IV. MATERIALS AND METHODS

In this chapter I will describe the methodology used for the study and characterization of human ODZ4 transcript in ovarian and breast tumor derived cell lines.

In the first sections the cell lines considered as source of ODZ4 messenger and their *in-vitro* culture conditions are described (section 4.1) as well as the general molecular protocols adopted for nucleic acid purification (sections 4.2) derived from them. cDNA synthesis and the conditions for its amplification by PCR are described in sections 4.3 and 4.4 respectively. The following sections are referred to procedures used for standard cloning of PCR-derived fragment (section 4.5) and for the analysis and databank submission of specific ODZ4 transcript sequences (section 4.6). Section 4.7 describes the technique adopted to define the extension of full-length ODZ4 transcript. Finally, in section 4.8 describes the procedure and conditions for the evaluation and the analysis of the ODZ4 messenger within mixed populations of RNA.

4.1 CELL LINES AND CULTURE CONDITIONS

In this study different human cell lines derived from breast and ovarian cancer tissues were used, as summarized below (Table 4.1).

Ovarian cancer cells (SKOV3), cervical cancer cells (HeLA) and breast cancer cells (MCF7, MDA-MB237) lines were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 2mM L-glutamine, 100U/mL penicillin and 100μg/mL streptomycin at 37°C in a 95% humidified incubator with 5% CO2. OVCAR3 and ZR75 cell lines were cultured in RPMI 1640 with the same supplements and under the same conditions as above. Culture media 5% DMSO was used for frozen storage of cell cultures in liquid nitrogen.

Cell cultures were subjected to routine testing for mycoplasma contamination using a PCR-based assay (EZ-PCR Mycoplasma Test kit) the following manufacturer's protocol.

			Source		
Cell Line	**growth properties**	**morphology**	**organ**	**disease**	**origin**
SKOV3	adherent	epithelial	ovary	adenocarcinoma	ascites
MCF7	adherent	epithelial	breast	adenocarcinoma	pleural effusion
MDA-MB237	adherent	epithelial	breast	adenocarcinoma	pleural effusion
HeLA	adherent	epithelial	utero	adenocarcinoma	cervix
ZR-75-1	adherent	epithelial	breast	ductal carcinoma	ascites
NIH : OVCAR3	adherent	epithelial	ovary	adenocarcinoma	ovary (n.d)

Table 4.1 Biological Characteristics of the studied cell lines *(adapted from ATCC catalogue)* [102].

4.2 NUCLEIC ACID PURIFICATION

The Easy-DNA Kit ™ (Invitrogen Life Technologies, MD USA; cat.n K1800-01) was used for DNA purification of trypsinized cell lines (10^3 to 10^7 total cells) following the manufacturer's protocol. Briefly, subconfluent cell cultures were pelleted at 1400 rpm for 5 minutes after trypsin treatment at 37ºC and then resuspended in 200 µl 1X Phosphate-Buffered Saline (PBS). The cells were then lysed and the DNA was isolated from the chloroform phase. Subsequently DNA was ethanol precipitated and RNA digestion (40µg/mL of RNAse) was performed prior to sample storage at 4ºC. DNA integrity was controlled by agarose gel electrophoresis as well as by spectrophotometric measurement of UV absorbance (A_{260} and $A_{260/280}$).

For RNA purification, subconfluent cell lines were lysed in 1mL TRIzol reagent and homogenized by repeated passing through a 1mL syringe (25G x 5/8"). Total RNA was isolated using Micro-to-Midi PureLink™ RNA Purification System (Invitrogen Life Technologies, MD USA; cat.n 12183-018) according to the manufacturer's instructions. To avoid contaminations with genomic DNA in downstream applications (i.e. RT-PCR), RNA samples were treated on-column with 10U of DNase I (1U per µg of RNA) for 20 minute at room temperature before sample elution.

