**OCT4 and downstream factors are expressed in human somatic urogenital epithelia and in culture of epididymal spheres**

David M. Kristensen, John E. Nielsen, Mark Kalisz, Marlene D. Dalgaard, Karine Audouze, Malene E. Larsen, Grete K. Jacobsen, Thomas Horn, Søren Brunak, Niels E. Skakkebaek, and Henrik Leffers

1University Department of Growth and Reproduction, Section GR5064, Rigshospitalet, Blegdamsvej 9, 2100 Copenhagen, Denmark 2Hagedorn Research Institute, Nielsen Steensensvej 6, 2820 Gentofte, Denmark 3Center for Biological Sequence Analysis, Department of Systems Biology, Technical University of Denmark, 2800 Lyngby, Denmark 4Department of Pathology, Rigshospitalet, Blegdamsvej 9, 2100 Copenhagen, Denmark 5Department of Pathology, Herlev Hospital, 2730 Herlev, Denmark

*Correspondence address. E-mail: david@moebjerg.com

Submitted on December 14, 2009; resubmitted on January 22, 2010; accepted on January 27, 2010

**ABSTRACT:** The transcription factor OCT4 plays a crucial role in the earliest differentiation of the mammalian embryo and in self-renewal of embryonic stem cells. However, it remains controversial whether this gene is also expressed in somatic tissues. Here, we use a combination of RT–PCR on whole and microdissected tissues, in situ hybridization, immunohistochemistry and western blotting to show that OCT4 and SOX2 together with downstream targets, UTF1 and REX1/ZFP42, are expressed in the human male urogenital tract. We further support these results by the analysis of DNA methylation of a region in the OCT4 promoter. In culture, human primary epididymal cells formed spheres that continued to express the investigated genes for at least 20 days. Transcriptomic analysis of cultured cells showed up-regulation of CD29, CD44 and CD133 that are normally associated with sphere-forming cancer stem cells. Furthermore, stimulation with retinoic acid resulted in down-regulation of OCT4 expression, however, without multilineage differentiation. Our results show that OCT4 and associated genes are expressed in somatic epithelial cells from the urogenital tract and that these cells can form spheres, a general marker of stem cell behaviour.

**Key words:** OCT4 / epididymis / prostate / CD133 / epididymal sphere

**Introduction**

The transcription factor OCT4 (also known as OCT3, OCT3/4 and POU5F1) is a Pou domain containing protein belonging to the homeobox gene family and plays a crucial role in maintaining pluripotency in mouse embryonic cells and in cells of the mouse germline (Nichols et al., 1998; Kehler et al., 2004). In human embryonic stem (ES) cells, OCT4 together with NANOG and SOX2 collaborates to form a regulatory network consisting of autoregulatory and feedforward loops (Boyer et al., 2005). A key target of OCT4 and SOX2 in both mouse and human ES cells is UTF1 (Nishimoto et al., 1999; Boyer et al., 2005), whereas Rex1/Zfp42 has been described as a target of both Oct4 alone and of Nanog together with Sox2 in mouse ES and embryonic carcinoma cells (Ben Shushan et al., 1998; Shi et al., 2006). However, although OCT4 is one of the main mediators of pluripotency, numerous studies have reported the expression of OCT4 in somatic stem cell compartments and in cultured somatic progenitor cells (for overview, see Supplementary Data in Lengner et al., 2007).

Another line of studies has, however, challenged this role of OCT4 in the soma. Lengner et al. (2007) showed that deletion of exon 1 in the soma, Lengner et al. (2007) showed that deletion of exon 1 in somatic tissues, including the intestinal epithelium and bone marrow most frequently reported positive for expression, revealed no abnormalities in homeostasis or regenerative capacity in the mouse. Other studies have shown differential expression patterns of three splice variants (OCT4A, OCT4B and OCT4B1) in human cells, where OCT4A and OCT4B1 are associated with pluripotency and with protein localization in the nuclei of cells, whereas OCT4B is detected in various non-pluripotent cell types with protein located in the cytoplasm (Lee et al., 2006; Atlasi et al., 2008). In addition to the
expression of different splice variants, Liedtke et al. (2007) reported
that the presence of several transcribed pseudogenes, which
have high similarity to the OCT4A sequence, can be a potential
source of false-positive results and might be misinterpreted in
reverse transcription–polymerase chain reaction (RT–PCR) (Liedtke
et al., 2007). In fact, Cantz et al. (2008) reported the absence of
OCT4 expression in somatic tumour cell lines and argued that
previous reports of OCT4 expression in various somatic cells could
be attributed to OCT4 pseudogene expression and misinterpretation
of background signals.

