V. RESULTS

In this chapter I describe the strategies chosen to carry out the experimental design, as well as the results obtained in the study of both coding and non-coding protein region of the ODZ4 mRNA derived from human tumor cell lines, which we used as an *in vitro* source for ODZ4 transcript.

5.1 *ODZ4* IS EXPRESSED IN HUMAN CELL LINES DERIVED FROM OVARIAN AND BREAST TUMORS

Since the ODZ2 mRNA expression was found in ovarian and breast human cancer as well as in tumor cell lines, as mentioned in a previous section 2.3 (Ziegler A. et al., unpublished results), and considering that the expression of ODZ4 was found to be up-regulated in human brain cancers [40][48], we decided to evaluate the presence of ODZ4 transcript in breast and ovarian human cancer-derived cell lines.

The avian ODZ2 transcript presents a splice variant consisting of an 8-amino-acid insert located between the seventh and eight EGF-like repeats [38]. Therefore, as a first step in our study we designed a primer pair that encompasses this region in the ODZ4 transcript, and we tested it by conventional PCR on cDNA derived from the ovarian tumor cell line, SKOV3. The corresponding region on the ODZ4 transcript is delimited by exons 16 and 18, thus the PCR amplification of this region in SKOV3 produced the expected 272bp band, which was subsequently cloned and sequenced (Fig. 5.1, Ex16-Ex18 PCR amplification product). Because the sequence analysis confirmed that the obtained PCR product corresponds to the ODZ4 transcript, this PCR become in later experiments a standard assay for detection of ODZ4 mRNA (Ziegler A et al., unpublished results). As evidenced in Figure 5.1, this transcript region was also detected in two different breast cancer cell lines (MCF7 and MDA-237), but not in the cervical cancer HeLA cell line.

Next, our aim was to determine which would be the approximated extension of the ODZ4 transcript. To this purpose, a screening searching to define the terminal regions of the ODZ4 transcript was performed. We evaluated the expression of the 5'-terminal exons five and seven (Ex7 and Ex8), which are localized within the coding region of the transcript; as well as the expression of the 3'- terminal exon (exon 34, Ex34 in the figure), both on cDNA templates

derived from ovarian and breast cell lines. As represented in Figure 5.1, expression of those exons compounding the 5' and 3' portion of the ODZ4 transcript was detected in SKOV3, MCF7 and MDA-273 cell lines, but not in HeLa cell lines.

Since we observed that the exons located on three diverse regions along the ODZ4 transcript might be detected (at 5',centered and at 3'), we postulate that the thirty coding-exons compositing the protein-coding region (CDS) would probably be expressed, and thus, in SKOV3, MCF7 and MDA-237, the ODZ4 "full-length" transcript could be present.

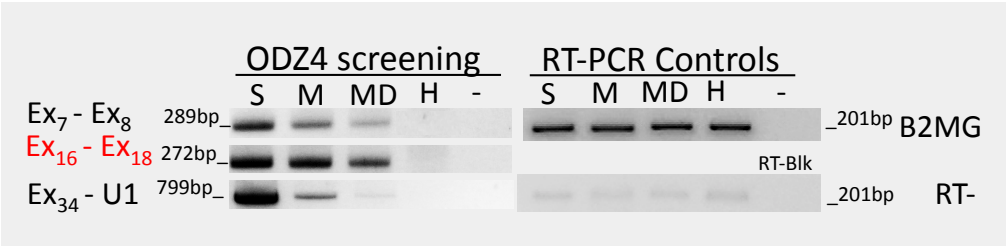


Figure 5.1. Screening of the ODZ4 Transcript in Human Cancer Cell Lines. The predicted full length of the ODZ4 transcript was tested by standard RT-PCR in different human cell lines derived from breast and ovarian cancer. At left, the presence of the 5'-region on cDNAs was detected by amplification of exons 7 to 8 (Ex7-Ex8 in the figure), whereas a central region of the transcript was detected by exon 16 to 18 amplification (Ex16-Ex18). Part of the 3'-UTR region was evidenced by 34-exon amplification. At the right of figure the RT-PCR controls which were used in the screening are shown. Amplification of the β2-microglobulin (B2MG) was used as RT-positive control; genomic contamination was tested by B2MG amplification without reverse transcriptase (RT-) addition. No-template controls performed during RT-PCR (RT-BLK) and B2MG PCR-amplification (-) are also reported. (*S*) SKOV3, (*M*) MCF7, (*MD*) MDA-237 and (*H*) HeLA cell lines were screened for the ODZ4 transcript. *For primes sequences see Table 4.2.*

5.2 CODING-STRUCTURE ANALYSIS OF THE ODZ4 TRANSCRIPT IN HUMAN CELL LINES DERIVED FROM OVARIAN AND BREAST TUMORS

Once some features of the transcript had been outlined, we were asked if all other protein-coding exons would be expressed in the ODZ4 transcript as well. Thus, the next steps of the study were addressed to characterizing the overall protein coding-structure of the ODZ4 transcript in ovarian and breast cancer-derived cell lines. For this, the experimental researching strategy chosen in this section as well as in section 5.5 was to generate increasingly long amplification fragments until the end of the coding region was reached. The amplicon length was extended going forward step by step, testing and checking the regions as a promenade along the ODZ4 transcript.

Figure 5.2 summarized the several exons expressed in the ODZ4 transcript derived from different human cancer cell lines. To determine the continuous expression of adjacent exons, we tested specifics regions using a constant reverse primer as "bait" with different forward primers and then, we compared the obtained amplicon sizes respect to database reference sequences predicted for ODZ4 RefSeq [55] amplification (for expected amplicon sizes see Table 4.3).

As showed in figure 5.2, we found the continuous 5'-expression from exon 4 to exon 8 in ovarian (SKOV3) and breast (MCF7 and ZR75) derived cell lines. The expression of our "ODZ4 specific fragment" was also detected as evidenced by exon 16 and 18 positive PCR-amplification. Furthermore, at the 3' coding-region we also found out the expression of exons 22, 28 and 32 in both cell lines, SKOV3 and MCF7.

Notably, we were able to define those exons from the 5'-termini to the 3'-termini within the predicted protein-coding region (CDS) that were consistently expressed in different breast and ovarian tumor cell lines. Moreover, our findings suggested the presence of different

splice variants between cell lines, since as evidenced in the figure 5.2, we found slight variations in size for the same amplification fragment in different regions examined. Additionally, as shown in the amplifications using the exon eight reverse primer (Ex8), we detected a "doublet" in SKOV3, MCF7 and ZR75 cell lines. This finding strongly suggests the presence of at least two different ODZ4 transcripts in these cell lines that can be distinguished from each other by their comparative different length.

