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RXRα and LXR activate two promoters in placenta- and tumor-specific expression of *PLAC1*

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Abstract

PLAC1 expression, first characterized as restricted to developing placenta among normal tissues, is also found in a wide range of tumors and transformed cell lines. To understand the basis for its unusual expression profile, we have analyzed the gene structure and its mode of transcription. We find that the gene has a hitherto unique feature, with two promoters, P1 and P2, separated by 105 kb. P2 has been described before. Here we define P1 and show that it and P2 are activated by RXRα in conjunction with LXRα or LXRβ. In placenta, P2 is the preferred promoter, whereas various tumor cell lines tend to express predominantly either one or the other promoter. Furthermore, when each promoter is fused to a luciferase reporter gene and transfected into cancer cell lines, the promoter corresponding to the more active endogenous promoter is preferentially transcribed. Joint expression of activating nuclear receptors can partially account for the restricted expression of *PLAC1* in placenta, and may be co-opted for preferential P1 or P2 *PLAC1* expression in various tumor cells.

Introduction

PLAC1 is an X-linked gene with expression restricted to the trophoblast lineage. In mice high expression is detected in ectoplacental cone, giant trophoblast cells, and labyrinthine layer along gestation days 7.5–14.5 dpc (days post coitum) [1]. In humans expression begins in placenta at 8 weeks and continues throughout pregnancy [2]. Follow-up studies by RT-PCR and compilations of expressed sequence tags further support the inference that the gene is expressed little if at all in non-placental cells [scattered reports suggest a very low level of mRNA in hematopoietic stem cells and testis [3]].

PLAC1 protein is inferred to be important during normal embryonic development, associated with instances of variable growth control. Mouse deletions spanning this region result in runty phenotype or stillbirth [4–6], and its level of expression has been implied to be critical during pregnancy [7]; and elevated *Plac1* transcription is also correlated with placentomegaly in cloned mice [8].

Interest in the possible action of *PLAC1* was augmented by the findings that although it is rarely expressed in normal cells, the gene is highly active in a wide variety of cancers [3, 9, 10]. Koslowski et al. [3] report 38% of primary tumor specimens express *PLAC1* at appreciable levels, with high levels in 82% of primary breast cancers and 55% of tumor cell-

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lines. They further present provocative evidence that anti-*PLAC1* antibody inhibits cell motility, proliferation and invasiveness of MCF-7 breast cancer cells, so that *PLAC1* might be involved in tumor progression (see Discussion).

How is the unusual expression profile of *PLAC1* achieved? Restricted expression could be conferred by specific transcription factors. To look for such factors, we began by reexamining *PLAC1* transcripts and structure. We have revised the gene structure to contain 6 exons, the last exon coding for the protein. A segment upstream of the first exon includes one active promoter (P1). A second promoter (P2), previously described, is located just upstream of exon 4, 105 kb 3' to P1, This gene structure is conserved in mouse, with the two promoters >100 kb apart, a structure unique among genes analyzed thus far.

To define regulatory elements, we resected the putative P1 and P2 promoter DNA regions. Binding sites for nuclear receptor transcription factor RXRα were identified in both human and mouse, and are active and stimulated by RXRα and LXR. Differential levels of these and possibly other nuclear receptors could at least partially rationalize the expression pattern of *PLAC1*.

Methods and Materials

Cell Culture

Human placental choriocarcinoma cell line BeWo (CCL-98™) and human breast adenocarcinoma MCF-7 (HTB-22™) cells were obtained from ATCC (Manassas, VA, USA). BeWo cells were cultured in Kaighn's modification of Ham's F-12 Medium (F12k) medium with 10% fetal bovine serum (FBS) and MCF-7, in Eagle's Minimum Essential Medium supplemented with 10% FBS and 0.01 mg/ml bovine insulin. All media were purchased from Invitrogen.

Transient transfections and Luciferase assay

BeWo and MCF-7 cells were transfected with Lipofectamine 2000 and Lipofectamine™ LTX with Plus reagent (Invitrogen), respectively, in 24-well plastic dishes. For promoter luciferase assay, cells (2×10^5) were transfected with 0.5μg/well DNA containing either pGL4.14 basic vector (Promega) or various constructs of pGL4.14 human or mouse *Plac1* promoter fusion plasmids along with renilla luciferase construct (10 ng/transfection) as a control for transfection efficiency. In assays with vectors expressing transcription factors, 0.3 μg/well of pGL4-plac1 promoter construct was mixed with 0.9 μg/well of expression vector and transfected. Controls included vector with no expression inserts. Luciferase activity was measured using Dual-Luciferase reporter assays (Promega) in a Victor 1420 Multilabel Counter (Perkin Elmer) 48 hours after transfection, normalized to renilla luciferase levels. Agonists were added to a final concentration of 0.1 μM T091713 or 10 μM 9 cis-retinoic acid (Sigma, St. Louis MO, US) 24 hr after transfection with the assayed plasmids. The linear ranges ranges for the agonist's concentration were determined by titration (results not shown). Luciferase activity was measured 24 hr later.

