplemented with 10% fetal bovine serum (ES Cell qualified), 2 mM L-glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 0.1 mM β-mercaptoethanol, 3 mM sodium bicarbonate, and 10^3 units per milliliter leukemia inhibitory factor (LIF). All reagents were purchased from Invitrogen (Melbourne, Australia, www.invitrogen.com) unless stated otherwise. mESCs were differentiated in N2B27 as described previously [14]; briefly, 2.0×10^3 cells were seeded into wells of a gelatin-coated 96-well plate (Corning, Melbourne, Australia, www.corning.com) in mESC maintenance media for 24 hours; differentiation was induced by switching to N2B27 media. The media was supplemented with the differentiation/growth factors at 34, 200, or 1,000 ng/ml of Shh, 20 ng/ml of FGF2, 20 ng/ml of FGF4, 100 ng/ml of FGF8 (all Peprotech, Rehovot, Israel, www.peprotech.com), or 300 ng/ml of retinoic acid (Sigma-Aldrich, Sydney, Australia, www.sigmaaldrich.com/australia.html) as indicated.

Cloning of Genes of Interest

Genes of interest (Supporting Information for primer sequences) were cloned into Gateway compatible entry vector pDonR201 (Invitrogen). After sequence verification, genes were shuttled into the viral expression construct.

Modification of Viral Expression Constructs

The original viral expression vector pLenti6.2/N-Lumio/V5-DEST (Invitrogen) was extensively modified (see Supporting Information). In brief, the blasticidin selection cassette of the vector was removed with a KpnI digest and a Woodchuck post-transcriptional regulatory element was ligated into the BstBI site to boost expression levels. In addition, site directed mutagenesis (Quikchange, Agilent Technologies, Melbourne, Australia, www.genomics.agilent.com) was used to introduce an AscI site between the cytomegalovirus promoter and the gateway cassette of the vector to allow the cloning of promoter fragments [ePet1, elongation factor 1 α (EF1 α), pNkx2.2, and pNkx6.1—see Supporting Information for primer sequences] after a NheI/AscI digest. Furthermore, a NdeI restriction site was introduced (Quikchange) at the start codon of gateway cassette (N-terminal expression vector) to allow the insertion of green fluorescent protein (GFP) or β-lactamase (BLA) trailed by a 2A-like (T2A) sequence at the 3′ end (see Supporting Information for primer sequences).

Viral Particle Production and Determination of Viral Titer

See Supporting Information for a detailed description.

Transduction of mESCs

Depending on the intended average integration number in the population (= mean occurrence of infection; MOI) an appropriate amount of viral concentrate was used, e.g., for MOI = 100, 2×10^4 cells were exposed to 2×10^6 transducing units. Five hours before transduction, 2×10^4 mESCs were seeded into each well of a gelatin coated 48-well plate. After cell attachment, the media was switched for 24 hours to mESC media containing the lentiviral particles and polybrene (6 μg/ml). For transduction with a single construct, cells were infected at MOI = 100, and for cotransduction with multiple constructs, mESCs were simultaneously exposed to viruses of each batch at MOI = 50.

Immunocytochemistry

Cells were fixed in 4% paraformaldehyde, blocked in 5% bovine serum albumin, and exposed to primary antibodies (see Supporting Information for a list of the primary antibodies used) overnight (4°C); and later labeled with secondary antibodies donkey anti-rabbit-AlexaFluor594 (1:500, Invitrogen), donkey anti-mouse-AlexaFluor488 (1:500, Invitrogen), and donkey anti-chicken fluorescein isothiocyanate (1:500, Millipore, Melbourne, Australia, www.millipore.com). After exposure to 4,6-diamidino-2-phenyindole at 0.5 μg/ml (5 minutes), wells were washed and used for imaging.

Immunoblotting

See Supporting Information for a detailed description.

Imaging/Cell Counting

Images were taken with a CoolSNAP-FX camera (Photometrics, Tucson, AZ, www.photometrics.com) attached to an Eclipse TE-2000E microscope (Nikon, Sydney, Australia, www.nikon.com). The total number of 5-HT positive cells per well from 96-well plates were manually counted in labeled day 12 cultures. On day 20, tyrosine hydroxylase (TH) and 5-HT positive cells were manually counted in at least two fields of view per well at ×4 magnification. Note: In experiments where transcription factors were expressed under control of the EF1 α promoter, four fields of view were counted (top, bottom, right, and left fields); in experiments where transgene expression was under control of the transcription factor promoter of *Nkx6.1*, two fields of view were counted (top and bottom fields).

Enzyme-Linked Immunosorbent Assay (ELISA)

For quantification of 5-HT content, cells were lifted from 96-well plates on day 20 of differentiation by vigorous titrating in a volume of 100 μl N2B27 media (per well) and sonicated (Vibra cell, Sonics & Materials Inc., Newtown, CT, www.sonicsandmaterials.com) for 30 second. Cell lysates were analyzed for 5-HT content using a 5-HT ELISA kit (Alpco, Salem, NH, www.alpco.com) according to manufacturer's instructions.

Calcium Imaging

Calcium imaging was undertaken as described previously [15]. Briefly, cells were washed twice with HEPES buffer (containing in mM; NaCl 145; 3 KCl 5; $MgSO_4$ 1; HEPES 10; D-glucose 10; $CaCl_2$ 2.5) at pH 7.4, and incubated with FLUO-4AM (10 μM, Invitrogen) in HEPES buffer for 30 minutes at 37°C. After incubation, cells were viewed using a Nikon Eclipse Ti camera (Nikon) coupled to an A1 Nikon confocal microscope (Nikon). Cells were illuminated at 488 nm and emission was recorded at ~515 nm. Cellular fluorescent intensities were captured with NIS-elements-AR software (Nikon) 30 times per second. Background fluorescence was subtracted from each neuron, and fluorescence intensity was calculated. During experiments, cultures were first exposed to vehicle control and then KCl (30 mM).

Draq5/Sapphire700 Labeling

To quantify well to well variability in cell density, cultures were labeled with fluorescent nuclear dyes Draq5 and Sapphire700 (both from Li-Cor Biotechnology, Lincoln, NE, www.licor.com) according to manufacturer's instructions after immunocytochemistry/imaging were performed. Emission values were recorded with the 700 nm channel of the Odyssey Infrared Imaging system (ODY-18090) (Li-Cor Biotechnology). Values are presented as a percentage of wild-type control.

Flow Cytometry

Single cell suspension analysis was performed with an FACS-Canto II analyzer (BD Biosciences, Sydney, Australia, www.bdbiosciences.com). Before analysis, cell suspensions were cleared of clumps with a 70-μm strainer (BD Biosciences). For assessing BLA activity, wild-type cells (BLA negative) were used to set gates.

Detection of BLA Activity

The LiveBlazer FRET–B/G Loading Kit (Invitrogen) was used to stain live cells at room temperature (2 hours). For flow cytometry, approximately one million cells were labeled in 500 μl LiveBlazer ×1 assay solution (dilutant: phosphate-buffered saline [PBS]). For visualization under a fluorescent microscope or detection with a EnVision 2,101 Multilabel fluorescence plate reader (PerkinElmer, Melbourne, Australia, www.perkinelmer.com/AU) cells were washed (PBS) and exposed to 100 μl LiveBlazer ×1 assay solution. For imaging and plate reader detection (bottom read mode), cells were excited at 405 nm and emission detected at 450 nm (product) and 520 nm (substrate). Background values were derived from untransfected wild type cells; these values were subtracted from those of the labeled cells. To normalize, plate reader data values are expressed as a ratio of 450:520 nm.