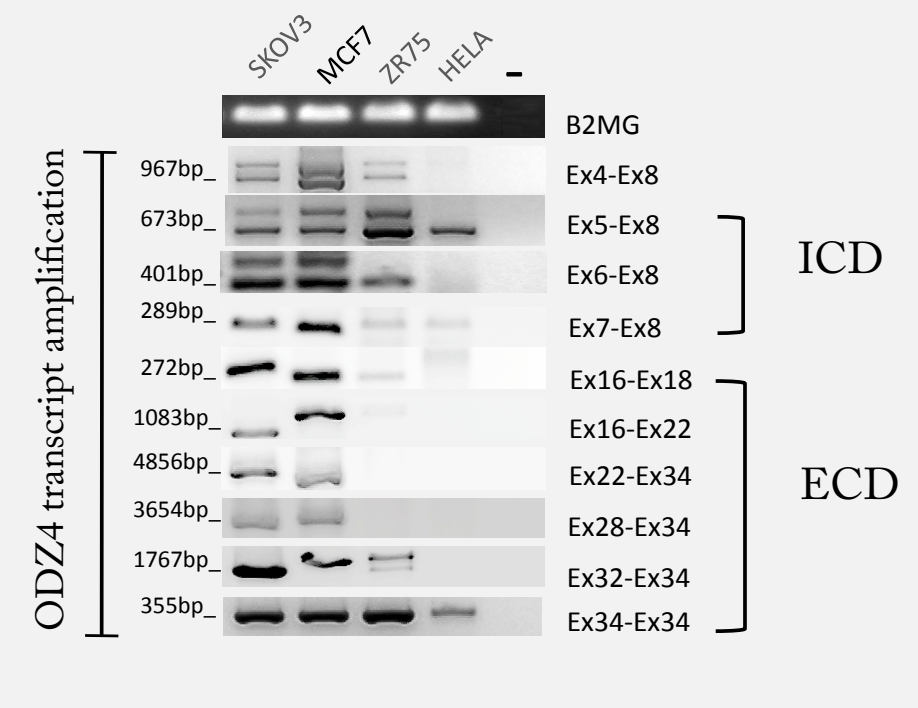


Figure 5.2. ODZ4-Exon expression in different human cancer-derived cell lines. At the right of the RT-PCR amplicons the exons are indicated which were being tested in the amplification. The primer pairs used have anelling on different exons spanning the overall length of the transcript [from exon 5 (Ex5) to exon 34 (Ex34)]. In the figure also the protein domains are schematized that result from the respective traslated regions [intracelular domain (ICD), and extracelular domain (ECD)]. β_2-microglobulin (B2MG) is the RT-PCR positive controls; genomic contamination was tested as described previously (section 4.4). PCR negative control (-) are also indicated.

5.3 5'-END: CDS DEFINITION AND SPLICE VARIANTS IN THE ODZ4 TRANSCRIPT

As described above, we found that at the 5'-end of the coding region in the ODZ4 transcript, two PCR-products were obtained when a reverse primer, which annels on exon eight (Ex8), was used as "bait". To determine the identity of products obtained in this PCR-amplification, the SKOV3 mRNA was reverse transcribed and the amplification products were cloned and sequenced. The sequencing results indicate that the region enclosed by exons 4 to 8 corresponds to the ODZ4 5'-coding end.

Any previous data that describe the expression of human exon four was found. In the predicted structure of the mature ODZ4 transcript, the start codon lies within exon five and we found that exon 4 is also expressed in the ODZ4 trascript derived from SKOV3 cell lines. Thus, we are suggesting that the start codon predicted within exon 5, at base position number 464, in ODZ4 RefSeq might determine the ORF for the protein translation and, in this context, we assume that exon five is the first exon to be translated. The region encompassing by exons 4 to 8 was submited to GenBank and EMBL-EBI database with the following accession numbers respectively: JN857068.1 and HE601753.1 (Figure 5.3A)

In addition to the above explained, the sequencing analysis also evidenced that the size variation observed in this region (exon 4 to exon 8) is due to a genomic insertion of about 200bp, and the insert sequences correspond to a central portion of the intron that lies between the sixth and the seventh exons (Figure 5.3B). The insert sequences found were submited on GenBank and EMBL-EBI database with the following accession numbers respectively: JN857071.1, JN857069.1 and JN857070.1; HE601754.1, HE601755.1 and HE601756.1.

Thus, in the mRNA region enclossed by exons 4 and 8 derived from SKOV3 cell lines, there is a "canonical" ODZ4 transcript which presents the expected size of 967bp according to the RefSeq

predicted amplification product and there are at least three other different splicing variants that range from 1177 to 1179 bp, depending of the insert length that they posses *[for insert details see figure 6.2].*

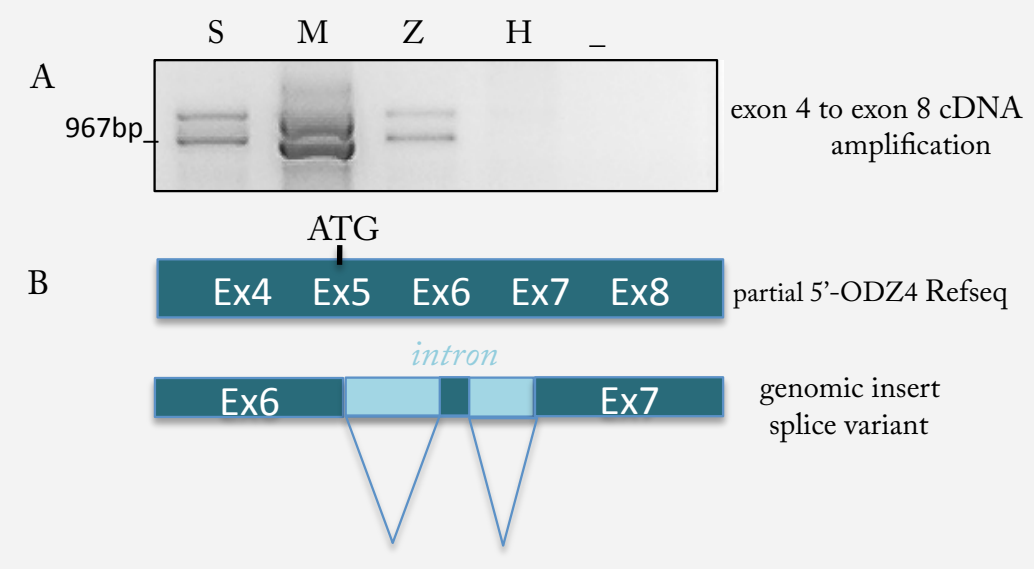


Figure 5.3. ODZ4 coding-end and Splice Variants at the 5'-terminus. A) Expected 967 bp PCR product and insertional variants obtained when exons 4 to 8 derived from human cancer cell lines were amplified . B) Sequenced of region encompassing exons 4 to 8 exon (5B Top) in SKOV3; and the insert fragment found in this region (5B down). *(S) SKOV3, (M) MCF7, (Z) ZR75, (H) HeLA, cell lines; (-) no-template amplification.* ***Significantly, we found continuous expression from exons 4 to 8 (IC domain) ressembling the "canonical" transcript, and a non-contiguous intronic-insertion of about 200bp in the splice variant forms.***

5.4 3'-END : CDS DEFINITION AND STUDY OF THE UNTRANSLATED REGION OF THE ODZ4 TRANSCRIPT

Following the same criteria applied to study the 5'-termini in section 5.2, we designed a set of primer pairs that span the ODZ4 transcript's predicted 3'-untranslated region (3'-UTR). We tested different reverse primer combinations using a constant forward primer on cDNA derived from human ovarian (SKOV3) and breast (MCF7) cancer cell lines.