In alternative to the above procedure, total RNA was isolated by isopropyl alcohol precipitation in those cases where more yield was required. As described above, subconfluent cell lines were lysed in 1mL TRIzol reagent and homogenized by repeated passing through a syringe. For each mL of TRIzol used, 200µL of chloroform were added. Tubes were shaken and centrifuged at 13000 rpm for 15 minutes at 4ºC. To the aqueous phase, 500µL of isopropyl alcohol were added and incubated for 20 minutes at -20ºC. Samples were then

centrifuged at 13000 rpm for 15 minutes at 4°C. The RNA pellet was washed twice with 300µL ice-cold ethanol 75% (in DEPC water) before resuspension in DEPC-treated water. Samples were treated with DNase I amplification grade and stored at -80°C.

To control RNA integrity after purification, samples were loaded on agarose gels. For intact RNA, gel electrophoresis must show at least two distinct ribosomal RNA bands (28S and 18S) and an mRNA smear. To determine RNA yield and sample purity, UV absorbance at 260nm and 260/ 280 (≥ 1.8) were measured.

4.3 REVERSE TRANSCRIPTION AND PCR-AMPLIFICATION (RT-PCR)

In order to obtain full-length mRNAs, reverse transcription conditions for long transcripts had to be optimized first. Thus, some modifications on the standard RT protocol were introduced, as reported below.

A standard RT protocol was used for priming of cDNA fragments ≤1000bp. Following the manufacturer's indications (MMLV High Performance Reverse Transcriptase; cat.n RT80125K), 1µg of total RNA in 10µL RNAse-Free water was reverse transcribed with oligo(dT) primer in 20µL of total reaction volume containing:

0.5µg oligo$(dT)_{15}$
1mM dNTPs
1X MMLV HP RT Buffer
100U MMLV High Performance Reverse Transcriptase (200U/µL)
10mM DTT
20U RiboLock (40U/µL) [19]
x µL RNAse-Free water
10µL

RNAs were denatured at 65°C for 2 minutes, chilled on ice for 1 minute and then incorporated into the RT reaction mix. Reactions proceed in a thermal cycler at 37°C for 90

minutes and then at 85ºC for 5 minutes to inactivate the reverse transcriptase. The cDNA was stored at -20ºC for future use.

The quality of cDNA was assessed by PCR using specific primers for ß2-microglobulin precursor transcript (for primers sequence see Table 4.3). These primers discriminate between cDNA and genomic DNA present in the sample because they are designed on two different ß2-microglobulin exons. In addition, other standard RT controls were included in the study such as the amplification of purified RNA without reverse transcriptase addition (RT-) and the amplification of no-template controls (RT-BLK), both with ß2- microglobulin primers.

Conditions for long RT were applied when amplicons >1200bp were expected. Thus, 2µg of total RNA were reverse transcribed with *ODZ4* specifics primers (see primer design and setup paragraph below), which annealed on the 3'-UTR region. RNAs were denatured at 75ºC for 5 minutes and cooled on ice for 2 minutes. The RNA sample was incubated for 5 minutes at annealing temperature determined experimentally and which varied depending on the primer used (generally the used Ta was -4ºC less than the calculated Tm), and then quenched on ice until thermo cycler started. The long RT reaction mix was as follows:

1µM specific primer
1mM dNTPs
1X MMLV HP RT Buffer
200U MMLV High Performance Reverse Transcriptase (200U/µL)
10mM DTT
40U RiboLock (40U/µL) [19]
x µL RNAse-Free water
10µL

Subsequently, cDNA priming was conducted at 37ºC for 150 minutes followed by enzyme inactivation at 85ºC for 5 minutes. The cDNA was aliquoted and stored at -20ºC until use.

4.4 POLYMERASE CHAIN REACTION (PCR) AMPLIFICATIONS

4.4.1 Primers Design

As previously mentioned, four members compose the teneurin family, which present high sequence homology in both amino acid and mRNA levels. Therefore, the four teneurin mRNAs sequences were aligned and their sequences were compared [103]. Thus, to assure the specificity of the subsequent PCR, those teneurin-4 regions having none or low cross-teneurin homologies were considered for primer design. These sequences were also subjected to BLAST (GenBank) and BLAT (UCSC Genome Browser) algorithms analysis [104][105]. Figure 4.1 shows an schematic of the different set of primers designed that lie throughout of entire predicted length of the transcript, including the UTR-regions, and Table 4.3 summarizes the corresponding primer sequence and their targets in the genome.