Contesting this line of studies, and supporting the original notion of
the expression of OCT4 in the somatic tissues, Mizuno and Kosakal
(2008) thoroughly characterized mouse Oct4 transcripts and showed
expression in the mouse soma. They reported that alternative Oct4
transcripts, transcribed from regions from intron 1 through exon 3,
were expressed in somatic cells and that two predicted proteins
could be translated (Oct4b and Oct4c). One of the proteins, Oct4c, transformed non-tumorigenic fibroblasts in vitro indicating a
functional role of the transcript. Thus, based on these findings, the
authors argue that Oct4 expression and function in adult tissue,
both in somatic and cancer cells, should be reconsidered.

By using the primers described by Liedtke et al. (2007) that only
amplify OCT4A transcript and immunohistochemistry (IHC) with an
antibody against OCT4A together with western blotting with two anti-
bodies, we here show that epithelial cells from the human male uro-
genital tract express OCT4. We support these findings with a
methylation analysis of the OCT4 promoter and show, by in situ hybrid-
ization (ISH) and laser-microdissection, that there is indeed transcript
localized to the epithelium. Moreover, we show that SOX2 together
with downstream factors from OCT4 and SOX2, UTF1 and REX1
are also expressed in the epithelia. We further show that in vitro
culture of human epididymal cells lead to the formation of spheres,
a marker of stem cells’ behaviour, that in a transcriptomic analysis
resemble epididymis but respond to retinoic acid (RA) by down-
regulation of OCT4. On the basis of these findings, we believe that
OCT4 is expressed in the human soma in the reproductive tract,
however, without conveying pluripotency.

Materials and Methods

Cell culture

Cultures of the human ES cell lines H7 and Shef5 were maintained as
described in Atlasi et al. (2008) and sorted using a fluorescence-activated
cell sorter (FACS) for cells expressing SSEA3.

Tissue samples

The regional committee for Medical Research Ethics in Denmark approved
the use of human tissue samples for this project and informed consent was
obtained from all human subjects. Samples of epididymis and prostate
were obtained from the Department of Pathology (Herlev Hospital,
Denmark) and the University Department of Growth and Reproduction
(Righospitalet, Denmark).

Derivation and culture of epididymal spheres

Whole human epididymal tissues were mechanically disrupted and enzym-
atically dissociated with 2.6 mg/ml collagenase IV (Sigma-Aldrich, St
Louis, MO, USA) in a knockout culture medium (Invitrogen, San Diego,
CA, USA) with 25 μg/ml DNase, 1% human serum albumin and
1.6 mM CaCl2 (Sigma-Aldrich) for 30–40 min at 37°C. To facilitate diges-
tion, the samples were shaken every 10 min during the incubation period.
The digested tissue solution was allowed to settle to remove any residual
pieces and/or debris not dissolved by the enzymes. The cell suspension
(supernatant) was centrifuged for 5 min at 1200g and washed twice in the
knockout culture medium with 10% knockout serum replacement,
100 μg/ml penicillin, 100 U/ml streptomycin, 1 mM l-glutamine, 1 mM
β-mercaptoethanol and Fungizone (1:500) (all from Invitrogen). Cells
were plated in 6-well plates (Nunc, Roskilde, Denmark) with half the
medium changed every day and incubated at 37°C in a humidified cell
culture incubator with 5% CO2. For RA stimulation, the epididymal
spheres were allowed to aggregate for a day before stimulation with 0.5 μM RA (Sigma-Aldrich) dissolved in DMSO.

Immunohistochemistry

The following primary antibodies were used: mouse monoclonal anti-OCT4
(POU5F1) (C-10, sc-5279; Santa Cruz Biotechnology Inc., Santa Cruz, CA,
USA) that recognizes amino acids 1–134 of the OCT4A protein and there-
fore recognizes only OCT4A isoform (Atlasi et al., 2008), goat polyclonal
anti-NANOG (AF1997; R&D Systems, Minneapolis, MN, USA), mouse
monoclonal anti-NANOG (14-5769-82; eBioscience, San Diego, CA,
USA), rabbit polyclonal anti-SOX2 (AB5770; Chemicon, Temecula, CA,
USA), mouse monoclonal anti-UTF1 (MAB4337; Chemicon) and biotinylated
sheep polyclonal anti-REX-1 (BASF3598; R&D Systems). Biotinylated second-
ary antibodies (Zymed Histostain kit, San Francisco, CA, USA) were applied,
and a peroxidase-conjugated streptavidin complex (Zymed Histostain kit) was
used as a tertiary layer. Visualization was performed with aminothiolcarbazole
(Zymed Histostain kit). Biotinylated anti-REX-1 was visualized with a
streptavidin-conjugated alkaline phosphatase complex (Roche Diagnostics,
Basel, Switzerland) and colour development was carried out with BCIP/
NBT (5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium chloride;
Sigma-Aldrich) with levamisol (Sigma-Aldrich) to inhibit endogenous phos-
phatases. Between incubation steps, the slides were washed with tris-buffered
saline (TBS). Tissue samples were fixed over night at 4°C in buffered formalin
or 4% paraformaldehyde (PFA). All experiments included control staining
without the primary antibody.

