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Regulation of gene expression by estrogen in mammary gland of wild type and estrogen receptor alpha knockout mice

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ABSTRACT

Using serial analysis of gene expression, we examined the effects of estrogen (E_2) replacement in gonadectomized wild type (WT) and E_2 receptor alpha knockout (ERαKO) mice on global gene expression in mammary gland. In WT mice, a total of 429,302 tags were sequenced, representing the expression level of 99,854 tag species. A total of ten transcripts were found to be modulated by E_2, such as sorting nexin 5 and two no match tags. In the ERαKO mice, a total of 459,439 tags were sequenced, representing the expression level of 120,149 tag species. Interestingly, the same three transcripts were inversely regulated by E_2 in ERαKO mice. In total, 78 transcripts were upregulated by E_2, while 29 transcripts were downregulated. In contrast to WT mice, the majority of transcripts related to immunity were repressed in ERαKO mice. Moreover, induction of transcripts involved in cell differentiation, Ca^{2+} response, cytoskeleton, protein biosynthesis and secretion, glycolysis, and oxidative phosphorylation were seen only in ERαKO mice. The current study will provide useful information to understand the cellular mechanisms of E_2-mediated gene regulation in tissues *in vivo* for the development of novel drugs targeting specific ER action in pathological conditions.

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1. Introduction

The sex steroid hormone estrogen (E_2) plays an essential role in the development of various tissues and the maintenance of numerous physiological processes such as the menstrual cycle and the functions of reproduction, as well as the regulation of growth by its powerful mitogen effects and proliferation in many target organs including breast, bones, cardiovascular system and genital organs. By its characteristics of growth and proliferation, E_2 is associated with numerous diseases such as breast cancer, Alzheimer's disease, atherosclerosis, osteoporosis and obesity [1]. In fact, the E_2 effects are mediated by two related nuclear hormone receptors (E_2 receptor, ER), namely ERα and ERβ, which are ligand-inducible transcription factors that act by regulating transcriptional processes. Thus, ERs belong to a large superfamily of nuclear hormone receptors sharing a well-conserved DNA-binding domain, a structurally conserved ligand-binding domain (LBD) and N-terminal domain. In addition, there are two activation functions, an N-terminal ligand independent activation function (AF-1) and a C-terminal ligand dependent activation function (AF-2) located within the LBD. Thus, AF-1 and AF-2, which synergically act in a promoter-specific and cell-specific manner, can independently activate transcription and contribute to E_2-mediated transcription. In fact, under identical conditions AF-1 seems more active in ERα on a variety of E_2-responsive promoters, while AF-1 activity in ERβ is minimal [2]. Furthermore, these receptors show a transcription activated by a number of distinct mechanisms. According to classical mechanism, after ligand binding there is dissociation of heat shock proteins from ER, hormone binding in the nucleus or cytoplasm, followed by conformational change within the LBD, dimerization of ERs, and binding to E_2 response elements (EREs) located within the promoters of E_2-responsive genes (ERGs) to finally activate transcription. This activation is achieved through the recruitment of specific transcriptional coregulators (coactivators and corepressors). Thus, coactivators bind to AF-2 or AF-1 and promote transcriptional activity by multiple functions including chromatin remodelling and recruitment of the basal transcriptional machinery, while corepressors inhibit transcriptional activity. In a way distinct of classical model, ERs can regulate the transcription without binding directly to DNA. Thus, ERs are tethered through protein–protein interactions to transcription factor complex that contacts the DNA and regulates the gene expression to large number of ERGs that do not contain EREs. Moreover, other E_2 actions are believed to be mediated through membrane associated ERs. In addition, other stimuli such as the activation of various protein kinase cascades stimulated by various growth factors can activate

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ERs in hormone-independent fashion. Consequently, natural gene promoters can be regulated by many transcription factors that bind to distinct regulatory sites [3].

The mammary gland is a highly E_2-dependent glandular tissue with important regulations of growth and differentiation which participate to the development and progression of breast cancer. In addition, the relative ERα and ERβ expression levels are significantly altered during the development of breast cancer with molecular details of receptors regulation poorly understood [4]. ERα and ERβ have unique roles in E_2-dependent action with different transcriptional activities with certain ligands, cell-types, and promoter contexts [3]. Intriguingly, the ERα-positive cells proliferate in ERα-positive human breast carcinomas and represent two third of all breast cancers [5]. Although there are subtle differences between ERs, ERα requires higher level of E_2 than ERβ for ERE activation. In the cells that have both ERs, the heterodimers are the dominant species in which ERβ acts as transdominant inhibitor of ERα with subsaturing hormone levels. At saturating hormone levels, ERβ does not interfere with transcription activated by ERα [6]. Thus, the effects of E_2 on homodimers and heterodimers ERs in a tissue require to be examined.

Serial analysis of gene expression (SAGE) is a powerful strategy to analyze quantitatively, simultaneously and differentially the expression of all mRNAs in a given tissue, including the expression of known and novel genes [7]. This method may constitute a great tool for the characterization of molecular mechanisms of interests [8]. To identify both known and novel genes regulated by or associated with E_2 action, we have performed the SAGE method in mammary gland of gonadectomized (GDX) wild type (WT) and ERα knockout (ERαKO) mice after injection of E_2.

2. Materials and methods

2.1. Sample preparation

Female C57BL6 mice (n = 24, 12–15 weeks old) were obtained from Charles River Laboratories (St. Constant, Canada). ERαKO mice (n = 19) were produced as previously reported [9]. The GDX mice had access to Lab Rodent Diet No. 5002 and water *ad libitum*. E_2 (50 ng) was injected 3 h (E_2 3 h) and 24 h (E_2 24 h) by a single subcutaneous injection preceding the sacrifice. The control group (GDX) received vehicle solution (0.4% (w/v) Methocel A15LV Premium in 5% ethanol). Although the ERαKO mice were heavier than WT mice, the bolus dose of E_2 was not adjusted to body weight, since the total blood volume was similar considering that the mice had few grams of difference in each group. Moreover, it is well established that a certain range of E_2 dose induces the same physiologic effect.

The mammary gland of *inguinal region* was dissected, and the samples from all mice of the same group were pooled to eliminate interindividual variations and to extract sufficient amount of mRNA. The samples were stored at −80 °C until the analysis. All animal experimentation was conducted in accord with the requirements of the Canadian Council on Animal Care.

2.2. Transcriptome analysis

The SAGE method was performed as previously described [7,10]. Total RNA was isolated by Trizol (Invitrogen Canada Inc., Burlington, Canada). The quality of total RNA was monitored by micro-capillary electrophoresis (Bioanalizer 2100, Agilent Technologies, Mississauga, Canada). Polyadenylated RNA was extracted with the Oligotex mRNA Mini Kit (Qiagen Inc., Mississauga, Canada), annealed with the biotin-5′-T_{18}-3′ primer and converted to cDNA using the cDNA synthesis kit (Invitrogen Canada Inc.). The resulting cDNA library was digested with NlaIII (New England BioLabs Ltd., Pickering, Canada), and the 3′ restriction fragments were isolated with streptavidin-coated magnetic beads (Dynal Biotech LLC, Brown Deer, USA) and separated into two populations. Each population was ligated to one of the two annealed linker pairs and extensively washed to remove unligated linkers. The tag beside the most 3′ NlaIII restriction site (CATG) of each transcript was released by digestion with BsmFI (New England BioLabs Ltd.).