The predicted length of the human ODZ4 exon 34 is arround 5.4Kb. The stop translation codon, TGA, is positioning at base number 8771 and therefore, the length of 3'UTR is about 4.7Kb (Figure 5.4A). We first examined the amplification products obtained using a forward primer that lies distal to the TGA whithin the coding-region of ODZ4 (Figure 5.4A, 34F cyan arrow and 5.4B, 34F *bait* column) followed by the examination of the amplification products obtained with a second forward primer located proximal to the stop codon (Figure 5.4A, 34S cyan arrow and 5.4B, 34S *bait* column). In these experiments, we observed that both the U1 and U2 regions were easily amplified with an standard PCR protocol and only one amplification band of the expected size was obtained in each case. However, when the amplicon size overcomes the 1.8Kb in length, no amplification was obteined, as observed in U3 region of both cell types and in regions U4/U5 on SKOV3 cDNA (Figure 5.4B, [34F *bait* and 34S *bait* columns]).

To verify that the missing amplificacion-bands in the 3'-UTR regions U3, U4 and U5 were not attributed to lack of amplification, we designed a third forward primer nearby these regions (Figure 5.4A, U2F cyan arrow and 5.4B, U2F *bait* column), and we tested again the reverse primers U3, U4 and U5 jointly with this last forward primer on cDNAs. In contrast with our previous mentioned finding, we obtained an strong PCR-amplification band in each of these regions (U3,U4,U5), as evidenced in the Figure 5.4B (U2F *bait* column).

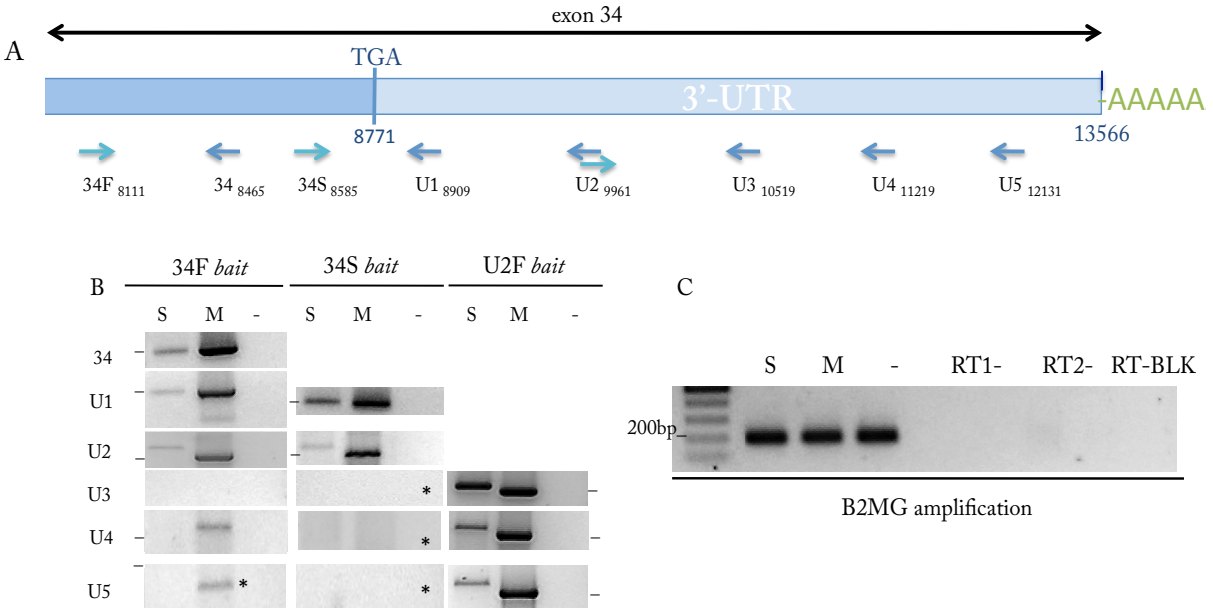


Figure 5.4. 3'-UTR region of ODZ4 Transcript derived from ovarian and breast cancer cell lines. To evaluate the length of the 3'UTR region three diverse forward primers were tested in combinations whith diverse reverse primer spanning the overall 3'-region length in ovarian (SKOV3) and breast (MCF7) derived cell lines. **A)** schematic rappresentation of the ODZ4 exon 34, the last predicted exon found on GenBank database; and the junction between coding and non-coding protein region at base position 8771 in the mature transcript. Arrows indicates forward (→) and reverse (←) primers orientation and numering respect to its localization on predicted mRNA structure [55]. **B)** The amplified cDNA-products obtained when a forward primer is used as "bait" whith the different reverse primers (left columns). Dashes (-) indicates the expected PCR-band migration. [For the amplicon expect size, see Table 4.3].(*) indicates that the PCR-obtained product/s present/s an inexpected sizing respect to the GenBank amplification prediction. C) Genomic contamination controls during samples preparation. cDNAs amplified with B2MG produce an expected 201bp band. No-template controls performed during RT-PCR (RT-BLK) and B2MG PCR-amplification (-) are reported. Lines RT1- and RT2- show the B2MG PCR-amplification of the samples where reverse transcriptase was not added. *(S) SKOV3, (M) MCF7 and (-) PCR negative control.*

The question at this point was if the "U3" region was expressed as part of the transcript as well. To resolve this question, an *in silico* search of amplicon sequences using database BLAT/BLAST algorithms was performed to find other similar sequences that might be also amplified with this set of primers, even if the PCR-product obtained with these same primer pairs and amplification conditions on genomic DNA derived from SKOV3 and MCF7 indicates that only one "gene" may be amplified (see PCR optimization condition in Figure 4.2, Material and Methods chapter). The *in silico* analysis shows that the putative mitocondrial asparaginyl-tRNA synthetase 2 precursor (NARS2) could potentially be amplified, since the amplification product enclosed by primers U2 and U5 presents an elevated sequence homology respect to the ODZ4 transcript (for alignment details see Figure A1, appendix section). Since amplicon size determined by 34F and U4 primers in MCF7 migrates above the expected range, we can not exclude the possibility that in the ODZ4 full-length transcript some of these "3'-UTR" regions may be not present, however these data should be subject to additional examination.