Examination was done on a Nikon Microphot-FXA microscope (Nikon,
Tokyo, Japan) and two investigators scored the results. Staining intensity
was assessed using an arbitrary semi-quantitative score of staining inten-
sity: +++, strong staining; ++, weak staining; +, weak staining; and neg, no staining.

Western blot

Protein extracts were prepared by Cell Lysis Buffer (Cell Signaling Technol-
ogy, Beverly, MA, USA) (20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM
Na2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate,
1 mM β-glycerophosphate, 1 mM Na3VO3, 1 μg/ml leupeptin) sup-
plemented with 1 mM phenylmercuransulphonyl fluoride followed by ultra-
sound homogenization. Protein concentrations were determined using the
Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, USA). A total of 20 μg
samples were separated on a 10% SDS–polyacrylamide gel according to
standard procedures and blotted onto a PVDF membrane (Millipore,
Schwalbach, Germany). Membranes were blocked for 1 h with blocking
buffer (5% skimmed milk powder in TBS + 0.1% Tween-20) and incu-
bated overnight with one of three antibodies in blocking buffer: mouse
monoclonal anti-OCT3/4 (POU5F1) (1:2000; C-10, sc-5279), goat polyclonal
anti-OCT3/4 (1:2000; N-19, sc-8628) and rabbit polyclonal anti-
TFIIIB (1:2000; sc-225). After washing in TBS Tween-20 (0.1%), mem-
branes were further incubated for 1 h with goat anti-mouse (1:2000;
sc-205), goat anti-rabbit (1:2000; sc-204) or donkey anti-goat
(1:2000; sc-2020) horseradish peroxidase-conjugated secondary
antibodies in blocking buffer. For chemiluminescence detection, ECL (ECL western blotting detection reagents, Amersham, Germany) was used according to the manufacturer’s instructions.

RT–PCR analysis

RNA was isolated with NucleoSpin RNA II purification kit with DNase I treatment as described by the manufacturer (Macherey-Nagel, Düren, Germany). Additional RNA samples were purchased: human testes total RNA (Ambion, Austin, TX, USA), human total Ductus Deferens (BioChain, Hayward, CA, USA), human total Epididymis (BioChain), human prostate total RNA (Ambion, Austin) and human ovary total RNA (Ambion, Austin). One microgram of DNase I-treated RNA was reverse transcribed with AMV reverse transcriptase (USB, Cleveland, OH, USA) using a dT20 primer and random hexamers and ultimately resuspended in 100 μl of TE buffer. RT–PCR was performed using the PAQ 5000 polymerase (Stratagene, La Jolla, CA, USA). The following primers were used for amplification for 35 cycles with OCT4A primers taken from literature and NANOG primers designed to avoid known pseudogenes: OCT4A AGGCCCTATCTCACAAGGCC and TGGGACTCCTCCGGTGTGTTTGG from Liedtke et al. (2007) and TCCCTTCGCAAGGCTCTCAT and TGACGTTGAGGGTCTCGGCG AGGCCCCTC from Suo et al. (2005); NANOG CTGTAAGAACACA ATATTGATAG and ATACAAAGACTTCTTTCTAAGAG; SOX2 GCC GAGTGGAAACTTTTTGCTG and GCCAGCTGTACTTTCTCTCTT; UTF1 GGCCGCTAAGTCCAATAA and GGATCTGCTGTC GAAAGG; REX1 GGAATGTCGAAAACTGGTCTGTG and CCGGTGTA GGCGGCACGCT; B2M (β2-microglobulin) AGTATGCTGCCGGTG AAGC and ATCCAAATGGGCGGCTC; RPS20 AGAAGTTGAAAGA CACTC and ATCCGCAATGTGCCTCACC. PCR products were run on 2% agarose gels and visualized by ethidium bromide staining. Representative bands from each primer combination were excised and sequenced for verification (DNA Technology, Aarhus, Denmark).

Quantitative RT–PCR analysis

RNA was isolated and reverse transcribed as described for RT–PCR. Quantitative RT–PCR (qRT–PCR) analysis was performed in triplicate in a Stratagene MX300P (Stratagene) with Brilliant SYBR Green QPCR Master Mix (Stratagene). The same primers were used for qRT–PCR as in a Stratagene Mx300P (Stratagene) with Brilliant SYBR Green QPCR Master Mix (Stratagene). The RNA quality was tested using Agilent 2100 Bioanalyzer with Agilent RNA 6000 pico kit. Agilent Whole Human Genome Microarray 4x44K chips (Agilent Technologies) for Amino Allyl MessageAmp II aRNA Amplification kit. Agilent RNA quality tested using Agilent 2100 Bioanalyzer with Agilent RNA 6000 pico kit. Agilent Whole Human Genome Microarray 4x44K chips (Agilent Technologies) were amplified once with MessageAmpTM II aRNA Amplification Kit (Ambion, Foster City, CA, USA) and the RNA quality was tested using Agilent 2100 Bioanalyzer with Agilent RNA 6000 nano kit (Agilent Technologies). Bisulphite modification of DNA was carried out with the EZDNA methylation Gold kit (Zymoresearch, Seattle, WA, USA), and PCR was carried out as described in Deb-Rinker et al. (2005) with the following primers amplifying a region with four known differentially methylated CpG dinucleotides: AAGTTTTTGTTGGGGATTTGTAT and CCCGCC ACTAACCCTAACTCT.

