Combined small-molecule inhibition accelerates developmental timing and converts human pluripotent stem cells into nociceptors

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Considerable progress has been made in identifying signaling pathways that direct the differentiation of human pluripotent stem cells (hPSCs) into specialized cell types, including neurons. However, differentiation of hPSCs with extrinsic factors is a slow, step-wise process, mimicking the protracted timing of human development. Using a small-molecule screen, we identified a combination of five small-molecule pathway inhibitors that yield hPSC-derived neurons at >75% efficiency within 10 d of differentiation. The resulting neurons express canonical markers and functional properties of human nociceptors, including tetrodotoxin (TTX)-resistant, SCN1A-dependent sodium currents and response to nociceptive stimuli such as ATP and capsaicin. Neuronal fate acquisition occurs about threefold faster than during in vivo development1, suggesting that use of small-molecule pathway inhibitors could become a general strategy for accelerating developmental timing in vitro. The quick and high-efficiency derivation of nociceptors offers unprecedented access to this medically relevant cell type for studies of human pain.

The in vitro derivation of postmitotic neurons from hPSCs requires extended culture periods typically lasting 30 d or more2,3. Protracted in vitro differentiation of hPSCs is thought to reflect the chronology of human development in vivo4. Identifying in vitro strategies to overcome this slow pace is a major challenge for realizing the full potential of hPSCs in basic biology and human disease modeling5. Here we describe a combinatorial small-molecule–based approach to rapidly coax pluripotent cells into nociceptors. Previously, we reported that dual-SMAD inhibition efficiently neuralizes hPSCs6. Follow-up studies have described the use of small molecules that replace Noggin7,8, and, similarly, we identified a bone morphogenetic protein (BMP) inhibitor LDN-193189 (ref. 9) that can replace Noggin for neuralization of hPSCs (Fig. 1a, abbreviated LSB for the three inhibitors) added on day 2 (Supplementary Fig. 2) abolishes Pax6 expression and induces TUBB3 in hPSCs at day 10 of differentiation (Fig. 1b). SU5402 is a potent inhibitor of vascular epithelial growth factor (VEGF), fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF) tyrosine kinase signaling12. CHIR99021 can act as a WNT agonist by selectively inhibiting glycogen synthase kinase-3β (GSK-3β) and thereby stabilizing β-catenin13, DAPT is a γ-secretase inhibitor that blocks Notch signaling14.

Upon maturation, neurons halt mitosis and lose expression of Ki67 (ref. 15) and phospho-histone H3 (pHH3)16. Compared with cells grown in LSB only, far fewer cells in LSB + 3i (LSB3i) expressed Ki67 and pHH3 (Fig. 1c–f), and we used FACS to confirm this decrease in cell cycle, starting at day 7 (Supplementary Fig. 3). Intracellular FACS for Nestin (a marker of neural progenitors) and neuronal TUBB3 (TUJ1) was used to quantify the efficiency of neuronal differentiation (Fig. 1g). In the presence of LSB, nearly all cells expressed Nestin (>95%), reflecting the high efficiency of dual-SMAD inhibition6. Conversely, when 3i was present, 75% of cells converted to a neuronal cell fate. We used FACS to examine combinations of 3i treatments for further mechanistic insight (Fig. 1g). Although none of the factors alone yielded high numbers of TUJ1+ neurons, treatment with CHIR99021 together with one of the other two factors generated robust numbers of neurons (53% for DAPT and 58% for SU5402), indicating that CHIR99021 is the key factor for inducing neuronal differentiation, whereas SU5402 and DAPT further enhance efficiency.