Chromatin immunoprecipitation and DNA capture assay

Chromatin immunoprecipitation was performed with anti-RXRα antibody (D-20, Santa Cruz, CA, USA) following the supplier's protocol. Briefly, 2×10^7 cells were fixed with formaldehyde at a final concentration of 1% for 10 minutes at room temperature. Cross-linking was terminated with glycine to 0.125 M. After two washes with PBS (137 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate dibasic, 2 mM potassium phosphate monobasic, pH 7.4), cells were lysed with 1 ml lysis buffer [5mM PIPES pH 8.0, 85mM KCl, 0.5% NP-40 and 1 tablet/50ml Protease Inhibitor Cocktail (SC-45000, Santa Cruz,

CA)], sonicated and centrifuged at 13,000 rpm for 15 minutes at 4°C. 300 μg of supernatant (chromatin) was incubated with 2 μg of anti-RXRα antibody overnight at 4°C. Antibody-protein-DNA complexes were recovered adding A/G PLUS-Agarose (Invitrogen). Beads were washed twice with lysis buffer and cross-linking reversed by incubation at 67°C overnight. Beads were removed by centrifugation. The supernatant was extracted with phenol/chloroform/isoamyl alcohol (25:24:1), vortexed thoroughly, and DNA precipitated with ethanol. Resulting DNA was quantitated using SYBR® Green PCR Master Mix (Applied Biosystems) and real-time PCR. Forward and reverse primer sequences amplifying across the P1 RXRα binding sites, respectively, were 5'TCACTATCCTATCAGAAATAGAACA, 5'GCACTCCTTGACAGACTCCTC (starting UCSC human assembly genomic coordinates forward primer: 133898272 and reverse primer: 133898767).

DNA-protein complexes were captured by suppliers' instructions using a Dynabeads kilobase BINDER kit (Invitrogen, CA, USA). Forward and reverse primers for human P1 promoter sequences between −120 to −150 bases, containing wild type or Mut2 RXRα binding sites (Supplementary Fig.3a and Supplementary Methods), were synthesized by IDT (USA). The reverse strand oligonucleotide for each primer-pair was biotin-tagged at the 5' end. Equimolar forward and reverse oligonucleotides were mixed and annealed by heating to 95°C and slow cooling. Annealed oligonucleotides (300 pmoles) were incubated with either 75ng of purified RXRα, LXRβ (Protein One, Bethesda, MD, USA) or 1.5 μgm BeWo cell nuclear extract (see Supplementary Methods) at 4°C with the binding buffer (Invitrogen, CA, USA) in a total volume of 300 μl. Protein-DNA complexes were captured using magnetic streptavidin beads (Dynabeads) and washed with the wash buffer (Invitrogen, CA, USA). Samples were loaded on an SDS-PAGE gel, followed by Western blotting with anti-RXRα (SC-553) or anti-LXRβ (SC-1001) antibodies (Santa Cruz, CA, USA).

Results

Revised gene structure and transcription start sites

The original study of *PLAC1* described a placental mRNA containing 3 exons, the last exon encoding the protein [1]. This has been extended by 5' RACE studies. Using a human RACE-ready placental RNA library and primers positioned in exon 2 yielded products that included 3 new non-coding exons with canonical splice donor and acceptor sites, extending the transcript much further upstream and implying the existence of a transcription start site ("P1" in Fig. 1a) between coordinates 133,898,352 and 133,699,873 (UCSC human genome assembly GRCh37/hg19, Feb. 2009). Notably, RACE products from this primer did not include the former "exon1" (now exon 4), suggesting that whenever the transcript originates from P1 this exon is skipped, but other exons are included, with the last exon again being the coding exon (Fig.1a). In contrast, 5'RACE with primers positioned in "exon1" (now exon 4) of the originally reported cDNA always terminated at genomic coordinate 133,792,552 (UCSC human genome assembly GRCh37/hg19, Feb. 2009), identifying a second start site ("P2" in Fig. 1a), corresponding to the initially inferred promoter, that is now placed more precisely 39 bases upstream of the previous report. Overall, we thus infer a revised gene structure spanning 198,479 bp of genomic DNA, with the two promoters separated by 105,800 bp. The exon-intron structures are diagrammed in Fig.1a, along with 5 alternative isoforms recovered by 5'-RACE. Their existence is independently supported by reported EST's (Fig. 1a). The genomic coordinates, splice sites and cDNA sequence are provided in Supplementary Table 1a.