Microarray analysis

The RNA quality was tested using Agilent 2100 Bioanalyzer with Agilent RNA 6000 nano kit (Agilent Technologies). Afterwards, the samples were amplified once with MessageAmpTM II aRNA Amplification Kit (Ambion, Foster City) and coupled to amino aliphyl-modified with Cy3 (Amersham Biosciences, Uppsala, Sweden) as described in the protocol for Amino Aliphyl MessageAmpTM II aRNA Amplification kit. Agilent Whole Human Genome Microarray 4x44K chips (Agilent Technologies) were used. Hybridization and scanning were performed as described by the manufacturer and analysed using the Agilent Feature extraction software (version 9.1.3.1) (Agilent Technologies). Data analyses were done using R software (www.r-project.org) with the Limma, G-Plots and Heatplus packages, and probe intensities were normalized with the q-spline algorithm as described in Workman et al. (2002).

Statistical analysis

Differences in gene expression between groups were evaluated by a Student’s t-test with a P-value of <0.05 considered statistically significant.
Results

Expression of OCT4A, NANOG, SOX2, UTF1 and REX1/ZFP42 in epididymis and prostate

The expression of OCT4, NANOG, SOX2 and downstream targets UTF1 and REX1/ZFP42 in the human urogenital tract was investigated in RNA from commercial vendors. In addition, we used RNA from human ES cells and human ovary as positive and negative controls, respectively (Fig. 1A). RT–PCR was performed, and for the OCT4A transcript, two different sets of primers were used as described in Suo et al. (2005) (results not shown) and Liedtke et al. (2007) designed to distinguish the OCT4A transcript from other OCT4 transcripts and pseudogenes. Furthermore, primers for NANOG were designed to avoid the many pseudogenes also known for this gene (Booth and Holland, 2004). The results showed that both OCT4A and NANOG transcripts were present in testis, duct deferens, epididymis and prostate together with the transcripts of SOX2, REX1 and UTF1. In addition, RT–PCRs on RNA which had been subjected to the cDNA synthesis protocol, but in which the RT enzyme was omitted to reveal possible residual DNA contamination, were all negative.

We next investigated the localization of NANOG, OCT4A and REX1 by ISH. The examination showed that these genes were expressed in the epithelial cells in the epididymis (Fig. 1B and C) and prostate. To further verify expression and localization of the mRNA, we laser-microdissected epithelial cells from epididymis and prostate and amplified the RNA. RT–PCR on the amplified RNA confirmed the presence of OCT4A and NANOG transcripts in the epithelia, whereas reactions without reverse transcriptase were all negative (Fig. 1D).

To examine whether the OCT4 expression was correlated with an unmethylated of the promoter, we employed bisulphite sequencing analysis. Consistent with previous results (Deb-Rinker et al., 2005), sequencing of bisulphite-modified DNA showed that four known differentially methylated CpG dinucleotides in the analysed OCT4 promoter region were generally methylated in human blood (15 replicates) and unmethylated in human NT2 cells (16 replicates). Moreover, when we analysed whole human epididymis (14 replicates) and prostate (16 replicates) for the methylation of the CpG sites, DNA from epididymis was in average 42.8% unmethylated, whereas DNA from prostate was in average 25% unmethylated (Fig. 1E).

To address expression of OCT4A, NANOG, SOX2, UTF1 and REX1 at the protein level, we used IHC with a chemical peroxidase development deliberately avoiding immunofluorescence and contra-staining that could result in false-positive signals and cover unspecific staining, respectively (Cantz et al., 2008). Furthermore, all experiments were performed with a control staining without primary antibody. The number of investigated samples together with staining intensity is depicted in Table I. Results showed that OCT4A was weakly expressed at the protein level in the nuclei of epithelia cells throughout both the epididymis and the prostate (Fig. 2A–C), whereas NANOG, with two separate antibodies, showed a cytoplasmic localization (results not shown). IHC for SOX2, UTF1 and REX1 showed a stronger expression in both epididymis and prostate epithelia than that of OCT4A (Fig. 2D–I). Furthermore, in the prostate some of the cells in the epithelia had a markedly stronger nuclear expression of SOX2, UTF1 and REX1 (indicated by arrows in Fig. 2G–I).

