Cell. Mol. Life Sci. 66 (2009) 495 – 503
1420-682X/09/030495-9
DOI 10.1007/s00018-008-8623-z

Cellular and Molecular Life Sciences

Research Article

Analysis of OCT4 expression in an extended panel of human tumor cell lines from multiple entities and in human mesenchymal stem cells

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Received 30 September 2008; received after revision 05 November 2008; accepted 10 November 2008
Online First 22 November 2008

Abstract. OCT4 is considered a main regulator of embryonic stem cell pluripotency and self renewal capacity. It was shown that relevant OCT4 expression only occurs in cells of embryonic pluripotent nature. However, several recent publications claimed to have demonstrated OCT4 expression in human somatic tumor cells, human adult stem or progenitor cells and differentiated cells. We analysed 42 human tumor cell lines from 13 entities and human bone marrow-derived mesenchymal stem cells (MSC). To validate OCT4 expression we used germ cell tumor (GCT) cell lines, derived xenografts and GCT samples. Analysis by RT-PCR, western blotting, immunocytochemistry and immunohistochemistry was performed. With exception of typical embryonal carcinoma cells, we did not observe reliable OCT4 expression in somatic tumor cell lines and MSC. We suggest that a high level of expression of the OCT4 protein together with its nuclear localization still remains a reliable and definitive feature of cells with embryonic pluripotent nature.

Keywords. OCT4, pluripotency, germ cell tumor, embryonal carcinoma cells, somatic tumor cells, mesenchymal stem cells.

Introduction

Currently, a discussion takes place in the literature concerning apparently discrepant data on the expression of the embryonic transcription factor OCT4 in various cell types (alternatively POU5F1, OCT3/4, OCT-4, OCT-3/4). As confirmed by many studies, OCT4 is considered a main regulator of embryonic stem cell pluripotency and self renewal capacity. Functional OCT4 expression was deemed a feature of cells of embryonic pluripotent nature, i.e. embryonal stem cells, embryonal germ cells as well as the embryonal carcinoma cells (ECC), seminoma cells, (dys)germinoma cells and precursor lesions of germ cell tumors (GCT) [1,2]. However, several recent publications claimed to have demonstrated OCT4 expression in human somatic tumor cells, in normal human adult stem or progenitor cells and in human differentiated cells [3–8]. In our view, the methodology used in these studies was insufficient and may thus have allowed misleading observations. In some cases, these observations resulted in premature conclusions either challenging the role of OCT4 as

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a marker of stem cell pluripotency or supporting the stem cell theory of carcinogenesis or determining OCT4 as a general tumor stem cell marker.

The activity of OCT4 as a transcription factor requires the nuclear localization of the functional OCT4 protein. Different mRNAs are generated by alternative splicing from the OCT4 gene [9] resulting in two OCT4 protein isoforms with different N-termini but identical POU DNA-binding and C-transactivation domains: the long OCT4A and the short OCT4B isoform. Only the OCT4A protein (i.e. OCT4) is localized in the nucleus, sustains stemness properties and confers self-renewal, whereas OCT4B does not seem to function as a transcription factor and is localized in the cytoplasm [10, 11]. In addition, transcription of OCT4 pseudogenes was recently demonstrated [12]. It has been suggested in the current discussion that an expression of OCT4 pseudogenes and splice variants may contribute artefacts regarding the expression and function of OCT4 in adult tissues and non-germ cell tumor malignancies [2, 13–16]. Given this discrepancy, a proof of functional OCT4 expression in a given cell type should not only rely on the analysis of transcription but also on the demonstration of nuclear localization of the OCT4A protein at relevant levels.

In the light of this discussion we investigated the expression of OCT4 in an extended panel of 42 human tumor cell lines from 13 entities and in human bone marrow-derived mesenchymal stem cells (MSC). As a control our panel of human nonseminomatous GCT cell lines, including OCT4-expressing ECC lines H12.1, H12.5, 2102EP, 833K, 1156Q and the OCT4-negative, more differentiated cell lines 1777NRpmet, 1411HP, H12.1D, GCT72 [17], was analyzed. To obtain reliable data on the expression of OCT4 we used a broad range of methods, comprising RT-PCR with various primer sets, immunocytochemistry, western blotting and immunohistochemistry. With the exception of typical ECC lines, we did not observe OCT4 expression in somatic tumor cell lines or MSC, despite some misleading signals obtained by RT-PCR and delusive signals produced by one antibody.