To further verify the inferred transcripts, poly $(A)^+$ RNA was isolated from a choriocarcinoma-derived cell line, BeWo, which expresses *PLAC1*. RT-PCR products with reverse primers positioned in the most distal exon and forward primers in newly defined 5'-

exons (see Supplementary Methods) directly demonstrated the presence of alternately spliced isoforms in BeWo cells (Fig 1a).

P1 and P2 account for nearly all the transcripts recovered from placenta and BeWo cells.

Mouse P1 promoter characterization

Mouse *Plac1* has an overall gene structure very similar to human. The previously reported promoter (now "P2") starts 5' of what proves to be exon 4; and a "P1" transcript, starting upstream of the equivalent exon 1 and including exons 1 and 3, was in evidence in ESTs in the NCBI database, supported by a placental-derived mRNA (AK145876, CK128283) starting at nucleotide position 50,593,288 (NCBI37/mm9). As with human P1 (Fig. 1a), using primers in exons 1 and 6, RT-PCR products were amplified from mouse placental RNA (Supplementary Methods), cloned and sequenced. Figure. 1b shows the structure of inferred isoforms 2 and 3, along with the originally defined transcript from the P2 promoter. Once again, transcripts originating from P1 skipped exon 4 during splicing. An EST (DV063536.1) supports the inclusion of exon 2 as diagrammed. The full gene in mouse thus spans 170,109 bases, with 125,585 bp between the two promoters. The exon sequences and their genomic coordinates and splice sites are listed in Supplementary Table 1b.

Human and mouse P1 and P2 promoter activity

To analyze P1 promoter activity, 5,241 bp of human DNA or a 3,323 bp mouse segment (including 78 bp of the most 5' exon between the *PLAC1* gene and the next upstream coding gene, FAM122B), was fused to a promoter-less luciferase reporter (Supplementary Methods) and transfected into BeWo cells, a choriocarcinoma cell-line that expresses *PLAC1*. Both fragments yielded high luciferase activity (Fig. 2a; p5241-LucF and p3323-LucF respectively). Sequential deletions defined a human promoter within 490 bp of 5' of exon 1 (Fig. 2b; p490-LucF), with ~ 80% of maximum luciferase activity. Further deletions led to sharp reductions in luciferase activity (p146-LucF, p98-LucF), and p20-LucF, retaining only 20 bp upstream of exon 1, showed only background luciferase levels. Consistent with critical promoter elements in a delimited region, an internal deletion of −725 to +18 bp completely abolished activity.

A similar analysis of mouse P1 promoter by resection of the p3323-LucF fragment showed that a minimal promoter activity for mouse "P1" lies within -163 bp of the putative transcription start site (Supplementary Materials, p163-LucF Supplementary Fig. 1a). Deletions extending further into this "critical" region led to a sharp loss of activity (p129-LucF through p88-LucF). We focus on the newly defined P1 promoter, but we similarly cloned human and mouse P2 promoters (Supplementary Materials), and sequential deletions defined a 161 bp minimal mouse promoter upstream of exon 4 (Supplementary Fig. 4) that is comparably activated by nuclear receptor agonists.

Mouse and human P1 promoters include critical RXRα sites

For P1, multiple blocks of homologies were seen across species [Vista plot [11] in Supplementary Fig.2a], most notably between human p146-LucF and mouse p163-LucF (alignments in Supp. Fig.2b). High scoring putative transcription factor binding sites, using Transfac [12] and Tess [13], included a core binding site for Retinoic Acid X receptor alpha (RXRα) [14]. Further analysis was focused in this region.

For human P1, the construct p146-LucF (Fig. 1b) that retains 60% of maximal activity includes two everted RXRα binding sites with 3 intervening bases between them -- AaGTCA ctc TcACCT-- (Supplementary Fig. 3a), a slight variant from consensus (9). Based on binding element classifications this can be considered an IR3-binding site. Single-

base mutations in these half-sites (Fig. 2c and Supplementary Fig. 3a) showed that the Mut1 mutation in the 5' RXRα half-site had little effect on luciferase activity, but the construct with Mut2 or Mut4 mutations at the 3' site reduced luciferase activity >50% compared to p146-LucF, to the level of the p98-LucF construct in which these sites are deleted (Fig. 2c). Thus, the results were again consistent with a requirement for an intact RXRα binding site for full promoter activity. Mutations in mouse P1 promoter had a similar effect (Supplementary Materials and Supplementary Figs. 1b and 3b).