Next we tested whether the neurons were of a particular subtype. Dual-SMAD inhibition of hPSCs generates a Pax6+ neuroepithelium coexpressing the anterior central nervous system (CNS) marker PAX6 (ref. 10) and induction of neuronal β3-tubulin (TUBB3+TUJ1+)11 at day 10 after addition of LSB. We discovered that a combination of three small molecules (SU5402, CHIR99021 and DAPT, called 3i for ‘three inhibitors’) added on day 2 (Supplementary Fig. 2) aboliishes Pax6 expression and induces TUBB3 in hPSCs at day 10 of differentiation (Fig. 1b). SU5402 is a potent inhibitor of vascular epithelial growth factor (VEGF), fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF) tyrosine kinase signaling12.

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Unexpectedly, we observed homogenous expression of ISL1 and BRN3A (Fig. 1h,i), canonical markers of sensory neurons
17,18, indicating that the resulting neurons have peripheral nervous system (PNS) rather than CNS identity at day 12. The emergence of BRN3A and ISL1 expression under these conditions was also observed for human induced pluripotent stem cells, such as line C14 (Fig. 1j,k). There are three main subsets of sensory neurons—proprioceptors, mechanoreceptor neurons and nociceptors—distinguished by the specific expression pattern of neurotrophic receptors19. More than 60% of all cells expressed neurotrophic tyrosine kinase receptor-1 (NTRK1) measured by FACS at day 10 (Fig. 1l; in contrast, NTRK2 and NTRK3 could not be detected by immunofluorescence or FACS (Supplementary Fig. 4), indicating that the majority of LSB3i-induced neurons were nociceptors.

We quantitatively assessed the reproducibility of LSB3i treatment across hPSC lines. Two hiPSC lines (C14 and C72), which have been shown to efficiently neuralize20, homogeneously gave rise to Nestin+ cells when treated with LSB (>95%), and formed TUJ1+ cells when treated with LSB3i (40% for C14 and 33% for C72; Fig. 1m). We obtained a further increase in neuronal yield upon passaging of bulk cultures (Supplementary Fig. 5), suggesting that the lower efficiency in those two hiPSC lines at day 10 is probably due to a slight delay in differentiation. Sorting based on NTRK1 expression can be used to further enrich for BRN3A+, ISL1+ and TUJ1+ neurons (Supplementary Fig. 6).

Nociceptors arise via two possible lineages during human development: SOX10+ neural crest21,22 can generate trunk nociceptors that flank the spinal cord23; alternatively, head placode contributes to the trigeminal nociceptors responsible for innervation of the face24,25. To distinguish between these possibilities, we generated a transgenic SOX10::GFP bacterial artificial chromosome hPSC line that enriches for neural crest markers in the GFP fraction (Supplementary Fig. 7). We used FACS to monitor GFP expression at 4 d, 8 d, 12 d and 16 d after starting differentiation when the SOX10::GFP cells were treated.
with LSB, LSB and CHIR99021 (LSB/CHIR), or LSB3i (Fig. 2a–c). A majority of the cells in culture became SOX10+·GFP+ by day 12 of differentiation when CHIR99021 was present (70% for LSB/CHIR and 80% for LSB3i; Fig. 2d). This result indicates that the LSB3i cells adopt a neural crest identity, supporting our earlier observation that CHIR99021-mediated neural crest induction is required for the generation of LSB3i nociceptors. Treatment with SU5402 accelerated neural crest cell fate choice, as LSB- or LSB/SU/CHIR-treated cells acquired neural crest identity more rapidly than did LSB/CHIR and LSB/CHIR DAPT–treated hPSCs (Fig. 2d and Supplementary Fig. 8).

To determine whether SOX10+·GFP+ cells can give rise to nociceptors, we sorted GFP+ cells at day 8 of LSB3i treatment (60% of total) and maintained them in 3i until day 11, resulting again in ISL1+ BRN3A+ TUJ1+ neurons (Fig. 2cf). These data show that LSB3i differentiates hPSCs toward nociceptors via a SOX10+ neural crest intermediate. Immunochemical studies confirmed that SOX10 was never coexpressed in neuronal β-tubulin (TU1) cells. Persisting SOX10+ precursor cells (Fig. 2d) could be largely eliminated (<5%) upon passage and replating of LSB3i cells (Supplementary Fig. 9).