The blunting kit from Takara Bio Inc. (Otsu, Japan) was used for the blunting and ligation of the two tag populations. The resulting ligation products containing the ditags were amplified by PCR with an initial denaturation step of 1 min at 95 °C, followed by 22 cycles of 20 s at 94 °C, 20 s at 60 °C and 2 s at 72 °C with 27 bp primers [10]. The PCR products were digested with NlaIII and the band containing the ditags was extracted from the 12% polyacrylamide gel with Spin-X microcentrifuge tube (Fisher, Pittsbergh, USA). The purified ditags were self-ligated to form concatemers using T4 ligase (Invitrogen Canada Inc.). The concatemers ranging from 500 bp to 1800 bp were isolated by agarose gel and extracted with Gene-Clean Spin (Qbiogene, Montreal, Canada). The resulting DNA fragments were ligated into the SphI site of pUC19 (Invitrogen Canada Inc.) and cloned into UltraMAX DH5αFT competent cells (Invitrogen Canada Inc.). White colonies were picked up with a Q-Bot colony picker (Genetix Ltd., Hampshire, UK). Concatemer inserts were sequenced by the Applied Biosystems 3730 (Foster City, USA).

2.3. Bioinformatic analysis

Sequence files were analyzed using the SAGEana program, a modification of SAGEparser [11]. In brief, SAGE tags corresponding to linker sequences were discarded and replicate concatemers were counted only once. Identification of the transcripts was obtained by matching the 15 bp (sequence at the last CATG + 11 bp tags) with SAGEmap, UniGene and GenBank databases. A minimum of one expressed sequence tag (EST) with a known polyA tail had to be in the UniGene cluster to identify the last NlaIII site on the corresponding cDNA. We have previously shown that the SAGE method is very reproducible with r^2 = 0.96 between two SAGE libraries generated from two cDNA libraries constructed from the same total RNA pool [11]. Classification of the transcripts was based upon the updated information of the genome directory [12] found at the TIGR web site (http://www.tigr.org/), the SOURCE (http://genome-www5.stanford.edu/cgi-bin/source/sourceSearch) and the OMIM (http://www.ncbi.nlm.nih.gov/) as well as previously published literatures.

2.4. Validation by the quantitative real-time PCR (Q_RT-PCR)

First-strand cDNA was synthesized using 5 μg of pooled RNA of each WT experimental group (GDX, E_2 3 h and E_2 24 h) in a reaction containing 200 U of Superscript III RNase H-RT (Invitrogen Canada Inc.), 300 ng of oligo-dT_{18}, 500 mM deoxynucleotides triphosphate, 5 mM dithiothreitol and 34 U of human RNase inhibitor (Amersham Pharmacia, Piscataway, USA) in a final volume of 50 μl. The resulting products after treating with RNase A were purified with Qiaquick PCR purification kits (Qiagen Inc.). The cDNA corresponding to 20 ng of total RNA was used to perform fluorescent-based real-time PCR quantification using the LightCycler real-time PCR apparatus (Roche Inc., Nutley, USA). Reagents were obtained from the same company and were used as described by the manufacturer. Reading of the fluorescence signal was taken at the end of the heating to avoid non-specific signal. A melting curve was performed to assess non-specific signal. Oligoprimer pairs that allow the amplification of approximately 250 bp were designed by GeneTools software (Biotools Inc., Edmonton, Canada) and their specificity was verified by blast in GenBank database. Gene name, GenBank accession numbers and regions used for the primer pairs were the following:

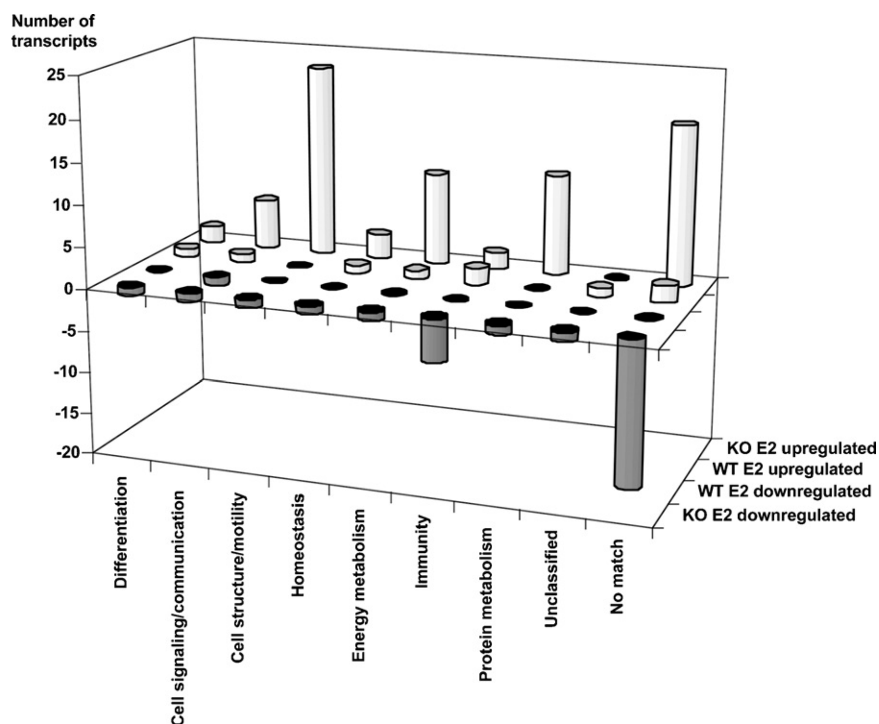

Fig. 1. Overview of the total number of estrogen responsive genes (ERGs) in wild type (WT) and estrogen receptor alpha knockout (KO) mice.

neuronatin (Nnat), NM_010923, 635/868; coagulation factor III (F3), NM_010171, 648/734. The expression levels of mRNA (number of copies/μg total RNA) were calculated using a standard curve of crossing point (Cp) versus logarithm of the quantity. The standard curve was established using known cDNA amounts of 0, 10^2, 10^3, 10^4, 10^5 and 10^6 copies of hypoxanthine guanine phosphoribosyl transferase 1 and a LightCycler 3.5 program provided by the manufacturer (Roche Inc.).

2.5. Statistical analysis

The identification of differentially expressed transcripts with more than twofold change ($p < 0.05$) was carried out by using the comparative count display test (CCD) between E_2 and GDX groups [13]. A normalisation of gene expression level was set at 100,000 in order to facilitate visual comparison in the tables.

Table 1
Effects of estradiol (E_2) on transcripts expressed in mammary gland of wild type mice.

