Pathol. Oncol. Res. (2015) 21:1045–1050
DOI 10.1007/s12253-015-9907-x

RESEARCH

Thymopoietin Beta and Gamma Isoforms as a Potential Diagnostic Molecular Marker for Breast Cancer: Preliminary Data

Daniel Marrero-Rodríguez · Keiko Taniguchi-Ponciano · Julio Lopez-Sleman · Pablo Romero-Morelos · Monica Mendoza-Rodríguez · Israel Garcia · Victor Huerta-Padilla · Alejandra Mantilla · Armando Duarte · Patricia Piña · Miriam Rodriguez-Esquivel · Ricardo Lopez-Romero · Jorge Parrazal-Romero · Salvador Tobias-Alonso · Florinda Jimenez-Vega · Mario Alvarez-Blanco · Mauricio Salcedo

Received: 18 March 2014 / Accepted: 16 February 2015 / Published online: 4 April 2015

Abstract Thymopoietin (TMPO) is an inner nuclear membrane protein, the coding gene named equally, can give arise to six isoforms by alternative splicing. This gene has been found up regulated in several types of cancer. At present work, we evaluated the TMPO isoforms generated by alternative splicing as well as the protein signal detection in breast cancer samples. TMPO expression was analyzed by immunohistochemistry in tissue microarray containing 46 breast tissue samples including normal ($n=6$), benign lesions ($n=18$) (fibroadenomas ($n=6$), fibrocystic changes ($n=6$), ductal hyperplasias ($n=6$)) and breast carcinoma ($n=22$). Isoforms -α, −β and -γ of TMPO were evaluated using RT-PCR; clinical-pathological correlation analysis were done by mean of X^2. Neither the normal nor the benign lesions of the breast showed positive TMPO immunodetection, whilst 45 % of the breast carcinomas were immunopositive ($p=0.000$), nine of ten positives carcinomas correspond to the Luminal A subtype. Further, alpha isoform was present in all breast samples analyzed; however, beta and gamma isoforms were only present in ten ($p=0.003$) and 17 ($p=0.000$), respectively, in the breast cancer samples. According with the present data, we suggest that TMPOβ and -γ isoforms could provide a potential reliable diagnostic marker for breast cancer.

D. Marrero-Rodríguez · K. Taniguchi-Ponciano · J. Lopez-Sleman · P. Romero-Morelos · M. Mendoza-Rodríguez · V. Huerta-Padilla · P. Piña · M. Rodriguez-Esquivel · R. Lopez-Romero · J. Parrazal-Romero · M. Salcedo (✉)
Laboratorio de Oncología genómica, Unidad de Investigación Médica en Enfermedades Oncológicas, Hospital de Oncología, CMN-SXXI. IMSS, Av. Cuauhtémoc 330, Col. Doctores, México, DF 06720, Mexico
e-mail: maosal89@yahoo.com

D. Marrero-Rodríguez · F. Jimenez-Vega
Laboratorio de Biotecnología, Universidad Autonóma de Ciudad Juárez, Chihuahua, Mexico