Statistical Analysis

All results are presented as mean ± SEM of at least three separate experiments. Statistical analysis was performed on raw data using PRISM v5.00 (GraphPad Software, La Jolla, CA, www.graphpad.com). Data were analyzed by Student's t test or one-way analysis of variance with post hoc Dunnett's test or Bonferroni's multiple comparison test. In all cases, $p < .05$ was considered to be significant.

RESULTS

To monitor the 5-HT neuronal differentiation process in the absence of forced transcription factor expression, mESCs were transduced with a construct expressing BLA under control of the *Pet-1* enhancer element (pPet1; Fig. 2A), a promoter element selectively active in 5-HT neurons in vivo [16]. Analysis of BLA activity showed increased construct activity beyond day 10 of the differentiation process (Fig. 2B). Consistent with this, immunolabeling revealed that 5-HT positive cells could be detected by day 12 in untransfected wild-type cells. Given that the occurrence of 5-HT neurons and motor neurons is closely linked in most rhombomeres in vivo, we investigated whether the ventral motor neuron marker *Islet-1* [17] was also expressed in developing wild-type cultures. Early 5-HT positive cells were found in proximity to Islet-1 positive cells (Fig. 2C, 2D). Areas of the culture devoid of Islet-1 positive cells also lacked 5-HT positive

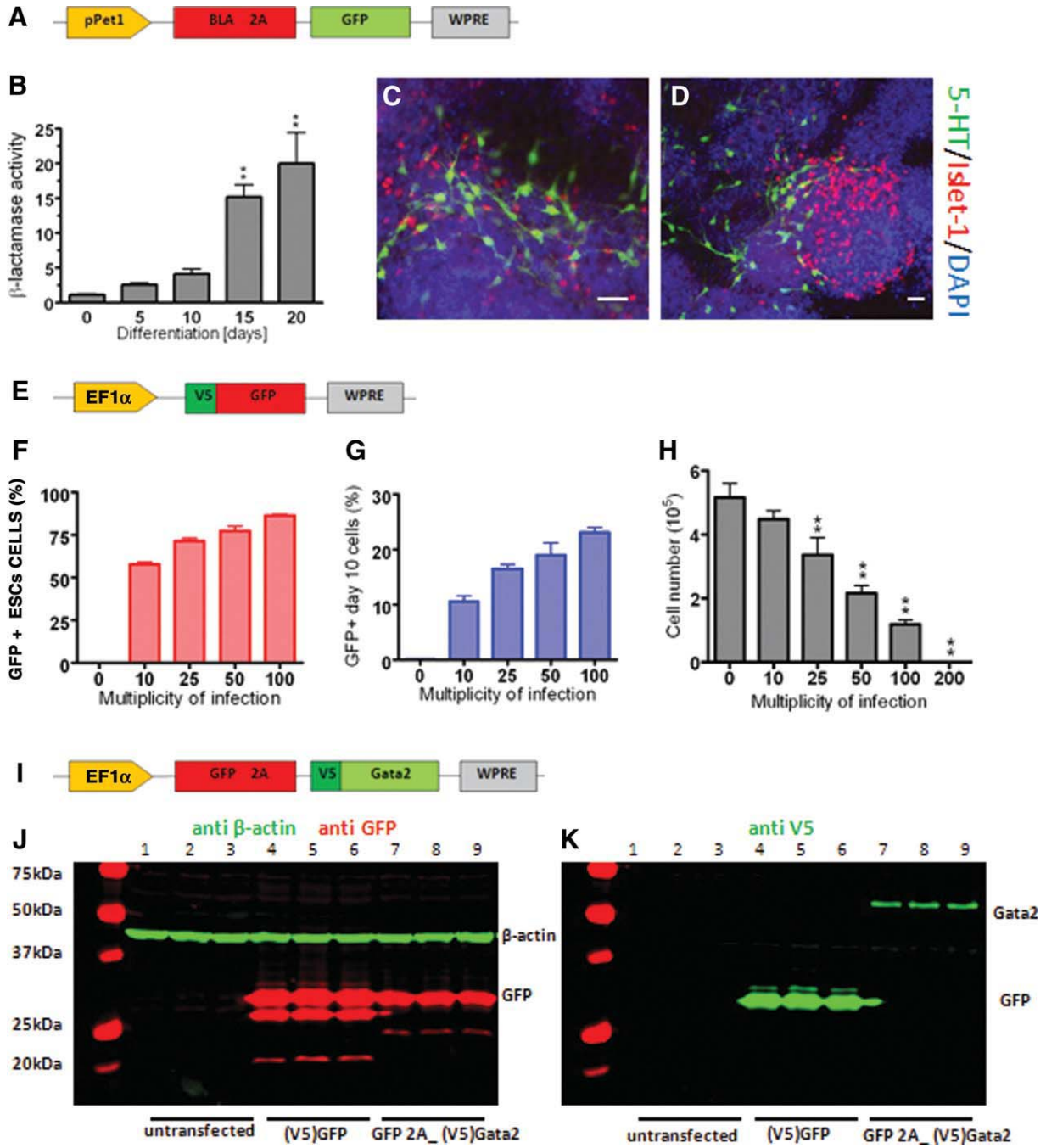

Figure 2. 5-Hydroxytryptamine (5-HT) neuron differentiation in N2B27 and transgene delivery into mouse embryonic stem cells (mESCs). **(A):** Outline of a lentiviral construct expressing β-lactamase (BLA) and green fluorescent protein (GFP) under control of the *Pet1* enhancer element (pPet1). **(B):** BLA activity on days 0, 5, 10, and 20 of the differentiation process after transduction of mESCs with the pPet1 construct (multiplicity of infection = 100). ** at $p < .01$, one-way analysis of variance (ANOVA) plus post hoc Dunnett's test when compared with day 0 values. **(C, D):** Early 5-HT neuron populations (day 14) were either **(C)** permeated by or **(D)** in proximity to clusters of cells positive for the motor neuron marker Islet-1. **(E):** Viral particles of a construct expressing GFP were used for testing transduction efficiency in mESCs. **(F):** Percentage of GFP positive cells at 5 days following transduction. **(G):** Percentage of GFP positive cells after 10 days of differentiation in N2B27 ($n = 3$). **(H):** Effect of lentiviral transduction on cell proliferation at 5 days after transduction. ** indicates $p < .01$, one-way ANOVA, post hoc Dunnett's test. **(I):** For over-expression of transcription factors, 2A-mediated bicistronic constructs expressing GFP (no tag) and a V5-tagged gene of interest (here: Gata2) were used. **(J, K):** Separate expression of protein products was verified by Western Blot analyses: lanes 1–3: control; lanes 4–6: monocistronic expression of V5-tagged GFP; lanes 7–9: bicistronic expression of untagged GFP and V5-tagged Gata2. **(J):** Probing with anti-GFP antibody (red) and for housekeeping protein β-actin (green); **(K):** Reprobing of the membrane with anti-V5 (green). GFP = 27 kDa, β-actin = 42 kDa, Gata2 = 53 kDa. Scale bars = 150 μm. Abbreviations: BLA, β-lactamase; DAPI, 4,6-diamidino-2-phenyindole; EF1 α, elongation factor 1 α; GFP, green fluorescent protein; 5-HT, 5-hydroxytryptamine; pPet1, *Pet-1* enhancer element; WPRE, Woodchuck post-transcriptional regulatory element.