SAGE tag	GDX	GDX + E_2			Abbreviation	Description (UniGene cluster, GenBank accession number)
		3 h	24 h			
Cell differentiation						
GGGGGAGTGGA[a]	22	69[b]	23	↑	**Nnat**	Neuronatin (Mm.233903, NM_010923)
Cell signalling/communication: transport proteins						
TGTACTTGAAA	0	11[b]	10	↑	**Atp1a2**	ATPase Na+/K+ transporting alpha 2 polypeptide (Mm.207432, BX528420)
TGAATAAATAT[a]	10	0[b]	3	↓	**Snx5**	Sorting nexin 5 (Mm.273379, BC002242)
Homeostasis						
TTAAATGCAGG	0	13[b]	4	↑	**F3**	Coagulation factor III (Mm.273188, NM_010171)
Energy metabolism						
AGTCAGGTCCA[c]	9	43[b]	45[b]	↑	**Lpl**	Lipoprotein lipase (Mm.1514, BC003305)
Immunity						
CTACCCTGAGC[c]	9	20	44[b]	↑	**H2-D1**	Histocompatibility 2 D region locus 1 (Mm.214762, NM_010390; Mm.33263, L36068; Mm.16771, M69069)
GACTTGGACAG	1	14[b]	9	↑	**Pxdn**	Peroxidasin homolog (Mm.251774, AK010185)
ATGATGATAGA[a]	6	11	29[b]	↑	**Slfn8/Slfn9**	Schlafen 9 (Mm.347694, NM_181545); Schlafen 8 (Mm.270253, NM_172796)
No match						
GAAAATGAGAA	492	356	1119[b]	↑		NM
GAAAATGATAA	17	16	63[b]	↑		NM

[a] Tag with disponible informations on promoter and transcription start site identification.
[b] Significatively different from gonadectomized (GDX) mice ($p < 0.05$).
[c] Tags with estrogen response elements known in the promoter region.

Table 2
Transcripts regulated by estradiol (E_2) in mammary gland of alpha estrogen receptor knockout mice.

SAGE tag	GDX	GDX + E_2			Abbreviation	Description (UniGene cluster, GenBank accession number)
		3 h	24 h			
Cell differentiation/division/growth						
CTCACTTAGTG[a]	0	17[b]	0	↑	**9530002B09Rik**	RIKEN cDNA 9530002B09 gene (Mm.157655, NM_023865)
CAATAAATGTT[a]	0	0	12[b]	↑	**Cdca7l**	Cell division cycle associated 7 like (Mm.281149, NM_146040)
CCTGAGCTCCT	38	9[b]	19	↓	**Gpnmb**	Glycoprotein (transmembrane) nmb (Mm.302602, BC026375)
TAAAAAGAAAG[a]	6	33[b]	12	↑	**Ndrg2**	N-myc downstream regulated gene 2 (Mm.26722, NM_013864)
Cell signalling/communication: transport proteins						
CATCTTCAGCC[a]	1	110[b]	2	↑	**Atp2a1**	ATPase, Ca^{2+} transporting cardiac muscle fast twitch 1 (Mm.35134, NM_007504)
TTGGAGACTCC[a]	0	17[b]	1	↑	**Hrc**	Histidine rich calcium binding protein (Mm.39968, NM_010473)
ACCGTGATGAC[a]	0	21[b]	0	↑	**Jsrp1**	Junctional sarcoplasmic reticulum protein 1 (Mm.45137, NM_028001)
TGGGCCACCTC[a]	0	70[b]	2	↑	**Pvalb**	Parvalbumin (Mm.2766, AK013561)
TTTTTCCTGAT[c]	0	14[b]	2	↑	**Ryr1**	Ryanodine receptor 1 skeletal muscle (Mm.226037, NM_009109)
ATGACAGCTTC[a]	13	0[b]	11	↓	**Scn7a**	Sodium channel voltage-gated type VII alpha (Mm.38127, AK051242)
TGAATAAATAT[a]	0	17[b]	6	↑	**Snx5**	Sorting nexin 5 (Mm.273379, BC002242)
Cell structure/motility						
CTTCTGAATAA[c]	0	38[b]	3	↑	**Actn3**	Actinin alpha 3 (Mm.5316, NM_013456)
GCCCCTCTCTT[c]	4	24[b]	4	↑	**Des**	Desmin (Mm.6712, NM_010043)
TGTTTAGTTCT[a]	21	3[b]	13	↓	**Klc1**	Kinesin 2 (Mm.278357, NM_001025360)
TTCTTTGGTGA[c]	1	22[b]	4	↑	**Krt5**	Keratin complex 2 basic gene 6a (Mm.302399, NM_027011)
AGAAACCAATA[c]	0	16[b]	0	↑	**Krt10**	Keratin complex 1 acidic gene 10 (Mm.22662, NM_010660)
CTGCTCAGGCT	0	12[b]	0	↑	**Krt14**	Keratin complex 1 acidic gene 14 (Mm.6974, NM_016958)
CAATGTGTCTG[a]	0	17[b]	0	↑	**Krt25**	Keratin 25 (Mm.268173, NM_133730)
TGGTGCACTTC[c]	0	12[b]	0	↑	**Krt71**	Keratin 71 (Mm.358677, NM_019956)
AAGATCCCAAA	2	24[b]	0	↑	**Mybpc2**	Myosin binding protein C fast-type (Mm.358888, NM_146189)
GAGCAGACCGT	0	144[b]	8	↑	**Myh4**	Myosin heavy polypeptide 4 skeletal muscle (Mm.297382, NM_010855)
GTGATGCTAAG[a]	3	373[b]	8	↑	**Mylpf**	Myosin light chain phosphorylatable fast skeletal muscle (Mm.14526, NM_016754)
CCTACAGTTGA[a]	14	193[b]	18	↑	**Myl1**	Myosin light polypeptide 1 (Mm.1000, NM_021285)
TGCATCATTTC	0	19[b]	0	↑	**Myoz1**	Myozenin 1 (Mm.141702, NM_021508)
AAGTGAAAGCA	0	12[b]	0	↑	**Tchh**	Trichohyalin (Mm.316671, AK132147)
CTCCCCAGAAG[c]	0	19[b]	0	↑	**Tnnc2**	Troponin C2 fast (Mm.1716, DV050221)
TGACAGAAGAG[c]	1	221[b]	4	↑	**Tnnc2**	Troponin C2 fast (Mm.1716, NM_009394)
GAGGGCCGGAA[a]	3	274[b]	4	↑	**Tnni2**	Troponin I skeletal fast 2 (Mm.39469, NM_009405)
ACTGTCCGGGC[a]	1	133[b]	3	↑	**Tnnt3**	Troponin T3 skeletal fast (Mm.389992, BC003747)
GGTGCCAACTA[a]	1	93[b]	0	↑	**Tnnt3**	Troponin T3 skeletal fast (Mm.389992, NM_011620)
AAAGTCATTGA[a]	8	158[b]	22	↑	**Tpm1**	Tropomyosin 1 alpha (Mm.121878, AK169188)
CACTGACCTCC[c]	0	48[b]	0	↑	**Tpm2**	Tropomyosin 2 beta (Mm.646, AK003186)
CCAGTATATGT[a]	0	14[b]	0	↑	**Ttn**	Titin (Mm.373672, AK003152)
GATAGCTTGGG[a]	0	25[b]	0	↑	**Ttn**	Titin (Mm.373672, BC025840)
Homeostasis						
CCAGCTTCCTC[a]	0	0	14[b]	↑	**Aldh2**	Aldehyde dehydrogenase 2 mitochondrial (Mm.284446, AK146286)
GTGGAGGCGCC[a]	0	17[b]	0	↑	**Cst6**	Cystatin E/M (Mm.36816, BC061036)
GAAGAGGGGGA[c]	228	92[b]	185	↓	**Hp**	Haptoglobin (Mm.26730, NM_017370)
TGCACAAATAA[a]	0	15[b]	0	↑	**Mettl6**	Methyltransferase like 6 (Mm.291731, AK005448)
Energy metabolism						
CCAGCCAGCGT[a]	1	34[b]	1	↑	**Ankrd23**	Ankyrin repeat domain 23 (Mm.41421, BC022973)
GAAAGTTGGCC	77	21[b]	50	↓	**Apod**	Apolipoprotein D (Mm.2082, AK157917)
CCCTGCCTTAA[a]	1	90[b]	8	↑	**Ckm**	Creatine kinase muscle (Mm.2375, NM_007710)
GAGGGGCAGGA	0	12[b]	0	↑	**Cox6a2**	Cytochrome c oxidase subunit VI a polypeptide 2 (Mm.43824, BC028514)
GACCTCATTCC[c]	1	40[b]	1	↑	**Eno3**	Enolase 3 beta muscle (Mm.251322, NM_007933)
CTCAGGTCTCC	0	44[b]	0	↑	**Mb**	Myoglobin (Mm.201606, NM_013593)
TGGTGTAAGCA	2	28[b]	7	↑	**MtCo1**	Cytochrome c oxidase subunit I (Mm.MTG.3, NC_005089)
CTGCGGCTTCA	5	30[b]	1	↑	**MtCo3**	Cytochrome c oxidase subunit III (Mm.MTG.7, NC_005089)
GTAGTGGAAGT	20	64[b]	42	↑	**MtNd3**	NADH dehydrogenase subunit 3 (Mm.MTG.8, NC_005089)
CCTGCAACCAG[a]	2	20[b]	6	↑	**Pfkm**	Phosphofructokinase muscle (Mm.272582, NM_021514)
GAAGCTGTTGC[a]	0	73[b]	2[b]	↑	**Pgam2**	Phosphoglycerate mutase 2 (Mm.219627, NM_018870)
TTTGTGTGCTT[a]	0	0	27[b]	↑	**Ucp1**	Uncoupling protein 1 (mitochondrial proton carrier) (Mm.4177, NM_009463)
Immunity						
TTTTCAAAAAT[a]	42	7[b]	24	↓	**B2m**	Beta-2 microglobulin (Mm.163, X01838)
GGGATGGACGG[c]	106	38[b]	102	↓	**C4b**	Complement component 4B (Childo blood group) (Mm.18845, NM_009780)
GTACCTGTGAG	0	16[b]	0	↑	**Defb50**	Defensin beta 50 (Mm.346928, NM_199067)
TGCCAACTGAT	0	37[b]	0	↑	**Fcgbp**	Fc fragment of IgG binding protein (Mm.292276, DV052978)