In addition, microarray analysis provided further evidence for nociceptor intermediate cell fates, distinct from mechanoreceptor neurons and proprioceptors (Supplementary Fig. 11). During mouse development, the neurogenin basic helix-loop-helix proteins mediate two sequential waves of neurogenesis to form the dorsal root ganglia (DRG)28,29. The first wave, marked by Neurog2 expression, gives rise to mechanoreceptor neurons and proprioceptors, and the second, marked by Neurog1 expression, gives rise to nociceptors. When hPSCs were treated with LSB, NEUROG2 expression was strongly induced by day 7 (Fig. 3c). In contrast, hPSCs treated with LSB3i showed a less pronounced induction of NEUROG2 by day 7 but selective induction of NEUROG1 by day 9 (Fig. 3c). We further observed that selective nociceptor generation with LSB3i depends on continuous CHIR treatment (days 2–14). Shorter CHIR pulses (days 2–4 or days 2–8) induced similar levels of SOX10 but high expression of ASCL1, NTRK1 and NTRK2, markers of autonomic neurons, mechanoreceptor neurons and proprioceptors, respectively (Supplementary Fig. 12).

Functional evidence, the ‘gold standard’ for demonstrating nociceptor identity, depends on ion channels and receptors that detect noxious stimuli26. We first assessed the expression of a broad range of mature nociceptive markers during LSB3i (Fig. 4a). The sodium channels SCN9A, SCN10A and SCN11A, the purinergic receptor P2RX3...
and the vanilloid receptors TRPV1 and TRPM8 were upregulated by day 15. SCN10A is selectively expressed in both rodent and human nociceptive sensory neurons30 and is thought to underlie the upstroke of the action potential and repetitive firing in C-fibers31. Using a current-voltage protocol, we observed voltage-gated currents in all LSB3i-derived cells with a neuronal morphology ($I = -23.7 \pm 3.5$ nA at 0 mV, $n = 30$). Currents were fully blocked in the majority of cells (24 of 30 recordings) by 500 nM TTX, but 500 µM CdCl$_2$ in the extracellular solution reduced the current by <5% ($n = 5$), suggesting that most of the voltage-gated current is carried by Na$^+$ rather than Ca$^{2+}$. We observed TTX-resistant (TTX-R) currents (mean current amplitude = −402 ± 112 pA), a characteristic feature of SCN10A$^+$ nociceptors, in 6 cells out of 24. Application of the selective SCN10A blocker A-803467 (ref. 32) blocked >90% of the TTX-R currents in all cases (Fig. 4b). To further test the functional expression of SCN10A, we applied A-803467 and recorded action potential activity in current clamp mode. Action potentials were detected in all cells with a neuronal morphology in response to suprathreshold current injections. In all cells with multiple action potentials, treatment with A-803467 at 500 nM ($n = 6$ cells) or 250 nM ($n = 5$ cells) decreased the repetitive firing without affecting the first action potential (Fig. 4c). Similarly, A-803467 had no effect on the cells with a single action potential (Supplementary Fig. 13, n = 6). These data suggest that SCN10A is commonly expressed in LSB3i-derived neurons and contributes to the repetitive action potential firing. The expression data and functional characterization of SCN10A strongly indicate that LSB3i yields a population of neurons with a nociceptive sensory phenotype.

We also evaluated calcium flux of LSB3i nociceptors in response to 1 µM capsaicin, the noxious component in chili peppers, known to activate a subset of nociceptive sensory neurons through binding to the TRPV1 vanilloid receptor33 and to 30 µM α,β-methylene-ATP, a selective agonist of P2RX3 (ref. 34); this activation mimics inflammatory pain (Fig. 4d). α,β-Methylene-ATP induced a robust calcium response. For capsaicin, we observed activation in a subset of neuronal processes and rare cell bodies (1–2% of cells), indicating either significant neurite arborization of a few responding cells, or selective calcium signaling sequestered to the processes (Supplementary Fig. 14).