Material and methods

Cell lines, xenograft tumors, tumor probes, preparation of MSC. The following human tumor cell lines were used:

H12.1, H12.5, 2102EP, 833K, 1156Q, 1777NRpmet, 1411HP, H12.1D, GCT72 (nonseminomatous germ cell tumor);
HCT8, HCT15, HCT116, HT29, DLD1, SW48, SW480, COLO205, COLO320DM (colorectal carcinoma);
8505C, SW1736, C643, HTK74, BCPAP, FTC133 (thyroid carcinoma);
A431, HeLa (cervical carcinoma);
A253, FADU, SCC15 (head and neck carcinoma);
A549, A427, H322, H358 (non-small cell lung carcinoma);
HepG2 (hepatoma);
MCF7, BT474 (breast carcinoma);
A2780 (ovarian carcinoma);
PC3 (prostate carcinoma);
SHSY5Y, SIMA (neuroblastoma);
U87MG (glioblastoma);
518A2 (melanoma);
In addition, the cell line HEK293T was used. The status of OCT4 expression in the used germ cell tumor (GCT) cell lines had been analysed previously by western blotting [17]. All cell lines were maintained as monolayer cultures in RPMI 1640 (PAA, Pasching, Austria) supplemented with 10 % fetal bovine serum (Biochrom AG, Berlin, Germany) and 1 % streptomycin/penicillin (PAA, Pasching, Austria). Cultures were grown at 37°C in a humidified atmosphere of 5 % CO_2/ 95 % air.

Subcutaneous xenograft tumors of the OCT4-positive GCT cell line H12.1 were established in athymic nude mice (Harlan Winkelmann, Germany). Xenografts were removed, fixed in 5 % Formalin/PBS (Carl Roth, Karlsruhe, Germany) and embedded in paraffin. Formalin-fixed and paraffin-embedded samples of patient-derived, primary testicular tumors were obtained from the archives of the Institute of Pathology, Martin-Luther-University Halle-Wittenberg.

MSC were isolated from the bone marrow (BM) of patients who underwent diagnostic BM-aspiration after informed consent according to institutional guidelines as described previously [18]. Briefly, mononuclear cells were separated by centrifugation over a Ficoll gradient (PAN, Aidenbach, Germany), suspended in growth medium and plated at an initial seeding density of 1 – 2.5 x 10^5 cells/cm^2. Growth medium was composed of DMEM (low glucose, PAA, Pasching, Austria) supplemented with 15 % FCS selected for optimal growth (Invitrogen, Carlsbad, USA) and 1 % penicillin/ streptomycin (PAA, Pasching, Austria). Media was exchanged every two days. Cells were passaged with 0.05 % trypsin/ 0.022 % EDTA (PAA, Pasching, Austria) after reaching confluence of 50 % within the colonies and replated at 200 cells/cm^2 directly or after cryopreservation in liquid nitrogen. Determination of MSC-characteristics was performed by flow cytometry and by analysis of differentiation capacity as described previously [18].

Antibodies. The following primary antibodies were used: sc-8628 goat polyclonal N-19, sc-5279 mouse monoclonal C-10, sc-9081 rabbit polyclonal H-134 (all from Santa Cruz Biotechnology, Santa Cruz, USA). Horseradish peroxidase (HRP)-conjugated and biotin-conjugated anti-goat, anti-mouse and anti-rabbit IgG (all from Santa Cruz Biotechnology, Santa Cruz, USA) were used as secondary antibodies.

RT-PCR analyses. Cells were harvested by trypsiniziation, rinsed twice with PBS, and RNA was isolated using the peqGOLD Total RNA Kit (Peqlab, Erlangen, Germany) following manufacturer instructions. RT-PCR was performed using the GeneAmp® RNA PCR Kit (Applied Biosystems, Foster City, USA) according to instructions of the manufacturer with the following modifications: reverse transcription was run in 10 µl sample volume containing 100 ng RNA. The transcription program consisted of a 10 min initial extension, 23°C; 30 min reverse transcription, 42°C; 5 min denaturation, 99°C. Negative controls were run without reverse transcriptase. Amplification was performed in 25 µl sample volume at 40 PCR cycles consisting of 60 s denaturation, 95°C; 45 s annealing, $T_{annealing}$; 80 s synthesis, 72°C; initial denaturation 5 min, 95°C; final synthesis 72°C, 5 min. The following primer pairs were used: β-actin (378bp): 5'-AGAAGAGCTACGAGCTGCCTGA and 5'-CATACTCCTGCTTGCTGATCCAC; OCT4-I (247bp): 5'-CGTGAAGCTGGAGAAGGAGAAGCTG and 5'-CAAGGGCCGCAGCTTACACATGTTC; OCT4-II (218bp): 5'-GACAACAATGAAAATCTTCAGGAGA and 5'-TTCTGGCGCCGGTTACAGAACCA; OCT4-full length (1133bp): 5'-TCATTTCACCAGGCCCC and 5'-GCAGGCACCTCAGTTTGAA; (all $T_{annealing}$ 62°C). 20 µl amplification product were mixed with 4 µl of 6x DNA Loading Dye (Fermentas, Vilnius, Lithuania) and run at 2% agarose (Biozym, Hessisch Oldendorf, Germany) gels including the molecular weight marker GeneRuler™ 50bp DNA Ladder (Fermentas, Vilnius, Lithuania).

