Ectopic Expression of Neurogenin 2 Alone is Sufficient to Induce Differentiation of Embryonic Stem Cells into Mature Neurons

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Abstract

Recent studies show that combinations of defined key developmental transcription factors (TFs) can reprogram somatic cells to pluripotency or induce cell conversion of one somatic cell type to another. However, it is not clear if single genes can define a cell’s identity and if the cell fate defining potential of TFs is also operative in pluripotent stem cells in vitro. Here, we show that ectopic expression of the neural TF Neurogenin2 (Ngn2) is sufficient to induce rapid and efficient differentiation of embryonic stem cells (ESCs) into mature glutamatergic neurons. Ngn2-induced neuronal differentiation did not require any additional external or internal factors and occurred even under pluripotency-promoting conditions. Differentiated cells displayed neuron-specific morphology, protein expression, and functional features, most importantly the generation of action potentials and contacts with hippocampal neurons. Gene expression analyses revealed that Ngn2-induced in vitro differentiation partially resembled neurogenesis in vivo, as it included specific activation of Ngn2 target genes and interaction partners. These findings demonstrate that a single gene is sufficient to determine cell fate decisions of uncommitted stem cells thus giving insights into the role of key developmental genes during lineage commitment. Furthermore, we present a promising tool to improve directed differentiation strategies for applications in both stem cell research and regenerative medicine.

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Introduction

During embryogenesis, all cell types of the body arise from a small pool of stem cells by differentiation – a complex process of defined sequential steps. Transcription factors (TFs) play an important role during this process by regulating the specific gene expression program of the various stages or triggering the transition to the next step. It has been shown that the ability of such key developmental genes to influence cell fates can also be operative outside of normal physiological development. Thus, ectopic expression of three defined genes can convert pancreatic exocrine cells into β-cells in vivo [1]. Similar in vitro experiments report the reprogramming of somatic cells to a pluripotent state [2,3] or the in vitro conversion of fibroblasts into neurons by specific combinations of defined TFs [4]. Furthermore, there are reports demonstrating that ectopic expression of lineage-specific genes can influence lineage decisions of in vitro differentiating stem cells. For example, ectopic expression of the neural TFs Sox1 or Sox2 in murine embryonic stem cells (mESCs) promotes the differentiation towards the neuroectodermal lineage upon induction of differentiation [5].

All these studies indicate that key developmental genes can define a cell’s identity outside of their physiological context. However, it is not clear if this cell fate defining potential depends on additional external signals or is only operative in certain cell types. Cell fate conversion by ectopic expression of certain genes in vivo is always performed on at least partially committed cells. Additionally, such processes may be influenced by unknown factors of the in vivo environment. In vitro cell conversion or reprogramming protocols are also performed with differentiated cells and generally include the addition of specific culture media components to enhance formation or survival of the desired cell type. Similarly, in reports describing directed differentiation of pluripotent stem cells by single defined TFs, differentiation itself was still induced and promoted by external signals like embryoid body formation or the addition of specific differentiation media [5–7].

Thus, until now, cell fate determination by key developmental TFs always includes the involvement of additional known or unknown factors. This makes it difficult to evaluate the strength of the cell fate defining potential of such genes.

Here, we wanted to investigate if a single key developmental gene is able to determine the cell fate of pluripotent stem cells without the need for any other external differentiation-inducing or lineage-promoting signals. We focused on the formation of neuronal cells types, as this differentiation pathway is of great
interest for many applications like potential clinical therapy of neurodegenerative diseases or drug screening. Neuronal differentiation of stem cells also offers a valuable tool to study neurogenesis in vitro as the development of the mammalian nervous system is hardly accessible for studies of the in vivo situation. Here, we show that ectopic expression of the neuronal basic helix-loop-helix transcription factor Neurogenin2 (Ngn2) is sufficient to induce and promote neuronal differentiation of mESCs towards the appearance of mature, functional neurons. Ngn2-mediated differentiation is fast and efficient and occurs even in the presence of external pluripotency-promoting signals like serum, leukemia inhibitory factor (LIF), or inhibitors of defined signaling pathways. Furthermore, our results indicate that Ngn2 induces a specific neuronal differentiation process that is – in certain aspects - reminiscent of the corresponding in vivo situation.

Materials and Methods

Plasmids

The Ngn2 expression construct was a kind gift from F. Guillermot and contains the coding sequence of Ngn2 with a N-terminal myc tag under control of the CMV promoter. As a transfection control, cells were co-transfected with pEGFP(C1)-Zeo, a vector coding for a fusion protein of the fluorescent protein EGFP and the zeocin resistance under control of the CMV promoter. Ratios were 1.5 μg expression vector +0.5 μg pEGFP(C1)-Zeo. Control cells were transfected with EBFP-N1 (kind gift of R. Campbell, P. Daugherty, and M. Davidson) instead of the Ngn2 expression construct.