RXRα along with other nuclear receptors augment P1 promoter activity

Nuclear receptors like RXRα function as homodimers, or as heterodimers with RAR, different PPAR isoforms, Vitamin D receptor or Liver X receptors [15]. To test for effects on P1 activity, we cotransfected human or mouse P1 constructs into BeWo cells along with vectors expressing these nuclear receptors. PPARγ or Vitamin D receptor had no detectable effect on transcription (results not shown). RAR was not considered to be a candidate because knock-out mouse studies show congenital malformations and fetal and postnatal Vitamin A deficient syndromes but with no placental defects [16]. By contrast, contransfected RXRα LXRα or LXRβ expressing constructs stimulated human and mouse P1 (Fig. 3a). Agonists increased the response somewhat for each receptor [1.5 fold higher for human P1 and RXRα with 9cis-RA (Fig.3a, cf. lane 1 vs. lanes 2 and 3)] and ~2-fold or more for LXRβ /LXRα in the presence of T091713 (Fig.3a, cf. lanes 4 vs. 5, and 6 vs. 7).

We also transfected expression vectors for RXRα in combination with LXRα or LXRβ. Human and mouse P1 transcription were both stimulated 6- to 7-fold in the presence of their corresponding ligands (Fig. 3a, cf. lanes 8 vs. 9 for LXRα and lanes 10 vs. 11 for LXRβ). We note that both LXRα and LXRβ are expressed in placenta, the latter particularly in invasive trophoblasts [17] and chorioallontoic placenta [18], and therefore are likely partners to interact with RXRα *in vivo*.

Mouse P2 promoter segments showed comparable responses to RXRα, LXR, and their agonists (Supplementary Fig. 5a).

Mutations in RXRα binding sites reduce response to RXR and LXR agonists

To see if mutated RXRα sites blunted the response to agonists *in vivo*, luciferase fusions of Mut2 and Mut3-modified human P1, and Mut2, Mut3, and Mut5-modified mouse P1 were tested for their activity in the presence of agonists 9cis-RA and T091713 independently. For human P1, wild-type construct p146-LucF responded to the addition of both 9cis-RA and T09713, although much more strongly to the former, and responses were impaired by Mut2 and Mut3 mutations (Fig. 3b). Similarly for mouse, wild-type P1 activity increased >3 fold in the presence of agonists 9 cis-RA and T091713, and Mut2, Mut3 and Mut5 showed reduced responses to agonists (2- to 5-fold lower than wild type; Fig.3c). We again infer that the P1 promoter responds to endogenous nuclear receptors, with the RXRα site and nearby 3' sequence affecting maximum activity.

RXRα interacts directly with P1 promoter sequences

RXRα interaction with human P1 was inferred from two independent methods. Chromatin immunoprecipitation (ChIP) [19] from BeWo cells, using anti-RXRα antibody followed by quantitative RT-PCR of upstream human promoter sequences (Methods), showed 3-fold enrichment of P1 promoter sequences compared to control ChIP assay with IgG alone (Fig. 4a). More directly, purified RXRα bound a 5' biotin-tagged oligonucleotide containing the human P1 RXRα binding site (Methods). Separated on a denaturing SDS-polyacrylamide gel, transferred to a nylon membrane, and tested for the presence of RXRα with anti-RXRα antibody, robust association was seen (Fig.4b, WT). Oligonucleotide with a "Mut2"-

mutation (Fig.4b, Lane Mut2) showed very weak binding, consistent with reduced capacity to support luciferase activity (cf. Fig 2c). A non-specific oligonucleotide did not bind RXRα (Fig. 4b, Lane, NS). Furthermore, the wild-type oligonucleotide, but not Mut2, bound endogenous RXRα when incubated with BeWo cell nuclear extract from BeWo cells (Fig. 4b, BeWo NE, Lanes 'WT' and 'Mut2').

Purified LXRβ also bound a wild-type oligonucleotide, and binding was slightly enhanced by added purified RXRα (Fig. 4b, lanes LXRβ +, and 'WT' and RXRα + and LXRβ +, respectively). Again, no binding to mutant oligonucleotide was seen (Fig 4b, LXRβ +, Mut2). The results were reproducible when BeWo cell nuclear extract was used (Fig. 4b BeWo NE, Lanes WT and NE).

Mouse wild-type P1 showed comparable interactions with both RXRα and LXRβ with nuclear extract from BeWo cells (Fig. 4c, BeWo NE, lane WT), and no interaction with a non-specific oligonucleotide. These results confirm direct interaction of RXRα and LXRβ with P1 promoter nuclear receptor sites of both human and mouse.

P1 and P2 promoter preferences *in vivo*

Relative promoter response varied in different cell types. Quantified by qRT-PCR and discriminated with a fluorescent reporter located in exon 5 (an exon shared by all isoforms), differential P1 and P2 promoter responses were seen in placenta (Supplementary Fig. 5a) and in several cancer cell lines (Supplementary Fig. 5b). Both human and mouse placenta contained transcripts from both promoters, but transcripts from P2 were 8-fold more abundant than those from P1 in human and 10-fold more in mouse placenta (estimated based on the number of clones recovered from RT-PCR products and sequenced).