Western blot analyses. Cells were harvested by trypsiniziation, rinsed twice with PBS and lysed in RIPA buffer (50 mM Tris HCl pH 8.0, 100 mM NaCl, 0.5% NP40, 0.5% DOC, 0.5% SDS) supplemented with a protease inhibitor cocktail (Sigma, St. Louis, USA). Insoluble components were removed by centrifugation and protein concentrations were measured (BIO-RAD protein assay, Bio-Rad, Hercules, USA). After boiling for 5 min in SDS-loading buffer (500 mM Tris HCl pH 6.8; 10% Glycerol, 2% SDS, 5% 2-mercaptoethanol, 0.05% Bromphenol blue) 40 µg protein per lane were separated by SDS-PAGE and electroblotted onto nitrocellulose transfer membrane (Whatman, Maidstone, UK). Equal protein loading was controlled by Ponceau S staining (Sigma, St.Louis, USA). Membranes were blocked with 5% non-fat dry milk in PBST for 1 h and probed for 2 h with the primary antibodies diluted (1:400) in PBST/ 5% milk followed by incubation with secondary HRP-conjugated antibodies (dilution 1:5000). Immuno-complexes were visualized by enhanced chemiluminescence (Carl Roth, Karlsruhe, Germany).

Immunohistochemistry. Sections of 5 µm were deparaffinized, rehydrated and pretreated with Target Retrieval Solution (DakoCytomation, Glostrup, Denmark). After washing, the slides were treated with Peroxidase Blocking Reagent (DakoCytomation, Glostrup, Denmark) for 15 min followed by blocking treatment with 3% BSA (Sigma, St. Louis, USA) in PBS for 20 min. Subsequently, slides were incubated with primary antibodies diluted 1:200 in 1% BSA/ PBS for 3 h. After washing three times with PBS, slides were treated with biotin-conjugated secondary antibodies diluted 1:800 in PBS for 1 h, rinsed three times, incubated with Streptavidin/HRP (DakoCytomation, Gostrup, Denmark) diluted 1:800 for 30 min and washed three times. Immuno-complexes were visualized using the Liquid DAB+ Substrate Chromogen System (DakoCytomation, Glostrup, Denmark). Slides were counterstained with ChemMate Hematoxylin (DakoCytomation, Glostrup, Denmark), mounted with DePeX (SERVA, Heidelberg, Germany) and analyzed by light-microscopy.

Immunocytochemistry. Cells were seeded in 24 well plates and fixed after 24 h with 2% Formalin/ PBS (Carl Roth, Karlsruhe, Germany) for 20 min at room temperature followed by washing 3 x 10 minutes in PBS. The cells were permeabilized in 0.5% Triton X-100 (Sigma, St. Louis, USA) / 0.5% BSA (Sigma, St. Louis, USA) / PBS for 5 min on ice followed by blocking with 3% BSA/ PBS for 20 min and were incubated with primary antibodies diluted (1:100) in 1% BSA/ PBS for 2 h at room temperature. After washing, the cells were treated with biotin-conjugated secondary antibodies diluted 1:800 in PBS for 1 h followed by incubation with Streptavidin/FITC diluted 1:2000 (Dako Cytomation, Glostrup, Denmark) for 30 min in the dark. Finally, the cells were washed three times with PBS and analysed by light- and fluorescence-microscopy.