I. Garcia
Servicio de Ginecología Oncológica, Hospital de Oncología CMN-SXXI, IMSS, Mexico, Mexico

A. Mantilla
Servicio de Patología, Hospital de Oncología CMN-SXXI, IMSS, Mexico, Mexico

A. Duarte
Clínica de Mama, Hospital General de Zona #35, IMSS, Cd. Juárez, Chihuahua, Mexico

S. Tobias-Alonso
Departamento de Patología, Hopital General, Cd. Juarez, Chihuahua, Mexico

M. Alvarez-Blanco
Unidad de Quimioterapia, Servicio de Oncología, Hospital General de Mexico, S.S., Mexico, Mexico

Keywords Thymopoietin · Isoforms · Molecular marker · Breast cancer

Introduction

Breast cancer has become the most ubiquitous malignancy among female population worldwide. In Mexico, it has been established as a priority health issue [1]. Basic molecular and cellular mechanism alterations seems to be implicated in the acquisition and progress of breast cancer [2], de-regulation of the cell cycle is one of the most important alterations in carcinogenesis. One of the proteins expressed in a cell-cycle manner is the thymopoietin (TMPO or lamina-associated polypeptide 2). This gene is located in the cytogenetic region 12q21.2 and comprises eight exons [3]. It can give rise to six different isoforms (α, β, γ, δ, ε and ζ) generated by alternative splicing, sharing a common N-terminal domain. The α-isoform is located through nucleoplasm whilst the remaining is anchored to the inner nuclear membrane (INM) [4]. At present, α, β, and γ isoforms are the best characterized. TMPOα has been shown to be up-regulated in cancer cell lines and several types of cancer such as larynx, lung, stomach [5], prostate [6], and colon [7]. Herein we report the expression of TMPO in protein and mRNA isoforms, in sporadic breast cancer samples, benign lesions (including fibroadenomas, fibrocystic changes, ductal hyperplasias), and normal breast tissue by immunohistochemistry (IHC) assay at protein level and RT-PCR for spliced isoforms at RNA level.

Patients and Methods

Tissues Collection

Forty six breast samples; 6 normal tissues, 6 fibroadenoma, 6 ductal hyperplasia, 6 fibrocystic changes, and 22 carcinomas were collected from Hospital de Oncología, CMN SXXI-IMSS and the Breast Clinic #35 Hospital-IMSS, Ciudad Juarez, Chihuahua, Mexico in the period from January of 2010 to February 2011. The tissues were collected before any chemotherapy and/or radiotherapy treatments. Normal breast tissues were collected from esthetical surgeries of patients who accepted to participate with resected tissue. These samples were previously evaluated for NF1 expression [8].