Table 2 (*Continued*)

SAGE tag	GDX	GDX + E_2			Abbreviation	Description (UniGene cluster, GenBank accession number)
		3 h	24 h			
AGGGCCTGCAC	92	17[b]	10[b]	↓	**Igh-6**	Immunoglobulin heavy chain 1a (serum IgG2a) (Mm.342177,BC057688)
TGACAGCTGCC[a]	16	5	1[b]	↓	**S100a9**	S100 calcium binding protein A9 (calgranulin B) (Mm.2128, BC027635)
GCTCCCGGCTC	23	2[b]	2[b]	↓	**Slpi**	Secretory leukocyte peptidase inhibitor (Mm.371583, NM_011414)
Protein metabolism						
TCTGCACCTCC[a]	0	15[b]	3	↑	**Eef1a2**	Eukaryotic translation elongation factor 1 alpha 2 (Mm.2645, NM_007906)
CAAACCTCCAT	0	13[b]	8	↑	**MtRnr1**	12S ribosomal RNA (Mm.MTG.14, NC_005089)
AATTTCTTCCT	28	163[b]	85[b]	↑	**Mup1**	Major urinary protein 1 (Mm.335875, NM_031188)
TTCTAATCGGT	0	70[b]	0	↑	**Pbsn**	Probasin (Mm.8034, NM_017471)
GGAAACAATGA	0	17[b]	0	↑	**Pbsn**	Probasin (Mm.8034, DV075247)
GGGTGGCCCAG	23	2[b]	10	↓	**Rps13**	Ribosomal protein S13 (Mm.381723, BC090397)
GCAGCGCCTCC	0	171[b]	2	↑	**Sbp**	Spermine binding protein (Mm.46428, AK075905; Mm.390958, NM_011321)
GCTCCTGAGGG	0	20[b]	0	↑	**Sbp**	Spermine binding protein (Mm.390958, DV072608)
CTACTCAGCTT	0	19[b]	1	↑	**EST Sbp**	EST Spermine binding protein (Mm.390958, DV057691)
AACCAACGATC	0	13[b]	0	↑	**Svs2**	Seminal vesicle secretory protein 2 (Mm.99395, BB807744)
CTTGTTTGTCT[a]	2	56[b]	0	↑	**Svs4**	Seminal vesicle secretory protein 4 (Mm.1286, NM_009300)
GGGCCTAGAAA	1	44[b]	1	↑	**Svs5**	Seminal vesicle secretory protein 5 (Mm.140154, NM_009301)
ATGGCCTGAGA[a]	0	14[b]	0	↑	**Svs7**	Seminal vesicle protein, secretion 7 (Mm.390143, NM_020264)
Unclassified						
CTAATATTTGC[a]	415	181	93[b]	↓	**Scrn3**	Secernin 3 (Mm.247457, AK008450)
No match						
AAAAATGAGAA	32	38	7[b]	↓		
AACATACAAGA	6	29[b]	8	↑		
AAGAAGGTAAA	0	15[b]	0	↑		
AAGGAGTCTCT	0	14[b]	1	↑		
AGATTTATTTC	0	42[b]	0	↑		
AGCACTCAGTA	0	32[b]	0	↑		
ATTGACGTGGA	0	41[b]	3	↑		
ATTTCCAGTTT	14	7	0[b]	↓		
ATTTTCACTTT	32	15	5[b]	↓		
ATTTTCAGGTT	11	4	0[b]	↓		
ATTTTCAGTTT	729	308[b]	105[b]	↓		
CACCTAATTGG	0	4	12[b]	↑		
CCAGACTTCTC	0	12[b]	0	↑		
CCCATCGTCCT	0	0	15[b]	↑		
CCGATGATCAG	1	15[b]	3	↑		
CCTCTCTCACT	1	20[b]	0	↑		
GAAAAAGAGAA	20	34	2[b]	↓		
GAAAACGAGAA	33	26	1[b]	↓		
GAAAATGAAAA	25	14	1[b]	↓		
GAAAATGAGAA	1983	1670	289[b]	↓		
GAAAATGAGAC	14	30	0[b]	↑		
GAAAATGAGAT	29	40	2[b]	↓		
GAAAATGATAA	124	120	13[b]	↓		
GAAAATGATGA	14	7	1[b]	↓		
GAACAGTCGAT	0	12[b]	2	↑		
GAATATGGCAA	24	19	3[b]	↓		
GAGCTCCAAGC	0	43[b]	2	↑		
GCAACAACACA	0	14[b]	1	↑		
GTGACCACGGG	3	36[b]	6	↑		
GTGGCGGTGGC	0	24[b]	0	↑		
TACCATCAATA	0	0	18[b]	↑		
TCCTAAAGTGT	23	15	3[b]	↓		
TCCTACAGTGG	90	44	16[b]	↓		
TTCCGTGATTT	12	8	0[b]	↓		
TTGGGGTTTCC	0	0	24[b]	↑		
TTTTTCATTGT	21	6	1[b]	↓		

[a] Tag with disponible informations on promoter and transcription start site identification.
[b] Significatively different from gonadectomized (GDX) mice ($p < 0.05$).
[c] Tags with estrogen response elements known in the promoter region.