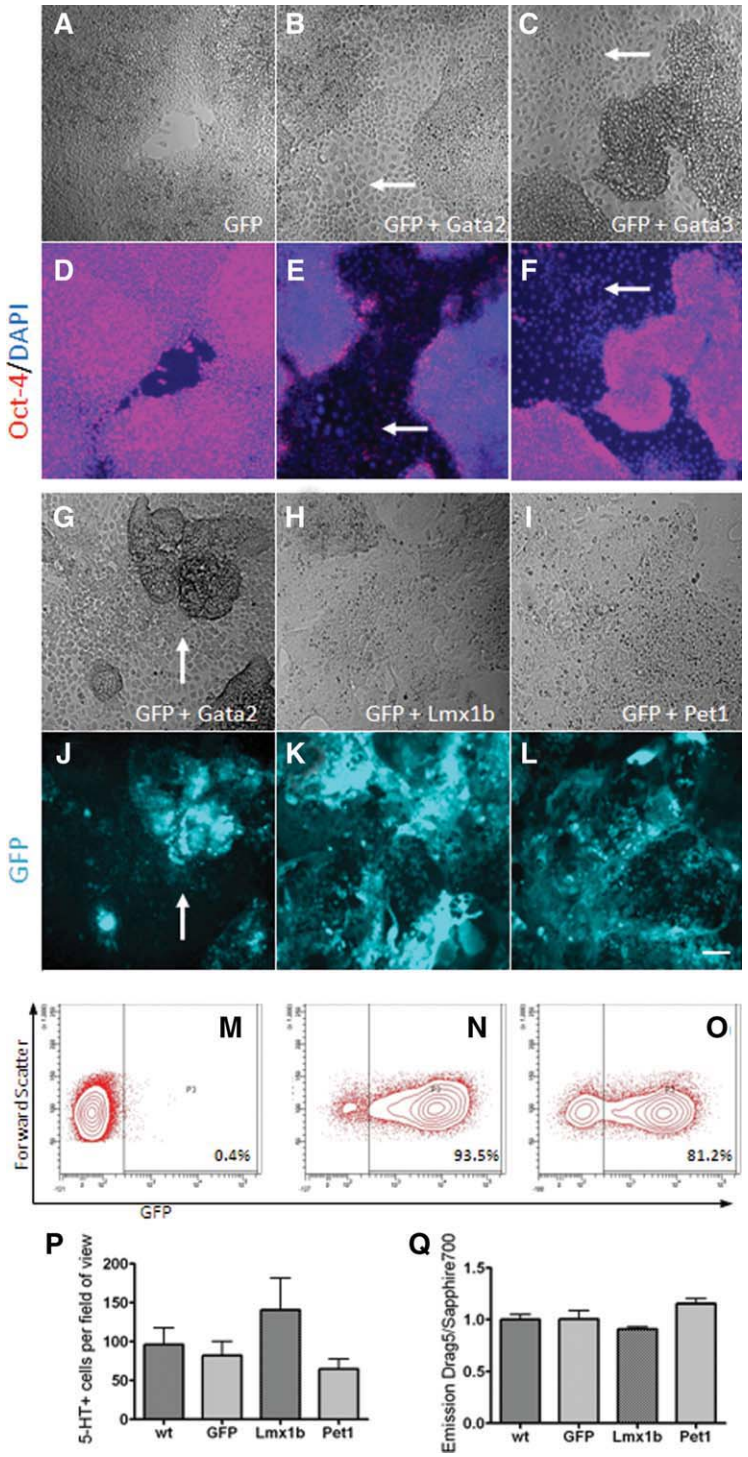

Figure 3. Transgene expression under control of elongation factor 1 α. Phase contrast images 5 days posttransduction with **(A)** the monocistronic green fluorescent protein (GFP) vector or with the bicistronic construct expressing **(B)** *Gata2* or **(C)** *Gata3* together with GFP; **(D, E)** colabeling with 4,6-diamidino-2-phenyindole/anti-Oct-4 on the same cells. White arrows indicate enlarged cells, morphologically distinct from mouse embryonic stem cells (mESCs). **(G–I):** White light images of mESCs transduced with the bicistronic vector expressing **(G)** *Gata2*, **(H)** *Lmx1b*, or **(I)** *Pet1* with corresponding images of GFP fluorescence below [Panels **(J)**, **(K)**, and **(L)**, respectively]. White arrows indicate culture sections in *Gata2* transduced cultures with mESCs morphology (*Gata3* transduced cultures looked identical, not shown). Panels **(M)**, **(N)**, and **(O)** show typical flow cytometry plots of GFP expression in wild-type, *Lmx1b* or *Pet1* transduced cells, respectively, 10 passages after transduction. Differentiation of these cells for 20 days did not affect numbers of 5-hydroxytryptamine positive cells **(P)** or the total number of cells **(Q)** when compared with wild-type cells and GFP only control. $p > .05$, one-way analysis of variance plus post hoc Dunnett's test. Scale bars = 150 μm. Abbreviations: DAPI, 4,6-diamidino-2-phenyindole; GFP, green fluorescent protein; 5-HT, 5-hydroxytryptamine; pPet1, *Pet-1* enhancer element.

cells. However, no Islet-1 positive cells could be detected in cultures on day 20 (data not shown), and we believe that the Islet-1 positive cells visualized during the early stages of differentiation might not have survived extended in vitro culture.

EF1 α Promoter Driven Expression of *Gata2* or *Gata3* Compromises ESC Pluripotency

To investigate the role of individual transcription factors previously linked to ectopic 5-HT neuron differentiation in vivo [5, 18], mESCs were separately transduced with viral constructs expressing *Gata2*, *Gata3*, *Lmx1b*, or *Pet1* under control of the constitutively active human EF1 α promoter. First, we established a reporter system to track construct activity. With a lentiviral monocistronic test construct (Fig. 2E), we routinely achieved GFP expression in over 85% mESCs (MOI = 100), although 10 days into the differentiation process, the GFP positive population dropped to under 25%. ESC cultures did not survive transduction at very high infection rates (MOI = 200) (Fig. 2F–2H). For over-expression of transcription factors, a picornaviral 2A-like sequence [19] was used to link GFP to genes of interest (Fig. 2I). This allowed the expression of two separate protein products (Fig. 2J, 2K) and enabled us to track construct activity before and during differentiation. Transduction of mESCs (at MOI = 100) with either *Gata2* or *Gata3* expressing constructs (unlike with a construct expressing GFP alone) resulted in gross morphological changes within 5 days under mESC maintenance conditions (Fig. 3A–3C), coinciding with reduced expression levels of the pluripotency marker *Oct-4* (Fig. 3D–3F). At this stage, GFP expression in *Gata2* or *Gata3* transduced cultures was mostly confined to areas retaining ESC morphology (Fig. 3G, 3J), potentially because of construct silencing in differentiated cells [20]. After 2–3 passages under ESC maintenance conditions, areas with ESC morphology were lost and, hence, cultures were not used for differentiation experiments. In contrast to the effects of *Gata2/3*, transduction with *Lmx1b* or *Pet1* did not induce morphological changes. GFP positive cells were also homogeneously distributed (Fig. 3H–3L) and still detectable in over 80% of mESCs after 10 passages (Fig. 3M–3O). Cultures of the *Lmx1b* or *Pet1* transduced mESCs did not produce more 5-HT neurons after 20 days of differentiation (Fig. 3P). To eliminate the possibility that transduction affected growth rates cultures were labeled with fluorescent nuclear dyes (Draq5 and Sapphire700) and resultant emission values were not different from wild-type controls ($p > .05$) indicating no major changes in cellular proliferation or survival (Fig. 3Q).