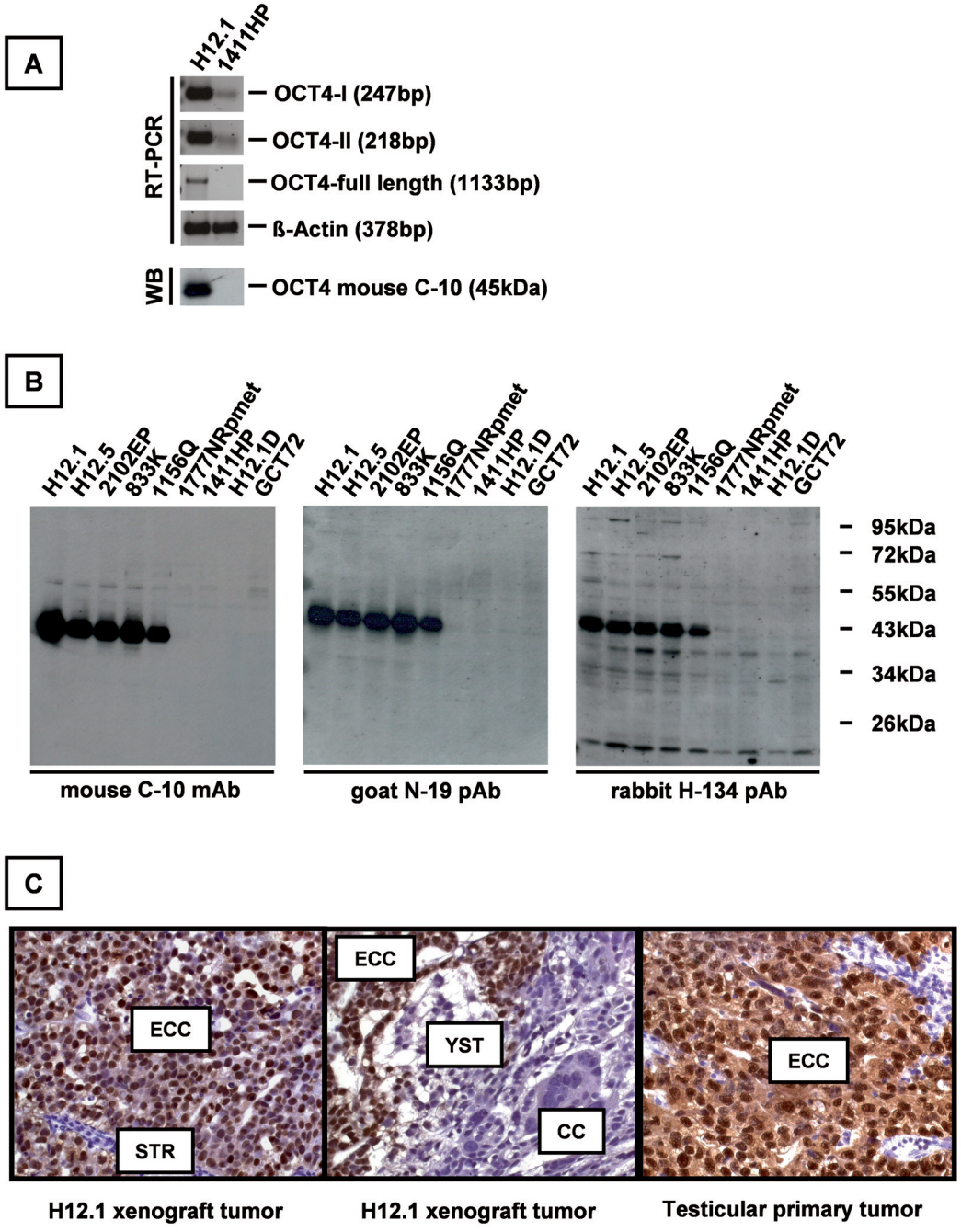

Figure 1. Validation of different methods for analyses of OCT4 expression using the GCT model. (*A*) RT-PCR and western blot analyses of OCT4 expression using all three primer sets and the antibody *mouse C-10* in OCT4-positive H12.1 and OCT4-negative 1411HP GCT cell lines. Note that the OCT4-I as well as the OCT4-II primer set provide clear signals in the 1411HP cell line. However, this is not confirmed by RT-PCR using the OCT4-full length primer set and by western blot analyses. (*B*) Western blot analyses of OCT4 expression comparing all three antibodies in different OCT4-positive- and OCT4-negative GCT cell lines. The first five cell lines with strong OCT4 expression represent typical ECC, the other four cell lines represent more differentiated cell types from nonseminomatous GCT. Note the additional bands yielded by the antibody *rabbit H-134* not only in cells where a 45kDa band is present but also in cells without a 45kDa signal. Equal protein loading was controlled by Ponceau S staining. (*C*) Immunhistochemical analyses of OCT4 expression in a nude mice xenograft tumor derived from the pluripotent GCT cell line H12.1 as well as in a testicular primary tumor sample. Note the strong nuclear signal in OCT4-positive undifferentiated ECC (brown) and lack of signal in OCT4-negative differentiated cells of yolk sac tumor (YST) and choriocarcinoma (CC) or tumor stroma cells (STR) demonstrating the high specificity of the antibody *goat N-19* (3,3'- diaminobenzidine, hematoxylin, x 400).