Primers around 18-22 base pairs in length and with a Tm ~ 56-66ºC were used. Thermodynamics parameters for each primer pair chosen were checked by the open-access software, Netprimer [106], following general considerations such as 40-60% GC primer content and an $\Delta G \geq -5$ kcal/mol for self dimer formation; repeats (as di-nucleotide) and runs in the primer sequence were also controlled. When were possible, 3'-end cross dimers were avoided, but sometimes a $\Delta G \geq -7$ kcal/mol was tolerated. Table 4.4 shows the estimated amplicon size for each primer pair set along the ODZ4 reference transcript (RefSeq Accession number: NM_001098816) [55].

4.4.2 ODZ4 Primer Specificity

The efficiency of the primer and their specificity for ODZ4 gene was tested by intra-exon amplification on genomic DNA derived from the studied cell lines. DNA was purified, (see section 4.2), and amplified with different primer sets that map to the same exon (for details see Figure 4.2).

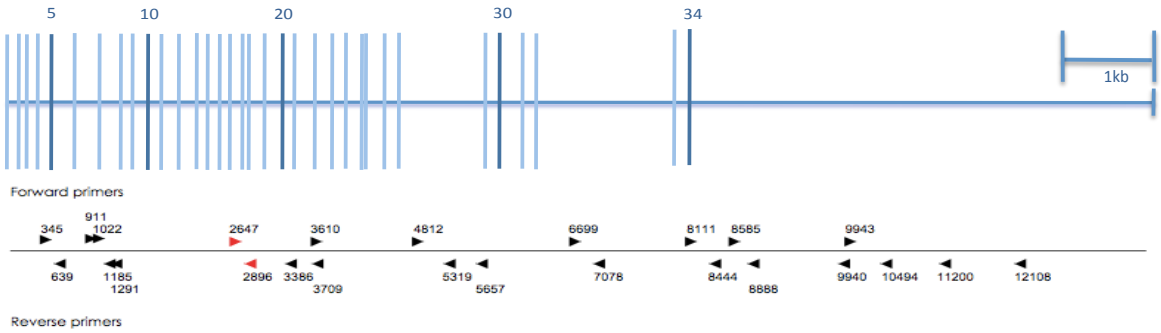

Figure 4.1. Relative alignment of the designed primers respect to the ODZ4 transcript. Primers described in Table 4.3 were aligned respect to the transcript. The top of the figure shows the thirty-four exons composing the transcript (vertical lines), whereas the relative positions of primers (arrowheads) along the complete transcript length are showed at the bottom. Numbers next to primers indicate the base position respect to the ODZ4 transcript.

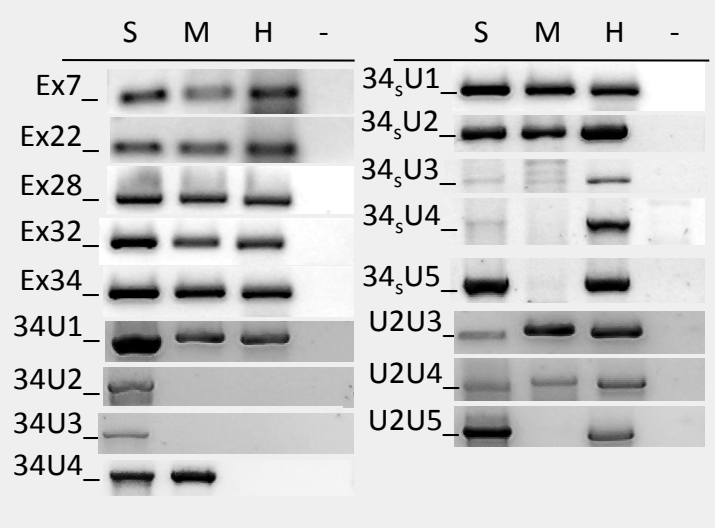

Figure 4.2. PCR-amplification efficiency and genomic specificity of the ODZ4 primers. The amplification efficiency of ODZ4 designed primers and their cross- amplification in the genome was first tested on genomic DNA derived from studied cell lines. Under our conditions of PCR amplification, it was observed that primers have no cross-amplification with others genomic sequences, since only the expected size in the amplification-bands was obtained in each cell line tested. After this optimization step, primers were uses for the ODZ4 exon-expression pattern analysis by RT-PCR experiments. The figure shows the different amplicons obtained when an intra-exons primer pair was used on cell line genomes. (-) dashes indicates the expected PCR-product size. (*S*) SKOV3, (*M*) MCF7, (*H*) HeLA, (-) PCR negative control. *For primer sequences see Table 4.3.*

