Original Paper

Expression of the *PDGF α-receptor* 1.5 kb transcript, *OCT-4*, and c-*KIT* in human normal and malignant tissues. Implications for the early diagnosis of testicular germ cell tumours and for our understanding of regulatory mechanisms

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Abstract

Human testicular germ cell tumours of adolescents and adults (TGCTs), including their precursor lesion carcinoma *in situ* (CIS), show expression of a 1.5 kb alternative transcript of the *platelet-derived growth factor* (*PDGF*) *α-receptor* gene. The so-called P2 promoter involved is located in intron 12 and its activity was found to be mutually exclusive with activity of the classical promoter (P1), which encodes the full-length receptor. The presence of the 1.5 kb transcript could be a putative marker for the early molecular diagnosis of TGCTs. In order to validate the RT-PCR approach, this study shows that not more than 100 transcripts are necessary to obtain positivity in the test used; moreover, samples from TGCTs or CIS-containing tissues can be diluted many-fold before resulting in false-negative findings. This study also shows that within TGCTs, as in TGCT-derived cell lines, expression of the 1.5 kb transcript is differentiation-dependent and positively correlated with expression of the embryonic transcription factor *OCT-4/POU5F1*. Furthermore, the results indicate that in some non-TGCT cancers and cell lines the 1.5 kb transcript is also expressed, but without concomitant *OCT-4/POU5F1* expression. The 1.5 kb transcript is also present in early B cells and derived leukaemias (B-ALL). In spite of similarities in chromosomal location, down-regulation upon differentiation of TGCTs, and *PDGF α-receptor* and c-*KIT* (the stem cell factor receptor) both being a tyrosine kinase receptor, no correlation was found between activity of the P2 promoter of the *PDGF α-receptor* gene and expression of c-*KIT*. In conclusion, the 1.5 kb transcript of the *PDGF α-receptor* is expressed in various cells and tissues, including particular blood cells. Although this may hamper the use of this transcript as a marker for malignancies in general, it does not appear to interfere with assays for the early detection of TGCTs. Copyright © 2002 John Wiley & Sons, Ltd.

Keywords: platelet-derived growth factor α-receptor; testis; germ cell tumours; haematopoiesis; glioma; melanoma; early diagnosis

Introduction

The platelet-derived growth factor (PDGF) can occur as three dimeric isoforms, PDGF AA, AB, and BB. Their biological function is dependent on binding to either of the existing PDGF receptors (α and β, which belong to the cell surface receptors of the protein-tyrosine kinase superfamily. The PDGF α-receptor has high affinity for both the A- and the B-chain, while the PDGF β-receptor has high affinity for the PDGF B-chain only [1,2]. PDGF has been implicated in several physiological and pathological processes, including embryogenesis and tumourigenesis [3–5].

Transcription from the *PDGF α-receptor* P1 promoter, located upstream of exon 1, results in a messenger of 6.4 kb, encoding the full-length receptor, as well as in a 3.0 kb transcript, potentially encoding a dominant-negative receptor isoform [6]. Besides the presence of this classical promoter, we identified an alternative promoter (P2), located in intron 12. From this promoter, both a 1.5 kb and a 5.0 kb transcript can be obtained, although no corresponding protein products have yet been identified [6–8]. Expression of the 1.5 kb and 5.0 kb transcripts has originally been found in undifferentiated Tera-2 clone 13 cells, a cell line derived from a non-seminomatous testicular germ cell tumour. Upon differentiation of these cells, the P2 promoter activity was silenced, while the P1 promoter became activated. Data from this cell line thus showed exclusivity between P1 and P2 promoter activities [7].

We previously studied a series of primary testicular germ cell tumours of adolescents and adults (TGCTs) for the presence of the different *PDGF α-receptor* transcripts [6,8]. Histologically and clinically, these tumours can be divided into seminomas and non-seminomas [9]. While seminomas mimic early germ cells, non-seminomas may show characteristics of early development: they can be composed of embryonal

Table 1. Summary of the results on *PDGF α-receptor* transcripts, c-*KIT* and *OCT-4* expression

Samples		#	α–R P1 mRNAs 6.4 kb	α–R P2 mRNAs 1.5 kb	OCT-4 mRNA	c-KIT mRNA
In vivo						
Testicular samples						
Parenchyma:	normal	4	+	−[a]	+	+
	atrophic	4	+	−[a]	nd	nd
	fibrotic	1	+	−	nd	nd
	with epididymitis	2	+	−	nd	nd
Epidermoid cyst and		1	+	−	nd	nd
adjacent parenchyma		1	+	−	nd	nd
Lymphoma		16	+	+	nd	nd
Leydig cell tumour and		8	+	+	nd	nd
adjacent parenchyma		5	+	−	nd	nd
TGCT-parenchyma:	without CIS	15	+	−	+	+
	with CIS	41	+	+	+	+
Seminoma		30	+	+	+	+
Nonseminoma:	EC	2	+	+	+	+
	TE	2	+	−[b]	−	+
	YS	2	+	−[b]	−	+
	YS xenograft	1	−	−	−	nd
	mixed	19	+	+	+	+
Haematopoietic samples						
Pheripheral blood:	PBL	2	−	+	nd	nd
	total lymphocytes	2	−	+	nd	nd
	B-lymphocytes	2	−	+	nd	nd
	T-lymphocytes	2	−	−	nd	nd
	granulocytes	2	−	−	nd	nd
	monocytes	2	−	−	nd	nd
EBV-B cells		5	−	+	nd	nd
Stem cells		1	−	−	nd	nd
0-ALL		1	−	−	nd	nd
C-ALL		2	−	−	nd	nd
Pre-B		3	−	−	nd	nd
B-ALL		2	−	+	nd	nd
PLL/CLL		3/5	−	−	nd	nd
Multiple myeloma		2	−	+	nd	nd
In vitro						
Tera2 clone 13	undifferentiated		−	+	+	+
	RA differentiated		+	−	−	+
S2			−	−	−	−
Melanocytes			−	+	−	+
Melanoma cell lines:	Mel 57		+	+	−	+
	1F6		+	−	−	+
	U1240		−	−	−	+
Glioma cell lines:	U251		+	+	−	+
	U1242		+	+	−	+
	U373		+	+	−	−