Cancer cell line NC1929, like MCF-7 and placenta (Supplementary Fig. 5b), preferentially transcribed P2, whereas BeWo and JAR cell lines, even though they are of placental origin (derived from choriocarcinoma), preferentially expressed P1. Other transformed/cancer cell lines, including HeLa, HTB122, LNCaP, and SV40-W138, all showed *PLAC1* expression from both promoters, but with a slight preference for P1. [The level of *PLAC1* transcription in these cells was also more than 10-fold lower than in BeWo, JAR, MCF-7 or NC1929 cells.]

Strikingly, when both mouse and human P1- or P2-luciferase constructs were transfected into high-expressing tumor cell lines, the cells expressed the transfected promoters in accord with their endogenous promoter use. BeWo cells, for example, showed far greater activity with P1 construct, whereas MCF-7 had higher activity with P2 (cf. Figs. 5b and 5c). Thus, promoter choice likely reflects the levels of endogenous factors rather than an inherited epigenetic state of DNA (see Discussion).

Evidence consistent with this notion came from the responses to nuclear receptor agonists after transfection of mouse P1 and P2 promoter-luciferase constructs into BeWo and MCF-7 cells. In BeWo, RXRα and LXRβ agonists each independently stimulated the P1 promoter 3.5 fold (Supplementary Fig. 7a). The P2 promoter was stimulated 2-fold by RXRα and 3.5 fold by LXRβ agonist (Supplementary Fig. 7, Panels a and b; cf. addition of T091713). For P1 and P2 (Supplementary Fig. 7a and b), simultaneous addition of both agonists yielded 6-fold and 5-fold stimulation respectively. Although the basal P2 expression was 4-fold lower than P1 expression in BeWo cells, maximal activity reached comparable levels for both P1 and P2 in the presence of agonists.

P1 and P2 promoters both had lower overall luciferase levels in MCF-7 cells (Supplementary Fig. 7c and d), but basal P2 activity was at least 1.6 fold higher than P1 (cf.

DMSO lanes in Supplementary Fig. 7c and 7d). Also, in contrast to BeWo cells, in which each agonist boosted luciferase levels, added RXRα agonist but not LXR agonist strongly stimulated both P1 and P2 3-fold (Supplementary Fig. 7c and d). Further, combined addition of 9cis-RA and T091713 achieved luciferase levels comparable to 9cis-RA alone, suggesting that RXRα itself is sufficient to up-regulate the P2 promoter.

In summary, both promoters responded to and were stimulated by receptor agonists, LXRβ in combination with RXRα having the strongest effect in BeWo cells, and only added RXRα agonist acting strongly in MCF-7 cells. Again, promoter preference could thus reflect the levels of endogenous nuclear receptors and endogenous agonists (see Discussion below).

Discussion

We have revised the gene structure to add 3 additional non-coding exons 5' to the existing 3 exons, resulting in both human and mouse Plac1 genes with 6 exons. Further, we detail P1 and P2 start sites based on 5'-RACE results and alternatively spliced transcripts. Very likely only exons 4–6 were identified in earlier studies of *PLAC1* [1] because the P2 promoter transcript predominates in placenta. However, both promoters are active to some extent in placenta as well as BeWo and several other cancer-derived cell lines (Supplementary Materials, Supplementary Fig.7). Transcripts starting at P1 may include exons 2 or 3 but always skip exon 4, whereas transcripts originating at P2 always include exons 4, 5 and 6; all transcripts include the sole coding sequence of exon 6.

RXRα in combination with LXRα or LXRβ stimulated both promoters. We review the evidence and possible actions on Plac1 transcription of other transcription factors in placenta and tumors.

Promoters and cognate transcription factors

Human or mouse P1- or P2-containing DNA was sufficient to activate a fused luciferase reporter gene (Fig.2a and Supplementary Figs. 1a and 4). And although nuclear receptor binding sites in P1 and P2 promoters vary somewhat from the consensus, the results support their participation in promoter activation by RXRα along with LXRα or LXRβ. Plasmids expressing RXRα stimulated P1- and P2-luciferase activity, and stimulated even more in presence of an agonist or likely heterodimeric partners (see below and Fig. 3a). Furthermore, activity was sharply reduced when the human or mouse binding sites were mutated (Fig.3b and 3c). Direct interaction of RXRα was supported by both DNA-protein complex capture and chromatin immuno-precipitation assays, which showed that the endogenous P1 promoter is indeed occupied by RXRα *in vivo* (Fig.4a).

RXRα (rather than RXRβ) is the major isoform expressed in placenta [20]. Knockout mouse studies have demonstrated that RXRα is critical for the normal development of placenta, with RXRα -null embryos showing multiple placental defects and embryo death as early as day 12.5 dpc. In RXRα and RXRβ double knockout mice the placental defects are even more severe [21] compared to RXRα ablation alone and embryo death at day 9.5 dpc. It is suggested that there is some functional compensation by RXRβ [22] in RXRα knockout mice.