4.4.3 PCR Cycle Conditions

As starting point, standard three-step protocols were used for cDNA amplifications following the manufacturer's indications for each enzyme utilized. Thus, a common enzyme (e.g MangoTaq™ DNA Polymerase) was used for routine amplifications (fragments up to 2Kb). In addition, and depending on downstream applications, longer fragments were amplified by proofreading (*PfuUltra* II HotStart and Accuprime™ *Pfx*) and/or high processivity enzymes (Accuprime™ *Taq* HF and Accuprime™ *Pfx*). The standard thermal cycler program used was:

- Initial Denaturation at 94ºC for 30"-to 5'
- 35-40 cycles of Denaturation at 94ºC for 30", Annealing* for 30"-to 1' and Extension at 72º-or-68ºC for 1 minute per Kb of PCR product.
- Final Extension at 72º-or-68ºC for 1 minute per Kb of PCR product.

(*) PCR Anneling Temperature (Ta) was estimated as follow:

$$T_a = 0.3 \times T_m(\text{primer}) + 0.7\, T_m(\text{product}) - 14.9$$

Where, T_m(primer) = Melting Temperature of the less stable primers-template pair

T_m (product) = Melting temperature of the PCR product

And program simulation determines the Tm as follow;

$$\text{Melting Temperature } T_m(^oC) = \{\Delta H/ \Delta S + R \ln(C)\} - 273.15$$

Where,

ΔH (kcal/mole), where H is Enthalpy

ΔS (kcal/mole) , where S is entropy

The available thermal cyclers used were Applied Biosystems®2720, Veriti® 96 well Fast Thermal Cycler (Life Technologies™), and GS482 Dual 48 well (G-STORM). PCR amplification products were controlled by gel (agarose or polyacrylamide) electrophoresis and staining with ethidium bromide (0.2 μg/mL).

Primer Target	primer ID name	Melting T (C°)	5' to 3' sequence
ODZ4 Transcript:			
5'- UTR	Ex4	56,06	CAAGGATTATTTGAAGGACTATTCT
exon 5	Ex5F	64,42	GCGTCAAGGACATTGTGCCG
exon 5	Ex5	63,52	TCCTGCGGCACAATGTCCTT
exon 6	Ex6	65,74	TCCAATCTCACACTCACCGACACC
exon 7	Ex7F	64,77	ACACCCCCAACCAGCACCAC
exon 7	Ex7	66,97	TCCAGGGGGATGTTGCTGTTGA
exon 8	Ex8	63,03	CCGTCACTGTAAGCCCCATCA
exon16	Ex16	66,81	CTGTGCTGCCGACTGTGGTGG
exon 18	Ex18	60,26	AGTGCCAACCATTCAGGTCTAAG
exon 22	Ex22F	60,74	GGAGGAAATCTCTATCTCTGGCTG
exon 22	Ex22	60,06	ATGAGGTTGAAGGGGATGGTC
exon 28	Ex28F	65,35	TCCCTGGCATTGACCACTTCCT
exon 28	Ex28	60,32	GCCATTGTTGTCTGTGATGAGTG
exon 29	Ex29	58,43	GTTATGGTGACATCATCCTTGCT
exon 32	Ex32F	59,06	CCCGTTTTGACTACAACTATGACA
exon 32	Ex32	59,50	GAGTGGCTTGTCATTGATGGAG
exon 34	Ex34F	67,01	TGCCAGCAGGCTCCAAAGACC
exon 34	34S	68,96	CAGAGACTGCGGGAAGGGGAGG
exon 34	Ex34	59,38	GCTGGATGTCTGTGTAGCGTCT
3'- UTR	U1	58,38	GCATGAGTTACAATGCAACCAG
3'- UTR	U2F	58,09	TCCTTCTAACCAGTTCCCAAAG
3'- UTR	U2	60,82	TGGGAACTGGTTAGAAGGATGC
3'- UTR	U3	59,81	TGTAAATGTAACCCATAAAAGTTCCA
3'- UTR	U4	68,27	GGAGGAGGGACAGGGCGTGG
3'- UTR	U5	64,08	GCCAACAATTCTGAGAGATGAGGG
γ-Heregulin*:			
exon 10	H-Fw	59,8	TTGGATTCAGGAATCTGGCAC
exon 11	H-Rv	52,21	CTGATCACTTTGCACATATACTC
β2-microglobulin			
exon 2	B2MGF	66,9	GTGGAGCATTCAGACTTGTCTTTCAGC
exon 4	B2MGR	72,72	TTCATCCAATCCAAATGCGGCATCTTC
amplicon size	201bp		
pGem-T easy vector	SP6	44,67	TAATACGACTCACTATAGGG
	T7	43,94	TATTTAGGTGACACTATAGAAT