An *Nkx6.1* Promoter Fragment Allows the Directed Expression of *Gata2* or *Gata3* Without Inducing Differentiation at the ESC Stage

To investigate the role of *Gata2* and *Gata3* transcription factors in 5-HT neuron differentiation in the absence of induced differentiation by high transgene levels at the stem cell stage we explored the use of *Nkx2.2* and *Nkx6.1* promoter fragments [21, 22]. Both homeodomain proteins are transiently expressed in serotonergic precursors [2, 23] but are suppressed in dopaminergic precursors [24] and we reasoned that their promoters might allow a more context-specific expression of *Gata* transcription factors during the differentiation process. BLA, a catalytic reporter that can be detected even at low levels of expression in live cells [25], was coexpressed with a gene of interest instead of GFP (Fig. 4A). This approach was taken because the *Nkx2.2* and *Nkx6.1* promoter fragments were not able to produce detectable levels of GFP expression at the ESC stage or during differentiation (data not shown). Initial experiments with test constructs (reporter expression only; Fig. 4B) showed that the *Nkx2.2* promoter fragment did not result in BLA expression during the first 12 days of differentiation (Fig. 4C). In contrast, BLA expression under the *Nkx6.1* promoter (pNkx6.1), showed increased activity during differentiation, with activity peaking on day 6 ($p < .01$) (Fig. 4C). At high infection rates (MOI = 100) up to 80% of cells registered BLA positive on day 6 of differentiation (Fig. 4D). Although Nkx6.1 is a class II homeodomain protein (e.g., expression is induced by Shh), the continuous presence of Shh (200 ng/ml) during differentiation did not increase the percentage of BLA positive cells on day 6 (Fig. 4E). Visualization of BLA activity in developing cultures (MOI = 35, day 6) showed positive cells in clusters rather than evenly distributed in the culture (Fig. 4F).

The pNkx6.1 vector construct allowed the expression of *Gata2* or *Gata3* in mESCs (MOI = 100) without inducing differentiation. Even after 10 passages under ESC growth conditions, virtually all cells continued to be Oct-4 positive (Fig. 4G–4J), although 24.9% and 39.7% of cells were positive for reporter BLA, respectively, indicating *Gata2* or *Gata3* expression (Fig. 4K–4M).

Directed Expression of *Gata2* or *Gata3* Affects 5-HT Neuron Differentiation After 20 Days of Monolayer Culture

In addition to *Gata2* and *Gata3*, the effects other genes implicated in serotonergic neuron differentiation were also investigated by introducing *Foxa2*, *Mash1*, *Nkx2.2*, *Lmx1b*, and *Pet1* into mESCs using the pNkx6.1 vector. mESCs were also cotransduced with *Lmx1b* and *Pet1*, a gene combination previously implicated with ectopic 5-HT neuron differentiation in vivo [18]. Expression of *Gata2* or *Lmx1b*/*Pet1* increased the number of 5-HT positive cells ($p < .01$) by day 12 of the differentiation process. In contrast, *Gata3* decreased 5-HT neuron differentiation ($p < .05$) when compared with both wild-type and GFP controls (Fig. 5A, 5C–5E). These changes were not due to differences in cell density (Fig. 5B). Observations for *Gata2* and *Gata3* cultures on day 12 were reflected in the numbers of 5-HT neurons at day 20, whereas *Lmx1b*/*Pet1* cultures did not produce significantly more 5-HT neurons when compared with controls at this stage (Fig. 5F–5G). Directed expression of *Gata2*, *Gata3*, or *Lmx1b*/*Pet1* did not affect the proportion of dopaminergic neurons in the differentiated cultures as shown by immunolabeling of day 20 cultures for TH (Fig. 5H).

Directed Coexpression of *Gata2*, *Mash1*, and *Foxa2* Synergistically Increases 5-HT Neuron Differentiation

Given that the directed expression of *Gata2* alone increased the number of 5-HT neurons, we explored whether coexpression with other transcription factors could further increase the number of serotonergic neurons. *Mash1* and *Foxa2* have been implicated in the activation of downstream determinants of the 5-HT neuron phenotype [7, 8]. Coexpression of *Gata2* with either one of *Mash1* or *Foxa2* did not further increase the occurrence of 5-HT neurons. However, the combined expression of all three transcription factors significantly increased the number of 5-HT neurons when compared with expression of *Gata2* alone ($p < .001$) (Fig. 6A, 6B), while the number of TH positive cells was unaffected (Fig. 6C–6F). Expression of *Gata2* alone or the combined expression of *Gata2*, *Mash1*, and *Foxa2* did not disrupt neural conversion as evidenced by the expression of early neural markers Pax6 and Nestin (day 6) when compared with wild-type controls (Supporting Information Fig. S1A–S1F). 5-HT positive cells produced by the combined expression of *Gata2*, *Mash1*, and *Foxa2* colabeled for neuronal markers TUJ-1, Map2, and neurofilament (Fig. 6G–6I). By day