For generation of the Ngn2 induction constructs the pmintol2/MCS vector (kind gift from S. Ekker), that contains the tol2 recognition sites, was modified by inserting a CMV promoter and a polyA tail resulting in the vector pMTCpA. For generation of the P2Angn2 construct the EGFP-Zeo coding sequence was amplified by PCR with primers containing flanking lox sites. PCR product was inserted in pMTCpA resulting in pMTC-EGFP-Zeo. Subsequently, the CMV promoter was replaced by ef1α promoter resulting in pMTE-EGFP-Zeo. Then, the coding sequence of Ngn2 was amplified by PCR and inserted in pMTE-EGFP-Zeo resulting in pMTE-EGFP-Zeo-ngo2. Puromycin resistance gene and 2A sequence were amplified by PCR and cloned into pMTE-EGFP-Zeo-ngn2 resulting in the final P2Angn2 construct.

For generation of the CreP2Angn2 construct the coding sequence of CreERT2 linked to a 2A sequence was amplified by PCR and cloned into pMTE-EGFP-Zeo-P2Angn2.

Cell Culture

Mouse ES cells were a subclone of an established ESC line originally named E14 [8]. ESCs were grown at 37°C, 5% CO₂ on gelatin coated wells in DMEM with stable glutamine (Invitrogen, 4 mM), 10% FBS, sodium pyruvate (1 mM), non-essential amino acids (0.1 mM), penicillin/streptomycin, B-mercaptoethanol (0.1 mM), and LIF (1000 U/ml). For all experiments in serum-containing medium, the same batch of fully defined FBS (PAA) was used. For medium culture conditions, cells were transferred to Knock-out DMEM (Invitrogen) supplemented with knock-out serum replacement (Invitrogen), stable glutamine (Invitrogen, 4 mM), penicillin/streptomycin, non-essential amino acids (0.1 mM), sodium pyruvate (0.1 mM), B-mercaptoethanol (0.1 mM), LIF (1000 U/ml), P00325901 (StemGent, 1 μM), and CHIR99021 (StemGent, 3 μM).

During all differentiation experiments, medium was changed every day. If differentiation was performed in the absence of LIF, cells were transferred to stem cell medium without LIF beginning from the day of transfection/recombination.

Transfections were performed using the Fugene HD transfection reagent (Roche) following the manufacturer’s instructions. Transfection was performed for 24 hours. Total amounts of transfected DNA were 2 μg per 6-well, 1 μg per 12 well and 0.6 μg per 24 well. Transfection efficiency estimated by expression of cotransfected fluorescent proteins was about 40-50 percent. Transiently transfected cells were selected with zeocin (100 μg/ml) starting at 2 days post transfection (dpt).

For electrophysiology, E14-P2Angn2 cell line was used that allows induction of Ngn2 expression and the puromycin resistance gene by addition of Cre recombinase as transducible protein.

For generation of E14-P2Angn2 and E14-CreP2Angn2 cell line, the tol2 transposase system [9] was used. Cells were transfected with a construct containing the coding sequence of the tol2 transposase under control of the CMV promoter and the P2Angn2 construct or the CreP2Angn2 construct, respectively, at ratio of 2:1. Subsequently, cells were grown at very low density and selected with zeocin (100 μg/ml) for 10 days to allow the formation of single colonies. Single colonies were picked manually under a microscope, expanded, and checked for correct function of the induction constructs. For each cell line, one clone was chosen for further analysis.

For protein transduction, E14-P2Angn2 cells were seeded as single cell suspension on gelatin coated wells. After cell adhesion, cells were treated with serum-free stem cell medium containing 2.5 μM TATCre recombinase [10] (kind gift from F. Edenhofer) for 4 hours. Then, cells were kept in LIF-free, serum-containing medium.

For induction of Ngn2 expression in E14-CreP2Angn2 cell line, CreERT recombinase [11] was activated by treating the cells with 4-hydroxytamoxifen (4OHT). 4OHT stock solution (1 mM, dissolved in 100% ethanol) was diluted 1:1000 in cell culture medium to a final concentration of 1 μM. Mock cells were treated with the same volume of 100% ethanol. After 18 hours, medium was changed for both 4OHT and mock treated cells. Medium consisted of complete ESC growth medium with and without LIF, respectively. For combination of medium-based and Ngn2-induced differentiation, cells were transferred to N2B27 medium (1:1 DMEM/F12 and Neurobasal supplemented with N2, B27 and penicillin/streptomycin) after 4OHT treatment.

For both E14-P2Angn2 and E14-CreP2Angn2 cell line, puromycin selection was started 2 days post recombination (dpr) at a concentration of 1 μg/ml and raised to 2 μg/ml 3dpr. Puromycin selection was continued until the end of the experiments.

For coculture experiments, E14-CreP2Angn2 cells were seeded on poly-D-lysine coated coverslips and Ngn2 expression was induced by treatment with 4OHT. Differentiating cells were cultured in serum-containing stem cell medium without LIF and selected with puromycin. Medium was changed every day. 5dpr, cells were labeled with CellTracker™ Green CMFDA (Invitrogen) according to the manufacturer’s instructions and cultured together with hippocampal neurons from C57Bl/6 mouse fetuses at E18 (breeding pairs from Harlan-Winkelmann, Borchen, Germany) in MEM with 2% B27, 0.22% sodium bicarbonate, 1 mM sodium pyruvate, 2 mM L-glutamine, 1% Penicillin/Streptomycin, and 0.6% glucose. Cultures were incubated at 37°C under 5% CO2/95% air and 90% humidity for 12 days. Medium was changed every second day.

