Transcriptome profiling of induced hair cells (iHCs) generated by combined expression of Gfi1, Pou4f3 and Atoh1 during embryonic stem cell differentiation

Citation for published version:
Costa, A & Henrique, D 2015, 'Transcriptome profiling of induced hair cells (iHCs) generated by combined expression of Gfi1, Pou4f3 and Atoh1 during embryonic stem cell differentiation' Genomics Data, vol 6, pp. 77-80. DOI: 10.1016/j.gdata.2015.08.017

Digital Object Identifier (DOI):
10.1016/j.gdata.2015.08.017

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Publisher's PDF, also known as Version of record

Published In:
Genomics Data

Publisher Rights Statement:
Under a Creative Commons license

General rights
Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy
The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.
Transcriptome profiling of induced hair cells (iHCs) generated by combined expression of Gfi1, Pou4f3 and Atoh1 during embryonic stem cell differentiation

Aida Costa ⁎,1, Domingos Henrique ⁎

Instituto de Medicina Molecular, Faculdade de Medicina da Universidade de Lisboa, Lisboa 1649-028, Portugal

Abstract

To gain new insights about the genetic networks controlling hair cell (HC) development, we previously developed a direct genetic programming strategy to generate an inexhaustible supply of HC-like cells (induced HCs, iHCs) in vitro, starting from mouse embryonic stem cells (ESC). We found that combined activity of three transcription factors, Gfi1, Pou4f3, and Atoh1, can program ESC-derived progenitors towards HC fate with efficiencies of 55%–80%. These iHCs express several HC markers and exhibit polarized structures that are highly reminiscent of the mechanosensitive hair bundles, with many microvilli-like stereocilia. Here, we describe the experimental design, methodology, and data validation for the microarray analysis used to characterize the transcriptome profile of iHCs at different stages of their differentiation. This approach based on FACS sorting and microarray analysis revealed a highly similar iHC transcriptome to that of endogenous HCs in vivo. The data obtained in this study is available in the Gene Expression Omnibus (GEO) database (accession number GSE60352).

© 2015 Elsevier Inc. All rights reserved. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

1. Direct link to deposited data

The deposit data can be found at: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE60352.

2. Experimental design, materials and methods

2.1. Cell culture and embryoid bodies (EBs) formation

The embryonic stem cell (ESC) line iGPA-Myo7a:mVenus used in this study was generated from the Ainv15 ESC line [1] by two genetic engineering steps: I) a DNA fragment containing the three genes Gfi1-Pou4f3-Atoh1 (GPA) was inserted into the doxycycline (dox) inducible locus of the Ainv15 cells as previously described in [2] to produce the iGPA line. II) The mVenus fluorescent reporter under control of the pro-moter and regulatory regions of the incipient HC marker, Myo7a [3] was randomly inserted into the iGPA genome as described in [4], to generate the iGPA-Myo7a:mVenus line. These ESCs were routinely grown on gelatin-coated (0.1%) Nunc dishes at 37 °C in a 5% CO2 incubator with Dulbecco’s Modified Eagles Medium 1× (DMEM, Gibco) supplemented with 10% of heat-inactivated fetal bovine serum (FBS) (Gibco, ES-qualified), 2 mM glutamine (Gibco), 1% penicillin/streptomycin (Gibco), 1 mM sodium pyruvate (Gibco), 1% MEM non-essential amino acids (Gibco), 2 ng/ml Leukemia inhibitory factor (LIF) and 7 μM 2-mercaptoethanol (Sigma). All solutions were mixed and filtered.
every other day, at constant plating density of $3 \times 10^4$ cells/cm². To generate embryoid bodies (EBs), ESCs were dissociated into single cells (using 002% trypsin at 37 °C for 3 min) and plated at low density (2 × 10⁴ cells/cm²) with the same supplemented DMEM medium but without LIF, in 10 cm bacterial grade petri dishes to prevent attachment. EB formation was checked on day 1 and medium was replaced every two days (days 2, 4, 6, 8 and 10). Supplementation with 2 μg/ml doxycycline (Sigma, diluted in sterile PBS and filtered through a 0.2 μm filter unit), 1 μm retinoic acid (RA) (diluted in 0.01% DMSO, Sigma) was initiated at day 4 and maintained until the required time point for analysis (day 8 or day 12) (Fig. 1).