To confirm that α,β-methylene-ATP was indeed activating the P2RX3 receptor, we carried out both calcium flux (Fig. 4e and Supplementary Fig. 14) and electrophysiological analyses with the selective P2RX3 antagonist A-317491 (ref. 32) (Fig. 4f). Pretreatment with A-317491 significantly (**$P < 0.01$; one-way ANOVA, Dunnett’s test) and in a dose-dependent manner decreased calcium flux response induced by 30 µM α,β-methylene-ATP (Fig. 4e). Focal application of 10 µM α,β-methylene-ATP to the LSB3i neurons resulted in typical P2RX3 currents (2.1 ± 0.46 nA, n = 6) with signature fast activation and desensitization ($n = 6$, Fig. 4f). Exposure to 1 µM A-317491 blocked the current in all cells tested (average reduction of 93 0.1 ± 2.3% ($n = 5$)).

The speed with which stable, mature and functional neuronal cell fates can differentiate from hPSCs using this combined small-molecule approach (Supplementary Fig. 15) remains the most unexpected finding. The time frame of 15 d for the generation of a functionally mature neuron phenotype is accelerated compared with estimates of nociceptor emergence during human development (30–50 d)35. hPSCs under LSB3i seem to transit through all the major intermediate stages expected for generating mature nociceptors, but at a much faster pace. Upregulation of ISL1 and BRN3A are concomitant with expression of SOX10, starting between days 5 and 7. The optimal time to add LSB3i is day 2 of dual-SMAD inhibition, similar to a previous finding from our laboratory showing an early requirement for sonic hedgehog treatment (day 2) for effective induction of FOXA2 and human floor plate differentiation22. The potent role of CHIR99021 in the derivation of neural crest–derived sensory neurons is probably related to activation of canonical WNT signaling, which is known to be essential during early neural crest specification16 and
capable of instructing naive neural crest precursors toward the sensory neuron lineage. We report efficient neural crest induction in zebrafish found that Notch signaling during neural crest development suppresses sensory neuron differentiation, consistent with the effects of DAPT observed in our study.

The functional data in the current study confirm differentiation of hPSCs into nociceptive sensory neurons. LSB3i differentiation and growth conditions drive the cells predominantly into a P2RX3-expressing phenotype, with some capsaicin-responsive cells. This is consistent with the expression profiling data (Fig. 4a), which show stronger upregulation of P2RX3 than of TRPV1 and may indicate the generation of a predominant P2RX3+, TRPV1− cell type, similar to the rodent nonpeptidergic IB4+ population. The downregulation of RUNX1 and upregulation of RET observed between days 8 and 14 in culture are consistent with this hypothesis (Fig. 2h), and may recapitulate the switch from the NTRK1 (TrkA)+ peptidergic population to the TrkA-negative, Ret+ nonpeptidergic population observed in rodent systems. Loss-of-function studies during mouse development suggest that early Runx1 expression is crucial for inducing ion channel expression and that late expression is a key determinant of the nonpeptidergic switch. We report broad RUNX1 expression at day 8 and robust ion channel expression. However, once ion channel expression is established by day 15, RUNX1 levels decrease in most cells (Fig. 2h), an observation that diverges with published RUNX1 data in the mouse. Future studies will be required to determine whether this result represents a true difference between human and rodent nociceptor development or an idiosyncrasy of our culture system.