RT-PCR

Total RNAs were isolated from cell cultures using the Total RNA Isolation Reagent (AB Gene). Samples were digested with
DNAasel (Fermentas) to exclude gDNA contamination followed by cDNA synthesis (Fermentas).

Polymerase chain reactions from 25 ng cDNA were run with the following primers: Ef1a 5'-GGTGCAACAGATGCAGGCACTGAGGTCACCCATGAAC-3', 3'-GGGGCTTGTGCTGTGCTGCT-3', add 5'-CCATGGCCCTGTCT-3', 3'-TGCTCAGCTTCCTCCTCCTCCT-3'; test and Figures display mean +/- SD.

To determine efficiency of neural differentiation in E14-CreP2Ang2 cells, total cells and Tuj1 positive cells were counted in an area of 3 mm². Data from three independent experiments were evaluated using Student's t-test and Figures display mean +/- SD.

For quantification of Nanog expression 3dpr, total cells and Nanog positive cells were counted in six to eight representative stem cell colonies for mock and 4OH Treated cells. Data from three independent experiments were evaluated using Student's t-test and Figures display mean +/- SD.

Microscopy
Light microscopy was performed using a Leica DMI6000B inverted microscope. Confocal microscopy was performed using either a Nikon C1 confocal microscope or a SP5 Confocal Microscope (Leica). All images were analysed using ImageJ software.

Electrophysiology
Whole-cell recordings [13] were performed at room temperature in a bath solution consisting of 125 mM NaCl, 2.5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, pH 7.4. Patch pipettes were pulled from borosilicate glass capillaries (Kimble Products, England), and heat-polished to give input resistances of 5–7 MΩ (pipette resistance, whole-cell). The pipette recording solution contained 140 mM KCl, 2 mM MgCl₂, 0.01 mM CaCl₂, 1 mM ethylene-bis(oxyethylenenitrilo) tetraacetate (EGTA), 1 mM Na₂ATP, 0.1 mM cyclic AMP, 0.1 mM ATP and 5 mM HEPES (pH 7.3, adjusted with KOH). Currents were recorded with an EPC9 (Heka) patch clamp amplifier and low pass-filtered at 1–2 kHz. Stimulation and data acquisition were controlled by the PULSE/PULSEFIT software package (Heka) on a Macintosh computer, and data analysis was performed with IGOR software (WaveMetrics, Lake Oswego, USA).

Results
Ngn2 is Sufficient to Induce Neuronal Differentiation
Murine ESCs were transiently co-transfected with a myc-tagged Ngn2 expression construct and GFPzeo, a fusion protein of GFP and the zeocin resistance protein allowing both visualization and selection of transfected cells. LIF, which prevents differentiation of mESCs under standard growth conditions, was withdrawn from the medium. Apart from LIF removal and zeocin selection, no other changes to either the medium or growth conditions were performed. Five days post transfection (dpt), cells with neuronal morphology could be detected in Ngn2-transfected cultures. These cells expressed pan-neuronal proteins Tuj1 and Map2ab (Fig. S1A-C). Ngn2 expression was analyzed. During

For all experiments, an equal number of cells were seeded for Ngn2 and mock treatment.

To determine efficiency of neural differentiation after transient transfection, Tuj1 positive cells were counted in an area of 15.04 mm². Data from three independent experiments were evaluated using Student’s t-test and Figures display mean +/- SD.

For staining of coculture experiments, 10% normal horse serum (NHS) instead of 5% BSA was used for blocking.

Quantification Experiments
For all experiments, an equal number of cells were seeded for Ngn2 and mock treatment.

To determine efficiency of neural differentiation after transient transfection, Tuj1 positive cells were counted in an area of 3 mm². Data from three independent experiments were evaluated using Student’s t-test and Figures display mean +/- SD.
embryonic neurogenesis, Dcx is expressed in migrating early postmitotic neurons [20,21] and NeuN in terminally differentiated postmitotic neurons [22]. All investigated genes were clearly upregulated in the Ngn2-transfected cells and were either not transcribed or only at background levels in mock-transfected cells. Furthermore, Ngn2-transfected cells showed a weak induction of endogenous Ngn2 at both 5 and 7dpt. This expression profile confirms at a molecular level the induction and progression of neural differentiation upon Ngn2 transfection.

To determine the efficiency of Ngn2-induced differentiation and to exclude the possibility of neurons arising due to random differentiation, we determined the number of Tuj1 positive cells in a wide field scan of Ngn2-transfected versus mock-transfected cells. After Ngn2 transfection, a significantly higher number (p = 0.0046 (5dpt), p = 0.0012 (7dpt)) of Tuj1 positive cells was detected compared to mock-transfected cells (Fig. S1L).

Next, we wanted to investigate if ectopic expression of Ngn2 induces the loss of stem cell identity which is a typical step during the early phase of differentiation processes. Immunofluorescence staining for the stem cell marker Nanog [23,24] 3dpt revealed a loss of Nanog expression specifically in Ngn2-transfected cells (Fig. S1E-J).