Here evidence for the interaction between nuclear receptors includes ~6 fold transcription stimulation after cotransfection of LXRα or LXRβ with RXRα in the presence of their respective agonists (Fig. 2a). While LXRβ directly interacted with the binding site (Fig. 4b), its binding was slightly elevated when RXRa was present. LXR α/β are expressed in yolk sac membranes and mouse placental labyrinthine layers [18], and LXRβ was especially

expressed in trophoblasts in early human placenta [23]. Thus we strongly suggest that in placenta LXRβ is the more likely partner for RXRα at the PLAC1 promoter.

Consistent with their involvement, microarray based expression profiling showed that the induction of Plac1 in mouse placenta [24] was accompanied by unusually strong induction of RXRα and LXRβ (25- and 7-fold, respectively).

Additional transcription factors are also likely to modulate the expression of *PLAC1*, both from our results on differential expression of the two promoters in various cells (see below) and especially from complementary studies of P2 promoter expression in cancer cells. Koslowski et al. [25] found that regulatory C/EBPβ and SP1 binding sites --further upstream of the P2 transcription start site than the RXRα sites□were critical for transcription in 3 breast cancer cell-lines. Further, they showed that estrogen receptorα augments transcription in a non-classical manner, with ligand-activated ERα interacting with DNA-bound C/EBPβ 2 and SP1.

P1 vs. P2 preference and possible *PLAC1* function in placenta and cancer cells

At least two a priori models fit the results on promoter specificity. In one, a repressor binds to cis-elements in other tissues, but is not expressed in placenta or tumor cells. In a simpler alternative, expression of a combination of nuclear receptors RXRα, LXR and possibly others all together may occur only in placenta and many tumors. The second model gains credence from the observed differential expression of the two promoters in various cells, and from transfection experiments that show that transfected promoter sequences are expressed according to the level at which the endogenous P1 vs. P2 promoters are expressed (Supplementary Fig.8). Trans-acting factors, including C/EBPβ-2 (see above), could affect the relative activation of P1 or P2.

As for its function, *PLAC1* localizes into membranous compartments in the syncytiotrophoblast apical region [26], and may influence the FGF-7 axis [27] as well as levels of cyclin D1 and pAKT (ser-473) [3]. Such actions would be consistent with recent speculation reviving the venerable idea that uncontrolled tumor cell invasiveness may subvert some features of highly controlled placental invasiveness [28]. But if tumor cells truly use PLAC1 to facilitate invasiveness, and *PLAC1* expression depends on co-expression of a set of nuclear receptors, then the basis for co-expression of the stimulatory transcription factors becomes of special interest.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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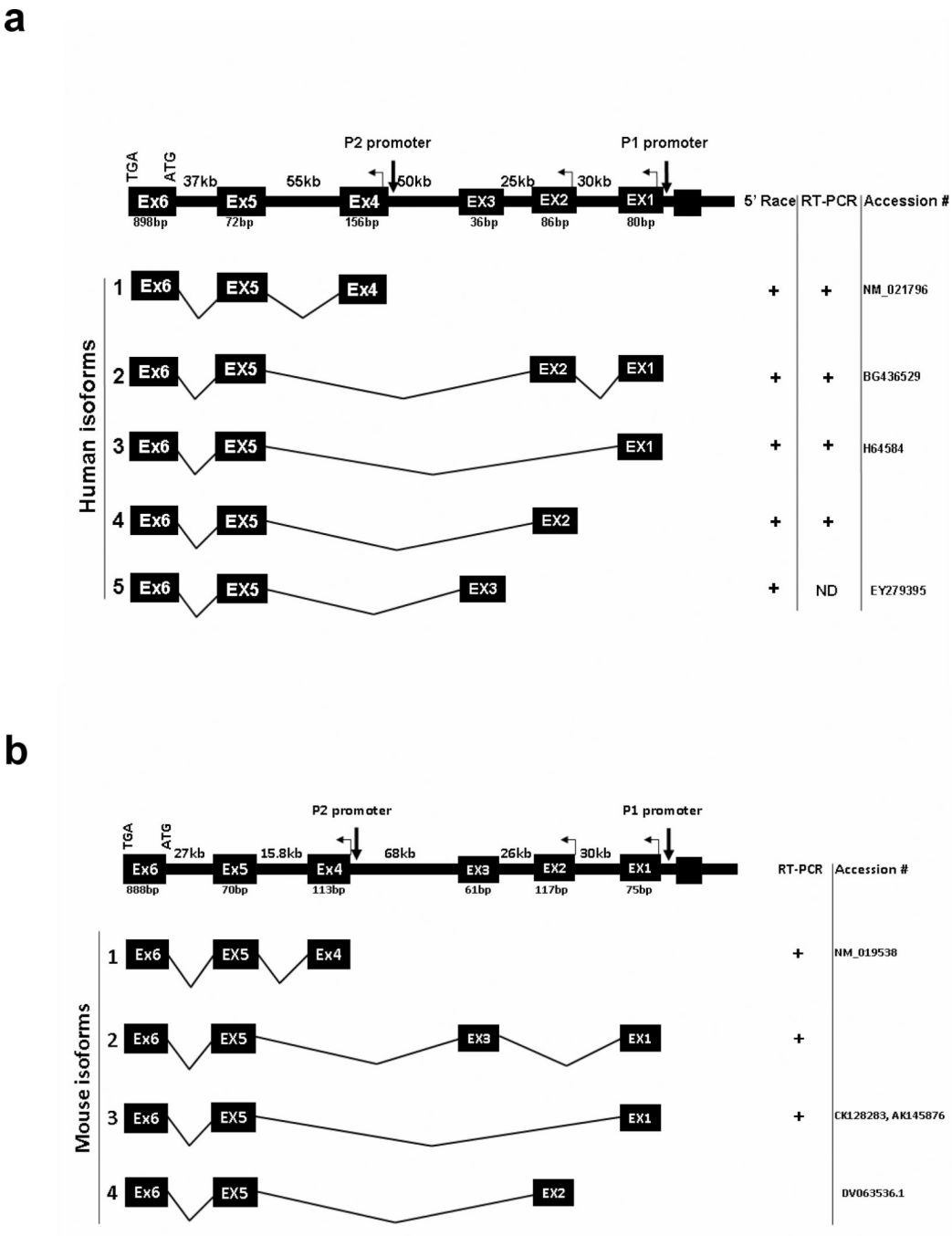