Table 4.2 List of Primers designed for this study and their corresponding sequences. The primers were synthesized by Integrated DNA Technologies Inc. USA, IDT [107]. (*) Primer sequences were obtained from Xiao-Zhong Wang et al., 1999 [108].

ODZ4 Forward \ ODZ4 Reverse	Ex5	Ex7	Ex8	Ex18	Ex22	Ex28	Ex29	Ex32	Ex34	U1	U2	U3	U4	U5
	Amplicon size (bp)													
Ex4	319	862	967	2574	3385	4997	5335	6755	8121	8565	9617	10175	10875	11787
Ex5F	25	568	673	2280	3091	4703	5041	6461	7827	8271	9323	9881	10581	11493
Ex6	---	296	401	2008	2819	4431	4769	6189	7555	7999	9051	9609	10309	11221
Ex7F	---	184	289	1896	2707	4319	4657	6077	7443	7887	8939	9497	10197	11109
Ex16	---	---	---	272	1083	2695	3033	4453	5819	6263	7315	7873	8573	9485
Ex22F	---	---	---	---	120	1732	2070	3490	4856	5300	6352	6910	7610	8522
Ex28F	---	---	---	---	---	530	868	2288	3654	4098	5150	5708	6408	7320
Ex32F	---	---	---	---	---	---	---	401	1767	2211	3263	3821	4521	5433
Ex34F	---	---	---	---	---	---	---	---	355	799	1851	2409	3109	4021
34S	---	---	---	---	---	---	---	---	---	325	1377	1935	2635	3547
U2F	---	---	---	---	---	---	---	---	---	---	---	577	1277	2189

Table 4.3. Expected amplicon size based on ODZ4 RefSeq Transcript. The table summarized the expected size of PCR with the different primer pairs designed for the ODZ4 transcript study (RefSeq NM_001098816)[50].

4.5 DNA FRAGMENT CLONING

The pGEM-T ® Easy Vector Systems II was used to clone cDNA amplification products, following technical manual instructions (Promega Corporation; Madison WI, USA. cat.n A1380). Briefly, JM109 high-efficiency competent cells (≥ 1x108 cfu/µg DNA), were transformed with 2µL of ligation product that was obtained as follows:

Reaction Component	Standard Reaction	Positive Control	Background Control
2X Rapid Ligation Buffer, T4 DNA Ligase	5µl	5µl	5µl
pGEM®-T or pGEM®-T Easy Vector (50ng)	1µl	1µl	1µl
PCR product	Xµl*	–	–
Control Insert DNA	–	2µl	–
T4 DNA Ligase (3 Weiss units/µl)	1µl	1µl	1µl
nuclease-free water to a final volume of	**10µl**	**10µl**	**10µl**

Table 4.4. Components for the Ligation Reaction. For cloning, the reaction was done following the manufacturer's instructions. Thus, 3µL of PCR product were added to the reaction. For gel-purified fragments, a Molar Radio of 3:1 (insert: vector) was used.

The recombinant's screening was performed by PCR amplification (with SP6 and T7 primers) of the colonies plated on selective media (LB-Agar[27] supplemented with ampicillin[28] 100µg/mL, 0.1mM/µL IPTG[29] and 0.05mg/µL X-Gal[30]). After that, the interested colonies were growth on liquid phase (LB medium[26] plus 100µg/mL ampicillin[28]) at 37ºC overnight and 225-rpm agitation for further applications.