2.2. iHC purification by FACS and isolation of total RNA

To determine the transcriptome profile of iHCs at different differentiation stages, we FACS-sorted Dox treated EBs at days 8 and 12 challenged with Dox or Dox + RA (Fig. 1). These EBs were collected into a conical tube and allowed to sediment (~5 min) at the bottom of the tube, the supernatant was removed and fresh PBS was added. EBs were washed twice in PBS, and after incubation with 1 ml of 0.25% trypsin for 5 min at 37 °C, DMEM media was added to stop the reaction. Cells were triturated 10–20 times using a P1000 Gilson pipette tip to obtain a single cell suspension. After centrifuge, cells were resuspended in FACS buffer (PBS with 4% FBS) and filtered using a cell strainer 70 μm (BD Bioscience). Cell sorting experiments were done on a FACS Aria cell sorter (Becton Dickinson). FACS-sorted cells were directly processed for RNA extraction using the High Pure RNA Isolation Kit (Roche), according to the manufacturer’s instructions. For synthesis of cDNA, 1 μg of total RNA was used as a template for the reverse transcription performed with the Superscript II Reverse Transcriptase system (Invitrogen) in a final volume of 20 μl. Transcription was conducted with random primers. The absence of contaminating genomic DNA was confirmed for each RNA extraction by PCR amplification of GAPDH-specific product from reverse transcriptase negative samples (without Superscript II enzyme).

2.3. Sample validation

Sorted Venus-positive cell populations from EBs were collected with high purity (~98%) and the Venus-negative cell fraction was discarded for the microarray analysis. However, these nonfluorescent cells were used for quantitative-real time PCR (qPCR) analysis, to validate samples and to determine the enrichment of the iHC population in the Venus-positive fraction. The qPCR reaction was performed on 96-well plates (MicroAmp; Applied Biosystems) or 384-well plates (MicroAmp; Applied Biosystems) covered with optical adhesive covers (Applied Biosystems). The instruments used were Applied Biosystems 7500 Real-Time PCR or Applied Biosystems Viia 7 Real-Time PCR. The Real-Time PCR was carried out using iTaq Universal Sybr Green Supermix (Bio-Rad), 2 μl of the retrotranscription cDNA template diluted 1:100 and 12.5 pmol of each primer. Reaction conditions were as follows: one step of 50 °C for 2 min, one 95 °C for 10 min, and 40 cycles of 95 °C for 15 s denaturation and 60 °C for 1 min annealing and extension. The cDNA was used as template for each pair of primers in a duplicate PCR reaction. GAPDH was used as a calibrator. Relative expression levels in the various Dox-treated samples were referred to the levels of expression in untreated control (without Dox), which were arbitrarily set to 1. Results are shown as averages ± standard error of mean (SEM) of three independent experiments. As expected, strong Venus expression was only observed among the sorted Venus-positive cells (Fig. 2). Myo7a expression was significantly higher in the Venus-positive sorted cells and HC markers such as Myo6 [5], Espin [6], and Cdh23 [7] were also enriched in this fraction (Fig. 2). Overall, the results confirmed the high degree of purity of the iHC-sorted cells.

3. Microarray sample preparation

RNA concentration and purity was determined by spectrophotometry, and integrity was confirmed using an Agilent 2100 Bioanalyzer with a RNA 6000 Nano Assay (Agilent Technologies, Palo Alto, CA). RNA was processed for use on Affymetrix (Santa Clara, CA, USA) Mouse Genome 2.1 ST Arrays Strip, by using the Ambion WT Expression Kit (Life Technologies, CA, USA) and Affymetrix GeneChip WT Terminal Labeling Kit, according to the manufacturer’s protocols. Briefly, 100 ng of total RNA containing spiked in Poly-A RNA controls (GeneChip Expression GeneChip Eukaryotic Poly-A RNA Control Kit, Affymetrix) was used in a reverse transcription reaction (Ambion WT Expression Kit) to generate first-strand cDNA. After second-strand synthesis, double-stranded cDNA was used in an in vitro transcription (IVT) reaction to generate cRNA (Ambion WT Expression Kit). 15 μg of this cRNA was used for a second cycle of first-strand cDNA synthesis (Ambion WT Expression Kit). 5.5 μg of single stranded cDNA was fragmented and end-labeled (GeneChip WT Terminal Labeling Kit, Affymetrix). Size distribution of the fragmented and end-labeled cDNA, respectively, was assessed using an Agilent 2100 Bioanalyzer with a RNA 6000 Nano Assay. 3.5 μg of end-labeled, fragmented cDNA was used in a 150 μl hybridization cocktail containing added hybridization controls (GeneAtlas Hybridization, CTAB-based Hybridization Controls, Agilent Technologies). RNA was fragmented and end-labeled using 100 μl of GeneChip Eukaryotic Poly-A RNA Control Kit, (GeneChip Eukaryotic Poly-A RNA Control Kit, Affymetrix) and incubated overnight at 37 °C. RNA was then labeled with biotin using 15 μl of RNA 3’ terminal labeling kit (Ambion). Hybridization cocktail contained fragmented and labeled cRNA (150 μg) and reference control RNA (30 μg). Hybridization reactions were performed on mouse genome 2.1 ST arrays (Affymetrix) following the manufacturer’s protocols. After hybridization, slides were washed at room temperature, 50 °C and 80 °C, and stained with streptavidin-Cy5 and Cy3 to visualize probes labeled with biotin and Cy3. After washing, slides were scanned at 14 μm resolution using an Agilent 2100 bioanalyzer with a GeneChip 2100 Scanner. The Affymetrix GeneChip Mouse 430 2.0 Genechip platform was used for hybridization on Affymetrix GeneChip Mouse 430 2.0 Genechip Arrays (Affymetrix).
Wash, and Stain Kit for WT Array Strips, Affymetrix), of which 120 µl were hybridized on array strips for 20 h at 48 °C. Standard post-hybridization wash and double-stain protocols (GeneAtlas Hybridization, Wash, and Stain Kit for WT Array Strips, Affymetrix) were used on a GeneAtlas system (Affymetrix), followed by scanning of the array strips.