ALL=acute lymphocytic leukemia (0-ALL=lymphoid progenitor cell leukemia; C-ALL=pre-pre-B cell leukemia; Pre-B=pre-B cell leukemia; B-ALL=early B cell leukemia); α–R=PDGF α-receptor; [a]=positive after an extra 12 cycles; [b]=remained negative after an extra 12 cycles; CIS=carcinoma *in situ*; EBV-B cells=Epstein Barr virus transformed B cells; EC=embryonal carcinoma; nd=not determined; PBL=peripheral blood; PLL/CLL=chronic lymphocytic and promyelocytic leukemias (intermediate B cell leukemias); P1=promoter 1; P2=promoter 2; RA=retinoic acid; TE=mature teratoma; TGCT=testicular germ cell tumours of adolescents and adults; YS=yolk sac tumour; #=number of samples tested.

carcinoma (the undifferentiated component), teratoma (the somatically differentiated component), yolk sac tumour, and choriocarcinoma (the extra-embryonally differentiated components). In concordance with the findings in the Tera-2 clone 13 cells, we demonstrated that embryonal carcinomas, but not teratomas, show expression of the 1.5 kb transcript [6,8]. In addition, seminomas and non-seminomas of mixed histology were also found to be positive. Moreover, expression was also detected in carcinoma *in situ* (CIS), being the precursor of all TGCTs [10], while normal and atrophic parenchyma, as well as semen, were negative [6]. Based on these data, we have proposed that the 1.5 kb transcript of the *PDGF α-receptor* may be an informative tool for the early diagnosis of TGCTs.

In vitro experiments using the Tera-2 clone 13 cells indicated that activity of the *PDGF α-receptor* P2 promoter is regulated by OCT-4, a POU-domain transcription factor that is expressed in undifferentiated cells only [11]. Whether this also accounts for the *in vivo* situation has not so far been investigated. In addition, particular developmental stages of TGCTs, including CIS and seminoma, express c-*KIT*, the stem cell factor receptor [12–15]. c-KIT is also a tyrosine kinase receptor, has structural similarities to the PDGF α-receptor, and their relative genes are arrayed in a 5′-*PDGF α-receptor* → c-*KIT*-3′ tandem on human chromosome 4, band q12 [16–18]. Interestingly, down-regulation of c-*KIT* expression is found upon differentiation of TGCTs, which suggests a possible correlation between c-*KIT* expression and activity of the *PDGF α-receptor* P2 promoter. We have therefore investigated the possibility that expression of the P2-derived 1.5 kb transcript might be associated with c-*KIT* expression.

To investigate further the applicability of the 1.5 kb transcript of the *PDGF α-receptor* for the early diagnosis of TGCTs, the sensitivity and specificity of the proposed RT-PCR detection were studied. In addition, to gain a better understanding of the possible regulatory mechanisms involved in *PDGF α-receptor* P2 promoter activation, expression of *OCT-4* and c-*KIT* was investigated in selected tumour samples and *in vitro* cell cultures.

Materials and methods

Tissue samples

Directly following orchidectomy, representative parts of the tumour and adjacent testicular parenchyma (when available) were divided into two fractions: one was snap-frozen in liquid nitrogen, while the other was fixed in 4% buffered formalin and embedded in paraffin for histological analysis. The tumours were diagnosed according to the World Health Organisation classification for testicular tumours [19]. The samples included are listed in Table 1.

Peripheral blood of two independent healthy volunteers was collected. One part was used, after lysis of erythrocytes, to isolate the cells by centrifugation,

which were snap-frozen until use (designated as 'total peripheral blood sample'). The other fraction was ficoll-purified, by which the lymphocytes/monocytes (designated as 'total lymphocyte sample') were separated from the erythrocyte/granulocyte fraction. The granulocyte fraction was obtained from the latter after blood lysis and centrifugation, and was also snap-frozen. The lymphocytes were separated from the monocytes by incubation for 2 h at 37°C in a Petri dish, to which the monocytes specifically attach. The medium was subsequently used to isolate the lymphocytes, whereas the monocytes were isolated by trypsinization and snap-frozen. The B- and T-cells present in the lymphocyte fraction were separated from each other as follows: the sample was treated with AET-treated sheep red blood cells (E-rosette test) and ficoll purification, at the end of which both fractions obtained were snap-frozen (Figure 4B).

CD34-positive haematopoietic stem cells were kindly provided by Dr R. Torensma (Department of Tumor Immunology, University Medical Center St. Radboud, Nijmegen, The Netherlands). The cells were isolated from a mononuclear fraction of normal bone marrow using CD34-coated magnetic beads (Dynal). The cells were detached, immunostained with a CD34 monoclonal antibody (HPCA 2; Becton & Dickinson, PE labelled), and subsequently the CD34-positive fraction was sorted with a Coulter Elite flow cytometer. This fraction was 99% pure.

Haematopoietic malignancies were kindly provided by Dr I. Touw (Department of Haematology, Erasmus University, Rotterdam, The Netherlands) and are listed in Table 1. All samples, except the multiple myelomas, were purified to obtain highly enriched populations of malignant cells as described before [20,21]. Routinely Epstein–Barr virus (EBV)-infected B cells ($n = 5$) were kindly provided by Dr H. Dolstra (Department of Haematology, University Medical Center St. Radboud, Nijmegen, The Netherlands).

Cell culture

Tera-2 clone 13 cells were cultured and differentiated by treatment with retinoic acid (RA) as previously described [7]. S2 cells were cultured similarly to the undifferentiated Tera-2 clone 13 cells.

RNA extraction and cDNA synthesis

Total RNA was isolated from sections of the frozen tissues and snap-frozen cells using RNA-STAT-60™ according to the supplier's protocol (Tel-Test 'B' Inc.). Parallel sections of the tissues were used for detailed histological analysis as previously described [7]. Total RNA was similarly isolated from the TGCT cell lines. Melanocyte total RNA, isolated from a routinely grown primary culture using the RNeasy mini kit (Qiagen), was kindly provided by Dr N. Smit (Department of Dermatology, Academic Hospital, Leiden, The Netherlands). Total RNA from the Mel 57 and 1F6 cell lines [22] was kindly provided by Dr William

Leenders. Poly(A+) RNA isolated from U-1240 MG, U-251 MGsp, U-1242 MG, and U-374 MG malignant glioma cell lines [23] was kindly provided by Dr Ester Piek (Ludwig Institute for Cancer Research, Uppsala, Sweden). Poly(A+) RNA from CD34-positive haematopoietic stem cells was isolated using the Micro-Fast Track Isolation Kit as recommended (Invitrogen). cDNA was synthesized using Expand RT (Boehringher Mannheim) according to the supplied protocol.