Results

We investigated OCT4 expression by RT-PCR, western blotting, immunocytochemistry and immunohistochemistry. For validation we used GCT cell lines of known OCT4 status, i.e. OCT4-positive lines H12.1, H12.5, 2102EP, 833K, 1156Q and OCT4-negative lines 1777NRpmet, 1411HP, H12.1D, GCT72. Moreover, xenograft tumors derived from selected lines of this GCT cell line panel as well as patient-derived GCT samples which contain OCT4-positive but also OCT4-negative cells were investigated. Therefore, this comprises an optimal model to investigate different methods of detecting OCT4. We performed experiments with three different primer sets for RNA analyses and three different antibodies for protein analyses. The first primer set (OCT4-I) was directed to amplify a sequence from exon 1 to exon 3 and was therefore deemed to be specific for OCT4A (i.e. OCT4). With the second primer set (OCT4-II) a sequence from exon 3 to exon 5 is amplified and should be able to detect expression of OCT4A and OCT4B. The third primer set (OCT4-full length) was designed to yield the cDNA of the entire OCT4 gene. All three antibodies (goat polyclonal N-19, mouse monoclonal C-10, rabbit polyclonal H-134) used have been raised against the N-terminal part of OCT4 protein and should therefore be specific for OCT4A. The analyses revealed that neither the OCT4-I nor the OCT4-II primer set but only the OCT4-full length primer set provided reproducible results reliably corresponding with OCT4 protein expression detected by western blotting (Fig. 1A). Western blot analysis showed that all three antibodies yielded a specific 45kDa band only in the GCT cell lines with positive OCT4-full length primer signals, i.e. OCT4-expressing cell lines with known typical ECC characteristics [17] H12.1, H12.5, 2102EP, 833K and 1156Q, but not in GCT cell lines negative for the OCT4-full length primer set, i.e. 1777NRpmet, 1411HP, H12.1D and GCT72. However, with the antibody *rabbit H-134* additional bands at other molecular weights were observed (Fig. 1B). The antibodies *goat N-19* and *mouse C-10* provided reliable results in immunocytochemical and immunohistochemical analyses. Figure 1C shows examples of immunohistochemical analysis. Based on these observations we feel confident that cells with a nuclear signal in immunohistochemical or immunocytochemical analysis, a 45kDa signal in western blot analysis and an additional positive PCR signal preferably from the full length primer set can be designated as truly OCT4-expressing cells. In contrast, we determined cells to be negative of functional OCT4 when only a positive PCR signal from a short amplicon primer set but no 45kDa immunoblotting signal and no nuclear signal in immunohistochemical or immunocytochemical analysis were observed.

We next analysed OCT4 expression in a panel 42 human tumor cell lines from 13 different entities and HEK293T cells. We found that in many cell lines the short amplicon primer sets yielded signals in RT-PCR analysis. This was mostly observed with the OCT4-II primer set, but in some cases also with the OCT4-I primer set. However, with the exception of the typical ECC lines known to be OCT4-positive, this never corresponded with OCT4 expression in western blot analyses and OCT4-full length primer signal. Figure 2 demonstrates this discrepancy as analysed by western blotting with two antibodies and by RT-PCR with the OCT4-I primer set.

For analysis of OCT4 expression in MSC we investigated four different preparations at passage 1. We did not observe any reliable expression of 45kDa OCT4 protein in MSC, despite some signals obtained by the OCT4-II primer set in RT-PCR analyses (Fig. 3).

As demonstrated in Figure 1, the antibody *rabbit H-134* yields additional bands at other molecular weights also in cells which showed neither an OCT4-full length primer signal nor a 45kDa protein signal. This inaccuracy for *rabbit H-134* was associated with false positive signals in OCT4-negative cells upon the use of this antibody for immunocytochemistry. Indeed, the signal is particularly delusive, since the antibody gives a distinct nuclear signal to a similar extent as observed in OCT4-expressing ECC. It is important to note that there are also antibodies commercially available which recognise both OCT4A and OCT4B and could thus also lead to false positive signals in immunocytochemical and immunohistochemical analyses. But in these cases a high cytoplasmic expression should exclude a genuine OCT4A expression. A comparison of cells with positive and negative OCT4 status proven by western blotting and full length primer signal, i.e. OCT4-negative and OCT4-positive cells using all three antibodies revealed the delusive, false positive nuclear signal produced by the antibody *rabbit H-134* (Fig. 4).