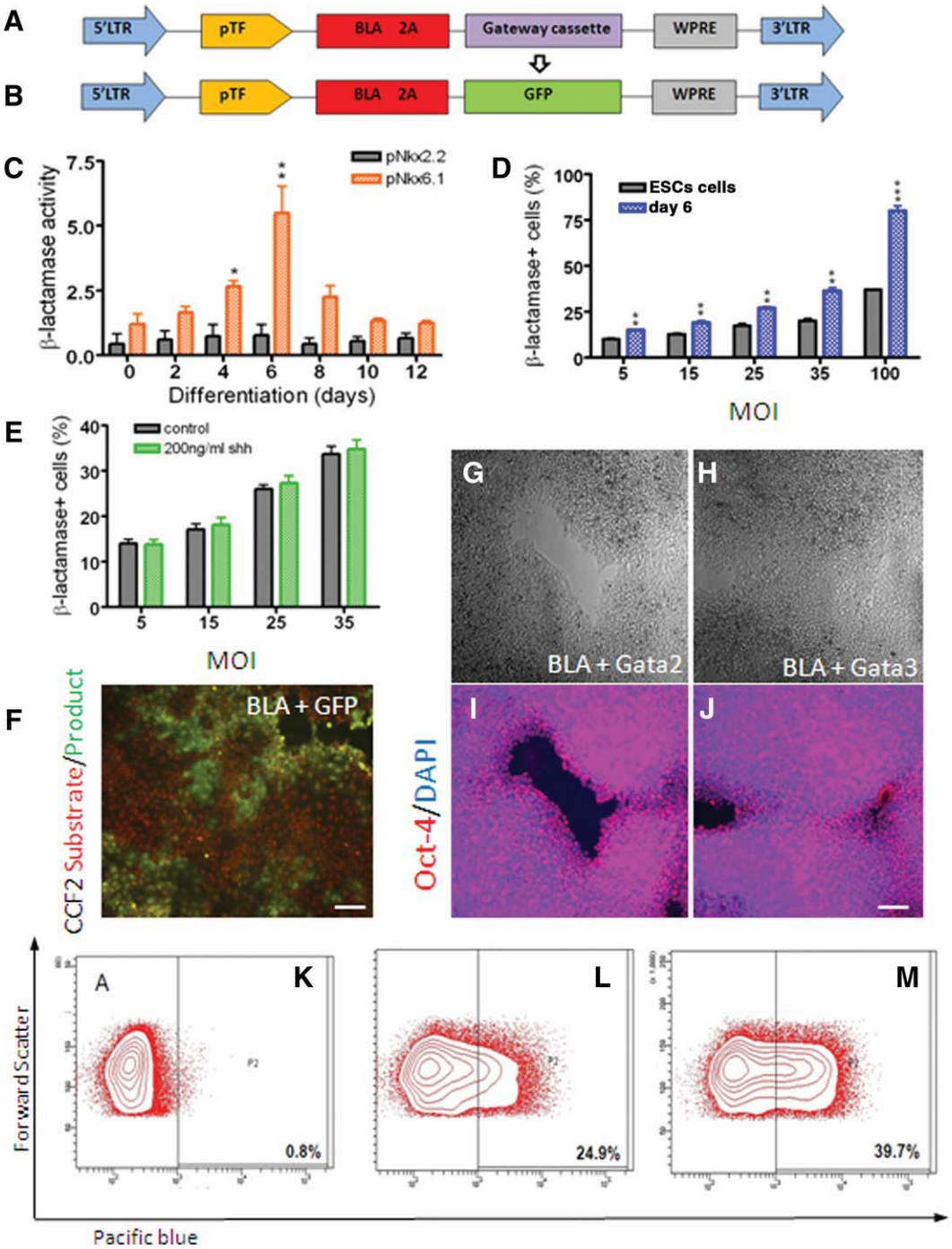

Figure 4. Transgene expression under the control of transcription factor promoters (pTF). **(A):** Vector outline of viral constructs with pTFs (e.g., fragments of the *Nkx2.2* or *Nkx6.1* promoter). The expression of *β*-lactamase (BLA) was 2A-linked to a gateway cassette. **(B):** Green fluorescent protein was cloned into the gateway cassette for initial experiments (test constructs). **(C):** BLA activity during differentiation of mouse embryonic stem cells (mESCs) transduced with the pNkx2.2 or pNkx6.1 test constructs at a multiplicity of infection (MOI) of 100. * and ** indicate $p < .05$ and $p < .01$, respectively, when compared with day 0 values with one-way analysis of variance plus post hoc Dunnett's test ($n = 4$). **(D):** Percentage of BLA positive cells in mESC cultures transduced with the pNkx6.1 test construct before, and on day 6 of the differentiation process. ** and *** indicate $p < .01$ and $p < .001$, respectively, Student's t test ($n = 3$). **(E):** The effect of continuous sonic hedgehog (200 ng/ml) exposure during differentiation of mESCs transduced with the pNkx6.1 construct on the percentage of BLA positive cells on day 6 when compared with control cells exposed to N2B27 only. $p > .05$, Student's t test ($n = 3$). In panels **(C)**, **(D)**, and **(E)** the bars represent the mean ± SEM of three replicate experiments. **(F):** Visualization of BLA activity (green) on day 6 of differentiation; the mESCs were transduced at MOI = 35. Panels **(G)** and **(H)** show phase contrast images of mESCs transduced with pNkx6.1 constructs expressing *Gata2* or *Gata3*, with respective anti-Oct-4/4,6-diamidino-2-phenyindole labeling below [Panels **(I)** and **(J)**]. Panels **(K)**, **(L)**, and **(M)** show typical flow cytometry plots of *β*-lactamase expression (CCF2 product detected via pacific blue channel) in wild-type, *Gata2* or *Gata3* transduced cells, respectively, after 10 passages. Scale bars = 150 *μ*m. Abbreviations: BLA, *β*-lactamase; CCF2, 7-hydroxycoumarin linked to fluorescein via a cephalosporin core; DAPI, 4,6-diamidino-2-phenyindole; GFP, green fluorescent protein; LTR, long terminal repeats; MOI, mean occurrence of infection; pTF, transcription factor promoters; shh, sonic hedgehog; WPRE, Woodchuck post-transcriptional regulatory element.

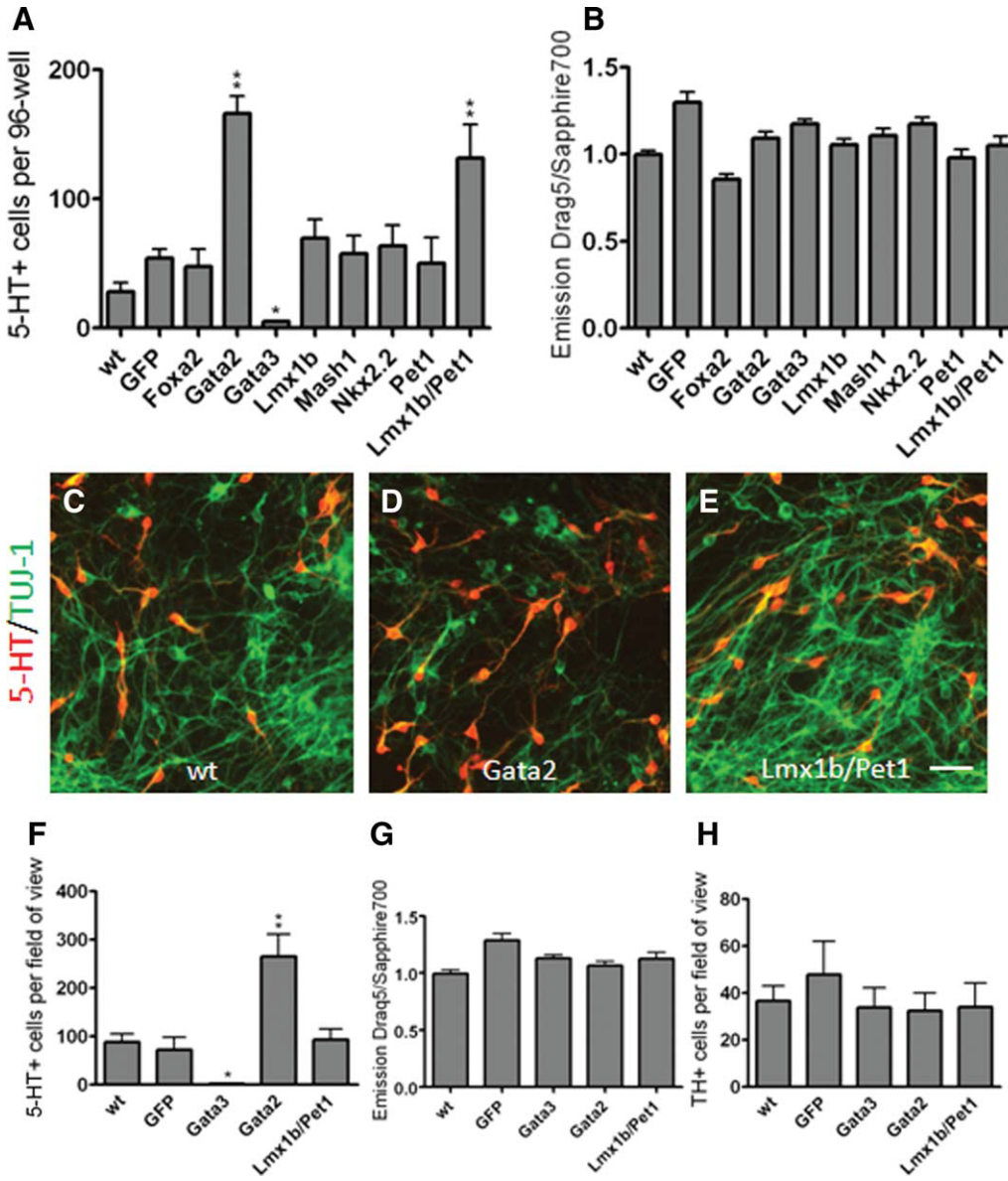

Figure 5. Impact of directed transcription factor expression (using the pNkx6.1 construct) on 5-hydroxytryptamine (5-HT) neuron differentiation. **(A):** Numbers of 5-HT/TUJ-1 positive cells per 96-well plate after 12 days of differentiation in N2B27 and **(B)** corresponding emission values for nuclear dyes Draq5/Sapphire700. **(C–E):** 5-HT/TUJ-1 coimmunolabeling of untransduced wild-type cells, *Gata2* or *Lmx1b/Pet1* expressing cells on day 12 of differentiation. **(F):** Numbers of 5-HT positive cells after 20 days of differentiation in N2B27. **(G):** Shows Sapphire700/Draq5 emission values and **(H)** the number of tyrosine hydroxylase positive cells in transduced cultures (day 20). ** at $p < .01$, * at $p < .05$ one-way analysis of variance plus post hoc Dunnett's test. All bars show the mean ± SEM of four replicate experiments. In panels **(F)**, **(G)**, and **(H)**, only bars that are significantly different from both wild-type and green fluorescent protein controls are indicated. Scale Bar = 100 μm. Abbreviations: 5-HT, 5-hydroxytryptamine; GFP, green fluorescent protein.