2.6. EREs

We have also integrated our results with a tool for accurate detection and analysis of elements in vertebrate genomes [14], and we have also used a large-scale mammalian promoter and transcription start site identification service (PromoSer) [15] by using the fasta sequence for our tags. Finally, we have used the ERGs database (ERGDB) which constitutes a tool for locating EREs in the promoter region of respective genes [16].

3. Results

Using the SAGE method, it was possible to quantify the expression frequency of known and novel genes. Six SAGE libraries (GDX,

E_2 3 h and E_2 24 h in WT or ERαKO mice) were generated to characterize the effects of E_2 on mammary gland transcriptome. In the WT mice, a total of 429,302 tags were sequenced, representing the expression level of 99,854 tag species. In ERαKO mice, a total of 459,439 tags were sequenced, representing the expression level of 120,149 tag species. Whereas more than 100 transcripts have rapidly responded either 3 h and/or 24 h following the administration of E_2 in ERαKO mice, only ten transcripts were modulated in WT mice. Number of transcripts up and downregulated by E_2 in WT and ERαKO mice was classified by function of gene products (Fig. 1).

The majority of the E_2-modulated SAGE tags represented known genes, whereas only two were novel transcripts in WT mice (Table 1). The upregulated transcripts were Nnat, ATPase Na^+/K^+ transporting alpha 2 polypeptide (Atp1a2), F3, lipoprotein lipase (Lpl), histocompatibility 2 D region locus 1 (H2-D1), schlafen 8 and/or schlafen 9 (Slfn8/Slfn9), peroxidasin homolog (Pxdn), and two novel transcripts. The downregulated transcript was sorting nexin 5 (Snx5).

Among 107 regulated transcripts in ERαKO mice, there were 78 transcripts upregulated by E_2, while 29 transcripts were downregulated (Table 2). An interesting effect of E_2 in mammary gland is the induction in cell differentiation, division or growth by the upregulation of three genes such as RIKEN cDNA 9530002B09 gene (9530002B09Rik), cell division cycle associated 7 like (Cdca7l) and N-myc downstream regulated gene 2 (Ndrg2), while glycoprotein nmb (Gpnmb) was downregulated. In cell signalling and communication, E_2 upregulated six transcripts, namely ATPase Ca^{2+} transporting cardiac muscle fast twitch 1 (Atp2a1), histidine rich calcium binding protein (Hrc), junctional sarcoplasmic reticulum protein 1 (Jsrp1), parvalbumin (Pvalb), ryanodine receptor 1 skeletal muscle (Ryr1), and Snx5, and downregulated only one transcript, sodium channel voltage-gated type VII alpha (Scn7a). In addition, E_2 increased the expression level of 22 transcripts and downregulated one transcript involved in cell structure and motility in cytoskeletal and extracellular matrix (ECM). Moreover, eleven transcripts were upregulated by E_2 in energy metabolism, whereas only one was downregulated. E_2 repressed five transcripts in cell defense, whereas two were upregulated. Finally, E_2 upregulated eukaryotic translation elongation factor 1 alpha 2 (Eef1a2) and 12S ribosomal RNA (MtRnr1) while it downregulated ribosomal protein S13 (Rps13) which are involved in protein biosynthesis. In protein secretion, nine transcripts were upregulated by E_2, namely major urinary protein 1 (Mup1), probasin (Pbsn), spermine binding protein (Sbp), seminal vesicle secretory protein 2, 4 and 5 (Svs2, 4 and 5), and seminal vesicle protein secretion 7 (Svs7). Furthermore, 36 of E_2-responsive SAGE tags did not match any known mRNA from public databases.

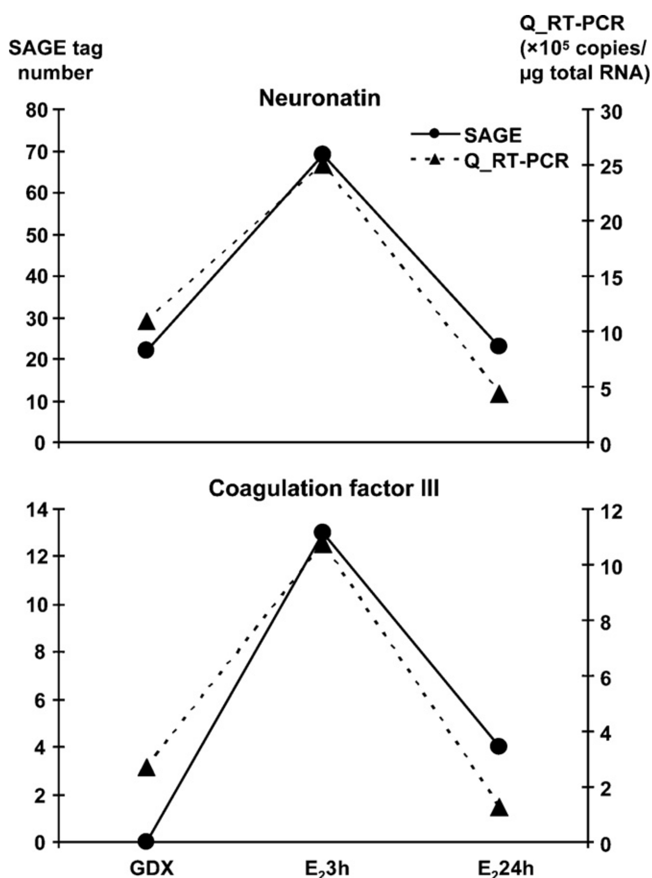

Fig. 2. Confirmation of regulated transcripts by E_2 with the quantitative real-time PCR (Q_RT-PCR) method comparatively with the serial analysis of gene expression (SAGE) method: (A) neuronatin and (B) coagulation factor III.

Using the Q_RT-PCR method, a confirmation of ERGs in the mammary gland of WT mice was performed. These data show that Nnat and F3 are upregulated by E_2 concordantly with the results from the SAGE method (Fig. 2).

4. Discussion

We have used the SAGE strategy to obtain a global view of the transcriptional responses in mammary gland after a single E_2 injection in WT or ERαKO mice. The main finding of this study is that more than 100 transcripts have rapidly responded either 3 h and/or 24 h following the administration of E_2 in ERαKO mice, whereas only ten transcripts were modulated in WT mice. In which, three transcripts were oppositely regulated in both mouse types, namely Snx5 and two unknown transcripts. In the ER α–β heterodimeric complex, AF-1 and AF-2 are required to achieve maximal transcriptional activity, and only AF-2 activity is entirely dependent of ligand binding [17]. In addition, the ERα–ERβ heterodimer can be activated when only one E_2-binding competent partner is present per dimer, and two functional AF-2 domains are required for transcriptional activity [17]. In cells that have both ERs, the heterodimers are the dominant species in which ERβ acts as transdominant inhibitor of ERα with subsaturing hormone levels [6]. Concordantly, E_2 had fewer effects in the current WT mice than in ERαKO mice, suggesting that E_2 might mediate gene expression in mammary gland mainly using ERβ. In addition, the present study showed downregulation of Snx5 in WT mice, while E_2 upregulated its expression in ERαKO mice. SNX5 functions not only in endocytic pathways to internalize extracellular components but is also involved in signal transduction pathways [18]. In a first view, Snx5 can be downregulated because both alpha and beta types of receptor are present in WT mice, and it is probably the result of heterodimer action under E_2 effect. It seems that, in normal mice, ERα is responsive for the *downregulation* of Snx5, whereas the antagonist effect of ERβ accountable for the *upregulation* of Snx5 in ERαKO mice. This could be an example of the opposing effects of E_2 according to ERs.