Discussion

In conclusion, with the exception of cell lines with typical ECC characteristics, we observed no functional OCT4 expression, neither in the investigated tumor cell lines nor in MSC from various donors. The positive signals obtained in tumor cells with the OCT4-I primer set as well as with one antibody in tumor cells and MSC were clearly demonstrated to be artefacts. The positive signals observed in MSC with

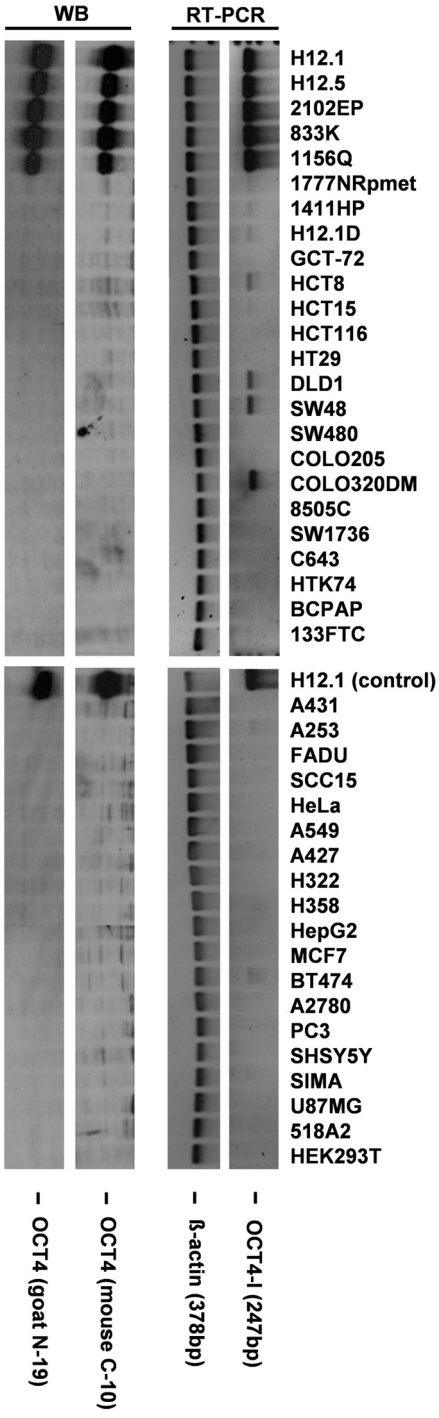

Figure 2. OCT4 expression in tumor cell lines. RT-PCR and western blot analyses using the OCT4-I primer set and the antibodies *mouse C-10* and *goat N-19* was performed in 42 tumor cell lines from 13 entities and HEK293T cells. The first five cell lines with strong OCT4 expression are typical ECC lines from non-seminomatous GCT. Note that the OCT4-I primer set provides clear signals in some cells for which no 45kDa signal is present in western blot analysis. (For this picture the films were purposely over-exposed to detect residual signals.) For Western blot analyses equal protein loading was controlled by Ponceau S staining.

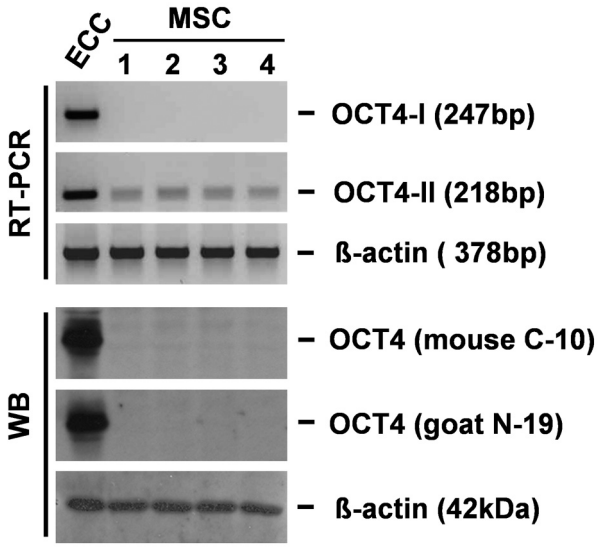

Figure 3. OCT4 expression in MSC. RT-PCR and western blot analyses of OCT4 expression using the OCT4-I and OCT4-II primer set and the antibodies *mouse C-10* and *goat N-19* was performed in preparations of MSC from four different donors in comparison with the ECC line H12.1. No expression of OCT4 is observed in MSC, despite some signals obtained by the OCT4-II primer set in RT-PCR analyses.

