SUPPLIMENTARY INFORMATION

Tolkunova et al. The Caudal-related Cdx2 Promotes Trophoblast Differentiation of ES cells.

Plasmid Constructs

The Cdx2 ORF was PCR amplified from a reversely transcribed mRNA from mouse E14 ES cells with the primers 5'-GGAATTCGGCGCGCCACCATGG CCTACGTGAGCTACCTTCTG-3' and 5'-TTCCGCTCGAGCTAGCTCACTGGGTG ACAGTGGAGTTTAAAACCCCTC-3' and the GR* ligand-binding domain coding sequence was amplified from pBKC-CreGR* plasmid (F. Stewart) with 5'-CATGCCATGGCCACTACAGGAGTCTCACAAG-3' and 5'-CATGCCATGGAC TTTTGATGAAACAGAAGTTTTTTG-3' primers. The Cdx2 PCR fragment was digested with EcoRI/XhoI and cloned to pBluescrptKS. Resulting plasmid was NcoI linearized and used to accept NcoI-cleaved GR* fragment to generate in-frame fusion ORF for the GR*Cdx2 protein. This ORF was then excised from pBS with AscI and XhoI and cloned into the pCX-IRES-Hyg vector. The latter was derived from the pCX-EGFP [19] by replacing the EGFP part with IRES-Hyg cassette (Clontech) at EcoRI sites.

Cells

E14 ES cells were cultured on gelatinized plates or hygromicin-resistant, mitomycin-inactivated mouse embryonic fibroblast (MEF) cell layer in DMEM supplemented with LIF (1000u/ml), 15% fetal calf serum (HyClone), non-essential amino acids, L-glutamine, and penicillin/streptomycin were electroporated with SalI-linearized pCX-GR*cdx2-IRES-Hyg vector. Selection was carried out for 10-12 days in the presence of 200μg/ml hygromycin. Approximately 5% of picked ES clones demonstrated the ability to differentiate into trophoblast-like cells in the following assay. The ES cells were seeded at low density ($1–3x10^3/cm^2$) on plates without MEFs. Dexamethasone (DEX, Sigma) was immediately added to the final concentration of 1μM. For derivation of TS_{ES} cells, pre-selected GR*cdx2 ES clones that have shown response to the DEX, were

induced as above in the presence of MEF-conditioned medium, recombinant Fgf4 (25 ng/ml, Peprotech) and heparin (1 μg/ml, Sigma), as described by Tanaka et al. (1998). TSL_{ES} were induced to differentiate into TGL_{ES} cells by plating them in the TS medium without Fgf4 and/or MEFs or MEF-conditioned medium.

Flow Cytometry

ES cells were harvested with trypsin, fixed, and stained with propidium iodide (PI) as per standard protocol (Darzynkiewicz Z, Juan G. DNA content measurement for DNA ploidy and cell cycle analysis. In: Robinson JP, ed. Current Protocols in Cytometry. New York: Wiley, 1997:7.5.1-7.5.24). PI fluorescence was measure by flow cytometry using FACSCalibur (Becton, Dickinson).

Steroid measurements

Supernatants from DEX-induced GR*cdx2 cultures were collected and subject to progesterone and androstenedione measurements as described in Tong MH, Christenson LK, Song WC.Aberrant cholesterol transport and impaired steroidogenesis in Leydig cells lacking estrogen sulfotransferase. Endocrinology 2004;145:2487–2497.

RT-PCR

Total RNA was extracted using the RNeasy Kit (Qiagen). Equal amounts of this RNA from various time point of culturing were reversely transcribed using a reverse transcriptase (MBI Fermentas) and amplified by PCR (22-25 cycles), using the following pairs of primers:

Hprt: 5’-CGTCATGCCGACCCGCAGTCC-3’, 5’-ATTCAACTTGCGCTCATCTTA-3’

Oct4: 5’-GAACAGTTTGCCAAGCTGCTG-3’, 5’-CCGGTTACAGAACCATACTCG-3’

GRcdx2*: 5’-GGAAAAGCCATTGTCAAGAGGG-3’, 5’-AAAGTTCTGCGGAGCCAGGTTC-3’

Cdx2**: 5’-GGAGAAGGAGTTTCACTTTAGTCGATAC-3’, 5’-CCAGGAATCACTTCGTTTGTCG-3’

Pl1: 5’-CCCTGTGTCATACTGCTTCCATC-3’, 5’-AACTCGGCACCTCAAGACTTTG-3’

Hand1: 5’-CAAGGCTGAACTCAAAAAGACGG-3’, 5’-AGATGGGTTGGAAGGGTGTGTC-3’

Hnf4: 5’-ATGCCTGCCTCAAAGCCATC-3’, 5’-CCACTCACACATCTGTCCATTGC-3’

Eomes: 5’-CCGCCCACTACAATGTTTTCG-3’, 5’-GAGAAGGTGAAGGTCTGAGTCTTGG-3’

Fgfr2b: 5’-AAGGTACGAAACCAGCACTGGAG-3’, 5’-TCCATCTCCGTCACATTGAACAG-3’

Fgfr2c: 5’-AAGGTACGAAACCAGCACTGGAG-3’, 5’-CAGAGTGAAAGGATATCCCGATAG-3’

Fgf4 5’-AAAGGCTTCGGCGGCTCTAC-3’, 5’-TGGTCCGCCCGTTCTTACTG-3’

Nanog: 5’-CAGAAGTACCTCAGCCTCCAG-3’, 5’-AAAGTCCTCCCCGAAGTTATG-3’

Sox2: 5’-GGGTGCAAAAAGAGGAGAGTAG-3’, 5’-CTTAAACAAGACCACGAAAACG-3’

Utf1: 5’-CGCCGTCGCTACAAGTTCC-3’, 5’-AGGCTCATTCGGGGTCTCC-3’

Id1: 5’-TTGGTCTGTCGGAGCAAAGC-3’, 5’-CAAAGTCTCTGGAGGCTGAAAGG-3’

Bmp4: 5’-GGAAGAAAAAAGTCGCCGAGATTC-3’, 5’-TGGGATGCTGCTGAGGTTGAAG-3’

Rex1: 5’-CCCGAGACTGAGGAAGATGG-3’, 5’-TTGCGTGGGTTAGGATGTGA-3’

* The forward primer in this pair annealed to the GR part of the fusion and the reverse primer – to the Cdx2-coding part.

** The forward primer in this pair annealed to the Cdx2-coding part and the reverse primer – to the Cdx2 3’UTR.

Immunofluorescent Labeling and In situ Hybridization

Cells were fixed directly in culture dishes in 4% PFA for 10-15 min at RT followed by 10-min treatment with 0.1% Triton X-100 and blocking in 2% normal sheep serum, 1%BSA in PBS for 15 min. Cells were then incubated for 1 hr at RT with monoclonal Oct4 antibody (1:100-200, Santa-Cruz) and rabbit polyclonal Cdx2 (1:100-200, gift of Felix Beck), diluted in the blocking solution plus 0.1% Tween-20, followed by extensive washing and 30-min incubation at RT with Alexa594-conjugated goat anti-mouse and Alexa488-conjugated goat anti-rabbit antibody (both at 1:200, Molecular Probes), diluted in the same buffer. In situ hybridization with Pl-1 probe was performed as described in Boiani M, Eckardt S, Scholer HR et al. Oct4 distribution and level in mouse clones: consequences for pluripotency. Genes Dev 2002;16:1209–1219.