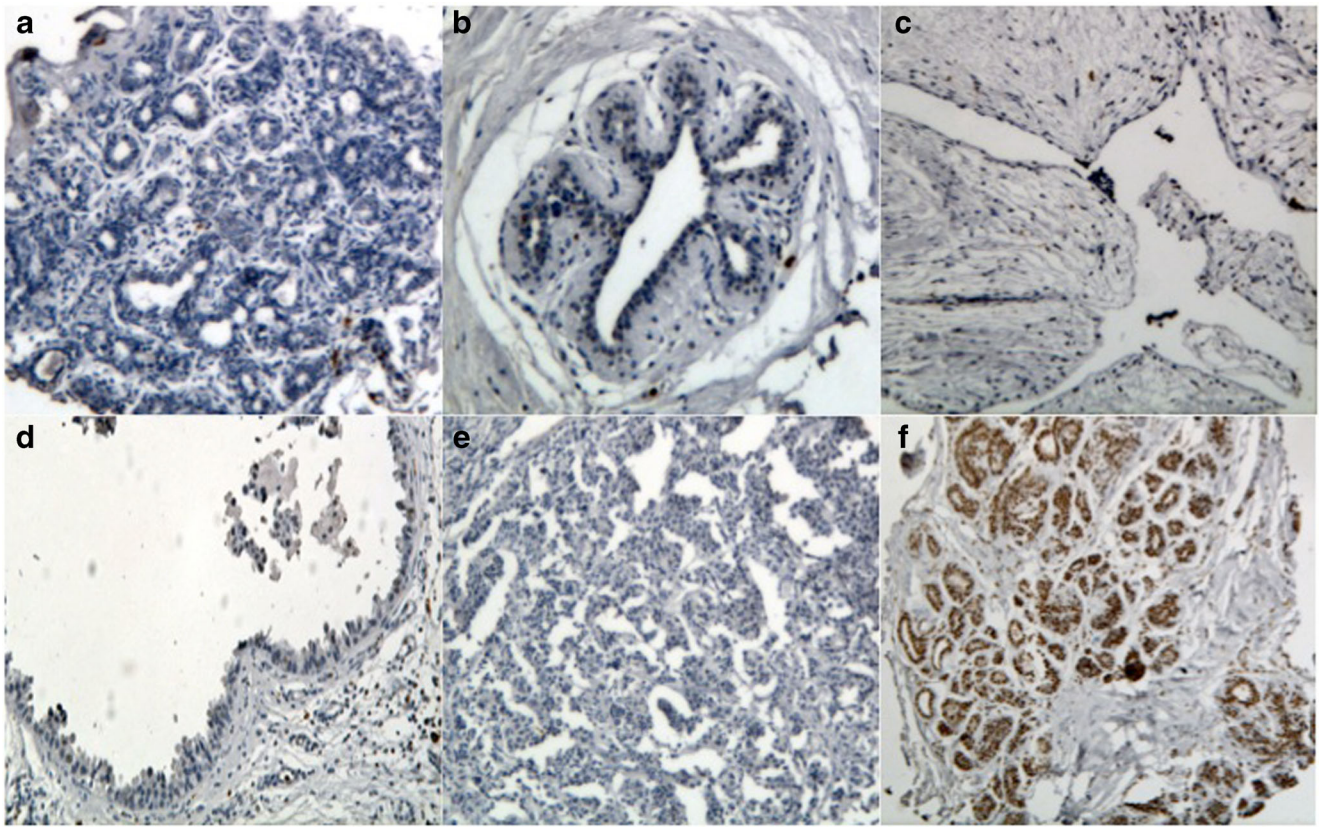

Fig. 1 Thymopoietin expression in breast tissues. Panels: **a** correspond to TMPO negative normal breast tissue, **b** show negative fibroadenoma, **c** show negative fibrocystic changes, **d** show negative ductal hyperplasia, **e** show a negative carcinoma, and **f**) show a strong positive immunoreaction for TMPO in breast cancer. All panels are viewed in a 20X original magnification

All the procedures for RNA extraction, reverse transcription reaction and immunohistochemistry (IHC) were performed as previously reported [8]. In the IHC incubation, the monoclonal antibody (mAB) anti-TMPO (L3414 Sigma-Aldrich, USA) was used, which recognized an epitope ranging from residues 29–50 of the N-terminal. Positive reaction was considered as an immunostaining in the epithelial cells of each tissue.

TMPO α, β and γ Transcripts Detection by PCR in Breast Tissue

PCR was carried using the following primers 5.-TTCTTCCA GGGAGGCAACACAGAT-0.3 and 5.-ATGGTATGGGCA GCCATCTTCACT-3 for alpha isoform (TMPOα) with a product of 363 bp and 5.-AGAGAACCACTAAAGGGCAG AGCA-0.3 and 5.-TTTGATTGGTCTGCGGCAACTAGC-0.3 for the beta (TMPOβ) and gamma (TMPOγ) isoforms producing 510 and 183 bp amplicons respectively with the program 94 °C 7 min, 94 °C 45 sec, 60 °C 45 sec, 72 °C 45 sec (35 cycles) 72 °C 7 min. RPS18 primers was used as an internal control [8].

Statistical Analysis

Clinical and pathological correlation analysis was performed by means of the X^2 test with Fisher exact test. All p values represent two-tailed test and were considered significant at $p < 0.05$. The parameters were dichotomized by expression positive or negative and each isoform present or absent and carried out the analysis against each clinical pathological variable. The statistical analysis was performed using the SPSS v15 statistical software.