Sequencing of the 34F-U1 amplification-fragment indicated that it corresponds to the ODZ4 transcript. Moreover, our findings regarding 3'-UTR region highlight that the region of about 1.1 Kb, enclosed by the 34F and U2 primer pair and proximal to the stop codon, is constantly expressed in both SKOV3 and MCF7 cell lines; thus, in analogy to section 5.3, we assume that the predicted stop codon located at base position 8771 might determine the end of the protein-coding region.

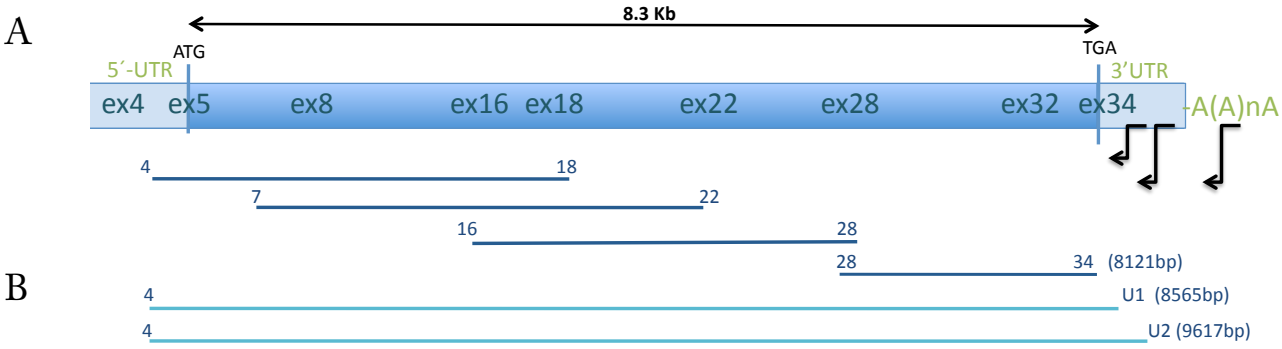
5.5 LONG RANGE INTER-EXON AMPLIFICATION OF ODZ4 TRANSCRIPT

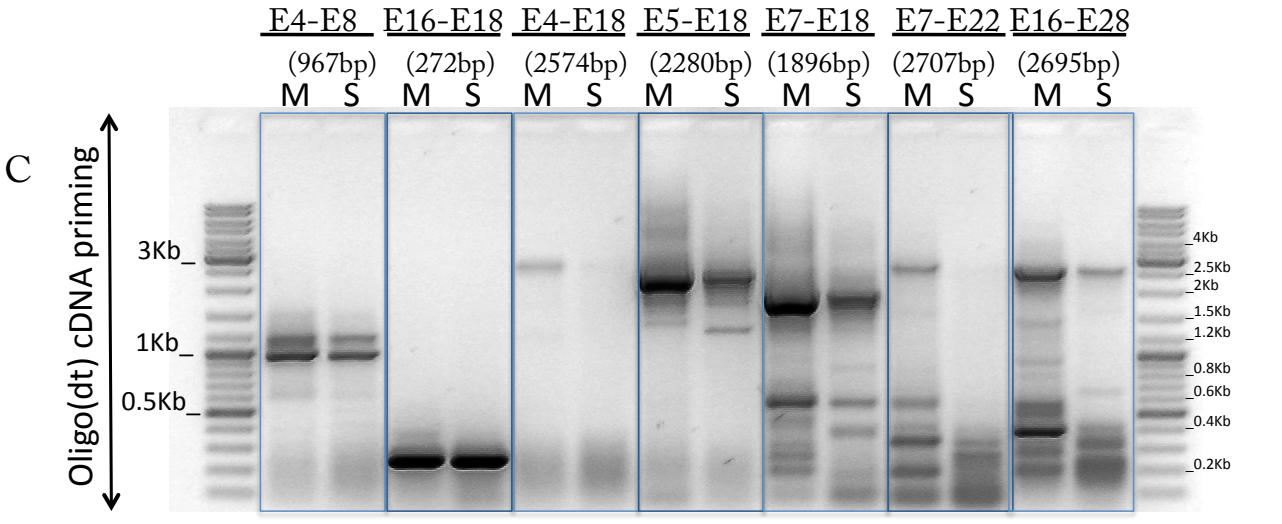
At this step of the study we are able to demonstrate that the ODZ4 transcript is expressed in different cancer cell lines derived from ovarian and breast tissues, and that the number of CDS-composing exons differs depending on the type of cell line analyzed. Furthermore, we detect the presence of splice variant located at 5'-end of the ODZ4 coding region. However, it is not clear if all of coding-exons are equally expressed to form a continuous transcript or some alternative splice process such as insertional and skipping-exon forms of the splicing might be also present. Thus, the main task was to define if the ODZ4 mRNA is a continuous transcript, and if is it not, what will be the extension of "longest" ODZ4 transcript.

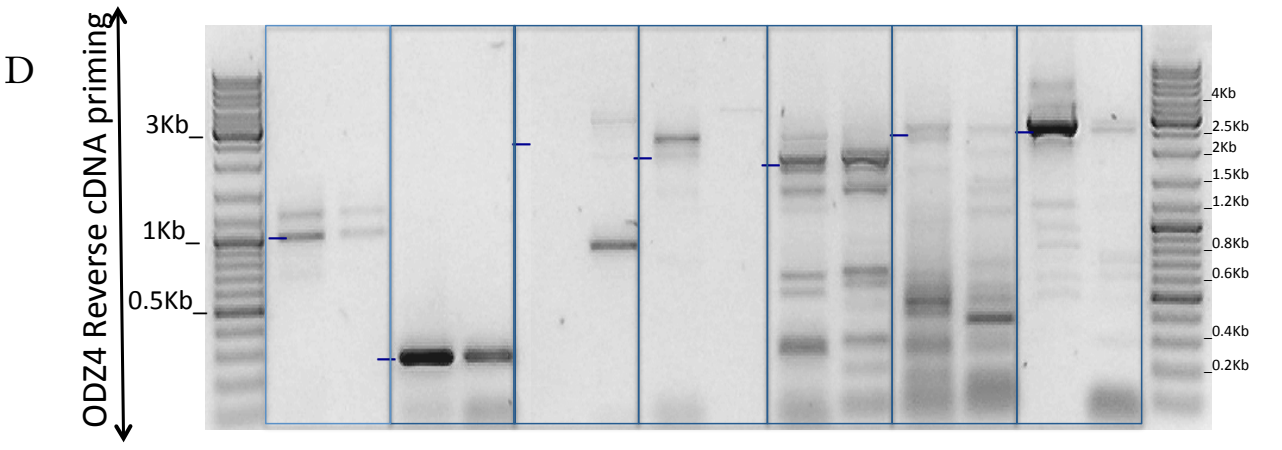
To this end, it was perform RT-PCR amplification on mRNA derived from ovarian (SKOV3) and breast (MCF7) tumor cell lines with a commercial oligo-dT primer. The exons expression analysis in both cell types is summarized in Figure 5.5 C. In Figure 5.5 is also schematized the ODZ4 transcript coding sequence and the corresponding regions amplified (Figure 5.5A and B).