Figure 1. Schematic representation of human and mouse *PLAC1* gene structures with dual promoters

Schematic of *PLAC1* gene structures for Human (a) and Mouse (b). The top line in 'a' and 'b' positions promoters P1 and P2 in relation to six exons, introns and their sizes. Five human and 4 mouse splice transcript isoforms detected by RACE and RT-PCR are diagrammed below. For both human and mouse only transcripts starting at P2 promoter include Exons 4–6; transcripts originating at P1 promoter include various other exons but always skip exon 4. The columns at right indicate isoforms detected from a human placental RNA library or in BeWo cells by 5' RACE experiments or RT-PCR. For mouse, splice isoforms confirmed by RT-PCR are indicated. Available Genbank database accession numbers supporting isoforms

are also indicated. [Exon 3 was identified as a potential exon by its homology to an EST, EY27935 derived, from a monkey cDNA library]. ND means not detected.

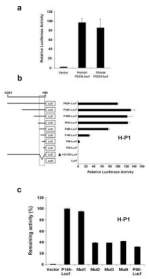

Figure 2. Luciferase yield in BeWo cells from human P1 promoter fusions

a) Luciferase activity driven by DNA fragments containing −5,241/+80 bp of human DNA or 3,323 bp of mouse DNA in pGL4 vector following transfection into BeWo cells. **b)** Luciferase activity from sequential deletions of the full-length construct. The plasmid number indicates the remaining number of nucleotides in each construct. Δ+18–725 is an internal deletion removing 725 bp upstream of exon1 and 18 bp in the first exon. Here and in the following figures H-P1 indicates to human P1 promoter. **c)** Effect on luciferase activity of mutations in the RXRα binding site and adjacent sequence in the minimal p146 promoter fragment. Mut 1, 2 and 3 indicate point mutations detailed further in Supplementary Fig. 3a. Luciferase levels in mutants are expressed as percent activity of wild-type p146-LucF construct.

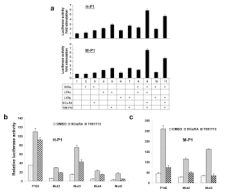

Figure 3. Luciferase levels from human and mouse P1 promoter: effects of transcription factors, their agonists, and mutations in nuclear receptor binding sites

a) Full length human P1 promoter construct (p5241-LucF, **panel a top**, Lanes 1–11) and full length mouse P1 promoter (p3323-LucF, **Panel a bottom**) were transfected along with nuclear receptor expression constructs, RXRα, LXRα and β, either alone or in combination, and with or without their respective agonists as designated under each bar. '+' indicates the presence of respective components in each transfection experiment. Levels are an average of 3 experiments with standard deviation indicated. Here and in other figures M-P1 refers to Mouse P1 promoter. **Panels b)** and **c)** The effect of mutations in the minimal human or mouse promoters on luciferase levels following transfection into BeWo cells along with independently added RXRα or LXR agonists. Comparative values are shown for the wild-type minimal human and mouse promoter constructs p146LucF and p163-LucF respectively. Mutations are described in the Supplementary Materials. See also Supplementary Figures 3a and b.