the OCT4-II primer set may indicate an expression of OCT4B. Our results are in line with two other recent reports by Kotoula et al. and Cantz et al, which also aimed to resolve the apparent discrepancy in expression of OCT4 using peripheral blood mononuclear cells and the two somatic tumor cell lines HeLa and MCF7, respectively [13, 14]. As shown by Cantz et al., a lack of expression of OCT4 corresponds to a promoter methylation-based epigenetic silencing of OCT4 in HeLa and MCF7 cells. Therefore, the thorough analysis of OCT4 expression by RT-PCR, western blot and immunocytochemistry is conclusive and corresponds well to the methylation status of the OCT4 promoter, which also can be analysed for further validation of OCT4 expression. In addition, our data support the role of OCT4 as a special and informative marker for germ cell tumors as originally stated [19].

Hence, our own data as well as the data from the literature show that in many cell types “any” OCT4-associated signals, regardless of their functional relevance, can be detected if RT-PCR methods amplifying short sequences are used. The use of a full length primer set as done in our experiments posses several technical problems, particularly when only a few copies of a complete OCT4 mRNA are present in the cell. Our primer set OCT4-I was directed to amplify a sequence from exon 1 to exon 3 and was therefore deemed to be specific for OCT4A,

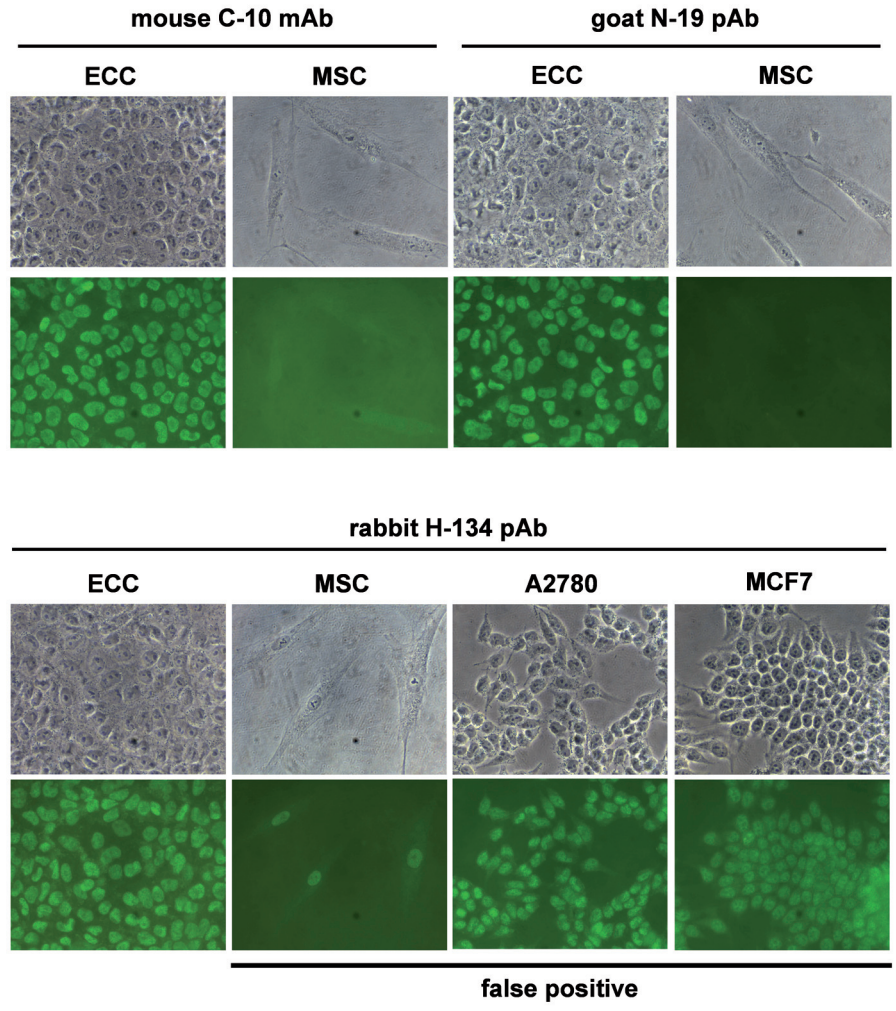

Figure 4. Comparison of antibodies for immunocytochemical analyses of OCT4 expression. Immunocytochemical analyses of OCT4 expression using all three antibodies was performed in OCT4-positive ECC line H12.1 and OCT4-negative MSC as well as the ovarian carcinoma cell line A2780 and breast carcinoma cell line MCF7. Note the false positive, particularly delusive, nuclear signals produced by the antibody *rabbit H-134* in OCT4-negative MSC and cells of A2780 and MCF7 (FITC, x 400).