Polymerase chain reaction (PCR)

The expression of the various genes, in the tissue samples, cell fractions, and cell lines, was determined by PCR amplification of the generated cDNA, using gene or transcript-specific primer combinations spanning intron sequences. The following primers and annealing temperatures were used: *c-KIT* sense: 5′-CTG AAC ACG CAC CTG CTG AA-3′; *c-KIT* antisense: 5′-AAG CTA CGT TGC TAT TGG GAA T-3′ (60°C); *OCT-4* sense: 5′-ACA CCT GGC TTC GGA TTT CG-3′; *OCT-4* antisense: 5′-GGC GAT GTG GCT GAT CTG CT-3′ (62°C); for the *PDGFα-receptor* P1-promoter-derived 6.4 kb transcript, the following were used: exon-12 sense: 5′-GAC CCG ATG CAG CTG CCT TA-3′ and exon-17 antisense: 5′-AAC TCC ATT CCT CGG GCA ACT-3′ (62°C); for the P2-promoter-derived 1.5 kb transcript the following were used: intron-12 sense: 5′-AAT GAT TCT GCC TGC CCA CAG-3′ and teratocarcinoma cryptic exon (TCCE) antisense: 5′-GAC TCA GGT TCC TCT GAC ATC TCG-3′ (66°C); for the P1 promoter-derived 3.0 kb transcript, exon-12 sense and TCCE antisense (62°C) were used; for the P2 promoter-derived 5.0 kb transcript, intron-12 sense and exon 17 antisense (66°C) were used [6]. *HPRT* RT-PCR was performed as a control for the quality of the cDNAs tested, using primers previously designated as 244 and 246 [24]. A negative control, in which cDNA was omitted, was used in every PCR assay. After an initial denaturation of 4 min at 94°C, the samples were amplified for 22–32 cycles (consisting of denaturation for 1 min at 94°C, annealing for 1 min as specified above, and an extension for 1 min at 72°C) and a final extension of 7 min at 72°C. Some samples were additionally boosted for 12 more cycles. The PCR was performed in a final volume of 25 μl containing 100 ng of each primer, 0.2 mM dNTPs, 1.5 mM MgCl$_2$ (except for the 6.4 kb *PDGFα-receptor* transcript, for which 2 mM MgCl$_2$ was used), and 1 U of Taq polymerase (Gibco BRL) in recommended buffer. In order to avoid detection interference by intron-less *OCT-4*-related genomic sequences [25], the RT-PCR analysis for *OCT-4* expression was performed on DNase-treated RNA as described before [26]. In addition, the obtained PCR-amplified samples were subsequently identified by an *Apa*-I restriction digestion, which positively discriminates the *OTF3A*-derived transcript, harbouring such a restriction site, from the *OTF3*-related, *Apa*-I negative sequences (C. E. P. van

Roozendaal, unpublished). The PCR products generated were subsequently analysed on an ethidium bromide-stained 1.5% agarose gel by UV exposure.

Sensitivity of the amplification method for the 1.5 kb transcript

The S2 cell line is found to be negative for all the *PDGF α-receptor* transcripts, even after extensive booster amplification (not shown). Therefore, 10^5, 10^4, 10^3, 10^2, 10^1, and 10^0 products obtained by PCR using the 1.5 kb transcript-specific primers (see above) on cDNA isolated from undifferentiated Tera-2 clone 13 cells were diluted in cDNA obtained from 250 ng of S2 cell RNA and used for PCR. The Tera-2-derived amplification products were checked by sequencing before they were used for the dilution experiments. In addition, undifferentiated Tera-2 clone 13 cells (10^1, 10^2, 10^3, 10^4, and 10^5, respectively) were diluted in 10^5 S2 cells, after which RNA was isolated, cDNA was prepared, and RT-PCR was applied. Samples with a known amount of transcripts were included as controls.

Additionally, the sensitivity of the amplification strategy was determined using serial dilutions (1:5, 1:10, 1:25, and 1:50) of RNA isolated from two seminomas, two embryonal carcinomas, and two CIS-containing parenchyma samples (80% of the tubules contained CIS) in RNA derived from S2 cells.

Results

PDGF α-receptor 1.5 kb transcript: sensitivity of detection method and expression in TGCTs

The 1.5 kb transcript of the *PDGF α-receptor* is present in undifferentiated Tera-2 clone 13 cells [6,7], but not in a cell line designated S2 (Table 1), which is most likely not derived from a TGCT [27], as reported originally [28]. In our initial study, we applied 32 amplification cycles on 250 ng of RNA input, associated with approximately 4.0×10^4 cells [6]. We thus serially diluted known concentrations of 1.5 kb transcript-specific amplification products in cDNA of S2 obtained from 250 ng of RNA. After 22 amplification cycles, 10^5 and 10^4 specific transcripts were required for visualization, while after 32 cycles, as few as 100 transcripts could be detected (Figure 1A). Dilution experiments of undifferentiated Tera-2 clone 13 cells in samples of S2 cells indicated that one Tera-2 clone 13 cell per 1000 S2 cells was required for detection (Figure 1B). This means that on average, a single 1.5 kb transcript is present in these cells. Based on these findings, we applied 32 amplification cycles in the experiments described further in this study, except where otherwise indicated.