To define if one exon is absent in the transcript, a double check for each single primer is needed. For example, in SKOV3 no-amplification was obtained when using the primer pair that anneals on exon 4 and 18 respectively ([Ex4-Ex18] in Figure 5.5C). However, since we detect an amplification band when these primers are used each in different combinations ([Ex4-Ex8] and [Ex16 and Ex18] amplifications), we infer that the region comprised from exon 4 to exon 18 is completely expressed in both cell type and the lacking in the amplification-band corresponding to amplicon Ex4-Ex18 in SKOV3 may be due to the low PCR efficiency. Additionally, it would appear that in the region comprised by exon 4 and 18 there is not an exon-skipping mechanism. The same is observed with the exon 22 ([E7-E22] Figure 5.5C and

[E22-E28] in 5.5E), the exon 28 ([E16-E28] in 5.5C and [E28-E32] in 5.5E), and with exon 32 ([E28-E32] and [E32-E34] in 5.5E).







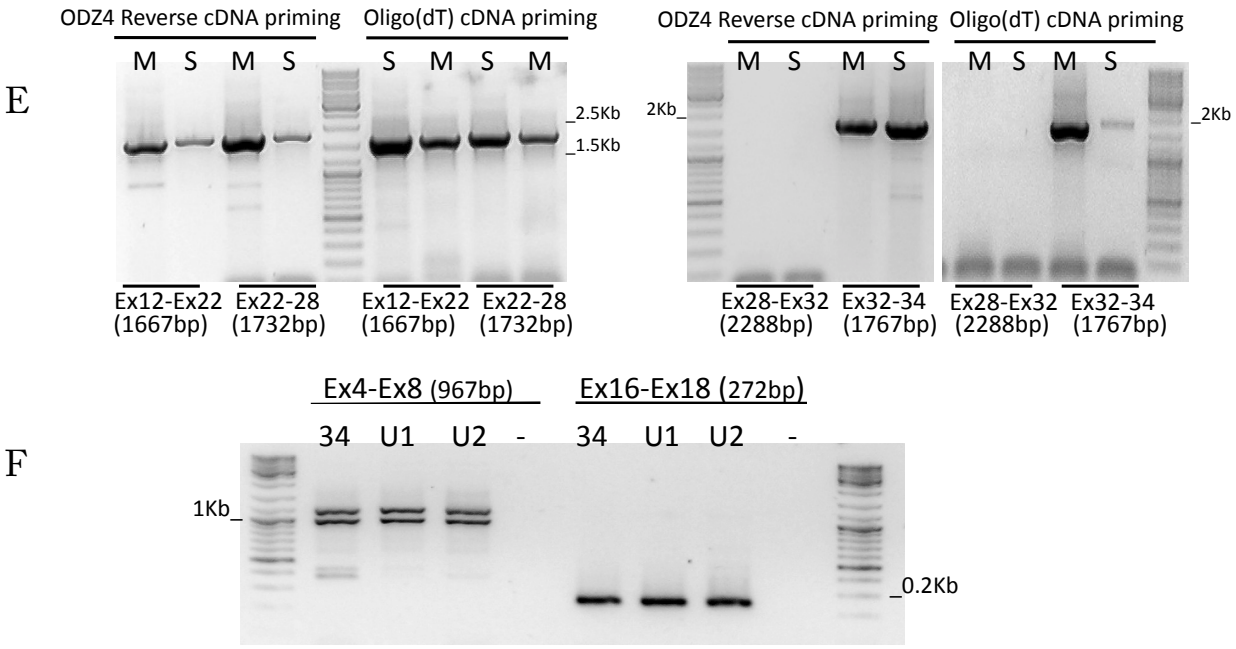


Figure 5.5. The ODZ4 mRNA Is a Continuous Expressed Transcript in Human Ovarian and Breast Cancer-Derived Cell Lines. The exon-expression patters in ovarian (SKOV3) and breast (MCF7) cancer cell lines obtained by RT-PCR with an oligo-dT and with a specific ODZ4 antisense were compared. **A)** Positioning of some predicted couponing-ODZ4 transcript exons in the partial depicted full-length mRNA. Broken arrows represent the antisense primer used in RT-PCR experiments. **B)** Straight blue lines represent the amplification-fragments spanning the 8121bp of the ODZ4 transcript. At the ends of these fragments are indicated the primer pair used by their amplification. Straight cyan lines represent the partial full-length transcripts found. **C)** The exon-expression pattern obtained in the studied cell lines when RT-PCR was performed with oligo-dT. The expected size of amplicons is also reported (). **D)** The exon-expression pattern obtained in the studied cell lines when RT-PCR was performed with a specific ODZ4 reverse primer. The fragments amplified and the expected sizes of amplicons are both aligned respect to the above positioned picture. (-) Indicates the amplicon expected size. **E) T**he exon-expression pattern obtained in the studied cell lines when RT-PCR was performed with an oligo-dT or with an ODZ4 specific reverse primers. The amplicon and its expected size are also reported (). **F)** Partial full-transcripts in SKOV3 obtained with different specifics ODZ4 antisense primers (34, U1 and U2). The amplification of the 5'-terminal region was used to check the continuity in the ODZ4 transcripts. This region is enclosed by the exon 4 and 8 (Ex4-Ex8) primer pair. Similar results were obtained in MCF7 cell lines (data not shown).

Moreover, the oligo-dT RT-PCR produces several amplifications bands that confuse the exon-expression pattern tracing along the transcript and as result, we could not identify any amplified single band. Nevertheless we suggest that some of the obtained band may reflect the presence of different splice variants in ODZ4 transcript whereas others may be assigned as PCR-artefacts.

In order to select only those ODZ4 transcripts and to overcome this "background noise" occurred when oligo-dT is used in RT-amplifications, we perform a specific ODZ4 RT with an antisense primer that is located on 3'-UTR. The obtained PCR-products were subsequently compared respect to those obtained with the common oligo-dT reverse primer (Figure 5.5D and 5.5E).

As observed in Figure 5.5D, a continuous expression from exon 4 to exon 34 was detected in both cell type: SKOV3 and MCF7 (Figure 5.5D and 5.5E). Furthermore, we were able to detect both: the canonical transcript that its PCR-amplification generates the expected band size, and the variant forms that respect to the canonical-transcript they differ by a higher size. Thus, it probably that these ODZ4 transcript variants might be derive from the insertional splice variant that we have been identified at 5'-end.