20 the majority of cells in these cultures labeled strongly for neuronal markers (Supporting Information Fig. S2A, S2B); only few GFAP and Nestin positive cells could be detected (Supporting Information Figure S2C, S2D). Cells positive for endodermal markers HNF-4 or Pdx-1 or mesodermal markers cardiac alpha myosin heavy chain or smooth muscle alpha actin could not be detected. An ELISA performed on cell lysates of terminally differentiated wild-type, *Gata2* and *Gata2/Mash1/Foxa2* transduced cells showed significant increases in 5-HT content for both transgenic cell lines when compared with control ($p < .001$) with *Gata2/Mash1/Foxa2* cultures containing significantly more 5-HT than *Gata2* cultures ($p < .01$) (Fig. 6J). To validate that the 5-HT neurons produced by cotransduction with three factors were functional, we performed calcium imaging to verify that depolarization (KCl 30 mM) produced Ca^{2+} influx into the cells, a hallmark of functional neurons [26]. Indeed KCl significantly ($p < .01$, Student's t test) elevated maximal intracellular Ca^{2+} (2.9 ± 0.8-fold, $n = 7$) when compared with vehicle control responses (1.4 ± 0.3-fold, $n = 7$); 5-HT positive neurons were identified retrospectively with immunocytochemistry (for typical immunolabeling and KCl responses see Fig. 6K, 6L).

To examine whether serotonergic neuron differentiation induced by *Gata2*, *Mash1*, and *Foxa2* expression could be further enhanced, we exposed differentiating cultures continuously or transiently to the growth factors implicated in the in vivo and in vitro differentiation of 5-HT neurons. Sequential exposure to Shh (days 4–6) and FGF2 (days 6-8) has been

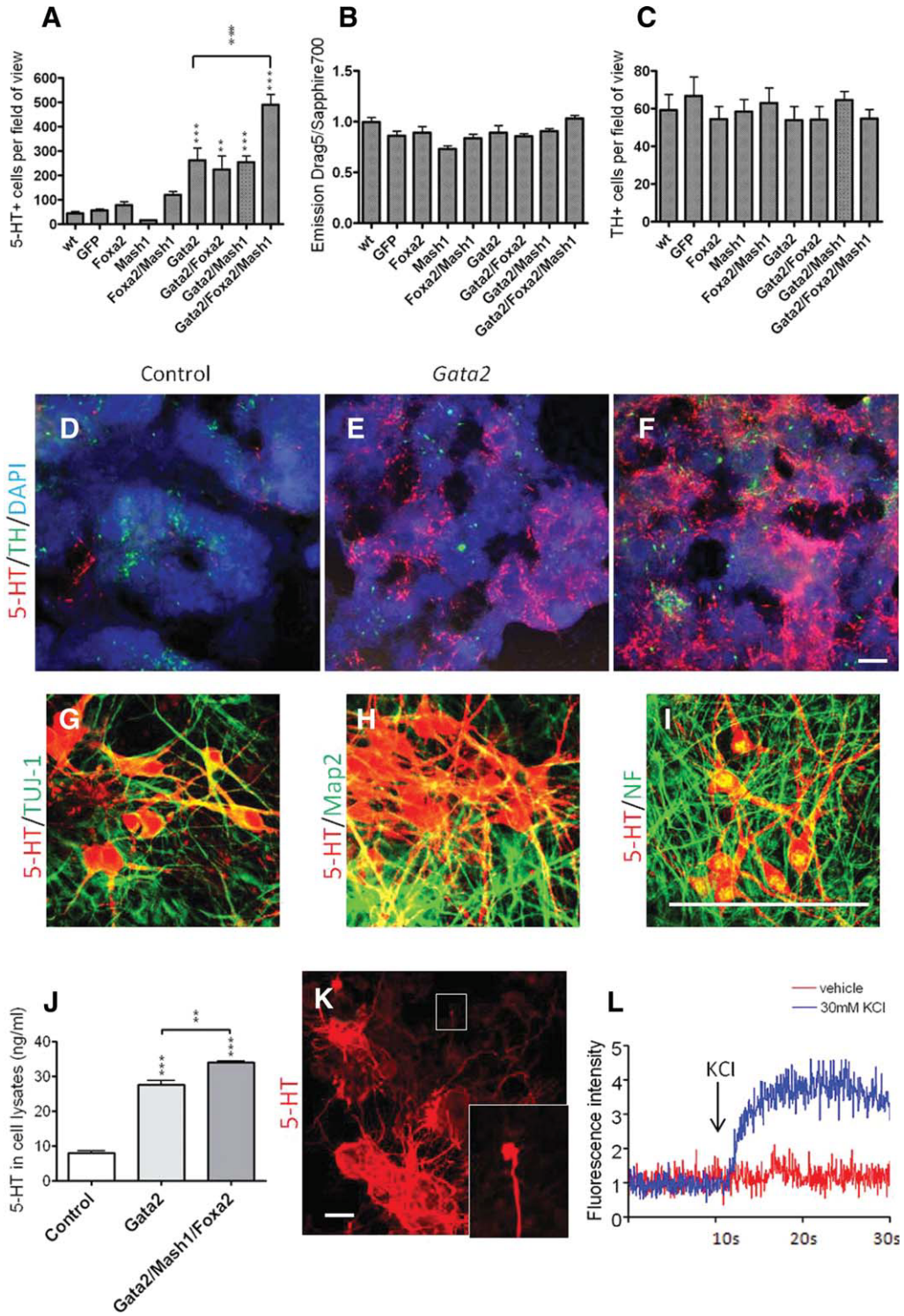

Figure 6. Directed coexpression of *Gata2* with *Foxa2* and *Mash1* using the pNkx6.1 construct. **(A):** Numbers of 5-hydroxytryptamine (5-HT) positive cells (day 20 of differentiation) for mouse embryonic stem cells (mESCs) transduced (alone or in combination) with vectors expressing *Gata2*, *Mash1*, and *Foxa2* and differentiated for 20 days in *N2B27*; **(B)** and **(C)** show corresponding graphs with Draq5/Sapphire700 emission values and numbers of tyrosine hydroxylase (TH) positive cells respectively. Immunolabeling with anti-5-HT (red), anti-TH (green), and 4,6-diamidino-2-phenyindole (blue) on day 20 of differentiation of **(D)** wild-type, **(E)** *Gata2*, and **(F)** *Gata2*/*Foxa2*/*Mash1* cultures. Immunolabeling of *Gata2*/*Foxa2*/*Mash1* cultures (day 20) with anti-5-HT (red) and **(G)** TUJ-1 (green), **(H)** Map2 (geen) or **(I)** neurofilament (green). **(J):** Wild-type mESCs (control) and mESCs transduced with *Gata2* and *Gata2*/*Foxa2*/*Mash1* were differentiated for 20 days in N2B27 and 5-HT content quantified from cell lysates. **(K, L):** Calcium imaging was performed on differentiated cultures of mESCs transduced with the three factors. Panel **(K)** shows a typical culture labeled for 5-HT with a high magnification insert of a single 5-HT neuron. Figure **(L)** depicts the neuron's response to vehicle (red) and 30 mM KCl exposure (blue). ***, $p < .001$ and **, $p < .01$ one-way analysis of variance plus post hoc Bonferroni's multiple comparison test. Note: All bars show the mean ± SEM of at least three replicate experiments. In panels **(A)**, **(B)**, and **(C)**, only bars that are significantly different from both wild-type and green fluorescent protein controls are indicated. Scale Bar = 250 μm. Abbreviations: DAPI, 4,6-diamidino-2-phenyindole; 5-HT, 5-hydroxytryptamine; GFP, green fluorescent protein; TH, tyrosine hydroxylase; NF, neurofilament.