Taken together, our data show that combined small-molecule inhibition of endogenous signals provides a rapid, efficient, nongenetic and cost-effective means to modulate hPSC cell fates. The scalable generation of hPSC-derived nociceptors using LSB3i (summarized in Supplementary Table 2 and Supplementary Fig. 15) provides a method for basic biology studies and for drug discovery related to human pain. We envisage that strategies similar to LSB3i can be developed for the rapid induction of other neuron subtypes in the PNS and CNS and may lead to a new generation of directed-differentiation protocols.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession code. GEO: GSE26867.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

S.M.C., experimental design, characterization experiments and manuscript; Y.Q., chemical screen to identify 3i; Y.M. and G.L., SOX10·GFP·BAC transgenic hPSC line generation and culturing; X.-Z. and L.N., initial LSB3i electrophysiology; L.B., L.C., E.S. and P.W., electrophysiology and calcium imaging experiments, PRPH characterization and manuscript; S.-H.S., electrophysiology experimental design; L.S., experimental design and manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

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ONLINE METHODS

Cells and culture conditions. hESC lines (WA-09, passages 32-50; SHEF1) and hiPSC lines (C14, C72; passages 10–20) were cultured with mouse embryonic fibroblasts (MEFs, Globalstem) pre-plated at 12,000–15,000 cells/cm². hiPSC lines were generated as reported. Medium contained DMEM/F12, 20% (vol/vol) Knockout Serum Replacement (Invitrogen), 1 mM L-glutamine, 100 µM MEM nonessential amino acids and 0.1 mM β-mercaptoethanol. We added 6 ng/ml FGF-2 after sterile filtration, fed the cells daily and passaged them weekly using 6 U/ml dispase. SHEF1 cells were maintained in mTESR1 (Stem Cell Technology). The online methods were initiated when the cells were confluent using KSR medium, which contained 820 ml of Knockout DMEM, 150 ml Knockout Serum Replacement, 1 mM L-glutamine, 100 µM MEM nonessential amino acids and 0.1 mM β-mercaptoethanol. To inhibit SMAD signaling, 100 nM LDN-193189 and 10 µM SB431542 were added on days 0–5. Cells were fed daily and N2 medium was added in increasing 25% increments every other day starting on day 4 (100% N2 on day 10). Nociceptor induction was initiated with the addition of the three inhibitors, 3 µM CHIR99021, 10 µM SU5402 and 10 µM DAPT, on days 2–10. Cell passage to lower density can promote maturation of SOX10+ progenitors, and long-term culture medium consisted of N2 containing 25 ng/ml human-b-NGF, BDNF and GDNF.

Microscopy, antibodies and flow cytometry. Cells were fixed with 4% (vol/vol) paraformaldehyde for 20 min, washed with PBS, permeabilized using 0.5% (vol/vol) Triton X in PBS and blocked using 1% (wt/vol) BSA in PBS. For glutamate staining, 0.05% (vol/vol) glutaraldehyde was added to the fixative. Primary antibodies used for microscopy included PAX6 (Covance, #PRB-278P, 1:50), TUBB3 (For Ig; Covance, #MM5-433P or MRB-433P, 1:1000), Ki67 (Sigma, p6834, 1:300), ISL1 (DSHB, #40.2D6, 1:100), BRN3A (Millipore, mab1858, 1:500), RET (R&D, #AF1485, 1:100), RUNX1 (Sigma), glial fibrillary acidic protein (GFAP) (Sigma, #G-6624, 1:1000), peripherin (Santa Cruz, #sc7604, 1:500), TRPV1 (Neuromics, #GB14100, 1:200), Substance P (Neuromics, #GP14103, 1:200), CGRP (Neuromics, #RA24112, 1:200) and SOX10 (Santa Cruz, #sc-17342, 1:500). For flow cytometry, cells were fixed using the BD Cytofix/Cytoperm Kit (BD), including the optional 4% (vol/vol) paraformaldehyde fixation step. Primary conjugated antibodies for flow cytometry were those NTRK1-APC (R&D, #EAB1751A, 1:50), Nestin-Alexa647 (BD Pharmingen, #560341, 1:50), TUJ1-Alexa488 (BD Pharmingen, #560338, 1:50).