Three biologically independent replicates at day 8 and day 12 were analyzed for the Dox EBs-treated conditions (Dox and Dox + RA), and two independent RNA preparations for unsorted EBs "no treatment." Thus, a dataset composed of 16 independent transcriptomes was generated.

4. Data analysis

The 16 scanned arrays were analyzed first with Expression Console software (Affymetrix) using RMA to obtain expression values, and for quality control. Control probe sets were removed and log2 expression values of the remaining 33,710 transcripts were imported into Chipster 2.4 [8]. Unsupervised hierarchical clustering (cluster method: average linkage; distances: Pearson correlation) and principal component analysis of the 16 transcriptome datasets were performed using Chipster 2.4 software. This analysis clearly shows two main branches that correspond to the untreated conditions and to Dox-treated conditions (Fig. 3). The clear segregation between “No Dox” and “Dox” was expected, and validates this global dataset. We next examined the microarray datasets to identify genes whose expression vary significantly among the 4 iHC stages, when compared to the non-induced cells (without Dox) at the same time point. An empirical Bayes two-group test with Benjamini-Hochberg multiple testing correction (P-value < 0.01, expression fold change > 2) [9] was used to identify lists of differentially expressed genes in each stage. These lists are organized accordingly to the variations in expression (up- or down-regulated in relation to the untreated cells) (Table 1). To correlate these differentially expressed gene lists with biological function, we searched for enrichment of gene ontology (GO) functional groups in the up-regulated and down-regulated gene lists using DAVID functional annotation tool (http://david.abcc.ncifcrf.gov/). We found that up-regulated genes are mainly involved in transmission of nerve impulses and sensory perception of mechanical stimulus in the 4 iHCs populations. In
contrast, down-regulated genes in all lists are connected to cell cycle and cell division.

Acknowledgements

We are indebted to Jörg Becker and IGC Gene Expression Unit for their technical support with microarray sample preparation and analysis. This work was supported by Fundação para a Ciência e Tecnologia, Portugal [PTDC/SAU-NEU/71310/2006, SFRH/BD/38461/2007 to A.C.]. A.C. was also a recipient of an EMBO Short-Term Fellowship.

References

[1] M. Kyba, R.C. Perlingeiro, G.Q. Daley, HoxB4 confers definitive lymphoid–myeloid engraftment potential on embryonic stem cell and yolk sac hematopoietic progenitors. Cell 109 (2002) 29–37.
[2] D.T. Ting, M. Kyba, G.Q. Daley, Inducible transgene expression in mouse stem cells. Methods Mol. Med. 105 (2005) 23–46.
[3] B. Boeda, D. Weil, C. Petit, A specific promoter of the sensory cells of the inner ear defined by transgenesis. Hum. Mol. Genet. 10 (2001) 1581–1589.
[4] A. Costa, L. Sanchez-Guardado, S. Juniat, J.E. Gale, N. Daudet, D. Henriques, Generation of sensory hair cells by genetic programming with a combination of transcription factors. Development 142 (2015) 1948–1959.
[5] M. Xiang, W.Q. Gao, T. Hasson, J.J. Shin, Requirement for Brn-3c in maturation and survival, but not in fate determination of inner ear hair cells. Development 125 (1998) 3935–3946.
[6] L. Zheng, G. Sekerková, K. Vranich, L.G. Tilney, E. Mugnaini, J.R. Bartles, The deaf jerker mouse has a mutation in the gene encoding the espin actin-bundling proteins of hair cell stereocilia and lacks espins. Cell 102 (2000) 377–385.
[7] J. Siemens, C. Lillo, R.A. Dumont, A. Reynolds, D.S. Williams, P.G. Gillespie, U. Müller, Caderhin 23 is a component of the tip link in hair-cell stereocilia. Nature 428 (2004) 950–955.
[8] M.A. Kallio, J.T. Tuimala, T. Hupponen, P. Klemela, M. Gentile, I. Scheinin, M. Koski, J. Kalli, E.J. Korpelainen, Chipster: user-friendly analysis software for microarray and other high-throughput data. BMC Genomics 12 (2011) 507.
[9] G.K. Smyth, Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. Stat. Appl. Genet. Mol. Biol. 3 (2004) 1544–6115.