The 1.5 kb transcript has been found in different histological types of TGCTs (with the exception of pure teratoma [8]), as well as in CIS-containing testicular parenchyma [6]. To assess the sensitivity of
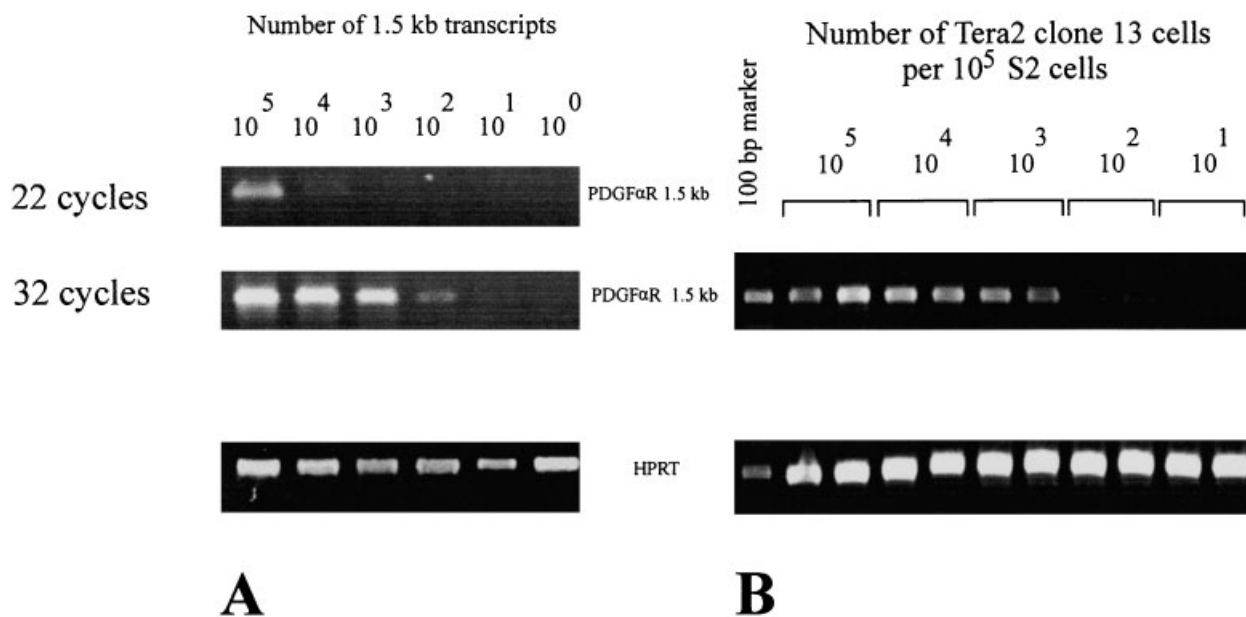
Figure 1. Analysis of the sensitivity of the RT-PCR amplification method used to detect the 1.5 kb transcript of the *PDGF α-receptor*. (A) Purified, sequence-verified, 1.5 kb transcript amplification products derived from the cell line Tera-2 clone 13 were diluted in cDNA from the S2 cell line, known to lack this specific transcript (see Table 1). (B) Thirty-two amplification cycles for the 1.5 kb transcript were used to detect undifferentiated Tera-2 clone 13 cells in a background of (non-expressing) S2 cells

our detection method in tissue samples, the effect of dilution of tumour cells was studied. cDNA from TGCTs and testicular parenchyma samples containing different amounts of CIS (20%, 50%, and 80% of the seminiferous tubules) was initially analysed. In accordance with previous studies, all seminomas, embryonal carcinomas, and parenchyma samples containing CIS were positive, whereas pure teratomas and yolk sac tumours were negative (Table 1 and Figures 2A and 2B), even after a booster amplification of an additional 12 cycles (not shown). The absence of the 1.5 kb transcript in yolk sac tumour is novel and was confirmed by analysis of a yolk sac tumour xenograft, derived from a mixed non-seminoma (Table 1 and Figure 2C). On normal and atrophic testicular parenchyma, weak positivity was observed only after a booster of an extra 12 cycles (see Table 1 and below). Subsequently, serial dilutions of RNA from seminomas, embryonal carcinomas, and CIS-containing testicular parenchyma (80% of the tubules contained CIS) in RNA from S2 cells were performed. The results (Figure 3) indicate that the 1.5 kb transcript can still be detected in both seminomas and in one of the embryonal carcinomas tested after a 1:50 dilution. The other embryonal carcinoma still showed positivity in a dilution of 1:25. The CIS-containing parenchyma samples (with 80% of the seminiferous tubules positive for CIS) could be diluted 10–25 times without losing positivity.

All testicular samples expressed the 6.4 kb transcript of the *PDGF α-receptor* (Table 1 and Figure 2A), in contrast to the results from the Tera-2 cell line, which can be explained by the presence of host tissue in the *in*

*vivo* samples, and possibly by heterogeneity of the tumour cells themselves.

Specificity of the *PDGF α-receptor* 1.5 kb transcript for TGCTs using *in vivo* samples

A series of testicular non-malignant lesions was investigated (see Table 1), showing no positivity, while both lymphomas and Leydig cell tumours contained the transcript.