### Ngn2-mediated Differentiation Is Not Counteracted by Pluripotency-promoting Signals

Hitherto, the data indicated that transient expression of Ngn2 is able to induce neuronal differentiation. Next, we wanted to determine the inductive strength of the signal mediated by Ngn2. Thus, mESCs were transfected with Ngn2 and, for the full duration of the following experiments, were kept in adherent culture in complete ESC medium containing serum and LIF. To confirm the presence of active LIF signaling, non-transfected mESCs were treated with conditioned medium from Ngn2-transfected cells and stained for STAT3, a known effector of LIF signaling [25] (Fig. S3). The staining showed a clear nuclear localization of STAT3 confirming the presence of active LIF in the medium of Ngn2-transfected cells.

Surprisingly, even under these pluripotency-supporting conditions, Ngn2-transfected cells adopted a neuronal morphology and expressed Tuj1 and Map2ab within 3dpt (Fig. 1A-H). Immunofluorescence staining for myc-Tag confirmed that Tuj1 positive cells expressed ectopic Ngn2 (Fig. 2D-F). Mock-transfected cells did not show any specific Tuj1 staining and continued to grow in stem cell like colonies (Fig. 1B, 1D). Quantification of Tuj1 positive cells confirmed that a significantly higher number of neurons (p = 0.0156 (3dpt), p = 0.0013 (7dpt)) was detected in Ngn2-transfected cells compared to mock-transfected cells (Fig. 1P). Moreover, even in the presence of LIF, loss of Nanog expression was detected specifically in Ngn2-transfected cells (Fig. 1I-N).

Analysis of the gene expression pattern of Ngn2-transfected cells in LIF-containing medium led to similar results compared to cells differentiated in the absence of LIF: Early and late neuronal marker genes were upregulated (Fig. 1O) except for endogenous Ngn2 which could neither be detected in Ngn2-transfected nor in mock-transfected cells (data not shown).

### Establishment of an Inducible Ngn2 Expression System for Efficient and Homogenous Neural Differentiation

A differentiation system based on transient transfection has several disadvantages: Differentiation directly correlates with transfection efficiency and probably with copy number of transfected plasmids per individual cell. Furthermore, complete elimination of non-transfected cells is difficult. To overcome these problems, we generated a clonal transgenic mESC line (E14-CreP2Angn2) allowing induction of Ngn2 and subsequent selection of Ngn2-expressing cells. Upon activation of CreERT by tamoxifen treatment, GFPzeo expression is replaced by expression of Ngn2 and the puromycin resistance gene (Fig. 2A-E). Thus, by allowing the generation of a homogenous culture of stem cells expressing Ngn2 the E14-CreP2Angn2 cell line enables a detailed analysis of the Ngn2-induced differentiation process.

First, loss of stem cell identity upon Ngn2 induction was analysed (Fig. 2F, Fig. S4). Three days post recombination (3dpr), Nanog positive cells represented about 65 percent in control cells in the presence of LIF. This result is in agreement with previous studies showing a fluctuation of Nanog expression in mESCs [26]. Induction of Ngn2 expression resulted in a significant decrease of Nanog positive cells (33 percent). Likewise, in the absence of LIF, the number of Nanog positive cells was significantly reduced in Ngn2 expressing cultures compared to control cultures (Fig. 2F, Fig. S4).

Next, we analysed whether induction of Ngn2 resulted in neuronal differentiation. Seven dpr, Tuj1 positive cells with neuronal morphology were detected in both LIF-free and LIF containing conditions (Fig. 2H, 2I). Tuj1 positive cells represented about 40 percent of total cells in the absence of LIF and about 16 percent in the presence of LIF (Fig. 2G, Fig. S5A). The lower percentage observed in the presence of LIF resulted from a higher number of total cells. Possibly, LIF promoted the higher proliferation of cells that did not respond to Ngn2 expression by neuronal differentiation. This interpretation is also supported by the fact that - in the presence of LIF - differentiating cultures still contained stem cell-like colonies (Fig. 2H). Immunofluorescence staining confirmed that these colonies predominately consisted of Nanog+/Tuj1- cells (Fig. S5B-D). To further determine the fate of cells that did not form neurons upon Ngn2 induction, the expression of pluripotency markers (Nanog, Oct4), mesodermal (MyoD1, PU.1) and endodermal markers (Alfp, Insulin) was analysed (Fig. S5E). These data revealed that 7dpr both in LIF-free and LIF-containing conditions, mRNA of pluripotency markers was still detectable with only a slight downregulation of Nanog in LIF-free conditions. Meso- and endodermal markers, however, were not specifically upregulated in 4OHT-treated cultures as it was the case for neuronal markers (Fig. 3A). This indicates that cells expressing ectopic Ngn2 either remained in a pluripotent state or underwent neuronal differentiation.

Next, we wanted to analyse in detail the neuronal differentiation process occurring in E14-CreP2Angn2 cells. Gene expression pattern of the resulting neurons differentiated without LIF was highly similar to that observed in transient transfection experiments. Early and late neuronal marker genes were activated, albeit Sox1 was not upregulated. Additionally, we detected activation of the early neural marker Pax6 [27] (Fig. 3A). In the presence of LIF, E14-CreP2Angn2 derived neurons showed a similar gene expression pattern with upregulation of Dcx, Math3, NeuN, Olig2, and Pax6 (Fig. S6).