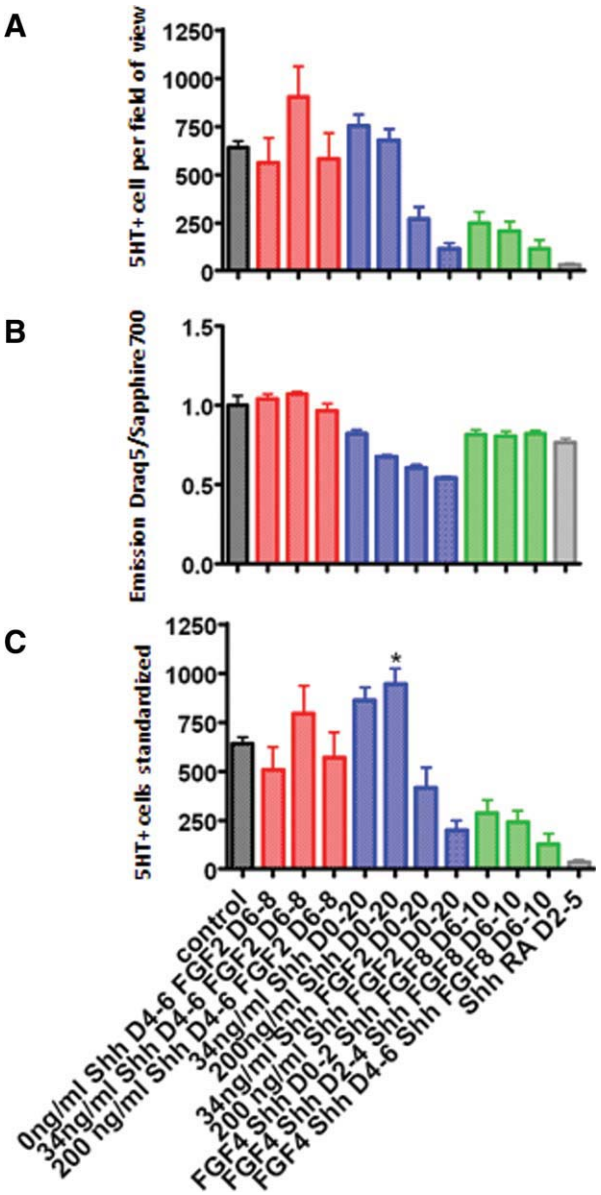

Figure 7. Effects of growth factor treatments on numbers of 5-hydroxytryptamine (5-HT) positive neurons derived from mouse embryonic stem cells cotransduced with *Gata2*, *Foxa2*, and *Mash1*. Growth factor paradigms tested from left to right: (red): Exposure to sonic hedgehog (Shh) (0, 34, or 200 ng/ml) from days 4–6, followed by fibroblast growth factor 2 (FGF2) (20 ng/ml) exposure on days 6–8. (blue): Continuous exposure to Shh (34 or 200 ng/ml) or to Shh (34 or 200 ng/ml) and FGF2 (20 ng/ml) for 20 days. (green): Exposure to Shh (34 ng/ml) and FGF4 (20 ng/ml) on days 0–2 or 2–4 or 4–6 followed by exposure to Shh (200 ng/ml) and FGF8 (100 ng/ml) on days 6–10. (gray): Exposure to retinoic acid (300 ng/ml) and Shh (1000 ng/ml) days 2–5. **(A):** Shows the numbers of 5-HT positive cells and **(B)** the corresponding Draq5/Sapphire700 emission values on day 20 of differentiation. **(C):** Compensation of 5-HT cell numbers with Sapphire700/Drag5 values revealed that only the continuous presence of Shh (200 ng/ml) significantly enhanced serotonergic differentiation when compared with control. * at $p < .05$ one-way analysis of variance plus post hoc Bonferroni's multiple comparison test. Abbreviations: 5-HT, 5-hydroxytryptamine.

suggested to increase serotonergic differentiation during monolayer culture [27]. We examined these conditions and also tested cultures with continuous exposure to Shh (34 or 200 ng/ml) ± FGF2 (20 ng/ml). To mimic in vivo differentiation of rostral 5-HT neuron clusters, we also tested whether exposure to low doses of Shh (34 ng/ml) and FGF4 (20 ng/ml) on days 0–2, 2–4, or 4–6 followed by exposure to Shh (200 ng/ml) and FGF8 (100 ng/ml) (days 6-12) increased 5-HT neuron numbers. Given the close link between 5-HT and motor neuron differentiation, we also tested a growth factor paradigm (300 ng/ml retinoic acid and 1,000 ng/ml Shh, days 2–5) suggested for motor neuron differentiation [28]. After normalizing for cell proliferation, immunolabeling and cell counting on day 20 showed that only the continuous presence of Shh (200 ng/ml) produced an increase (1.5-fold) in 5-HT cell number when compared with controls ($p < .05$) (Fig. 7D–7F).

Discussion

We have shown that directed expression of a combination of transcription factors implicated in the activation of downstream determinants of the 5-HT neuron phenotype (e.g., *Gata2*, *Foxa2*, and *Mash1*) [5–8] are able to increase the yield of ESC-derived 5-HT neurons approximately 10-fold. These transduced neurons can respond to KCl with depolarization and, consistent with evidence of an increase in cell number, there is a concomitant increase in 5-HT content in these cultures. Initial experiments demonstrated that enhanced 5-HT neuron differentiation was not achieved by expressing 5-HT developmental genes under control of the EF1 α promoter, which drove expression in ESCs and at all stages during differentiation. We observed that either *Gata2* or *Gata3* expression compromised ESC pluripotency even in the presence of LIF and serum. Consistent with our findings Gata transcription factors have been implicated in extra-embryonic endoderm differentiation of ESCs [29]. The use of the tissue-specific promoter pNkx6.1 allowed the transduction of mESCs with *Gata2* or *Gata3* expressing constructs without compromising ESC pluripotency, despite detectable BLA activity in mESCs. To link expression of *Gata* factors to BLA, the open reading frames of both genes were joined by a 2A sequence. Multicistronic expression of transgenes results from a translational skip induced by the 2A sequence [19, 30, 31]. In view of the fact that high, EF1 α mediated, expression of *Gata2/3* (2A-linked to GFP) induced obvious morphological changes and impacted on *Oct-4* expression, we strongly assume that *Gata2/3* transcripts get properly processed and produce active protein in the cellular context of mESCs. We conclude that the low levels of *Gata2* or *Gata3* expression produced by the pNkx6.1 construct in ESCs were not sufficient to induce ESC differentiation.