Next, we investigated the specificity of the antibodies and the level of OCT4 expression in the epididymis and prostate compared with ES cells. Loading 20 μg protein and using both a mouse monoclonal (C-10, sc-5279) and a goat polyclonal (N-19, sc-8628) raised against the N-terminal part of OCT4A, we detected very strong bands of ~41 and 43 kDa in the ES cell preparation (Fig. 3A and C). A much weaker but similar band to the lower band from ES cells (~41 kDa) was detected in both epididymis and prostate protein extracts (Fig. 3A and C). Further ECL development revealed that in the tissue preparation from epididymis head, a double band was detectable similar to the much stronger double band seen in the protein extract from ES cells (Fig. 3B and D). Surprisingly, we also detected with both antibodies a band of ~51 kDa in the epididymis and prostate preparations, which was not seen in the preparation from ES cells.

In vitro culture of primary epididymal cells continued expression of pluripotency associated genes

Expression of members of the presumed pluripotency-associated factors in the epithelia of the urogenital tract raised the question of whether these cells can sustain the expression when placed in culture and form sphere characteristic of stem cells. To test this, we made a collagenase-based enzymatic digestion of whole epididymis tissue and placed the cells in culture. The epididymis was obtained from patients who had undergone surgery for small localized testicular germ cell tumours. Although contamination with cells from the tumour was unlikely, we performed the same procedure with material from tumours as a control (results not shown). Twenty-four hours after the collagenase treatment, the epididymal cells had aggregated into sphere-like structures (Fig. 4A and B). qRT–PCR analysis of the epididymal spheres showed that the spheres continued transcriptional expression of the pluripotency-associated factors throughout 20 days in culture. In three repeated experiments with tissue from three different patients, OCT4A expression increased compared with Day 4 and stayed higher throughout the 20 days in culture, whereas NANOG and SOX2 expression decreased after Day 8 to ~50% of the Day 4 expression level (Fig. 4C). Furthermore, qRT–PCR analysis for early lineage differentiation markers BACHYURY, SOX1 and GATA6 (D’Amour et al., 2005) showed that the endodermal marker GATA6 was not expressed, and the mesodermal marker BRACHYURY was evenly expressed whereas ectodermal marker SOX1 varied largely among the samples throughout the 20 days (Fig. 4D). Staining for the transcription factors in the spheres revealed that OCT4A, SOX2 and downstream targets UTF1 and REX1 were all evenly expressed in the nuclei of the cells dispersed throughout the spheres (Fig. 4E–H), whereas staining for NANOG showed variable results with two different antibodies (results not shown). Our results show that epididymal cells can be placed in culture in which they form spheres that continue to express the pluripotency factors. Moreover, although there was a large variation in SOX1 expression, qRT–PCR results suggest that there is no de novo differentiation into endo-, ecto- or mesoderm in the epididymal spheres within the first 20 days in culture.
Figure 1 Transcription of pluripotency associated genes in the adult human urogenital tract. (A) RT–PCR showing the expression of pluripotency-associated genes in the testes (TE), duct deferens (DD), epididymis (EPI), prostate (PR) and ovary (OV) together with human ES cells (ESC) as a positive control. (B) Anti-sense ISH showing staining of OCT4 transcript in the epithelium from epididymis. Insert shows negative sense control to ISH shown in B. (C) Anti-sense ISH showing staining of NANOG transcript in epithelium from epididymis. Insert shows negative sense control to ISH shown in C. (D) RT–PCR for OCT4, NANOG and RPS20 in RNA amplified from laser-microdissected epithelia cut from epididymis (EPI), prostate (PR), seminal vesicle (SV) together with FACS sorted human ESCs as positive control. (E) Analysis of DNA methylation at four known differentially methylated CpG sites in the OCT4 promoter region done in 14–16 replicates with DNA from whole epididymis, prostate, blood and human NT2 cells. Black and white circles represent methylated and non-methylated sites, respectively. Scale bar 50 μm.
Transcriptional profiling of epididymal cells in culture showed closest resemblance with epididymis

To address whether epididymal cells in culture had reversed to a more embryonic state after being placed in culture, we performed genome-wide transcriptional profiling comparing the cells with FACS sorted human ES cells and whole epididymis. We centred our attention on genes known to be specifically expressed or overexpressed in human ES cells (Assou et al., 2007). The profiling revealed that whole epididymis and cultured epididymal cells clustered together and showed weak expression of pluripotency-associated genes compared with FACS sorted human ES cells (Fig. 5A). Furthermore, spheres from the individual days in culture clustered together indicating a development in the cells in vitro, but there were no indications of the cultured cells having an ES cell-like phenotype. However, the epididymal spheres did up-regulate CD29, CD44 and CD133 that are markers associated with sphere-forming cancer stem cells (Klonisch et al., 2008; Fig. 5B).