Quantitative real-time PCR was performed to determine the expression levels of ectopic and endogenous Ngn2 in both transient transfection assays and in the inducible cell line. In both setups, endogenous Ngn2 was virtually not expressed. Expression levels of ectopic Ngn2 at day 7 were significantly higher in the inducible cell line compared to transient transfection assays. The presence of LIF did not influence the levels of ectopic or endogenous Ngn2 (Fig. 3B).

More detailed characterization of differentiated cells showed that Ngn2-derived neurons stained positive for Tau (Fig. 3C). Furthermore, expression of vesicular glutamate receptors eGLUT1
and 2 mRNAs was detected while markers for other neuronal subtypes like Tyrosine hydroxylase (Th) and Gad1 were not expressed (Fig. 3G). On protein level, cells were positive for vGLUT1 and NMDA receptor 1 (NR1) (Fig. 3D-F, 3H-J) indicating the formation of glutamatergic neurons. In Ngn2-derived neurons differentiated in the presence of LIF, specific activation of vGLUT2 expression was observed on mRNA level together with a slight activation of Th (Fig. S6). On protein level, cells stained positive for vGLUT1 and NR1 (Fig. S7A-F). As a weak induction of Th mRNA was detectable, we also analysed TH protein expression. Very rarely, cells weakly expressing TH protein could be detected (Fig. S7G-I). These data indicate that Ngn2-induced differentiation preferably results in the appearance of glutamatergic neurons. However, the formation of other neuronal subtypes like dopaminergic neurons in the presence of LIF or of a mixed, artificial phenotype cannot be completely excluded.

**Functional Features of Ngn2-induced Neurons**

To test if Ngn2-derived neurons show functional features of terminally differentiated neurons whole-cell patch-clamp recordings were performed. In the voltage-clamp mode voltage-gated currents typical for terminally differentiated neurons could be observed. Depolarizing pulses from a holding potential of −70 mV elicited fast-activating transient inward currents typical for voltage-activated Na⁺-currents (Na⁺ channels) followed by a delayed outward current indicative of voltage-activated potassium currents (K⁺ channels) (Fig. 4A). In the current-clamp mode injection of depolarizing current elicited an action potential with a duration of 5 ms and an amplitude of 105 mV (Fig. 4B).

Next, we wanted to investigate whether Ngn2-induced neurons can form contacts with primary neurons in co-culture. In coculture with primary mouse hippocampal neurons, the E14-CreP2Atngn2 cells expressed the presynaptic marker synapsin-1 and formed morphologically tight contacts with the hippocampal neurons (Fig. 4C-H).

**Ngn2-induced Neuronal Differentiation in Chemically Defined Media**

Hitherto, our findings strongly suggest that Ngn2 is sufficient to induce a neuronal differentiation process even under pluripotency promoting signals mediated by the combination of serum and LIF. To further strengthen this hypothesis, differentiation of E14-CreP2Atngn2 cell line was performed in 2i medium. This chemically defined stem cell medium contains LIF, knock-out serum replacement and two small-molecule inhibitors that are able to maintain pluripotency and self-renewal of ESCs by the inhibition of defined signaling pathways [28]. Seven days post 4OHT-mediated induction of Ngn2 expression, few Tuj1 positive neurons could be detected in 4OHT treated cultures. Mock treated cultures did not contain any Tuj1 positive cells (Fig. S8A, S8B, S8E, S8F). This indicates that Ngn2 induces neuronal differentiation also in 2i medium conditions albeit at lower frequency compared to LIF and serum containing culture conditions (Fig. S8C, S8D). Thus, one can conclude that the induction of a neuronal fate by Ngn2 is influenced by media conditions but does not depend on them. Ngn2-induced neuronal formation did not require any additional signals nor was it prevented by contradictory cues. This highlights the strength of the signal mediated by Ngn2 and suggest that this might be beneficial in conventional differentiation protocols. To test this idea, E14-CreP2Atngn2 cells were cultivated after Ngn2 induction in N2B27 medium which is used in many neuronal differentiation protocols. Immunofluorescence staining for Tuj1 5dpr revealed that in these culture conditions neuronal cells were more frequent compared to LIF conditions. Furthermore, already 5dpr, neurons cultured in N2B27 showed mature neuronal morphology and stained positive for Tau and Synapsin (Fig. S9). This indicates that Ngn2-induced differentiation is accelerated by the presence of a neuronal differentiation medium.

**Discussion**

Exact cell fate determination is of crucial importance for correct embryogenesis. Numerous studies indicate that certain genes are essential for this process. However, it is still not clear if such genes can determine cell fate on their own, or they require a specific environment or auxiliary factors.

**Ngn2 Induces Formation of Mature Neurons**

In this study, we demonstrate that the bHLH transcription factor Ngn2 is sufficient to induce efficient and rapid formation of mature neurons from pluripotent stem cells. These results indicate that certain genes have the potential to determine the cell fate choice of totally uncommitted stem cells outside the normal process of development. Several earlier studies report that ectopic expression of certain neuronal genes like Ngn1, Ngn2, or Axin1 can induce the formation of neural cells from different cell types [29,30,7,4]. In these studies, ectopic expression of the fate-inducing genes was accompanied by changes of culture conditions like e.g. the addition of media components known to support the formation and survival of neuronal cells. As especially changes in medium compositions frequently have a considerable but often immeasurable influence on cellular processes it is difficult to evaluate the potential of single genes to determine cell fate decisions. Our findings prove that the cell fate defining potential of Ngn2 is independent of additional external or internal signals.