Total peripheral blood was subsequently tested for the various *PDGF α-receptor* transcripts and found to be weakly positive for the 1.5 kb transcript (Table 1 and Figure 4A). To determine the origin of this positivity, different enriched sub-populations were investigated (see Figure 4B and the Materials and methods section), of which the B-cell-containing fraction was positive (Table 1 and Figure 4A). None of the samples showed expression of the 6.4 kb transcript (Table 1 and Figure 4A). Moreover, EBV-transformed B cells were also positive for the 1.5 kb transcript (Table 1). To substantiate these findings further, several haematological malignancies reflecting various stages of B-cell development were tested (see Table 1 and Figure 5). No 6.4 kb transcripts were found in any of the samples. B-ALL samples, representative of the early B-cell stage, were exclusively positive for the 1.5 kb transcript. Two myelomas, representative of the plasma cell stage, were also found to be positive, which might be explained by the fact that these samples had not been purified (see the Materials and methods section). The results confirmed the previous finding of co-expression of the 1.5 kb and 5.0 kb transcripts, as
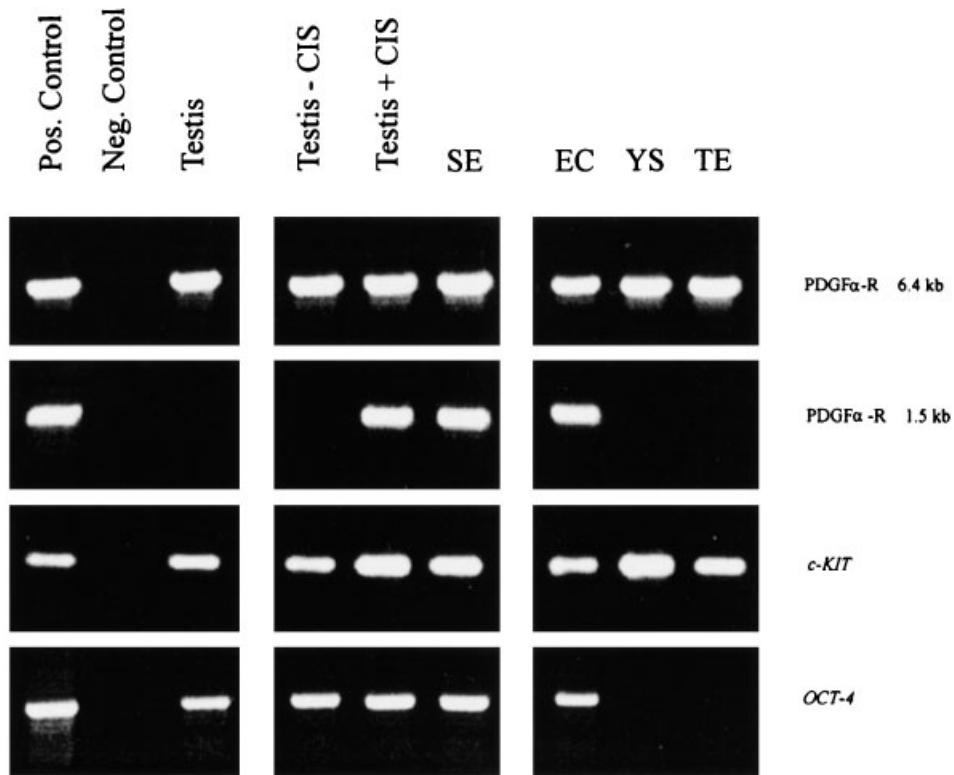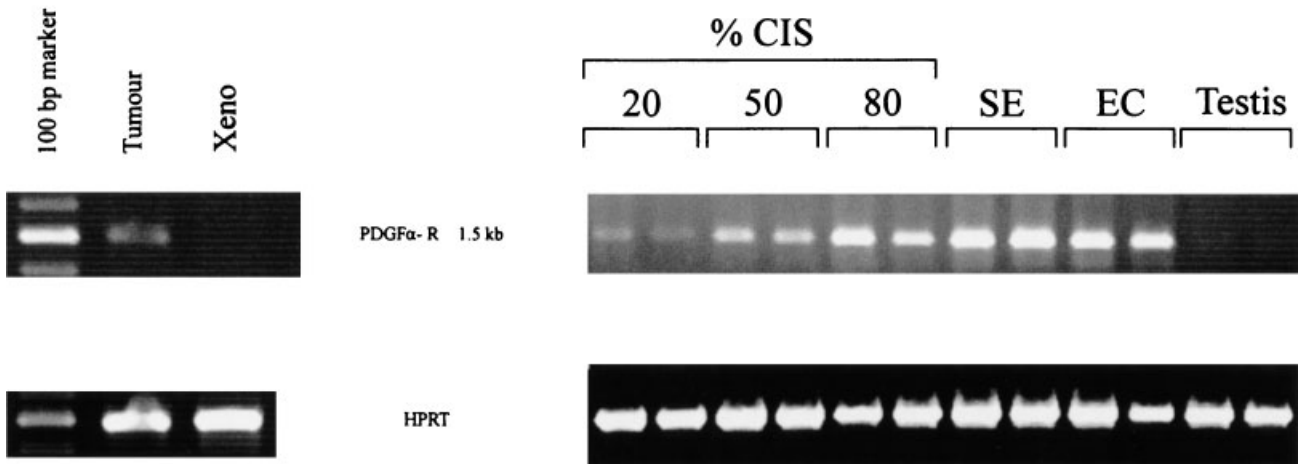
Figure 2. (A) Representative results of the detection of the 6.4 kb and 1.5 kb transcripts of the *PDGF α-receptor*, c-*KIT* and *OCT-4* expression in normal testicular parenchyma (Testis), testicular parenchyma without CIS adjacent to a seminoma (Testis − CIS), testicular parenchyma with CIS adjacent to a non-seminoma (Testis + CIS), a seminoma (SE), an embryonal carcinoma (EC), a yolk sac tumour (YS), and a teratoma (TE). (B) Results of the detection of the 1.5 kb transcript of the *PDGF α-receptor* in testicular parenchyma with 20%, 50%, and 80% CIS-containing seminiferous tubules, seminoma (SE), embryonal carcinoma (EC), and normal testicular parenchyma (Testis) after 32 amplification cycles. All experiments were carried out in duplicate (not shown) on two independent cases. (C) Results of the detection of the 1.5 kb transcript of the *PDGF α-receptor* in a primary mixed non-seminoma (Tumour), containing embryonal carcinoma, teratoma and yolk sac tumour components, and absence of detection in a derived xenograft only composed of yolk sac tumour (Xeno)
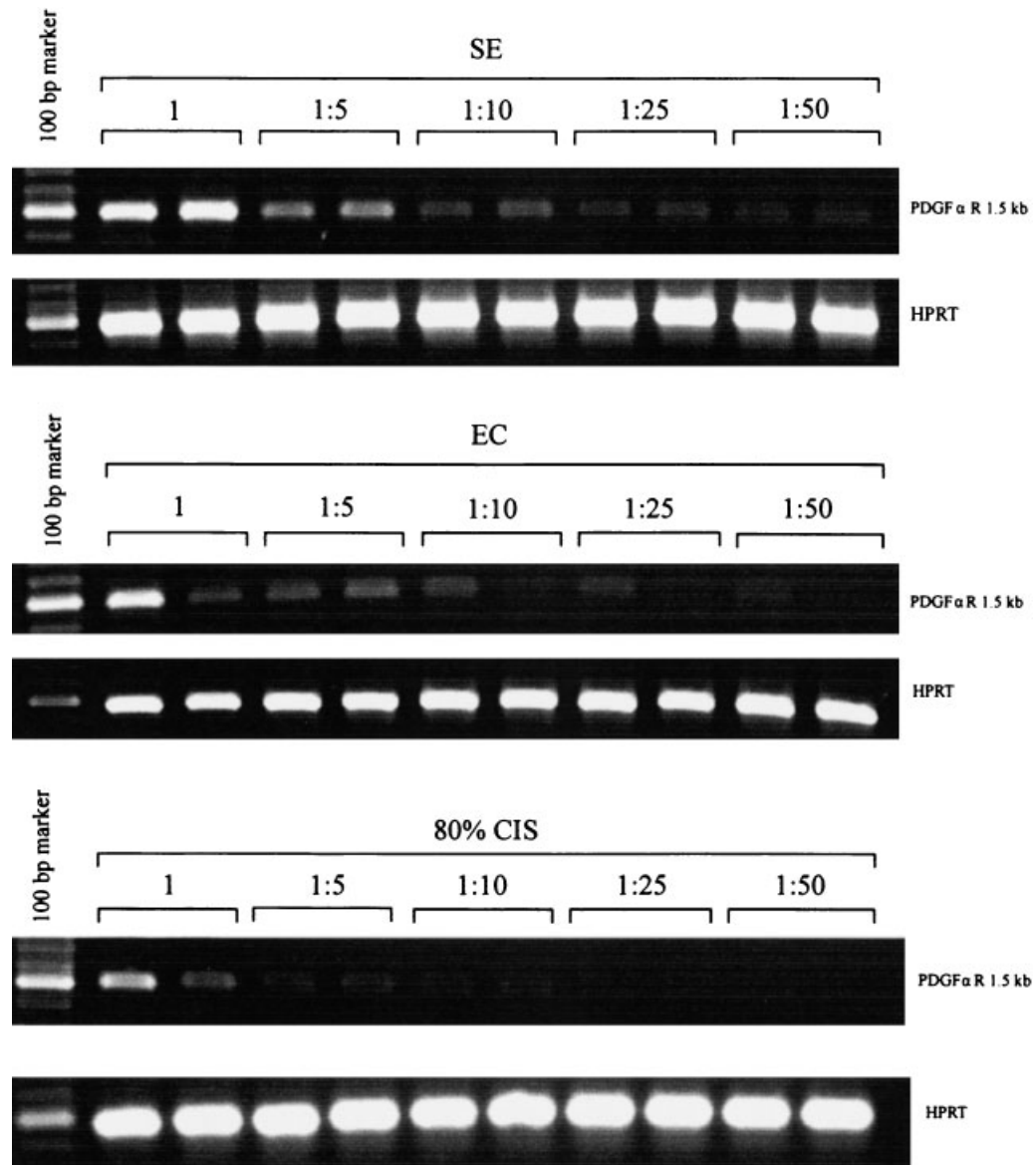
Figure 3. Results of the detection of the 1.5 kb transcript of the *PDGF α-receptor* in serially diluted RNA derived from two seminomas (SE), two embryonal carcinomas (EC), and two samples of testicular parenchyma with 80% CIS-containing seminiferous tubules