RA stimulation of primary epididymal cells in culture

To further investigate whether epididymal cells in culture had stem cell properties, we stimulated spheres from four different donors with 0.5 μM all-trans RA from Day 2 to Day 8 in culture. qRT–PCR

<table>
<thead>
<tr>
<th>Histology</th>
<th>OCT4</th>
<th>n</th>
<th>SOX2</th>
<th>n</th>
<th>UTF1</th>
<th>n</th>
<th>REX1</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epididymis</td>
<td>+</td>
<td>5</td>
<td>+</td>
<td>4</td>
<td>+++</td>
<td>4</td>
<td>+++ to +</td>
<td>6</td>
</tr>
<tr>
<td>Prostate</td>
<td>+</td>
<td>5</td>
<td>+</td>
<td>3</td>
<td>+ + to +</td>
<td>5</td>
<td>+ + to +</td>
<td>5</td>
</tr>
<tr>
<td>Seminal vesicle</td>
<td>+</td>
<td>3</td>
<td>+</td>
<td>2</td>
<td>+ + to +</td>
<td>3</td>
<td>+ + to +</td>
<td>3</td>
</tr>
</tbody>
</table>

Table 1 Description of human specimens included and the assessment of OCT4, SOX2, UTF-1 and REX-1 expression

Figure 2 Expression of pluripotency-associated factors in the human adult epididymis and prostate. (A) Expression of OCT4A in the nuclei of epithelial cells of the epididymis. (B) Enlargement of boxed area in A. (C) Expression of OCT4A in nuclei of epithelial cells from the prostate. (D) Expression of SOX2 in nuclei of epithelia cells from epididymis. (E) Expression of UTF1 in nuclei of epithelial cells from the epididymis. (F) Expression of REX1 in nuclei of epithelial cells (indicated by arrows) from the epididymis. Expression of (G) SOX2, (H) UTF1 and (I) REX1 in nuclei of epithelial cells from the prostate with arrows indicating strongly stained nuclei. Inserts show staining with no primary antibodies. Scale bar 50 μm.
In the present study, we report expression of OCT4 and other pluripotency-associated genes in human somatic epithelia from the urogenital tract. Furthermore, we show that this expression continues in culture, where epididymal cells form spheres, and that stimulation with RA results in a significant down-regulation of OCT4 transcripts without differentiation into multiple germ layers.

The expression of OCT4 is supported by freely available data from the GEO database at NCBI (http://www.ncbi.nlm.nih.gov) (Edgar et al., 2002). Data from a study of splice variants performed by Johnson et al. (2003) (accession number GSE740) on 52 tissues and cell lines showed that the expression of OCT4 was highest in the epididymis among all the investigated samples. Probes designed to span exons 2–3 and 3–4 of the OCT4 locus revealed similar results with high intensity, whereas a probe spanning exon 1–2 showed lower intensity. The lower intensity of the exon 1–2 spanning probe could be due to different splice variants in the tissue; however, a more likely scenario is that it is due to the amplification process. In addition, expression data for NANOG showed high expression in the prostate for probes spanning exons 1–2 and 2–3.

ISH showed that the origin of the transcripts detected by RT–PCR were cells of the epithelium, which was further supported by the microdissection of epithelia from both epididymis, prostate and the seminal vesicle. Avoiding misinterpretations, as is possible with immunofluorescence (Cantz et al., 2008), we used IHC to investigate whether OCT4A was also expressed at the protein level. Low expression of OCT4A and comparable higher expression of SOX2, UTF1 and REX1 were seen in nuclei of epithelial cells from both epididymis and prostate. Using western blotting, we detected OCT4A bands with both a monoclonal antibody (C-10, sc-5279) and a polyclonal (N-19, sc-8628) antibody raised against the N-terminal part of OCT4 in preparations from epididymis and prostate. Not surprisingly, the bands from the tissue preparations were much weaker than the comparable bands detectable in ES cell protein extract. Interestingly, both antibodies also revealed a stronger band of ~51 kDa in the preparations from epididymis and prostate, which was not detectable in the ES cell extract. A similar band for OCT4 is described by the manufacturer (www.scbt.com) for the polyclonal antibody (N-19, sc-8628); however, another possibility is that the additional band represents a post-translational modification. Hence, although purely speculative, the band could be due to ubiquitination increasing protein weight from ~41 to ~51 kDa.

Recently, Cantz et al. (2008) argued that the most appropriate control to determine the expression of OCT4 in cells is to analyse the methylation status of the promoter. Epigenetic control of OCT4 gene has been investigated in the upstream enhancer and promoter regions of exon 1 in cell lines and somatic tissues and the evidence show a negative correlation between the level of methylation and OCT4 expression (Hattori et al., 2004; Deb-Rinker et al., 2005; Feldman et al., 2006). We decided to focus on a region with four well-known differentially methylated CpG dinucleotides (Cowen et al., 2005; Deb-Rinker et al., 2005). Results showed that 42.8% of the analysed promoter region was unmethylated in whole epididymis tissue, but the level of methylation in the epithelial cells could be lower due to the interference of DNA from the interstitial cells. In addition, we found that 25% of the CpG dinucleotides in the prostate were unmethylated, which is comparable with previous studies reporting the expression of OCT4 in the prostate together with unmethylation of the promoter, where ~22% was found unmethylated (Hoffmann et al., 2006; Sotomayor et al., 2009).