The process of differentiation induced by Ngn2 was confirmed by morphology, induction of early and late neural marker genes, expression of neuron-specific proteins, and electrophysiological activity unique to neurons. Furthermore, Ngn2-derived neurons formed contacts with hippocampal neurons in co-culture signifying their ability to interconnect with physiological neuronal networks. Although more in-depth analyses would be useful to fully determine the subtype as well as the status of functionality.
Figure 2. Directed neuronal differentiation using E14-CreP2Angn2 cell line. (A) Inducible construct of E14-CreP2Angn2 cell line and method of induction. (B-E) Loss of GFP signal in 4OHT treated cells (D,E) 1 day post recombination (dpr) (B,C) Mock treated cells. Scale bars: 20 μm. (F) Nanog positive cells in mock and 4OHT treated cells 3dpr in the presence and absence of LIF. Columns represent mean ± SD of three independent
of the Ngn2-derived neurons, our data prove that Ngn2 induces the formation of mature and at least partially functional neurons.

Ngn2- induced Differentiation Shows Features of in vivo Neurogenesis

The gene expression pattern observed after Ngn2 transfection revealed particularly interesting information regarding the mechanism of Ngn2-induced differentiation.

First, the upregulation of Dcx and NeuN indicates the progression of the differentiation process until the mature neuron stage. Dcx is a microtubule-associated protein that is transiently expressed in early postmitotic migrating neurons [21,20]. Like NeuN, it is a marker for postmitotic neurons. NeuN is a nuclear protein that can be detected in terminally differentiated neurons throughout the nervous system [22].

Another important observation is the upregulation of Math3 and Olig2 during the differentiation process. Math3 is expressed in the developing nervous system [15] and it has been shown that in vivo Ngn2 and Math3 are expressed in a temporal sequence with Ngn2 expression preceding that of Math3 [31]. Possibly, Ngn2-mediated differentiation in vitro follows at least partially its physiological genetic cascade. It has also been demonstrated that in vivo Math3 augments Ngn2 activity [32]. Thus, one can speculate that in our in vitro experiment Ngn2 does not induce unspecific neuronal differentiation, but activates defined differentiation pathways of in vivo neurogenesis with directed activation of its interaction partners.
Figure 4. Functionality of Ngn2-derived neurons. (A, B) Whole-cell patch clamp recordings of neurons after Ngn2-induced neuronal differentiation 10 dpr in Ngn2-inducible cell line. Expression of Ngn2 was induced by delivery of Cre recombinase as transducible protein. (A) Voltage-
This hypothesis is also supported by the observed upregulation of Olig2 because Ngn2 and Olig2 have combinatorial roles in the generation of motor neurons [33,34]. Interestingly, Olig2, together with the related Olig1, regulates expression of Ngn2 in motor neurons [35] in vivo. This raises the question how and why Olig2 expression was induced by ectopic Ngn2 expression in our in vitro experiments.

Similarly, an interesting observation was the upregulation of Sox1 and Pax6. Sox1 is known to be the earliest marker of neural precursors and its expression precedes that of Ngn2 in vivo [14]. Pax6 was shown to directly regulate Ngn2 [36,37]. In our study, both Pax6 and Sox1 were upregulated during Ngn2-induced differentiation processes, namely Sox1 in transient transfection assays and Pax6 after induction of Ngn2 in a transgenic cell line. It is surprising that three upstream genes, Sox1, Pax6, and Olig2, are activated in a differentiation process based on an inducing factor which, during physiological differentiation, is located genetically downstream in the cascade.

One hypothesis explaining this phenomenon would be that differentiating cells recapitulate the sequence of steps documented for neurogenesis in vivo, including stages that precede activation of the here employed inducing factor Ngn2. This idea is supported by a similar observation reported in another study describing cell conversion of medaka fish spermatogonia into various somatic cell types by ectopic expression of lineage-specific TFs [38]. In that study, the activation of genes that in vivo are located upstream of the inducing factors was also detected for three different processes of cell fate conversion. Likewise, TF-induced differentiation of medaka fish ESCs into melanocytes included the activation of upstream marker genes [39]. Thus, the here reported upregulation of Sox1, Pax6, and Olig2 during Ngn2-mediated differentiation could be a conserved part of the in vitro differentiation process.

Alternatively, Ngn2-induced differentiation resulted in the formation of mature Pax6 and Olig2 positive neurons which is indicated by the increased expression of these genes in later stages of differentiation (Fig. 3A). This idea is in line with previous findings demonstrating that Pax6 enhances the differentiation of neuroepithelial cells into radial glial cells and neurons [40]. Olig2 has also been shown to be expressed in the adult brain albeit only in a small subpopulation of progenitor cells [41]. This is contradictory to the assumption that Ngn2 expression led to the formation of mature neurons. However, it cannot be excluded that Olig2 has an unknown function in postmitotic neurons that has still to be determined. Furthermore, in vitro generated neurons exist in an artificial environment that might induce phenotypical features – like an unusual gene expression - that are not displayed by neurons arising during physiological development.