well as of the 3.0 kb and 6.4 kb transcripts (Table 1 and Figure 4A).

Expression of *PDGF α-receptor* transcripts in melanocytes, melanoma, and glioma cells

Expression of the *PDGF α-receptor* transcripts was also studied in primary melanocytes, and melanoma and glioma cell lines (Table 1 and Figure 6), which show expression of c-*KIT* (see below). While three out of the four glioma cell lines (U251, U1242, U373) and the normal melanocyte primary culture expressed the 1.5 kb transcript, of the two melanoma cell lines only Mel 57 showed positivity. The 6.4 kb transcript was present in both melanoma cell lines, but not in normal melanocytes, and in all glioma cell lines with, again, the exception of U1240. The co-expression of the

1.5 kb and 6.4 kb transcripts is a novel finding, and since it is found in cell lines, this cannot be due to histological heterogeneity. Expression of the 1.5 kb transcript in normal melanocytes again indicates that the 1.5 kb transcript is not a tumour cell-specific marker.

Correlation between *PDGF α-receptor* 1.5 kb transcripts, *OCT-4*, and c-*KIT* expression

We investigated *OCT-4* expression in both our *in vivo* and *in vitro* samples. A direct correlation was observed between expression of the 1.5 kb transcript and *OCT-4* in TGCTs (Table 1 and Figure 2A). This is in contrast to the non-TGCT cell lines and melanocyte primary culture (Table 1 and Figure 6). Moreover, normal testicular parenchyma showed *OCT-4* expression, but