Although OCT4 and NANOG are expressed at the transcript level in adult human testes, it is impossible to detect any of the two proteins in the germline, which is in contrast to, for example, UTF1 and REX1.

**Discussion**

In the present study, we report expression of OCT4 and other pluripotency associated genes in human somatic epithelia from the urogenital tract. Furthermore, we show that this expression continues in culture, where epididymal cells form spheres, and that stimulation with RA results in a significant down-regulation of OCT4 transcripts without differentiation into multiple germ layers.

The expression of OCT4 is supported by freely available data from the GEO database at NCBI (http://www.ncbi.nlm.nih.gov) (Edgar et al., 2002). Data from a study of splice variants performed by Johnson et al. (2003) (accession number GSE740) on 52 tissues and cell lines showed that the expression of OCT4 was highest in the epididymis among all the investigated samples. Probes designed to span exons 2–3 and 3–4 of the OCT4 locus revealed similar results with high intensity, whereas a probe spanning exon 1–2 showed lower intensity. The lower intensity of the exon 1–2 spanning probe could be due to different splice variants in the tissue; however, a more likely scenario is that it is due to the amplification process. In addition, expression data for NANOG showed high expression in the prostate for probes spanning exons 1–2 and 2–3.

ISH showed that the origin of the transcripts detected by RT–PCR were cells of the epithelium, which was further supported by the microdissection of epithelia from both epididymis, prostate and the seminal vesicle. Avoiding misinterpretations, as is possible with immunofluorescence (Cantz et al., 2008), we used IHC to investigate whether OCT4A was also expressed at the protein level. Low expression of OCT4A and comparable higher expression of SOX2, UTF1 and REX1 were seen in nuclei of epithelial cells from both epididymis and prostate. Using western blotting, we detected OCT4A bands with both a monoclonal antibody (C-10, sc-5279) and a polyclonal (N-19, sc-8628) antibody raised against the N-terminal part of OCT4 in preparations from epididymis and prostate. Not surprisingly, the bands from the tissue preparations were much weaker than the comparable bands detectable in ES cell protein extract. Interestingly, both antibodies also revealed a stronger band of ~51 kDa in the preparations from epididymis and prostate, which was not detectable in the ES cell extract. A similar band for OCT4 is described by the manufacturer (www.scbt.com) for the polyclonal antibody (N-19, sc-8628); however, another possibility is that the additional band represents a post-translational modification. Hence, although purely speculative, the band could be due to ubiquitination increasing protein weight from ~41 to ~51 kDa.

Recently, Cantz et al. (2008) argued that the most appropriate control to determine the expression of OCT4 in cells is to analyse the methylation status of the promoter. Epigenetic control of OCT4 gene has been investigated in the upstream enhancer and promoter regions of exon 1 in cell lines and somatic tissues and the evidence show a negative correlation between the level of methylation and OCT4 expression (Hattori et al., 2004; Deb-Rinker et al., 2005; Feldman et al., 2006). We decided to focus on a region with four well-known differentially methylated CpG dinucleotides (Cowen et al., 2005; Deb-Rinker et al., 2005). Results showed that 42.8% of the analysed promoter region was unmethylated in whole epididymis tissue, but the level of methylation in the epithelial cells could be lower due to the interference of DNA from the interstitial cells. In addition, we found that 25% of the CpG dinucleotides in the prostate were unmethylated, which is comparable with previous studies reporting the expression of OCT4 in the prostate together with unmethylation of the promoter, where ~22% was found unmethylated (Hoffmann et al., 2006; Sotomayor et al., 2009).