Nevertheless, the gene expression pattern observed during Ngn2-mediated differentiation showed some similarities to processes of in vivo neurogenesis that are marked by Ngn2 expression. These similarities included the activation of interaction partners and potential upstream regulators of Ngn2. Furthermore, the preference towards the formation of a glutamategic phenotype is also reminiscent of in vivo corticogenesis where Ngn1 and Ngn2 specify glutamategic cortical neurons [42]. The findings presented here indicate that Ngn2 alone can activate parts of a genetic cascade in uncommitted stem cells. Further experiments will be required to test this idea and to analyse the underlying mechanisms. Nevertheless, these observations provide a new insight in the role of key developmental transcription factors in genetic networks.

Ngn2 Breaks Intra- and Extracellular Pluripotency Signals

Another important finding of this study is that Ngn2 is able to induce and promote differentiation under conditions normally enhancing pluripotency. Cells were grown in medium with serum, which is normally omitted during in vitro neuronal differentiation protocols [43,44] and in the presence of LIF. LIF was initially considered as a strong factor for the maintenance of pluripotency [45,46] and has been shown to have an inhibitory effect on neural determination of stem cells [47]. Other studies, however, report that LIF enhances the differentiation of ESCs into neural progenitor cells albeit mainly as a permissive factor [43]. Thus, the role of LIF during the formation of neural cell types from ESCs is not definitely clear and probably depends on other environmental factors. In our study, addition of LIF was combined with conditions normally used to enhance the proliferation of undifferentiated stem cells. Our results prove that even under these conditions Ngn2 expression leads to the loss of pluripotency markers like Nanog suggesting that the network maintaining pluripotency can be overcome by single defined signals. This idea is also strengthened by the fact that Ngn2-induced neuronal differentiation did also occur in chemically defined 2i medium. Thus, although these pluripotency promoting conditions affected the efficiency of the Ngn2-induced differentiation process, they were not sufficient to prevent it. This proves that the signal mediated by Ngn2 does not depend on extracellular cues.

Interestingly, our data indicate that not all cells that express ectopic Ngn2 differentiate into neuronal cells. Gene and protein expression analyses suggest that these non-responsive cells are Nanog and Oct4 positive and – in the presence of LIF – form stem-cell like colonies. Thus, Ngn2-expressing cells either undergo a neuronal differentiation process or retain features of pluripotent stem cells. One possible explanation for this varying responsiveness to Ngn2 could be the heterogeneity of mESC cultures. Indeed, it has been shown that mESCs oscillate between various states that are more or less prone to various differentiation cues [26,48]. These oscillations could influence the ability of mESCs to undergo a differentiation process upon Ngn2 expression.

Alongside, the here demonstrated potential of Ngn2 to define cell fate decisions in vitro is in line with the dominant role of Ngn2 during neurogenesis in vivo. During mouse embryogenesis, Ngn2 expression is detected widely in the developing CNS and PNS [49] and Ngn2 knockout mice exhibit severe neural defects and die shortly after birth [31]. Interestingly, it has been shown that the Ngn2-related Ngn1 can specify a neural fate in a non-physiological context in zebrafish embryos [50]. Although it cannot be excluded that in that study the effects of Ngn1 were influenced by unknown factors of the in vivo environment one can assume that Neurogini have the ability to define a neuronal identity in non-neuronal cells.

This hypothesis is confirmed by our data demonstrating that Ngn2 can induce and promote a complete neuronal differentiation process.
Conclusion

In summary, our data prove that ectopic expression of Ngn2 is sufficient to induce the formation of mature neurons from stem cells. This is, to our knowledge, the first study reporting that a single TF determines the fate of totally uncommitted stem cells without the need for additional signals and independent of culture conditions.

TF-induced differentiation therefore constitutes a promising alternative or expansion to conventional differentiation protocols. Ngn2-mediated differentiation is fast and robust and furthermore differs from standard differentiation protocols in the fact that both induction of differentiation and lineage commitment depend on a single factor and are totally independent of external signals. Therefore, this system is less susceptible to variability compared to approaches depending on several parameters which each can have unexpected effects. Thus, single gene mediated differentiation lends itself to a model for differentiation studies that require a very robust and reproducible differentiation process. Alternatively, single gene mediated differentiation can be combined with medium-based strategies to improve rapidness, efficiency, and the levels of control over the direction of differentiation.

In addition, our findings are a proof of concept for the feasibility of single gene mediated differentiation. We suggest that this approach can be extended to generate other cell types if the appropriate TFs are identified. Indeed, we were recently able to use this approach for the formation of myoblasts by ectopic expression of MyoD1 [51]. Importantly, this process could be combined with the here presented Ngn2-induced differentiation allowing the formation of neurons and myoblasts in parallel. Thus, the concept of single gene mediated differentiation enables the simultaneous generation of unrelated cell types as mixed cultures.

Moreover, the here presented system provides a valuable tool for studies of neural development, the loss of pluripotency, and effects of key developmental genes on cell fate decisions.