Gene expression profiling. Total RNA was isolated at days 2, 3, 5, 7, 9 and 15 of differentiation of LSB- or LSB3i-treated hPSCs using Trizol LS. All samples were processed by the MSKCC Genomics Core Facility and hybridized to the Illumina Human 12 Oligonucleotide array. Normalization and model-based expression measurements were calculated using the Illumina analysis package (LUMI) from the Biociconductor project (http://www.biociconductor.org/) within the statistical programming language R (http://cran.r-project.org/). Expression values are log2 of the fold change. Pair-wise comparison cut-off was significant if, after multiple test corrections, P < 0.05.

Electrophysiology. Patch-clamp experiments were performed in whole-cell configuration at room temperature (20–22 °C) using a Multiclamp 700B patch-clamp amplifier controlled by pClamp 10 software (Molecular Devices). Patch pipettes had resistances between 1.5 MΩ and 2 MΩ. Extracellular solution contained 140 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl2, 1.2 mM MgCl2, 10 mM HEPES and 10 mM glucose; pH was adjusted to 7.4 with NaOH. The intracellular (pipette) solution for voltage-clamp contained 100 mM CsF; 45 mM CsCl, 10 mM NaCl; 1 mM MgCl2, 10 mM HEPES and 5 mM EGTA; pH was adjusted to 7.3 with CsOH. For current-clamp, the intracellular (pipette) solution contained 130 mM KCl, 1 mM MgCl2, 5 mM MgATP, 10 mM HEPES and 0.5 mM EGTA; pH was adjusted to 7.3 with KOH. The omolarity of all solutions was maintained at 320 mOsm/L for extracellular solution and 300 mOsm/L for intracellular solutions. All chemicals were purchased from Sigma. Cells were sampled at 25 kHz and filtered at 10 kHz. Series resistance was compensated by 80–90% to reduce voltage errors; however, space-clamp artifacts occurred because of the presence of processes emanating from the cell bodies. A current-voltage protocol was applied using a 20-ms pulse from −120 mV to +50 mV in 10-mV increments from a holding potential of −120 mV. The voltage protocol used to assess pharmacological block of voltage-gated sodium channels consisted of a voltage step to −85 mV for 8 s, a subsequent voltage step to −120 mV for 100 ms, followed by a test voltage at 0 mV for 20 ms, from a holding potential of −120 mV (Fig. 4b). Interstimulus intervals were 15 s. Action potentials were recorded by holding the membrane potential at −60 mV and injecting increasing currents to elicit action potentials. P2X receptor currents were measured at a holding potential of −80 mV. Whole-cell patch-clamp data were analyzed using Clampfit 10 (Molecular Devices) and Origin 8.1 (Originlab). Results are presented as mean ± s.e.m. Tests for statistical significance were performed using Student's t-test or nonparametric ANOVA, as noted.

Calcium imaging. For calcium-imaging studies, differentiated cells at 3–4 weeks after addition of growth factors were loaded with calcium 5 dye (Molecular Devices) for 1 h at 37 °C/5% CO2. Calcium 5 dye was added at 2× stock to 100 µl DMEM/F12 1:1 medium in well. Calcium flux was monitored using the BD Pathways 855 Bioimager platform to quantify calcium flux at the single-cell level. To monitor stimulation by capsaicin or α,β-methylene-ATP, 5 spherical images were taken, and then compounds were added at 10× directly to the medium in the well to give the final stimulation concentration (1 µM capsaicin, 30 µM α,β-methylene-ATP). Calcium flux was monitored for 30 s, with 0.3-s exposure time and 0.25-s delay between exposures. In a subset of wells, 20 mM KCl was added as a second stimulation to confirm the neural identity of responsive cells. For experiments using the selective P2RX3 antagonist A-317491, the compound was added to the wells 15 min before calcium imaging, and then the protocol was followed as above.

Following data collection, we analyzed the images to quantify calcium flux. Segmentation was carried out based on intensity and event size. Segmentation was carried out on one control image, and held constant across all wells and images in each experiment. Calcium flux was determined by subtracting the mean basal intensity value per cell from the peak intensity following stimulation to determine the change in intensity for each individual cell.

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