4.1. Cell differentiation and breast cancer

A study with knockout of ERβ has suggested that ERβ is essential for the fully differentiated phenotype of the mammary gland by contributing to cellular differentiation, growth control and homeostasis, in addition to the participation in the protection offered by pregnancy and lactation against breast cancer [19]. ERβ may also play a role in adipocytes development although ERα is the predominant modulator of E_2 effects in adipose tissue [20]. Concordantly, more transcripts related to differentiation, growth control and tumorigenesis were regulated by E_2 in the ERαKO mice than in

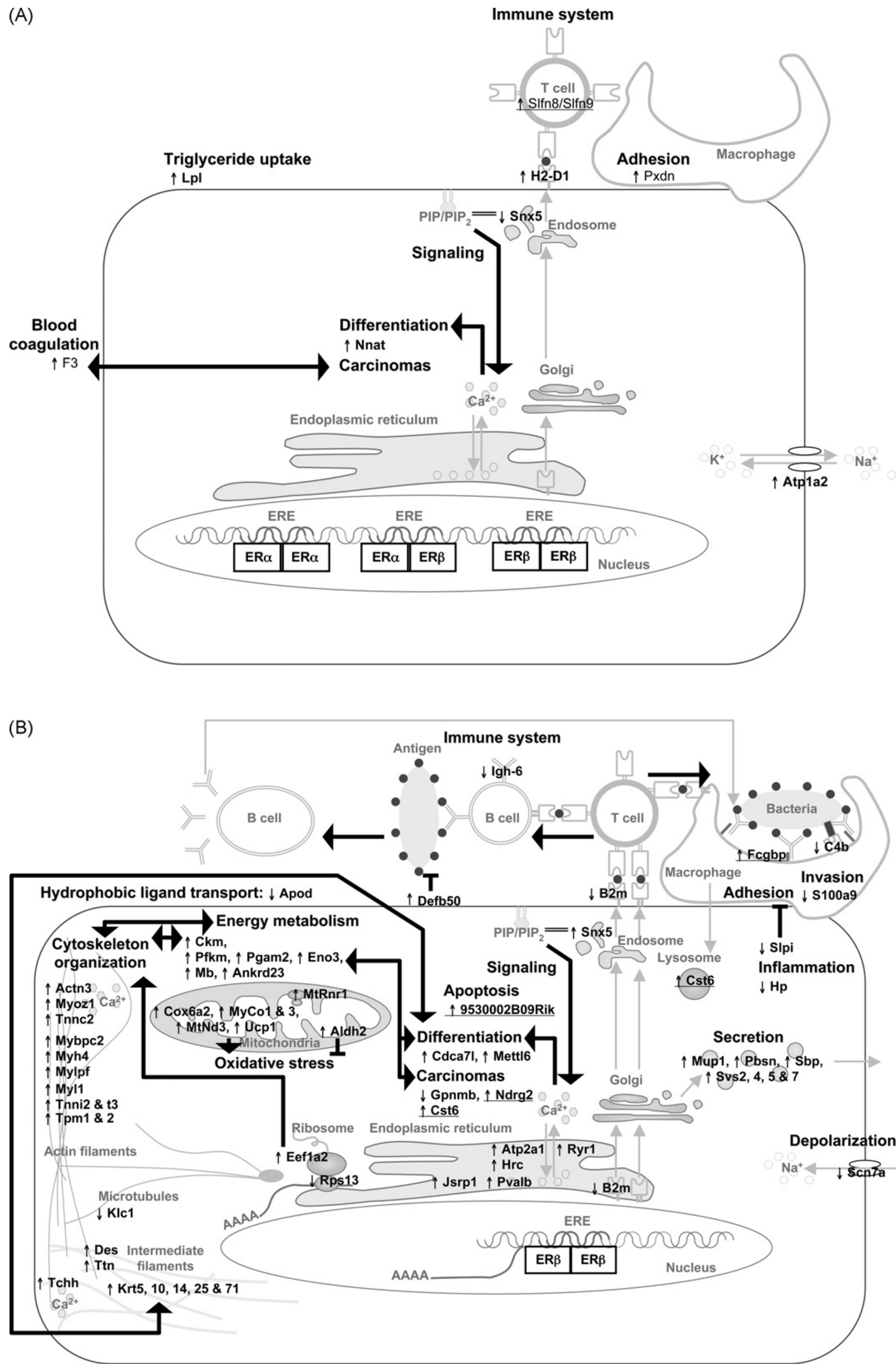

Fig. 3. Overview of general pathways of estrogen (E_2) actions *in mammary gland of mice*. The up and downregulated transcripts by E_2 treatment are shown, respectively, with ↑ and ↓ in wild type (WT) mice [A] and in E_2 receptor alpha knockout (ERαKO) mice [B]. Gray arrow shows movement of molecules. One side arrow, both sides arrow and T line in bold show activation, relation and inhibition, respectively. *Abbreviations*: **9530002B09Rik**, RIKEN cDNA 9530002B09 gene; **Actn3**, actinin alpha 3; **Aldh2**, aldehyde dehydrogenase 2 mitochondrial; **Ankrd23**, ankyrin repeat domain 23; **Apod**, apolipoprotein D; **Atp1a2**, ATPase Na^+/K^+ transporting alpha 2 polypeptide; **Atp2a1**, ATPase, Ca^{2+} transporting cardiac muscle fast twitch 1; **B2m**, beta-2 microglobulin; **C4b**, complement component 4B; **Cdca7l**, cell division cycle associated 7 like; **Ckm**, creatine kinase muscle; **Cox6a2**, cytochrome c oxidase subunit VI a polypeptide 2; **Cst6**, cystatin E/M; **Defb50**, defensin beta 50; **Des**, desmin; **Eef1a2**, eukaryotic translation elongation factor 1 alpha 2; **Eno3**, enolase 3 beta muscle; **ER**, estrogen receptor; **ERE**, E_2 response element; **F3**, coagulation factor III; **Fcgbp**, Fc fragment of IgG binding protein; **Gpnmb**, glycoprotein nmb; **H2-D1**, histocompatibility 2 D region locus 1; **Hp**, haptoglobin; **Hrc**, histidine rich calcium binding protein; **Igh-6**, immunoglobulin heavy chain 1a; **Jsrp1**, junctional sarcoplasmic reticulum protein 1; **Klc1**, kinesin light chain 1; **Krt5, 10, 14, 25 & 71**, keratin complex 2 basic gene 6a, complex 1 acidic gene 10, complex 1 acidic gene 14, 25 and 71; **Lpl**, lipoprotein lipase; **Mb**, myoglobin; **Mettl6**, methyltransferase like 6; **MtCo1 & 3**, cytochrome c oxidase subunit I and III; **MtNd3**, NADH dehydrogenase subunit