Results

Expression of the Thymopoietin in Human Breast Tissues

First, the expression of TMPO in breast cancer samples, normal breast tissue and benign lesions were analyzed by immunohistochemistry. Positive immunoreaction was observed in 45 % (10/22) of breast cancer samples analyzed, while no signal was detectable in the benign lesions or normal tissue (Fig. 1). The statistical analysis shows significant correlation between the expression of TMPO in breast cancer compared against the benign lesions and normal tissues ($p = 0.000$). Subgroups of the breast cancer samples were made according to their histological type (Ductal and/or lobular in situ and/or infiltrating carcinomas) and the receptor expression (ER, PR and Her2/Neu) [9]. Nine of the ten positive carcinomas correspond to the Luminal A subgroup, and the last one fits into the Luminal B subgroup. We did not observe any statistical correlation between the subgroups and the ER, PR and Her2/Neu. The analysis of the clinical-pathological variables

Table 1 Correlation between TMPO (IHC) expression and clinical-pathological variables

	TMPO positive	TMPO Negative	*p* value	95 % CI
Sample type				
Cancer	10	12	0.000*	0.372 to 0.799
Non-cancer	0	24		
Age				
≤45	3	25	0.017*	0.033 to 0.729
>45	7	9		
Clinical stage				
I/II	9	10	0.571	0.139 to 23.374
III/IV	1	2		
Histological type				
∞IDC/ISDC	9	9	0.368	0.260 to 34.575
∞∞ILC/ISLC	1	3		
Oral contraceptive use				
Positive	3	3	0.12	0.733 to 26.744
Negative	7	31		
Smoke				
Positive	4	5	0.101	0.795 to 18.802
Negative	6	29		
Alcohol				
Positive	3	6	0.328	0.398 to 10.051
Negative	7	28		
Cancer history				
Positive	3	1	0.032*	1.276 to 156.782
Negative	7	33		
Family cancer				
Positive	4	5	0.639	0.169 to 5.151
Negative	6	7		
Lymph node status				
Positive	4	4	0.546	0.233 to 7.626
Negative	6	8		
Estrogen receptor				
Positive	10	32	0.461	0.643 to 0.902
Negative	0	3		
Progesterone receptor				
Positive	9	26	0.39	0.261 to 22.493
Negative	1	7		
Her2/neu				
Positive	1	3	0.639	0.113 to 13.208
Negative	9	33		

∞*IDC* Infiltrating Ductal Carcinoma and/or *ISDC* In Situ Ductal Carcinoma, ∞∞*ILC* Infiltrating Lobular Carcinoma and/or *ISLC* In Situ Lobular Carcinoma

*Statistical significance at $p<0.05$ parameters associated with TMPO expression highlighted in *italics*

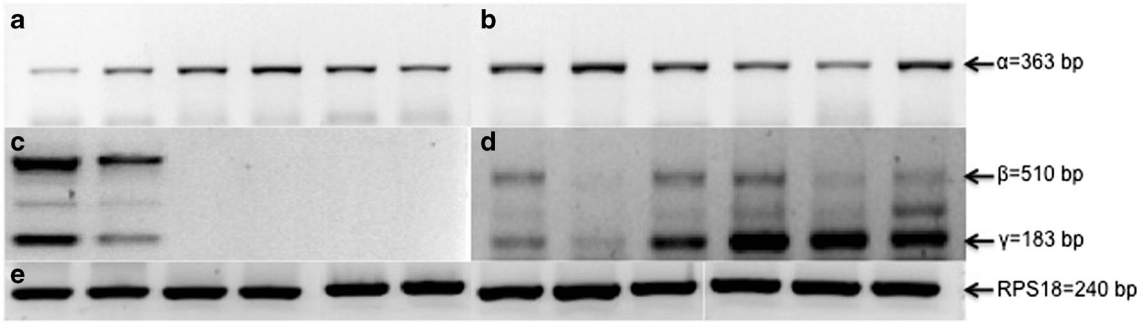

Fig. 2 Thymopoietin isoforms detection in breast tissue. Panel **a**) and **b**) shows the presence of alpha transcript in normal and carcinoma breast tissue respectively, first two lanes of panel **c**) shows positive HeLa and CaSki controls for beta and gamma isoforms and the four remaining lanes show absence of these two transcripts in normal breast tissue whilst **d**) panel show positive reaction for beta and gamma isoforms present in breast carcinomas only. **e**) show RPS18 PCR product as an internal control

showed that the common risk factors such as smoking (p= 0.101), oral contraceptive consumption (p=0.120) and alcohol intake (p=0.328) are not correlated with the TMPO expression, and only a positive correlation was observed with the age (p= *0.017*) and previous cancer history (any type of cancer) in the patient (p= *0.032*; Table 1).