For plasmid DNA extraction were used the PureYield ™ plasmid Miniprep System (Promega Corporation; Madison WI, USA. cat.n A1222), following technical bulletin instructions. To -80ºC storage, glycerol stocks (1mL culture 15% glycerol) were done for cultures.

4.6 DNA SEQUENCING AND SEQUENCE ANALYSIS

For sequencing reactions, samples were sent to Macrogen Inc. (908 World Meridian Venture Center, #60-24, Gasan-dong, Geumchum-gu. Seoul, Korea) [109]. Here an automated capillary sequencing service is performed for DNA plasmid or PCR product using the customers provided or synthesized primers. The sequencing reaction is conducted under BigDye ™ terminator cycling conditions and samples are running in a 3730XL Automatic DNA Sequencer. The whole experimental process is monitored through Laboratory Information Management System. Final results are provided in .ab1 and PDF files formats.

For sequencing data analysis DNASTAR™ Lasergene v.8 software package was used; and for alignment and homology data analysis, the UCSC Genome Bioinformatics Browser [110] and the Ensembl Genome Browser [111] were used.

Transcript variants sequence submission on GenBank and EMBL-EBI databanks was conducted by stand-alone software Sequin™ up-load [112]. The EMBL-EBI and GenBank sequences accession numbers are HE601756.1, HE601755.1, HE601754.1 and HE601753.1; JN857071.1, JN857069.1 JN857068.1 and JN857070.1, respectively.

4.7 RAPID AMPLIFICATION OF cDNA ENDS

The Rapid Amplification of cDNA Ends (RACE) technique was chosen to obtain the ODZ4 full-length mRNA through the definition of the untranslated regions [113]. To this end, the 5'/3' RACE Kit, 2nd Generation (Roche Applied Science; Indianapolis IN, USA. cat.n 03353621001) was used.

5'-RACE Experiments. Following the manufacturer's instruction, 2μg of total RNA were reverse transcribed with an ODZ4 specific primer at 55°C for 60 minutes. Prior to the poly-adenylation reaction, cDNA was purified using the High Pure PCR Product Purification Kit and tailed with 80U of TdT at 37°C for 30 minutes. In the first PCR round (named as Qt PCR), the dA-tailed cDNA was amplified using Expand High Fidelity DNA polymerase in conjunction with a second ODZ4 reverse primer and the provided forward primer.

The amplification program consisted of an initial denaturation of 94°C for 2 minutes and ten standard cycles using a T°a = ~ T°m. In the next 25 cycles, the elongation time was prolonged in each successive cycle by the addition of 2 seconds such that different size of the transcripts could be amplified.

Nested PCR (second round of amplification, or Q1 PCR) was performed using a third ODZ4 reverse primer and the respective provided forward primer. In this case, a T°a = 60°- up to 65°C was used in the amplification program.

3'-RACE Experiments. Purified mRNA (0.5μg) or total RNA (2.5μg) was reverse transcribed with a commercial reverse oligo-dT primer for 60 minutes at 55°C. The obtained cDNA was purified through column chromatography and then amplified by using ODZ4 forward primer jointly to the provided reverse primer (Qt). The annealing temperature for this amplification ranged from 62° to 65°C. Subsequently, 3μL of the Qt amplification product were subject to a nested PCR; thus, another ODZ4 forward and provided reverse (Q1) primers were used. Some

protocol variations were introduced in these experiments; their reason will be discussed later (see Results Chapter).

RACE products were analyzed by 1% agarose gel electrophoresis and cloned into pGEM-T ® Easy Vector Systems II (Promega Corporation; Madison WI, USA. cat.n A1380), for further analysis and sequencing.

4.8 NORTHERN BLOT ANALYSIS

Northern hybridization was used to visualize ODZ4's transcripts that may be present in the cell lines. To this aim, the following protocol was adopted [114].