the WT mice of the present study. Indeed, Ndrg2, a new candidate gene for cancer suppressor, is involved in cell differentiation [21], and 9530002B09Rik might have a role in suppression of apoptosis [22]. Moreover, Gpnmb is overexpressed in breast carcinomas [23]. Furthermore, CDCA7L, also known as JPO2, is a novel c-Myc NH2-terminal domain (MycNTD) binding protein. MycNTD, whose activated state promotes tumor formation and metastases by modulating differentiation, cell adhesion and tumor angiogenesis, is essential for cellular transformation as well as mediates critical protein interactions that modulate c-Myc oncogenic properties [24]. Ou et al. [25] demonstrated the induction of CDCA7L by the oncogene c-Myc and their interaction upon exposure to the E_2 analogue 4-OH-tamoxifen. Taken together, these emphasize the role of ERβ in cell differentiation as well as breast cancer. In the WT mice of the present study, E_2 also induced Nnat which is related to adipogenesis [26], mammary tumorigenesis and tamoxifen-resistant mammary carcinoma [27]. The NNAT-induced adipocyte differentiation is accompanied by an elevation of intracellular Ca^{2+} level, enhanced cyclic AMP-response element binding protein phosphorylation and the subsequent induction of adipogenic transcription factors [26]. In the WT mice, Atp1a2, whose product is structurally and functionally linked to the Na^+/Ca^{2+} exchanger and thereby helps to modulate Ca^{2+} transport and signalling [28], was also upregulated by E_2. Thus, it seems that there is a modulation of Ca^{2+} transport and Ca^{2+} signalling in WT mice via Atp1a2 protein which is probably indispensable for the E_2 action via ERs.

In ERαKO mice, we have identified five ERGs, such as Atp2a1, Hrc, Jsrp1, Pvalb and Ryr1, which suggest a unique response of Ca^{2+}. Interestingly, we have found that Ryr1 possesses an ERE. Ryr1, Atp2a1, Hrc and Jsrp1 proteins are responsive for the management of internal Ca^{2+} from endoplasmic reticulum in cytosol, and most of Ca^{2+} bind to the Pvalb protein for buffering [29]. Then, a proportion binds to the effectors that activate various cellular processes that accomplish their functions in a wide temporal spectrum. Moreover, while some channels formed by transport proteins are continuously open, others open only transiently in response to a change in the membrane potential, so-called voltage-gated channels such as Scn7a protein. Thus, the downregulation of this gene by E_2 in the ERαKO mice might permit the cell to avoid a possible depolarization of the membrane which allows the sodium entry [29].

The cytoskeleton is involved in essentially all structural and dynamic aspects of living cells, including maintenance of cell shape, cell movement, cell replication, apoptosis, cell differentiation and cell signalling [30]. In the present study with WT mice, no regulated transcript was intervening in the functions associated to cell structure and motility as well as in cytoskeletal and ECM. However, in the ERαKO mice, numerous genes involved in these functions were regulated by E_2. Among them, we have found EREs which permit to link the direct action of E_2 on ERGs, such as actinin alpha 3 (Actn3), desmin (Des), keratin complex 2 basic gene 6a, keratin complex 1 acidic gene 10 (Krt10), keratin 71, tropomyosin 2 beta (Tpm2) and troponin C2 fast (Tnnc2). Depending on their differentiation state, keratinocytes express different sets of keratins [31]. The loss of Krt10 reduces the tumor formation by an accelerated turnover of keratinocytes, possibly mediated by activation of mitogen activated protein kinase (MAPK) pathways which is probably mediated by Ca^{2+} signalling [31]. In the present study, the trichohyalin (Tchh) was also upregulated. Tchh protein is a multifunctional cross-bridging protein that functions in the inner root sheath and perhaps in other specialized epithelial tissues by coordinating mechanical strength between their peripheral cell envelope barrier structures and their cytoplasmic keratin filament networks [32]. Thus, the upregulation of keratins genes by E_2 seems reflect the activities of cell differentiation. In addition, the only downregulated gene by E_2 in this category, kinesin light chain 1 (Klc1), is involved in binding of cargos permitting the transports of various cargos along microtubules [33]. Thus, the inhibition of Klc1 by E_2 via ERβ could contribute in a process selection of the keratins type which constitutes an indicator of the differentiation state of cells [33]. Moreover, actin influences the organization, the assembly and the movements of keratin intermediate filament networks [33]. In the present study, numerous regulated transcripts were encoding proteins involved in cell motility including ACTN3 which links the ECM with the actin cytoskeleton inside the cell. The suppression of several key cytoskeletal proteins, such as ACTN3 and tropomyosin 1 alpha (TPM1), during neoplastic transformation contributes to the altered cytoskeleton and neoplastic phenotype [34]. Taken together, since Actn3, Des, Tnnc2, Tpm1 and Tpm2 proteins as well as myosin binding protein C fast-type, myosin heavy polypeptide 4 skeletal muscle, myosin light chain phosphorylatable fast skeletal muscle, myosin light polypeptide 1, myozenin 1, titin, troponin I skeletal fast 2 and troponin T3 skeletal fast are contributing in cell reorganization, they can be also implicated in cellular differentiation and constitute some diagnostic makers in neoplastic transformation of E_2 pathway by ERβ. In addition, spermine can enhance E_2-induced cell signalling and cyclin D1 transcription by activation of the p38 MAPK and phosphorylation of activating transcription factor-2, contributing to breast cancer cell proliferation [35]. In the present study, three SAGE tag species corresponding to Sbp are upregulated by E_2 in ERαKO mice probably via ERβ.

4.2. Protein biosynthesis and secretion

In the category of protein biosynthesis, we found only three ERGs, namely Eef1a2, MtRnr1 and Rps13. Eef1a2 protein is a potential oncoprotein in breast cancer [36]. In addition to its role in protein biosynthesis, Eef1a2 protein is also associated with the regulation of cytoskeletal organization forming a variety of sources that bind to F-actin and depolymerise α-tubulin microtubules [37]. The MtRnr1 protein contributes to the structural integrity of the ribosome and allows a ribosome-mediated process in which the information in mRNA is used to specify the sequence of amino acids in the protein [38]. By an upregulation of these genes, E_2 contributes to the process of protein biosynthesis, which is probably realized by ERβ. On the other hand, E_2 downregulated Rps13 whose product is a surface protein at the interface of the ribosomal subunit, which cross-links to initiation factors and is thought to be involved in the initiation of translation, and which is correlated with tumor aggressiveness [39]. In the category of protein secretion, we have identified seven ERGs, such as Mup1, Pbsn, Sbp, Svs2, Svs4, Svs5 and Svs7. Although the physiological functions of these genes in the mammary gland are largely undefined, Pbsn and Svs2 are under androgen regulation [40].

3; **MtRnr1**, 12S ribosomal RNA; **Mup1**, major urinary protein 1; **Mybpc2**, myosin binding protein C fast-type; **Myh4**, myosin heavy polypeptide 4 skeletal muscle; **Myl1**, myosin light polypeptide 1, **Mylpf**, myosin light chain phosphorylatable fast skeletal muscle; **Myoz1**, myozenin 1; **Ndrg2**, N-myc downstream regulated gene 2; **Nnat**, neuronatin; **PIP/PIP$_2$**, phosphatidylinositol 3-phosphate/phosphatidylinositol 3,4-bisphosphate; **Pbsn**, probasin; **Pfkm**, phosphofructokinase muscle; **Pgam2**, phosphoglycerate mutase 2; **Pvalb**, parvalbumin; **Pxdn**, peroxidasin homolog; **Rps13**, ribosomal protein S13; **Ryr1**, ryanodine receptor 1 skeletal muscle; **S100a9**, S100 calcium binding protein A9; **Sbp**, spermine binding protein; **Scn7a**, sodium channel voltage-gated type VII alpha; **Slfn8/Slfn9**, schlafen 8/9; **Slpi**, secretory leukocyte peptidase inhibitor; **Snx5**, sorting nexin 5; **Svs2, 4, 5 & 7**, seminal vesicle secretory protein 2, secretory protein 4, secretory protein 5 and protein secretion 7; **Tchh**, trichohyalin; **Tnnc2, i2 & t3**, troponin C2 fast, I skeletal fast 2 and T3 skeletal fast; **Tpm1 & 2**, tropomyosin 1 alpha and 2 beta; **Ttn**, titin; **Ucp1**, uncoupling protein 1.