Since the PCR-amplification of one region produces several bands with different intensity, we suggest that when the U1 template (i.e the cDNA that has been generated by the use of the U1 antisense primer) is PCR-amplified the main product generated during amplification correspond to the insertional splice form, as evidenced by the strong band intensity of those fragments that posses a larger size respect to the "canonical" sizing fragment. Because we wanted to test which would be the approximate length of transcripts that we have be found, it was performed specifics ODZ4 RT-PCR by using others reverse primers localized at the end of transcript (Figure 5.5F) and the transcripts length was verified by the 5'-region

amplification, a very distant terminal region. *Again, both the canonical and the insert- splice variant were obtained in SKOV3 cell line even though different 3' region are selected as priming site. Since an exon-deletion was not evidently detected, these transcripts showed the potential to generate the complete teneurin-4 protein if they could be translated.*

5.6 FULL-LENGTH ODZ4 TRANSCRIPT DERIVED FROM TUMOR CELL LINES

5.6.1 RACE Experiments

To obtain the full-length transcript of ODZ4 gene in SKOV3, we perform RACE experiments by the using of RT-PCR procedures, where the unknown 5'/3' UTR-sequences present on mRNA under examination may be identified. An explicative overview of the 5'-UTR and the 3'-UTR RACE experiments is depicted in this section (Figure 5.6.1).

5'-RACE Experiments. In order to amplify the overall transcript variants, the cDNA synthesis was carried out using a reverse primer that lies on exon 8 (Ex8), an exon that seem to be commonly expressed between the ODZ4 transcripts. After poly-A tail addition, the generated template was amplified with an antisense primer annealing on exon 7 (Ex7) and the provided oligo-dT-adaptor forward primer. The obtained product was subsequently amplified by nested PCR using the primer pair that lies on exon 5 (Ex5) and Qt. An example of 5'-RACE product obtained on our hands is show in Figure 5.6.2

The RACE reaction kinetics is complex and many parameters have to be first optimized experimentally. The starting material integrity control in purified mRNA or total RNA samples is critical, as well as to determine the optimal PCR amplification conditions and to posses the appropriate controls for the monitoring in each step. Moreover, the reproducibility of obtained amplification product is not always predictable. However we did not found a more faster and convenient technique to identify the full-length ODZ4 transcript and its splicing variants. The main technical limitation in 5'-RACE approach was to generate the full-length cDNA during reverse transcription process. It happens because all premature termined cDNAs will be also tailed by terminal transferase at their 5' end, as consequence in the first round of amplification (Qt PCR) they will be amplified and during recovery they will be captured as

well. As result no really full-length transcripts will be obtained. In addition, a mixture of the largely heterogeneous fragments sizing could be obtained that might difficult the right data interpretation. The achievement of premature termined transcript during cDNA priming is encouraged by the presence of GC rich traits at the 5'-ends of vertebrate mRNAs that hinder the reverse transcription completing termination.

On our hands the major problems found were: 1) the self Qt primer dimer priming that result in the recovery of other sequences no-11q14.1 related; 2) incomplete 5' cDNAs priming end. In this case, the 5' end of the obtained sequences mapped on exon 5 and 4. Thus, no additional information regarding 5'-UTR region was generated.

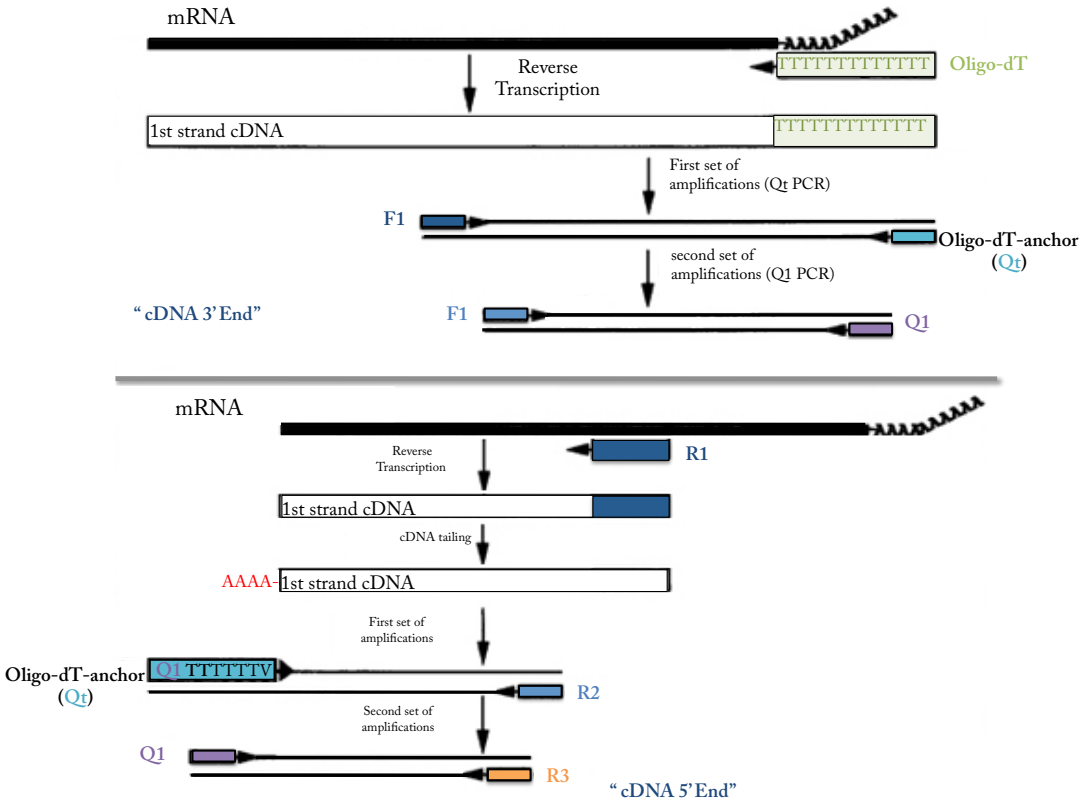


Figure 5.6.1. RACEs Experiments. To obtain the ODZ4 full-length transcript RACE experiments were performed. In order to define the length of untranslated regions (UTRs), specific gene primers were located proximal to the UTR regions and the unknown sequences were then amplified by using the provided anchor primers (Qt and Q1). To generate 5'-end cDNAs, reverse transcription was carried out using an antisense primer that lies on exon 8 (R1 in the figure). Then, a poly(A) tail was added using terminal deoxynucleotidyltransferase (TdT) and dATP. First amplification round is achieved using the primer Qt that anneals on poly-A added-tail in conjunction with a second reverse primer (R2) that is 5'-complementary to exon 7. Finally, Nested-PCR is performed using nested primers (Q1 and Ex5 [R3]) to increase specificity.