TMPO Transcripts Detection in Breast Tissue

In order to define the presence of TMPO isoforms, the cDNA was subjected to PCR. Interestingly, TMPO mRNA was detected in all kind of breast tissues. Thus, despite the absence of protein expression in normal and benign breast samples, mRNA was present in all the tissues.

In the normal and benign breast tissues the TMPOα isoform was the only detected, lacking the beta and gamma isoforms. TMPOα was also present in all breast carcinomas samples, in contrast, 17/22 invasive samples presented TMPOγ and 10/22 invasive samples presented TMPOβ isoforms (Fig. 2). The analysis showed a good correlation between TMPOβ (p= *0.003*) and TMPOγ (p= *0.000*), and cancer (Table 2).

Discussion

The breast cancer has become as one of the most important health problems regarding female neoplasias worldwide. This indicates the need for the knowledge of the molecular basis of this type of neoplasia as well as reliable molecular markers. In the present work TMPO was evaluated in breast tissues. TMPO is a nuclear protein that binds to components of the nuclear lamina and DNA implicated in the regulation of cell cycle [10] and has been shown to be up-regulated in several types of cancer [5–7, 11]. In order to know the TMPO protein expression, the breast samples were subjected to immunodetection assay. TMPO was only observed in almost half of the breast carcinomas samples, suggesting that TMPO could be related with a subgroup of breast cancer samples. In the case of negative immunoreaction, we did not discard that epitopes could be masked or a low concentration of the protein

Table 2 Statistical correlation between TMPO isoforms expression and clinical pathological variables

	°α	°β	°γ
Cancer vs non cancer	§N/C	0.003*	0.000*
95 % CI	§N/C	0.372 to 0.799	0.105 to 0.491
Age	§N/C	0.057	0.002*
95 % CI	§N/C	0.43 to 1.058	0.18 to 0.454
Clinical stage	§N/C	0.571	0.558
95 % CI	§N/C	0.139 to 23.374	0.134 to 26.320
Histological type	§N/C	0.632	0.675
95 % CI	§N/C	0.091 to 7.002	0.094 to 14.518
Oral contraceptive use	§N/C	0.229	0.301
95 % CI	§N/C	1.165 to 1.932	0.319 to 36.828
Smoke	§N/C	0.565	0.112
95 % CI	§N/C	0.123 to 4.556	0.690 to 24.244
Alcohol	§N/C	0.154	0.112
95 % CI	§N/C	0.634 to 17.518	0.690 to 24.244
Cancer history	§N/C	0.067	0.301
95 % CI	§N/C	0.880 to 110.425	0.319 to 36.828
Family cancer	§N/C	0.639	0.293
95 % CI	§N/C	0.169 to 5.151	0.326 to 38.777
Lymph node status	§N/C	0.156	0.233
95 % CI	§N/C	0.037 to 1.703	0.035 to 2.204
Estrogen receptor	§N/C	0.636	0.457
95 % CI	§N/C	0.060 to 9.319	0.034 to 5.057
Progesterone Receptor	§N/C	0.645	0.434
95 % CI	§N/C	0.153 to 5.942	0.115 to 3.225
Her2/neu	§N/C	0.057	0.04*
95 % CI	§N/C	0.959 to 119.696	1.619 to 3.742

§N/C Not Calculated, value not calculated by SPSS software due to α isoform is a constant

°Thymopoietin α, β, and γ splicing isoforms

*Statistical significance at $p<0.05$ parameters associated with TMPO isoforms highlighted in *italics*

[12, 13]. Regarding to positive samples, most of them were Luminal A subtype, being the most common subtype of breast cancer [14]. There is some evidence that, TMPO expression could be regulated by ER [15]. We did not observe statistical correlation between TMPO and ER, probably due to small number of samples. In this case it should be necessary to increase the sample number to strength this finding.