Although OCT4 and NANOG are expressed at the transcript level in adult human testes, it is impossible to detect any of the two proteins in the germline, which is in contrast to, for example, UTF1 and REX1.
Figure 4 Expression of pluripotency-associated factors in spheres derived from human epididymal cells placed in culture. (A) Formation of epididymal spheres after 24 h of culture (magnification ×20). (B) Epididymal spheres from A after 20 days in culture (magnification ×20). (C) qRT–PCR on RNA from experiment done three times in triplicates showing expression patterns of pluripotency-associated genes in epididymal spheres from Day 8–20 in culture normalized to Day 4 in culture and with B2M as internal control (bars show SD). (D) qRT–PCR on RNA from experiment done three times in triplicates showing expression pattern of differentiation marker genes BRACHYURY and SOX1 from Day 8–20 in culture normalized to day 4 in culture and with B2M as internal control (bars show SD). (E) Strong expression of OCT4 in the nuclei of cells from epididymal spheres after 20 days in culture. (F) Expression of SOX2 in the nuclei of cells from epididymal spheres after 12 days in culture. Expression of (G) UTF1 and (H) REX1 in the nuclei of cells from epididymal spheres after 20 days in culture. Scale bar 50 μm.
Interestingly, a recent study showed that it is possible to derive pluripotent cells from the adult human testes that after initial derivation begin to express OCT4, and later also NANOG, with the capacity to develop teratomas consisting of all three germ layers when implanted into immunodeficient mice (Conrad et al., 2008). Using a similar approach, however, without growth factors leukaemia inhibitory factor and glial cell-line-derived neurotrophic factor, we derived spheres from the epididymis that express OCT4 and several other members of the pluripotency network, but not NANOG. Although the epididymal spheres consist of a heterogeneous cell population with only a subset of cells expressing OCT4A, it was possible to detect a significant decrease after stimulation with low levels of RA, whereas there was no indication of differentiation into germ layers other than the mesodermal. Nevertheless, taking the recent study by Conrad et al. (2008) into consideration, it cannot be dismissed that further isolation and propagation in vitro of epididymal cells may result in cells capable of differentiation into all three germ layers. However, we stress that we do not believe the expression of OCT4 in the epididymis and prostate is directly associated with multilineage differentiation in vivo since none of the residing cells show this kind of potential in vivo or initially in vitro and NANOG is not expressed at the protein level.

It is also important for this discussion that ectopic activation of Oct4 in the intestine or skin results in rapid expansion of progenitor cells...
and invasive tumour formation indicating that Oct4 might act as an oncogene in somatic cells (Hochdilinger et al., 2005). It has therefore been argued that if Oct4 functioned in somatic stem cells and the Oct4 genomic locus existed in a state permissible for transcriptional activation, one might expect that it would be more frequently activated oncogene in cancers of somatic origin (Lengner et al., 2007). Interestingly, Mizuno and Kosaka (2008) showed that multiple OCT4 transcripts exist in the mouse somatic tissues and that overexpression of at least one of the transcripts, OCT4C, resulted in transformation of NIH-3T3 cells, indicating oncogenic potential of this isoform. So either the OCT4 gene is active in somatic cancers in an isoform not yet detected or it is simply not oncogenic in somatic cells except when being overexpressed.

The epididymis is rarely the localization of primary cancer development, metastasis or invasive growth from nearby OCT4 expressing testicular tumours (Rix et al., 1990; Ganem et al., 1998; Tilki et al., 2008). This has led to the hypothesis that the epididymis is protected from the development of tumours through high expression of endogenous inhibitors such as endostatin (Tilki et al., 2008). Hence, it is possible that OCT4, at least when it comes to the epididymis, does not lead to tumour formation because the organ is well protected against such a scenario. The culture of epididymal spheres could be a tool to further investigate this possibility in a manner similar to the use of mammo-, neuro- and prostate spheres in cancer research (Becher et al., 2008; Goldstein et al., 2008; Mani et al., 2008). Following this line of research, it is also interesting that the transcriptional profiling revealed that epididymal spheres up-regulate CD29, CD44 and CD133, which are markers associated with sphere-forming cancer stem cells from breast and prostate (Klonisch et al., 2008). Whether the up-regulation of these markers indicates that the epididymal cells are converting to a more embryonic cell-like phenotype or the genes solely are associated with forming the spheres remains to be investigated.

Our data show that OCT4 and other members of the pluripotency network are expressed in somatic cells of the reproductive tract, but the physiological role of the genes in the adult human remains unclear. Although purely speculative, a role in the self-renewal of the epithelia or in a program associated with the nurture of developing spermatozoa is possible. Another tempting possibility is that OCT4 could work anti-apoptotic in the epithelia similar to the scenario described by Kehler et al. (2004) in primordial germ cells, which could also explain the expression of the protein in meiotic oocytes (Kristensen et al., 2008). We are at the moment using the established culture of epididymal spheres to isolate the OCT4 expressing cells and to knockdown the gene in culture. We believe that this approach will reveal clues to the role OCT4 and associated genes in somatic cells.

**Authors’ role**

D.M.K., J.E.N., and H.L. initiated the project. D.M.K., J.E.N., and H.L. conceived and designed the experiments. D.M.K., J.E.N., and M.K. performed PCR, IHC, ISH and western blotting, whereas D.M.K. analysed the data. D.M.K. performed cell cultivation. D.M.K. and M.D.D. performed microarray, whereas D.M.K., K.A. M.E.L. and S.B. analysed the data. G.K.J. and T.H. supplied tissues. D.M.K., H.L., and N.E.S. prepared the manuscript. All authors approved the final manuscript.

**Acknowledgements**

The authors wish to thank Paul J. Gokhale and Peter W. Andrews for kindly providing ES cell RNA and Brian Vendelbo Hansen for excellent technical assistance. We furthermore like to thank Si B. Sonne for valuable advice regarding laser-microdissection.

**Funding**

The study was supported by grants from the Villum Kann Rasmussen Foundation and the Danish Advanced Technology Foundation.

**References**