Qt : GACCACGCGTATCGATGTCGACTTTTTTTTTTTTTTTV with V = A, C or G

Q1: GACCACGCGTATCGATGTCGAC

The 3'-cDNA ends were generated when RNAs are reverse transcribed using a common oligo-dT primer. Amplification is then performed using a provided oligo-dT anchor primer (Qt) jointly with the exon 34 forward primer (34F or 34S, F1 in figure). This oligo-dT additional step was taken to encourage the amplification of transcripts that their expression could be lower. Last, a second set of amplification cycles was carried out using "nested" primers (Q1 and F2) to prevent the amplification of nonspecific products. *(Adapted from MA Frohman. 1994)* [113].

3'-RACE Experiments. In these experiments, standard cDNA priming was done with commercial oligo-dT-primer. Because our first 3'-RACEs experiments indicate that the provided oligo-dT-anchor primer anneals on several non-ODZ4 polyA traits, and since we suspect that the ODZ4 transcript abundance is lower in the cell, some modifications to protocol were introduced. Thus, rather than use immediately the oligo-dT-anchor primer, we chose a common oligo-dT to prime the first strand in the cDNA synthesis in order to increase the overall ODZ4 messenger quantity. The obtained cDNAs were subsequently amplified with forward primers that anneals on exon 34 (34F or 34S) in combination with the provided oligo-dT-anchor primer, whereas nested PCR-amplifications were carried out using 34S or U2F forward primer with Q1 reverse primer. [For relative forward primers positioning see section 5.4, Figure 5.4A]. As result, a difference of about 474bp in length will be expected between the 3'-RACE products generated using the 34F-Qt primer pair respect to the 3'-RACE products that are obtained with the 34S-Qt primer pair (Figure 5.6.2, F).

Cloning and sequences analysis of 3'RACEs products failed to detect a reliable polyA consensus site on ODZ4 transcript. Moreover, non-specifics products were obtained for 11q14.1 transcripts. Nevertheless, interesting amplification fragments were obtained as nested-PCR products (Figure 5.6.2 F, Q1 lane), which suggest the presence of more than two splice variants, however we have not the sequencing data that supports our findings. Additionally, these experiments have served to confirm that both, exon 4 and 34 are compounding of the ODZ4 transcripts. Accordingly to section 5.4, it may be possible that the end of predicted full-length ODZ4 transcript could be localized between U2 and U3 region, in such way that when we move to the 3'-end, surpassing the U2 region, other transcripts are detected and thus no specific ODZ4 product is obtained.

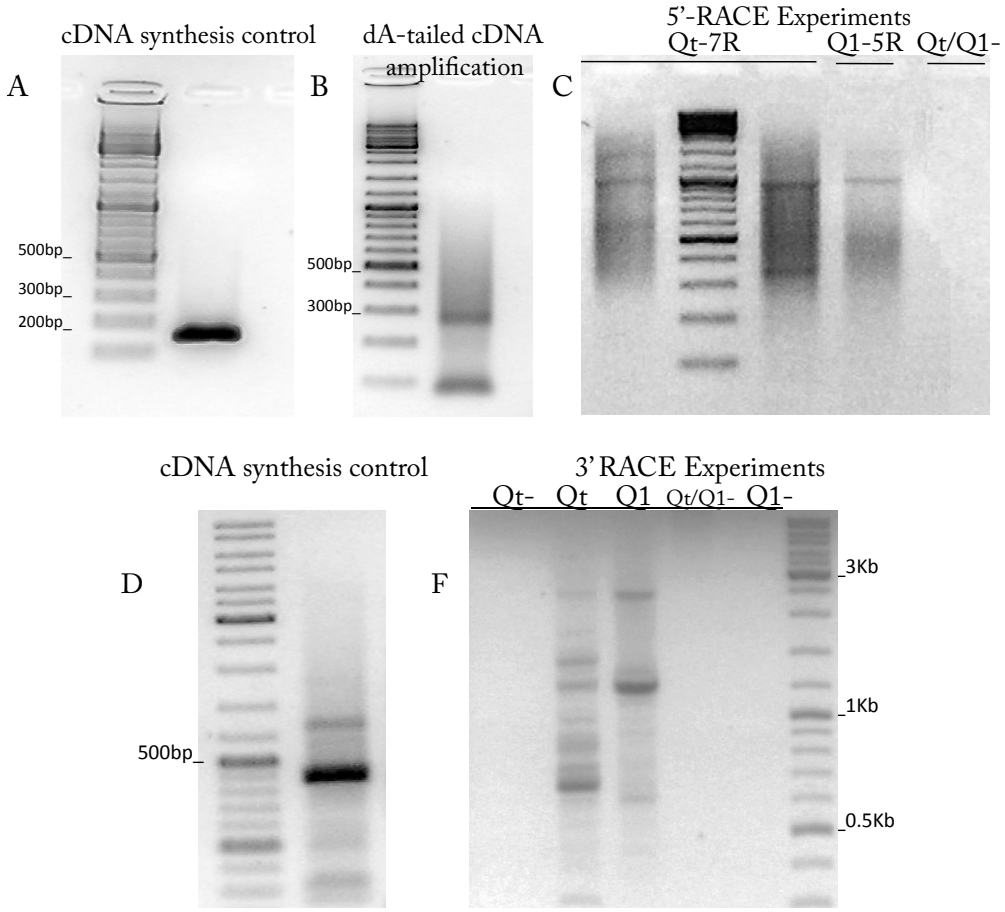


Figure 5.6.2. Representation of the obtained RACEs products in ovarian cancer-derived cell line. cDNA-ends obtained from ovarian cancer cell line, SKOV3 were analyzed by Rapid Amplification of the cDNA Ends (RACE) technique. The RT and the tailing completion of samples were evaluated respect to the positive controls (kit provided). The RT-PCR positive-control amplification produces a expect band of 157bp in 5'-RACE (A) and one of 655bp in 3'-RACE (D), whereas the amplification of the polyA-tailed control-cDNA produces a expect amplicon band of 293bp (B). C) Two different PCR-amplification conditions were tested on SKOV3 cDNA (Qt-7R lanes) during 5'RACE performance and from one of they, a nested PCR was done (Q1-5R lane). The no-template control obtained from the first round of amplification was subsequently amplified with the nested primer pair (Qt/Q1-). If the ODZ4 predicted full-length transcripts exists, the expect PCR-nested product will be ranged between 700 and 800bp in length supposing that 30 to 150 dATP residues have been added by TdT during tailing reaction. F) 3'-RACE-generated products by 34F-Qt (Qt) and 34S-Q1 (Q1) primer pairs. No-template controls for each round of PCR-amplification are also indicated (Qt-, Q1-, Qt/Q1-).