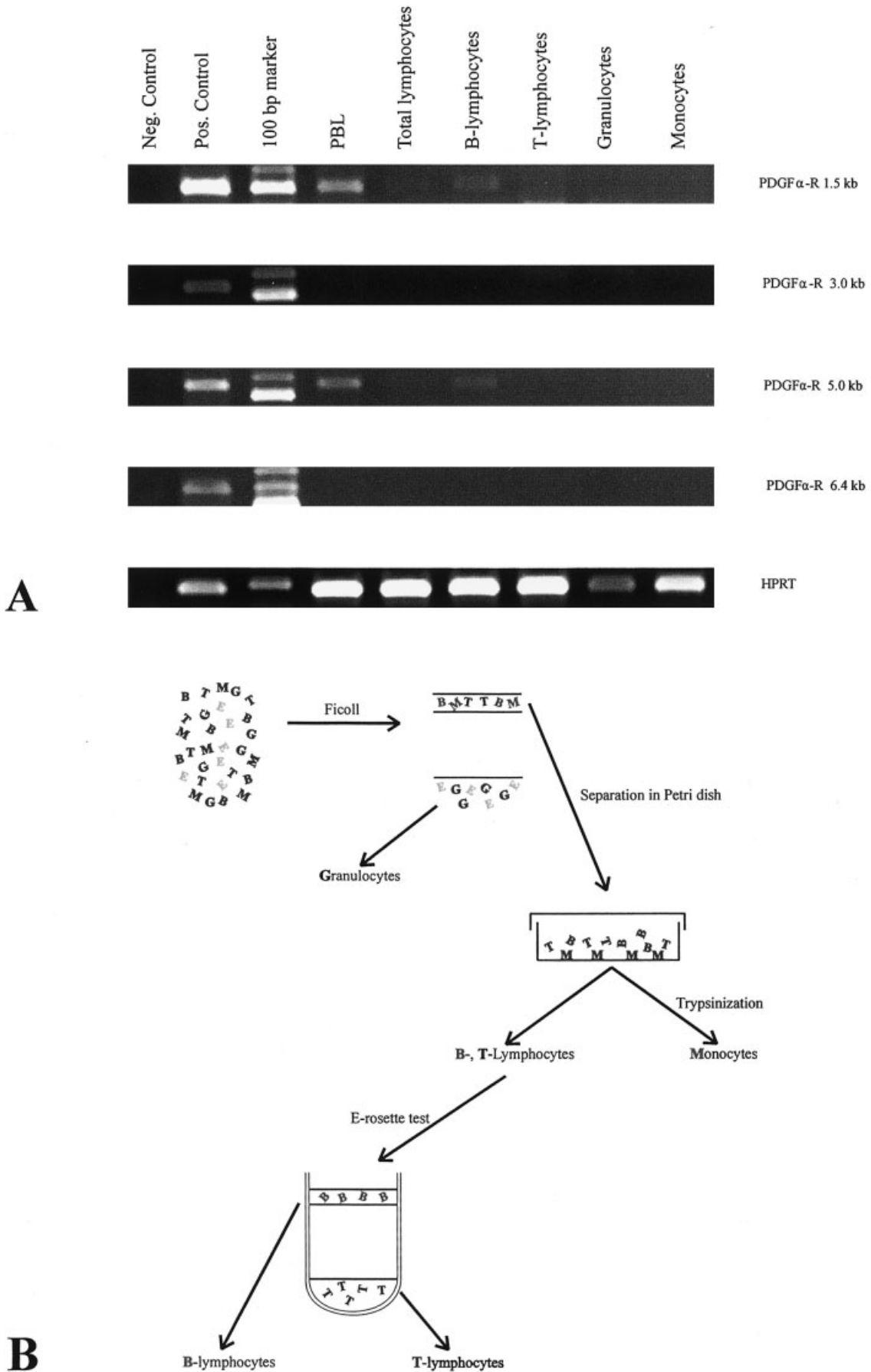
Figure 4. (A) Results of the detection of all transcripts of the *PDGF α-receptor* (1.5, 3.0, 5.0, and 6.4 kb) by means of RT-PCR on RNA isolated from total peripheral blood (PBL) and different enriched cell populations. (B) Schematic representation of the purification strategy used to enrich specific cell populations from normal peripheral blood, i.e. B- and T-lymphocytes, granulocytes, and monocytes (see the Materials and methods section for more details)
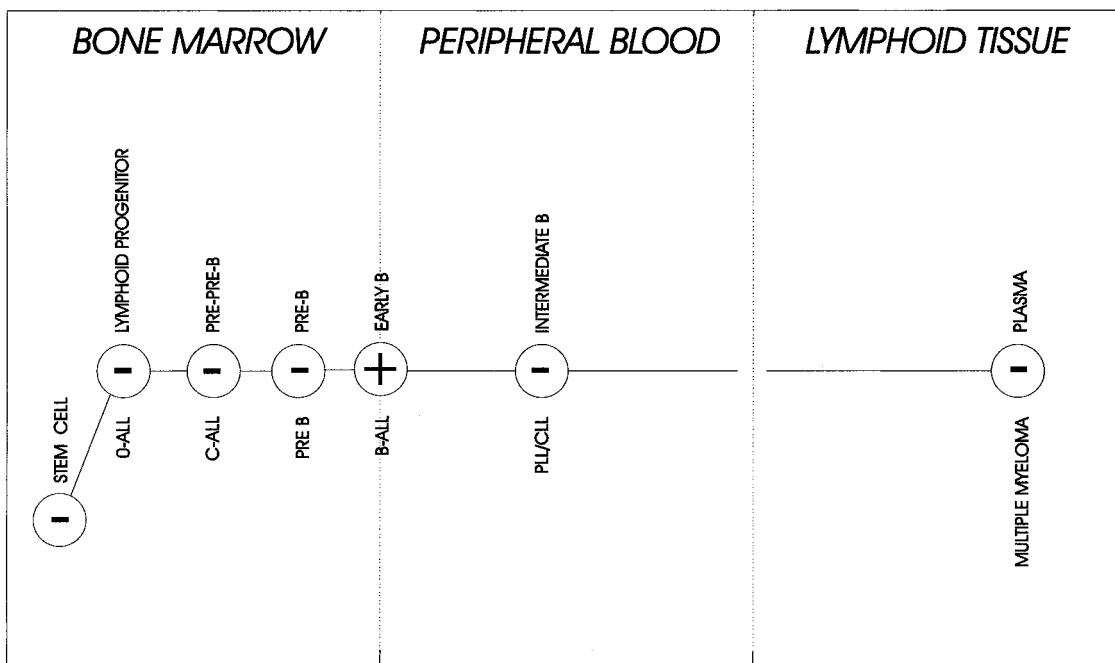
Figure 5. Schematic representation of the detection of the 1.5 kb transcript of the *PDGF α-receptor* in haematopoietic malignancies representative of different developmental stages of the B cell (upper panel). The compartment in which the cells are normally situated is indicated. In addition, their malignant counterparts are shown (lower panel), which can be found in either compartment
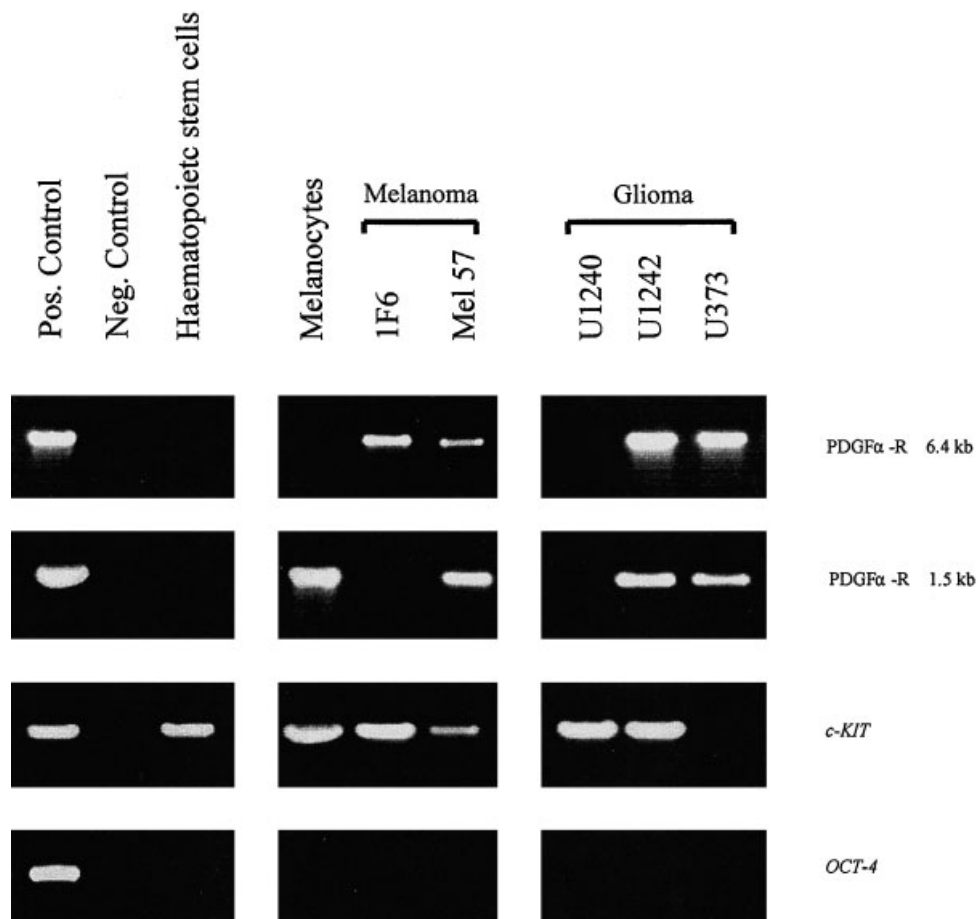

Figure 6. Representative results of the detection of the 6.4 kb and 1.5 kb transcripts of the *PDGF α-receptor*, expression of c-*KIT* and *OCT-4*. Shown are haematopoietic stem cells (CD34 +), normal melanocytes, two cell lines derived from melanomas, and three from gliomas

did not express the 1.5 kb transcript (Table 1 and Figure 2A).