RNA Isolation and Electrophoresis. Total RNA was precipitated as described above (see section 4.2) and its integrity was controlled by gel electrophoresis. Then, 15µg of each sample were denatured at 65°C for 10 minutes in 25-30µL of Denaturation Buffer (1X MOPS, 2.4M formaldehyde, 50% v/v formamide) before mixing with 2µL of Formaldehyde gel-loading buffer (50% glycerol v/v, 10mM EDTA pH 8, 0.25% w/v bromophenol blue and 0.25% w/v xylene cyanol FF). Samples were run on a denaturing 1.2% agarose gel containing 0.22 M formaldehyde, and electrophoresis separation was conducted at 5-7V/cm for ~4 hours in 1X MOPS/formaldehyde 2.7% (0.9M) Buffer.

Transfer and Fixation of RNAs to the solid matrix. Before transfer, RNAs were partially hydrolyzed, thus, the gel was rinsed with DEPC-water and incubated for 20 minutes in a solution containing 0.01N NaOH/3M NaCl at 25°C, and then pre-incubated for 5 minutes in 20X SSC solution at room temperature. Manufacturer's indications were followed for membrane preparation prior to its use (Amersham Hybond-N+ GE Heathcare Life Science. cat.n.RPN203B). Upward transfer was conducted for 16 hours in neutral conditions (20X SSC).

To assess the transfer efficiency, the gel was stained with ethidium bromide (0.5µg/mL in 0.1M ammonium acetate) and digitally recorded on a UV transilluminator.

After blotting, the membrane was rinsed with 300-400mL of 6X SSC for 5 minutes at room temperature with lower agitation. For cross-linking the RNA to the membrane, the latter was UV irradiated at 254nm, 1 J/cm^2, for 1 minute (Bio-Rad GS Gene Linker ®UV Chamber).

Hybridization Conditions. A low-stringency hybridization protocol was applied. Therefore, preheated ULTRAhyb solution (6-8 mL at 68ºC) was added to the membrane and they were prehybridized for 60 minutes at 42ºC in the hybridization bottle. Next, the denatured probe (5 minutes at 100ºC) was introduced into solution and the membrane was hybridized overnight (14-16 hr) at 42ºC. The probe solution was recovered whereas the membrane was subject to the following washing steps before detection:

-2X 5 minutes in 2X SSC, 0.1% SDS at 48ºC
-2X 15 minutes in 0.1X SSC, 0.1% SDS at 48ºC

Hybridization signals were acquired using X-ray film autoradiography and/or digitalized by Cyclone ® Plus Phosphor Imager (Perkielmer, cat.n. C431200).

Probe's Selection and Radiolabeling Conditions. To select the sequence to be used as a probe, the following criteria were used: amplicons ≥ 300bp, presenting less than 50% of cross-homology with other teneurins and <8% of homology with other transcripts on a genomic BLAST/BLAT. The primer pairs chosen for probe generation mapped on 5'-region (the region encompassing by exon 6 and 8), and 3'-region (a region within exon 34) in the ODZ4 transcript.

The radiolabeling reaction of double strain DNA probes was done by PCR and the use of the high specific activity nucleotide, [αP^{32}] dCTP (3000 Ci/mmole). A dNTP hot-mix (dATP, dTTP, dGTP, each at 10mM and 0.1mM of dCTP) was prepared and 1µL was added to 50µL of the amplification reaction containing:

10ng of purified PCR product or plasmid preparation
1X Mango Taq Colorless Reaction Buffer
1.5mM $MgCl_2$
1µM Forward primer
1µM Reverse primer

10mCi/mL [αP^{32}] dCTP (5µL)
2.5U Mango Taq polymerase (5U/µL)

The DNA was then amplified on Applied Biosystems® 2127 thermal cycler as follows.

Cycle Number	Denaturation	Anneling	Polymerization
1	**45s at 94°C**		
20	**45s at 94°C**	**45s at 58°C**	**2 min at 72°C**
1	**1 min a 94°C**	**30s at 58°C**	**2 min at 72°C**

Subsequently, the PCR product was purified through spin-column chromatography (Micro Bio-Spin), to remove the unincorporated radiolabelled nucleotides. Finally, the probes radioactivity was measured in a beta-counter (Beckman LS 6500 Scintillation Counters, WS-BKLS65), and more than 0.1µg having a specific activity >$2x10^8$ cpm/µg were used for membrane hybridization.