On the other hand, it has been previously described that TMPO is regulated by E2F [5]. The Rb/E2F pathway is generally disrupted in cancer; particularly in breast cancer it has an intrinsic heterogeneity regulation [16]. An increased expression of E2F has been reported [17, 18]. Thus, it is possible that the TMPO expression in part could be due to increased expression of E2F.

One mechanism by which a protein's function and diversity can be regulated is alternative splicing. This process has been associated with cell cycle, immune response, organ development and malignant transformation [19], and it is known that several proteins that can affect splicing events are up-regulated in breast cancer [20]. TMPO gene can give arise to six different isoforms, but only three of them has been well described in humans, α, −β and –γ [21]. All breast samples expressed alpha isoform, nevertheless only a subgroup of the carcinomas presented beta and gamma isoforms.

Alpha isoform lack the C-terminus transmembrane domain and is localized throughout the nucleus and it can bind to type-A lamins and DNA and has been implicated in apoptosis, nuclear assembly [22] and cell cycle regulation trough the binding of Rb/E2F protein complex [10]. Beta and gamma isoforms conserve a C-terminus transmembrane domain and are anchored to the INM and binds with B-type lamin and DNA. TMPOβ is capable to reduce E2F transcription activity and it can affect nuclear assembly [23].

On the other hand, it could favor the efficiency of the semi-conservative DNA replication and a high proliferation rate cells [23]. Although the three isoforms are expressed in the majority of the mammalian tissues, a differential expression pattern has been described [11]. Taylor and colleagues (2005) suggest that TMPOγ is up-regulated in differentiated tissues while TMPOα and –β are highly expressed in proliferating tissues and cell cultures [12, 23]. This could support in part our observations.

Here we are reporting the expression of TMPO in breast cancer but not in the benign lesions or normal breast tissue; the presence of alpha transcript in normal and benign lesions, and beta and gamma isoforms only in breast cancer samples. This scenario suggest that TMPO β and γ as could represent a potential molecular marker in a subgroup of breast cancer. It is crucial to understand the fine mechanism of regulation and role of TMPO in breast cancer and currently we are increasing the sample number to validate the potential as a diagnostic and prognostic marker. At the same time we are carrying the proper experiments to elucidate its role in breast cancer cells.

Acknowledgments This work was partially supported by a grant FIS-IMSS (Mexico). During this work DMR, KTP, PRM, MMR, LPG and MRE were recipients of a CONACyT fellowship.

Competing Interests The authors declare that there is not conflict of interest.