Supporting Information

Figure S1 Induction of neuronal differentiation by transient transfection with Ngn2 in the absence of LIF. (A-D) 5dpt, Ngn2-transfected cells display neuronal morphology and express neuronal marker proteins like Tuj1 (A,B) and Map2ab (C,D). (B,D) Overlays of immunofluorescence staining and Hoechst staining. Scale bars: 20 μm. (E-J) Loss of Nanog expression (arrowheads) 3dpt in Ngn2-transfected (E,G,I), but not in mock-transfected cells (F,H,J). Transfected cells are visualized by expression of cotransfected GFP (E,F). Scale bars: 20 μm. (K) Gene expression pattern of untreated (ut), Ngn2-transfected (d5+), d7+, and mock-transfected (d5-, d7-) mESCs 5 and 7dpt. b: Brain cDNA. Dashed lines indicate grouping of different parts from the same gel. A representative result from three independent experiments is shown. (L) Tuj1 positive cells in Ngn2-transfected and mock-transfected cells 5 and 7dpt. Absolute numbers are shown as non-differentiating cells continue proliferating. Therefore, the relative number would not really reflect the increase of neurons upon Ngn2 compared to mock transfection. Columns show mean +/- SD of three independent experiments.

Figure S2 Expression of ectopic Ngn2 specifically in developing neurons differentiated in the absence (A-C) or the presence (D-E) of LIF. Immunofluorescence staining for myc-tagged Ngn2 (A, D) and Tuj1 (B, E) in Ngn2-transfected mESCs 5dpt Overlays (C, F) reveal that neurons express ectopic Ngn2. Scale bars represent 20 μm.

Figure S3 STAT3 immunofluorescence staining proving active LIF signaling. (A) Colony of non-transfected mESCs treated for 24 hours with conditioned medium from Ngn2-transfected cells. (B) STAT3 staining. (C) Nuclei visualized by Hoechst staining. (D) Overlay of B and C showing nuclear localization of STAT3. Scale bars: 20 μm.

Figure S4 Loss of Nanog protein expression in E14-CreP2Angn2 cells upon induction of Ngn2 expression by 4OHT treatment. 4OHT (D-F) and mock treated cells (A-C) 3dpr in the presence of LIF. 4OHT (J-L) and mock treated cells (G-I) 3dpr in the absence of LIF. Scale bars: 20 μm.

Figure S5 Neuronal differentiation in E14-CreP2Angn2 cells. (A) Efficiency of neuron formation 7 days post recombination in the presence (16.7%) and absence of LIF (41.8%). Columns show mean +/- SD of three independent experiments. (B-D) ESC-like colonies (arrow) remaining in Ngn2-expressing cultures in the presence of LIF. Immunofluorescence of E14-CreP2Angn2 cells 7dpr for Nanog (B) and Tuj1 (C). (D) Overlay showing Nanog+/Tuj1- cells. Scale bars: 50 μm. (E) Expression of non-ectodermal differentiation and of pluripotency markers in 4OHT (d7+) treated and mock treated (d7-) E14-CreP2Angn2 cells 7dpr differentiated in the presence or in the absence of LIF. A representative result from three independent experiments is shown. (ut) untreated. (+) positive control.

Figure S6 Neuronal marker expression of E14-CreP2Angn2 derived neurons differentiated in the presence of LIF. (A) untreated, (d7+) 4OHT treated, (d7-) mock treated, (b) Brain cDNA. Dashed lines indicate grouping of different parts from the same gel. A representative result from three independent experiments is shown.

Figure S7 Neuronal differentiation of E14-CreP2Angn2 cell line in the presence of LIF. Expression of vGLUT1 (A-C) and NR1 (D-F) indicating the formation of glutamatergic neurons. (G-I) Very rarely, cells positive for TH (arrows) could be detected. Scale bars: 20 μm (A-C), 10 μm (D-F), 50 μm (G-I).

Figure S8 Neuronal differentiation of E14-CreP2Angn2 cells in chemically defined 2i medium. Overlays of Tuj1 and Hoechst staining 7dpr. (A, B) Induction of Ngn2 results in neuron formation in 2i medium (A, arrowheads) with no neurons detectable in mock-treated cells (B). (C, D) Ngn2-induced neuron formation is more efficient in LIF and serum containing medium. (E, F) Close-up of representative Tuj1 positive neuronal cells detected 7 days post recombination in 2i medium condition. Scale bars: 100 μm (A-D), 20 μm (E, F).

Figure S9 Enhanced neuronal differentiation of E14-CreP2Angn2 cells in neuronal differentiation medium N2B27. (A, B) Wide field scans of Tuj1 staining of cells differentiated in LIF-free ESC medium (A) or N2B27 (B) 5dpr. Images were taken with exposure time and gain settings. Neurons formed under N2B27 culture conditions are more frequent and show a more mature phenotype. Scale bars: 200 μm. (C, D) Close-ups of neurons differentiated in N2B27 medium 5dpr. Cells show morphology of mature neurons and stain positive for Tau and Synapsin. Scale bars: 20 μm.
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Author Contributions

Conceived and designed the experiments: ECT EW NO AS. Financial and administrative support: MS. Contributed reagents/materials/analysis tools: AS MS. Wrote the paper: ECT EW NO AS. Financial and administrative support: MS.
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