5.6.2 Detection of ODZ4 Transcript in Human Cancer Derived Cell Line

In order to determine approximately how many ODZ4 transcripts could be present in the cell and what will be their estimated average size, we perform a Norther blot analysis. However we were not be able to detect a hybridization signal in any cell line analyzed with both, the 5' and the 3' probe (data not shown). Since we known that the sequences used as probes correspond to ODZ4 transcript, we have associated this no-hybridization signal to the limited transferring of longer mRNA transcripts from gel to the membrane. As mentioned previously, ODZ4 could be a non-abundant transcript; furthermore, it is known that molecules over 2Kb shows an increasing low transfer efficiency during traversal passing toward nylon membrane, thus it is possible that molecules of 13.6Kb, as ODZ4 mRNA, may be scarcely transfers and consequentially non-detected.

To overcome to the difficult likely associated to the transcript abundance; we perform a set of long-PCR amplifications spanning the partial full-length transcript on cDNA derived from MCF7 cell lines. The different amplicons obtained were blotted onto a membrane and hybridized with the same probes used in Northern experiments. As evidenced, both the 5' and 3' probes generated stronger hybridizations signals on different templates (Figure 5.6.3). Thus, the 5'-probe distinguished two groups of transcripts with approximately 6-5Kb and ~2Kb sizing in the 7-28 templates which were obtained with the Ex7 forward and Ex28 reverse primers. This primers lie respectively, on the exon 7 and on the exon 28.

Also the 4-18 templates were positive to the 5'-hybridization, generating hybridization signals of about 2.5-3Kb in length (Figure 5.6.3C). On the other hand, the 3'-probe detected both, the 7-U1 and the 7-U2 templates.

Then, in conclusion our results indicate that many ODZ4 transcripts are presents in MCF7 cell line and two main groups of transcripts containing the ODZ4 3'-end can be

differentiated largely by their size (Figure 5.6.3D, lane n.2). Notably, we were able to characterize the partial ODZ4 full-length expressed by MCF7 and SKOV3 cell lines. It is possible that the predicted ODZ4 full-length transcript exist in these cells as well, however due its high dimension and lower expression, conventional strategies failed to detect it. In addition, techniques with elevated resolution will be needed to see one or another form of the transcript selectively. Furthermore remain to be elucidated which will be the functionality of these many different expressed-transcripts in the cells.

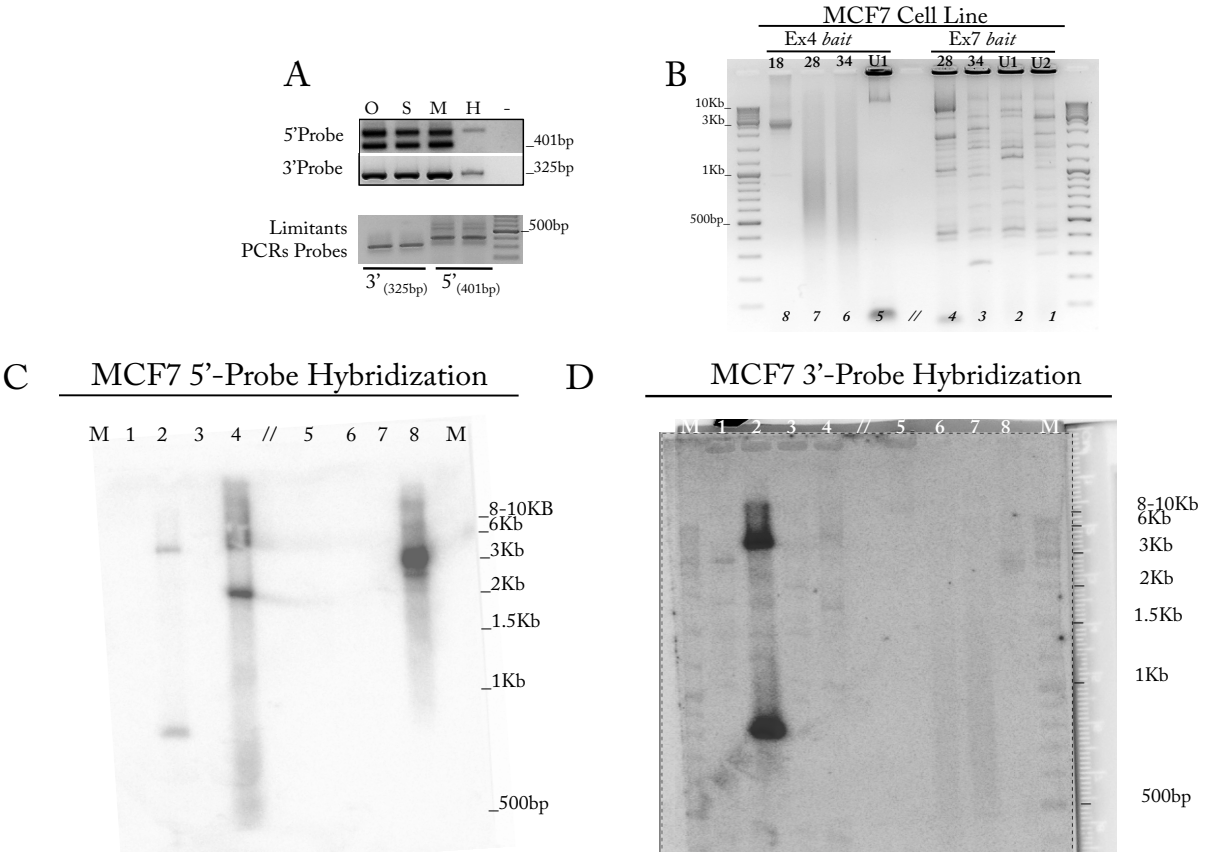


Figure 5.6.3. The ODZ4 Transcript Blotting Derived From Human Breast Cancer Cell Line. As ODZ4 may be a low abundance transcript, we decided to evaluate its length after PCR-amplification. A) The amplified regions selected to be used as probe in the hybridization experiments. The 5'-probe was generated with the Ex6-Ex8 primer pair whereas 3'-probe was generated with the 34S-U1 primer pair. A limited PCR-condition respect to dCTP content was first checked in order to miming the radioactive-condition during the ^{32}P-probes PCR-amplification. B) PCR-products subject to the transferring onto the membrane. A constant forward primer ("bait") was used in combination with different reverse primers generating amplicons spanning the ODZ4 partial full-length transcript. At button of the gel, the *numbers* indicate the respective orientation of lanes onto the membrane. C) Hybridization experiment performed with a 5'probe. Hybridization signals around 8, 5-3 and 2Kb (lane 4) and from 2.5 to 4Kb (lane 8) were detected. The expected amplicon sizing were ~4.9Kb and ~2.5Kb in lane 4 and 8, respectively. D) Hybridization experiment performed with a 3'probe. Hybridization signals around 4Kb (lane 1) and 5-3Kb and 800bp (lane 2) were detected. The expected amplicon sizing were ~7.8Kb and ~8.9Kb in lane 1 and 2, respectively. *(O) Ovcar3; (S) SKOV3; (M) MCF7; (H) HeLA cell lines. (-) PCR Negative-control.*