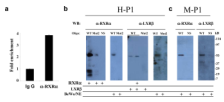

Figure 4. RXRα interacts with human and mouse P1 promoters

a) Measurements by qRT-PCR of enrichment of promoter sequences spanning the RXRα binding site in human P1 in BeWo cell chromatin immunoprecipitate and control immunoprecipitate with IgG. **b)** Western blot (WB) analysis with antibodies as indicated above, following incubation of the 5' biotin tagged wild type human P1-RXRα binding sequence, Mut2 or a non-specific oligonucleotide, with (see Methods and Supplementary Methods for experimental details and oligonucleotide sequence) and pure RXRα protein, BeWo cell nuclear extract or LXRβ as indicated, followed by capture of the protein-DNA complex with magnetic Streptavidin beads. Capture was done (1st panel), after incubation with pure RXRα protein and wild type oligonucleotide, with Mut2 oligo or control non-specific oligonucleotide as specified. (Low molecular weight bands seen with pure RXRα were due to protein degradation). In the 2nd panel wild-type or Mut2 oligonucleotide was incubated with BeWo cell nuclear extract. In the 3rd panel, wild-type oligonucleotide was incubated with pure LXRβ itself or along with RXRα and LXRβ, and Mut2 oligonucleotide with LXRβ. In the 4th panel BeWo cell extract was used with the same oligonucleotides. **c**) Mouse P1 sequences incubated with BeWo cell nuclear extract, similar to Human P1 (b). WT indicates wild-type and NS indicates a non-specific oligonucleotide in the reaction mix.

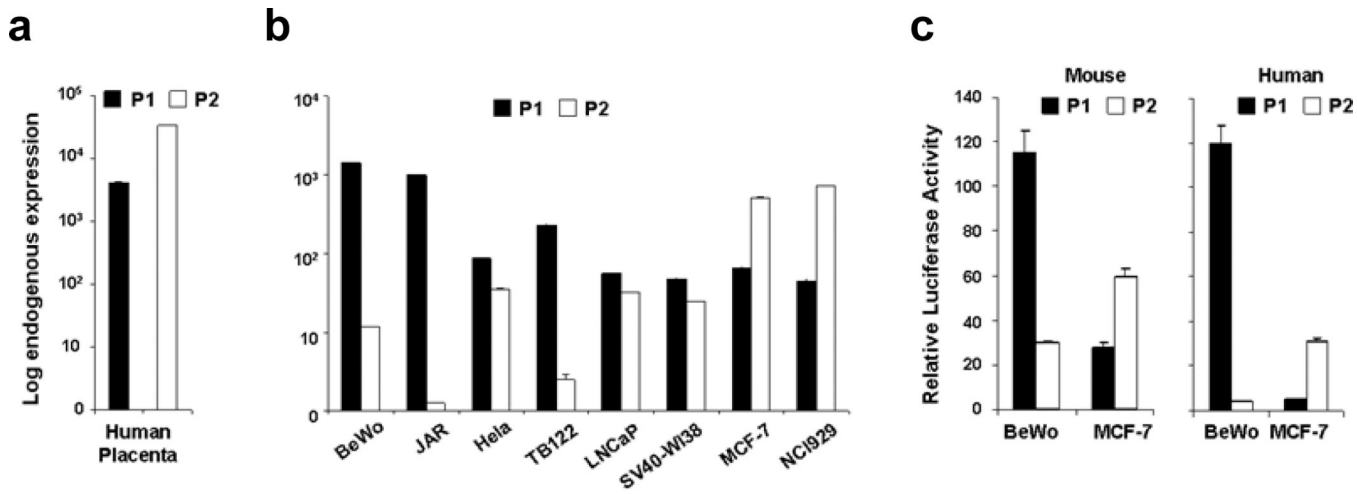

Figure 5. P1 and P2 promoter activities in placenta and cancer cell lines assessed by q-RT PCR

a) P1 and P2 promoter transcript levels from human placental RNA by q-RT PCR (see Supplementary Methods for details). A fluorescent reporter primer was located within exon 5, and transcript levels originating from P1 and P2 promoters were compared using a reverse primer in exon 6 and forward amplification primers in exon 1 or in exon 3 (see Fig.1a for exon/intron structures). Fold expression levels were calculated from the $2^{\Delta\Delta ct}$ values. Values are an average of 3 replicates, with error bars indicating standard deviations. **b)** Transcript levels from P1 (filled bars) and P2 (open bars) promoters in several human cancer cell-lines. All expression levels were normalized to control actin levels. **c)** Relative luciferase yields after transfection of mouse and human P1 and P2 promoters into BeWo or MCF-7 cells. The plasmid constructs used are as follows: mouse P1 promoter p3324-LucF, mouse P2 promoter p1174-LucF, human P1 promoter p5241-LucF, and human P2 promoter p2456-LucF.