The finding that most of the tissue samples and cell lines, both malignant and non-malignant, show expression of c-*KIT* irrespective of the presence or absence of the 1.5 kb transcript (Table 1 and Figures 2A and 6) indicates that there is not a common regulatory mechanism that drives expression from c-*KIT* and the P2 promoter of the *PDGF α-receptor* gene.

Discussion

We proposed the 1.5 kb transcript of the *PDGF α-receptor* as an informative parameter for the early diagnosis of TGCTs [6,8], the most common malignancy in Caucasian adolescent and adult males [29]. This was based on the finding that no expression was detected in normal and atrophic testicular parenchyma and semen, while it was detectable in testicular parenchyma containing CIS and in invasive TGCTs [6,8]. *In vitro* experiments showed that expression is differentiation-dependent [6–8]. Our results confirm that this regulation is also present in primary TGCTs.

The dilution experiments indicate that for positivity, at least 100 copies of this transcript are required within a single experiment, consisting of 32 cycles with a total input of 250 ng of RNA. This was found to correlate with approximately 100 Tera-2 clone 13 cells, meaning that on average a single 1.5 kb transcript is present in these cells. The calculated single transcript per cell is, however, probably an underestimate, because Tera-2 clone 13 cells are known to differentiate spontaneously, thereby losing the 1.5 kb transcript [7]. We have shown here that RNA isolated from seminomas and embryonal carcinomas can be diluted at least 25 times without losing positivity. In addition, our results indicate that positivity is still found when fewer than 10% of the seminiferous tubules included in the samples under investigation contain CIS.

To assess specificity, we investigated a series of testicular non-TGCT lesions, as well as samples of the parenchyma of normal and atrophic testis. In accordance with our earlier findings [6,8], no expression of the 1.5 kb transcript was found in non-malignant testicular lesions, or in testicular parenchyma without CIS. The observation that 1.5 kb transcripts can be detected, using additional cycles, in normal and atrophic testicular parenchyma samples is most likely related to our finding that at least a subfraction of B cells, i.e. early B cells, express the transcript. The early B cells can in fact indeed be found both in the bone marrow and in the circulating lymphocyte pool, representing a small fraction of the circulating B-cell pool. Our investigation of 1.5 kb transcript expression in haematopoietic malignancies suggests that it might be restricted to specific steps during normal B-cell development, being present at the early B-cell stage, of which B-ALL leukaemic cells are representative (Figure 5). Moreover, EBV-transformed B cells were also found to be positive for the 1.5 kb transcript and it is noteworthy that CD21, which is expressed on B-ALL and early B cells, is required for EBV transformation [30]. All primary testicular lymphomas and Leydig cell tumours were also positive. Because of the clear presence of the 1.5 kb transcript, the relatively low level of expression in early B cells and the absence of a correlation between positivity of the sample after 32 amplification cycles and the presence of lymphocytes, it is highly likely that the positivity found is mainly due to the presence of the transcript in the tumour cells, as supported by our *in vitro* data (see below).

We demonstrated that some glioma and melanoma cells, as well as normal melanocytes, showed expression of the 1.5 kb transcript. These samples also expressed the 5.0 kb transcript of the *PDGF α-receptor*, and the samples showing expression of the 6.4 kb transcript also showed positivity for the 3.0 kb transcript, as found before [6–8]. These data indicate that co-expression of the 1.5 kb and 5.0 kb transcripts from the P2 promoter and the 3.0 kb and 6.4 kb transcripts of the P1 promoter is independent of the cell of origin. Although it was originally found that the activities of the P1 and P2 promoters were mutually exclusive [6,8], it is now shown that both promoters can be active simultaneously. The mutual exclusivity of the P1 and P2 promoter activities observed in the undifferentiated versus differentiated Tera-2 cells is therefore most likely to be due to a different availability of differentiation-specific transcription factors. Our results do not support the model that activity of the P2 promoter might be associated with expression of the nearby c-*KIT*.

In vitro experiments showed that activity of the *PDGF α-receptor* P2 promoter is dependent on the POU-domain transcription factor OCT-4 [11]. This also holds true in TGCTs, because all components that are positive for the 1.5 kb transcript also express *OCT-4*, while both teratomas and yolk sac tumours are negative for the 1.5 kb transcript, as well as for *OCT-4*. Noteworthy is the finding that loss of *OCT-4* from mouse embryonic stem cells prevents them from forming somatic tissues, and that their differentiation is consequently restricted to the trophoblastic lineage [31]. Indeed, human yolk sac tumours were found to be negative for *OCT-4* (this study). Our results show, however, that expression of *OCT-4* is not necessary or sufficient to activate the P2 promoter: 1.5 kb transcripts can even be found without expression of *OCT-4*. From these data it appears likely that P2 transcript expression in TGCTs is driven by *OCT-4*, in cooperation with other transcription factors. It remains to be investigated which other members of the large *OCT* family are involved in regulation of the P2 promoter in non-TGCT cells.

This study has demonstrated that expression of the 1.5 kb transcript, i.e. activity of the *PDGF α-receptor* P2 promoter, is not restricted to TGCTs, but can be found in a number of malignant and non-malignant

tissues and cells. At least two mechanisms seem to regulate P2 promoter activity, one partially OCT-4-dependent, which is found in embryonal tissues and derived tumours, and one completely independent of OCT-4. The present data validate the usefulness of detection of the 1.5 kb transcript for the early diagnosis of TGCTs, and possibly of other testicular malignancies, such as lymphomas and Leydig cell tumours. It must, however, be performed in a well-controlled set-up, in which the presence of contaminating early B cells is reduced as much as possible.

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