It could be argued that the cloned pNkx6.1 fragment (size: 1.9 kb) may not fully recapitulate native *Nkx6.1* promoter expression, because construct activity was detectable at the stem cells stage, and is highly dependent on viral infection rates, and unaffected by the presence of Shh during differentiation. However, despite these limitations, the pNkx6.1 promoter allowed a more context-specific expression of Gata transcription factors for 5-HT neuron differentiation, circumventing unwanted differentiation at the stem cell stage. Experiments with the pNkx6.1 constructs showed that expression of *Gata2*, *Gata3*, or *Lmx1b*/*Pet1*, after removal of LIF and serum, influenced 5-HT neuron development in vitro. Interestingly, the latter gene combination increased the number of 5-HT neurons on day 12 but not on day 20 of differentiation. Directed expression of *Lmx1b* and *Pet1*, essential for the 5-HT neuron phenotype [18], potentially induced the premature maturation (e.g., 5-HT synthesis) of prospective 5-HT neurons on day 12 but did not impact on total number at the end of the differentiation period. In contrast, expression of *Gata2* or *Gata3* significantly changed the proportion of 5-HT neurons on both day 12 and day 20 of differentiation. *Gata2* increased the number of 5-HT neurons, while *Gata3* disrupted the development of 5-HT neurons. This

was surprising as *Gata3* expression is a hallmark of all 5-HT neurons in vivo. Ablation of this gene completely eliminated the occurrence of caudal 5-HT neurons and reduced the size of rostral populations in a transgenic mouse model [6]. It is possible that the timing of expression of *Gata3* under the pNkx6.1 promoter was out of context, i.e., before native Gata2 and/or other essential factors were present. Moreover, *Gata3* has been implicated in the differentiation of other neuronal subtypes like V2-interneurons [32], noradrenergic neurons [33] and inner ear efferent neurons [8]. Consequently, it is possible that directed *Gata3* expression enriched differentiating cultures for cell types other than 5-HT neurons. Our observation, that directed expression of *Gata2* increased 5-HT neuron differentiation in vitro, supports published in vivo experiments, which demonstrated that *Gata2* can induce ectopic 5-HT neurons in r1 [5]. Although this observation is encouraging, we do not expect that *Gata2* expression will drive serotonergic development in a high proportion of all differentiating ESCs, because this transcription factor has very context-specific effects. *Gata2* is required for the proper specification of extraembryonic endoderm [29] and is a vital regulator for hematopoiesis [34], urogenital [35], and inner ear development [36]. Even within the neural lineage, it has been implicated in the specification of four neuronal subtypes, namely 5-HT neurons [5], V2-interneurons [37], midbrain GABAergic neurons [38], and sympathetic noradrenergic neurons [39]. These considerations led us to investigate whether coexpression of *Mash1* and/or *Foxa2* would boost *Gata2*-induced increases in the proportion of 5-HT neurons. *Mash1* was deemed a good candidate because over-expression in mESCs reportedly increased motor neuron differentiation [40, 41], which is linked to 5-HT neuron differentiation in r2–r3 and r5–r7 in vivo [2]. Moreover, *Mash1* has been implicated in the activation of *Gata3* [8], a transcription factor required for the normal development of caudal 5-HT neurons [6]. *Foxa2*, a target of Shh signaling [42], was chosen because its early expression is implicated in the absence of motor neuron generation in r1 while subsequent expression in r2–r3 and r5–r7 facilitates the switch from motor neuron to 5-HT neuron production [7]. Interestingly, only the combined expression of *Gata2* with both *Mash1* and *Foxa2* was able to significantly increase the number of 5-HT neurons when compared with the directed expression of *Gata2* alone, demonstrating that the presence of both genes is required to make a larger population of cells susceptible to the effects of the downstream gene *Gata2*.

As the pNkx6.1 construct was active in >80% of cells on day 6 of differentiation, the observation that dopaminergic differentiation was unaffected was surprising, as the differentiation of rostral 5-HT neurons and dopaminergic neurons is closely linked in vivo: Ye et al. [4] showed that FGF4 or FGF2 is able to induce 5-HT neurons in midbrain explants with endogenous sources of Shh and FGF8 at the expense of dopaminergic neurons. Similarly, FGF4 can promote serotonergic differentiation at the expense of dopaminergic differentiation in vitro [10, 12]. This implies that, during differentiation, genetic modifications took effect in a population of cells that were beyond the decision-point representing dopaminergic or nondopaminergic commitment. In addition, it is possible that the transcription factors found to impact on 5-HT cell number on day 20 (*Gata3* and *Gata2*—alone or in combination with *Mash1*/*Foxa2*) promoted the loss or generation of 5-HT neurons in vitro that correspond to caudal (and not rostral) cells in vivo. We suggest that differences in 5-HT number on day 20 are unlikely to be due to the conversion of dopaminergic precursors toward a serotonergic fate or vice versa. A further indication that the 5-HT neurons generated correspond to the caudal subpopulation is provided by the response of the differentiating population to growth factors. Shh alone, but not in combination with FGF family members FGF2, FGF4, and FGF8, enhanced serotonergic differentiation of our engineered mESCs. In fact, the addition of FGFs, which are associated with rostral 5-HT neuron differentiation in vivo [3], was of no benefit. It is possible that monolayer differentiation is not favorable for the differentiation of more rostral 5-HT subpopulations, because during wild-type differentiation, early 5-HT neurons arise only in proximity to Islet-1 positive cells, suggesting that these cells do not correspond to rostral r1 5-HT neurons, which themselves originate in vivo from a pool of progenitor cells that do not produce motor neurons [2].

We decided to use mESCs to explore basic gene function in 5-HT neuron differentiation for two reasons [1]. Reduced transduction efficiency has been reported for human ESCs (hESCs). Even infection with high titer viral concentrates results in <15% of the cell population expressing reporter GFP [43]. Over-expression of multiple genes in hESCs would be a time consuming process requiring sequential rounds of viral transduction with dual promoter constructs followed by periods of antibiotic selection. In contrast, infection with lentiviruses readily produces transgene expression (GFP) in >80% of mESCs [43], allowing simultaneous cotransduction with multiple constructs [2]. The vast majority of clinically active antidepressants ranging from tricyclics antidepressants, selective serotonin reuptake inhibitors to novel pharmacological compounds, such as agomelatine produce a detectable behavioral effect in rodents in animal tests for depression [44]. Accordingly, mESC-derived serotonergic neurons have the potential to be used for drug screenings aimed at modulating the human system.

Conclusion

5-HT (also known as serotonin) neurons are involved in the coordination of complex sensory and motor patterns that are associated with different behavior states, and their abnormal function has been implicated in affective illnesses such as, schizophrenia, aggression, and depression [3]. In addition, abnormality of the serotonin network has been strongly implicated in several complex developmental disorders such as Autism and Down's syndrome [45]. Derivation of enriched cultures of serotonergic neurons from ESCs could yield valuable disease models and would be useful in drug discovery [46]. In particular, the forced expression of transcription factors is a powerful tool in stem cell engineering and previous studies have demonstrated increased differentiation of dopaminergic neurons [9, 11, 47–49] and motor neurons [40, 41]. In this study, we highlight the importance for context-specific transgene expression at the right stage of ESC differentiation to increase generation of a desired cell type. Only the directed expression of the transcription factor combination *Gata2*, *Mash1*, and *Foxa2* synergistically resulted in widespread differentiation of 5-HT neurons from ESCs under chemically defined conditions. This work potentially lays the ground for further studies aimed at producing pure, homogeneous cultures of 5-HT neurons in vitro.

Acknowledgments

We thank our colleagues, Fred Hollande, Helen Irving, and Erica Sloan, for their useful comments on the manuscript. This work was supported by Ph.D. scholarship from Monash University (to C.M.N.).

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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