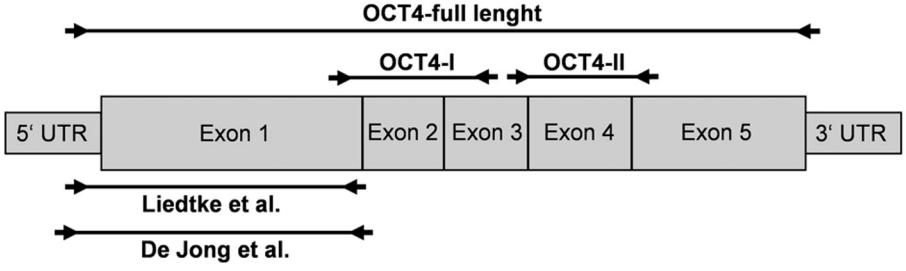

Figure 5. Schematic representation of OCT4 mRNA and location of primers and amplicons used in this study compared to those of Liedtke et al. [15] and De Jong et al. [20].

since the exon 1 is missing in OCT4B. However, the use of this primer set yielded signals in some cell lines. In a recent report Liedtke et al. demonstrated the complexity of OCT4 gene sequences by summarizing all OCT4 splice variants and pseudogenes found in a database search [15]. They designed primers that are able to exclude amplification of all non-functional unwanted transcripts. In another recent report De Jong et al. used primers that are similarly directed as those of Liedtke et al. [20]. In both studies the forward primer is located in the 5' untranslated region of OCT4 mRNA and the reverse primer spans the removed intron region between exon 1 and exon 2. In contrast, most of the studies with ambiguous data regarding OCT4 expression used primers located in exons 3–5, which share high homology to most of the pseudogens and splice variants [15, 16]. This is in line with our observations when using the primer set OCT4-II, which yielded signals in almost all cell types analysed in this study.

Figure 5 shows a schematic representation of location of primers and amplicons used in our study compared to those of Liedtke et al. and De Jong et al. Conclusively, for analysis of the functional OCT4 transcript, a primer set directed at exon 1 as done in both cited studies or a full length primer set as in our study should be applied.

Given these risks of potential artefacts with PCR-analyses, the investigation of OCT4 protein is important to evaluate the functional expression of OCT4. However, analyses based solely on immunocytochemistry may also lead to false positive signals, depending on the antibody. Therefore, both immunocytochemistry as well as western blotting should be performed to validate the obtained signal through determination of molecular weight and nuclear localization. The choice of the antibody used is critical. In our view the most reliable results can be obtained when one single antibody can be used for all applications as done in this study. At any rate, a comparison with cells of known pluripotent nature such as ECC is required. This will allow an evaluation of the extent of OCT4 expression and the exclusion of a possible over-interpretation of spurious signals. Taken together, when investigating OCT4 expression the careful choice and use of adequate controls and appropriate materials and methods is of utmost importance.

Our results do not exclude the possibility that a very small fraction of MSC as well as of cells of the respective tumor cell lines may express traces of functional OCT4. However, our data suggest that the frequency of such cells in the respective total population and the amount of resulting RNA and protein is not sufficient to yield reliable signals in RT-PCR with different primer sets or relevant signals in western blot analysis. This putative cell population can therefore not account for the ambiguous data reported by others. We suggest that incorrect detection of OCT4 expression in some recent reports may be attributed to expression of the isoform OCT4B or other splice variants, to expression of OCT4 pseudogenes, to other unknown sequences with OCT4 homology or to the use of non-appropriate antibodies. Distinct sequences of OCT4 genes or pseudogenes may be involved in OCT4-related regulation processes and detection of their genuine expression may possibly help to identify more undifferentiated cells such as tumor stem cells and adult stem cells. However, a high level of expression of the OCT4 protein together with its nuclear localization still remains a reliable and definitive feature of cells with embryonic pluripotent nature.

Acknowledgements. The study was supported in part by grants from the Federal State of Saxonia-Anhalt and the German Ministry of Education and Research through the Wilhelm-Roux-Program at the Medical Faculty of the Martin-Luther-University Halle-Wittenberg (FKZ SI/05 to T.M. and SI/09 to L.P.M.) and by additional grants from the Federal State of Saxonia-Anhalt (FKZ 3646A/0907 to H.J.S.)

Declaration of interest: None of the authors has to declare a commercial interest related to the contents of the study.
Statement of author contribution: T. M. and L. P. M. designed the study; T. M., J. L. and K. N. performed the research; T. M., H. J. S. and L. P. M. analyzed data and wrote the paper.

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