References

1. Knaul FM, Nigenda B, Lozano R, Arreola H, Langer A, Frenk J (2008) Breast cancer in Mexico: a pressing priority. Reprod Health Matters 16:113–123
2. Cantile M, Pettinato G, Procino A, Feliciello I, Cindolo L, Cillo C (2003) In vivo expression of the whole HOX gene network in human breast cancer. Eur J Cancer 39:257–264
3. Harris C, Andryuk P, Cline S, Mathew S, Siekerka J, Goldstein G (1995) Structure and mapping of the human thymopoietin (TMPO) gene and relationship of human TMPO B to rat lamin-associated polypeptide 2. Genomics 28:198
4. Ward M, van der Watt P, Tzoneva G, Leaner V (2011) Deregulated LAP2a expression in cervical cancer associates with aberrant E2F and p53 activities. IUBMB Life 11:1018–1026
5. Parise P, Finocchiaro G, Masciadri B, Quarto M, Francois S, Mancuso F et al (2006) Lap2α expression is controlled by E2F and deregulated in various human tumors. Cell Cycle 12:1331–1341
6. LaTulippe E, Satagopan J, Smith A, Scher H, Scardino P, Reuter V et al (2002) Comprehensive gene expression analysis of prostate cancer reveals distinct transcriptional programs associated with metastatic disease. Cancer Res 62:4499–506
7. Agrawal D, Chen T, Irby R, Quackenbush J, Chambers A, Szabo M et al (2002) Osteopontin identified as lead marker of colon cancer progression, using pooled sample expression profiling. J Natl Cancer Inst 94:513–521
8. Marrero D, Peralta R, Valdivia A, De la Mora A, Romero P, Parra M et al (2012) The neurofibromin 1 type I isoform predominance characterises female population affected by sporadic breast cancer: preliminary data. J Clin Pathol 5:419–423
9. Hugh J, Hanson J, Chon U, Nielsen T, Perou C, Dumontet C et al (2009) Breast cancer subtypes and response to docetaxel in node-positive breast cancer: use of an immunohistochemical definition in the BCIRG 001 trial. J Clin Oncol 8:1168–1176
10. Dorner D, Vlcek S, Foeger N, Gajewski A, Makolm C, Gotzmann J et al (2006) Lamina-associated polypeptide 2α regulates cell cycle progression and differentiation via the retinoblastoma–E2F pathway. J Cell Biol 1:83–93
11. Kim H, Hwang H, Han M, Baek S, Sim H, Yoon S et al (2012) LAP2 Is widely overexpressed in diverse digestive tract cancers and regulates motility of cancer cells. Plos One 6:e39482
12. Taylor MR, Slavov D, Gajewski A, Vlcek S, Ku L, Fain PR et al (2005) Thymopoietin (Lamina-Associated Polypeptide 2) gene mutation associated with dilated cardiomyopathy. Hum Mutat 6:566–574
13. Dechat T, Gotzmann J, Stockinger A, Harris C, Talle M, Siekierka J et al (1998) Detergent-salt resistance of LAP2α in interphase nuclei and phosphorylation-dependent association with chromosomes early in nuclear assembly implies functions in nuclear structure dinamics. EMBO J 17:4887–4902
14. McCafferty M, Healy N, Kerin M (2009) Breast cancer subtypes and molecular biomarkers. Diagn Histopathol 15:485–489
15. Staples E, Gasiewicz T, Fiore N, Lubahn D, Korach K, Silverstone A (1999) Estrogen receptor is necessary in thymic development and estradiol-induced thymic alterations. J Anat 163:4168–4174

16. Ertel A, Dean J, Rui H, Liu C, Witkiewicz A, Knudsen KE et al (2010) RB-pathway disruption in breast cancer: differential association with disease subtypes, disease-specific prognosis and therapeuti responsse. Cell Cycle 20:4153–4163
17. Zhang S, Liu S, Al-Saleem L, Holloran D, Babb J, Guo X et al (2000) E2F-1: a proliferative marker of breast neoplasia. Cancer Epidemiol Biomarkers Prev 9:395–401
18. Moroy T, Heyd F (2007) The impact of alternative splicing in vivo: mouse models show the way. RNA 13:1155–1171
19. Venables J, Klinck R, Bramard A, Inkel L, Dufresne-Martin G, Koh C et al (2008) Identification of alternative splicing markers for breast cancer. Cancer Res 22:9525–9531
20. Vlcek S, Korbei B, Foisner R (2002) Distinct functions of the unique C terminus of LAP2 in cell proliferation and nuclear assembly. J Biol Chem 21:18898–18907
21. Martins S, Eikvar S, Furukawa K, Collas P (2003) HA95 and LAP2 mediate a novel chromatin–nuclear envelope interaction implicated in initiation of DNA replication. J Cell Biol 2:177–188
22. Gant T, Harris C, Wilson K (1999) Roles of LAP2 proteins in nuclear assembly and DNA replication: truncated LAP2b proteins alter lamina assembly, envelope formation, nuclear size, and DNA replication efficiency in Xenopus laevis extracts. J Cell Biol 6:1083–1096
23. Dechat T, Vlcek S, Foisner R (2000) Review: lamina-associated polypeptide 2 isoforms and related proteins in cell cycle-dependent nuclear structure dynamics. J Struct Biol 129:335–345