4.3. Energy metabolism

E_2 markedly decreases the amounts of fat accumulation and Lpl mRNA in adipocytes stably expressing ERs [41]. Although no classical ERE has been found in the search of Lpl promoter, an AP-1-like TGAATTC sequence is responsible for the suppression of the Lpl gene transcription by E_2 [41]. We have reported that DHT induces Lpl mRNA levels in adipose tissue [42]. In the current WT mice, we found an ERE domain and induction of Lpl by E_2 in the mammary gland. These results should be integrated in the comprehension of E_2 action in Lpl regulation. Moreover, in the ERαKO mice, E_2 downregulated apolipoprotein D (Apod) whose protein plays an important role in the homeostasis and housekeeping, by transporting a hydrophobic ligand [43]. Zhou et al. [44] have demonstrated that the E_2 has no effect on Apod while E_2/androgen suppresses it via androgen effect on ERα. Concordantly, the downregulation of Apod by E_2 in the current study was seen only in the ERαKO mice. On the other hand, all other transcripts related to creatine metabolism (creatine kinase muscle), glycolysis (enolase 3 beta muscle: Eno3, phosphofructokinase muscle and phosphoglycerate mutase 2), oxygen transport (myoglobin), oxidative phosphorylation (cytochrome c oxidase subunit I, III and VIa polypeptide 2, and uncoupling protein 1) as well as ankyrin repeat domain 23 which is a novel nuclear protein highly expressed in type 2 diabetes and insulin resistance model animal [45]. A reduction of creatine kinase levels or its ATP-generating potential has been reported to inhibit the cellular shape changes as well as a signalling pathway that relays signals to the cytoskeleton [46]. Moreover, Eno3 is a gene associated with differentiation and is regulated in tumorigenesis [47].

4.4. Immunity

E_2 leads to the survival and activation of autoreactive cells in the naive repertoire and contributes to the increased frequency of autoimmune disorders in mammals [48]. In immunity, we found three induced ERGs in WT mice, namely H2-D1, peroxidasin homolog (Pxdn) and Slfn8/Slfn9. In contrast, the majority of this class of ERGs in ERαKO mice were downregulated, such as beta-2 microglobulin (B2m), complement component 4B (C4b), immunoglobulin heavy chain 1a (Igh-6), S100 calcium binding protein A9 (S100a9) and secretory leukocyte peptidase inhibitor (Slpi). Pxdn protein is a cell adhesion molecule which is thought to participate in immunological process [49], and Slfn8/Slfn9 belongs to a new family of genes whose proteins have an important role in the regulatory networks governing T cell development and growth [50]. Moreover, B2m protein is a smaller chain partners in a non-covalent way of major histocompatibility complex (MHC) class I resulting from pathogen to cytotoxic T cells. B2m protein plays an essential role allowing endogenous peptide binding at the endoplasmic reticulum from the cytosol by a first association with one H chain to induce a peptide-receptive conformation [51]. We have seen opposite effects in immunity at the level of MHC class 1 with H2-D1 whose protein participates in developing immunity via ERα, contrary to B2m which attenuates it via ERβ. C4b contains an ERE region and is a fragment formed following enzymatic cleavage in the classical pathway of complement [52], and Fc fragment of IgG binding protein has IgG binding activity in several fluids and could inhibit a complement mediated reaction of IgG [53]. Furthermore, a balanced functioning of innate immunity is realised by SLPI, a product of the inflammatory cells that helps to attenuate excessive inflammatory responses [54] as well as by Igh-6 protein which is an antigen receptor on B lymphocytes that regulates B-cell growth and differentiation [55]. In addition, S100a9 positive leukocytes belong to the first group of cells invading inflammatory sites [56]. By microarray studies, S100a9 was identified as a gene inversely correlated to ERα expression in breast tumors [57]. Taken together, our results indicate that E_2 suppresses immunity in ERαKO mice in contrast to WT mice.

4.5. Homeostasis

In the homeostasis, one and four genes were modulated in WT and ERαKO mice, respectively. In the later, we have found a downregulation of haptoglobin (Hp) which has an ERE region. White adipose tissue is known to secrete Hp protein which is implicated in inflammation and stress responses, consequently raised levels are found in diabetes and obesity [58]. On the other hand, aldehyde dehydrogenase 2 mitochondrial (Aldh2), methyltransferase like 6 (Mettl6) and cystatin E/M (Cst6) were found upregulated in ERαKO mice. It was reported that ALDH2 plays a major role as protector against oxidative stress such as acetaldehyde detoxication, which is suspected to influence the risk for Alzheimer's disease [59]. The gene family of Mettl6 plays a variety of roles in different cancer types and probably at different stages of oncogenesis [60]. Moreover, the Cst6 protein activity has been implicated in progression of breast cancer [61]. In WT mice, F3 was upregulated by E_2. Extracellularly, this interaction triggers the blood coagulation cascade, while intracellularly it leads to profound changes in gene expression including several effectors of angiogenesis. In addition, F3 is strongly upregulated by transformed epithelium in cancers of the breast, pancreas, colon and other organs, and acts as a receptor for the coagulation factor VIIa present in plasma [62].

4.6. No match

Interestingly, two SAGE tags (GAAAATGAGAA and GAAAATGATAA) in WT mice were induced by E_2, while downregulated in ERαKO mice. This is again an example of the opposite effect on the common ERGs between WT and ERαKO mice. Moreover, the SAGE tag GAAAATGAGAA was highly expressed in both types of mice, while the SAGE tag GAAAATGATAA was highly expressed only in ERαKO mice. It was reported that ERβ mediated effects on adipose tissue are opposite to those of ERα, although E_2 effects are predominately through ERα [63]. This could be a good example for the opposite effect of E_2 and is in conformity with the fact that the E_2 effect is depending on the presence of various types of ERs in a tissue and promoter context. Despite our results, the exact role of E_2 is complex and numerous mechanisms appear to be cell type specific with many differences due to the different ratios of epithelial versus stromal tissue. Further studies performing immunostaining and in vitro assays, such as luciferase assay or chromatin immunoprecipitation are needed to complement the current study aimed to investigate the complexities of gene expression on a global scale.

5. Conclusion

We have presented ERGs in mammary gland of WT and ERαKO mice in order to improve the mechanistic comprehension of numerous roles of E_2 via ERs. We have made the comparison between the WT and ERαKO mice, considering that the ERαKO group represents E_2/ERβ signalling, while the WT mice represent numerous possibilities such as E_2/ERα, E_2/ERβ and E_2/ERα/ERβ heterodimers signalling which depends of the selection of ER type or antagonist effect of ERβ on ERα. An overview of these data is presented in Fig. 3. In WT mice, E_2-induced transcripts related to immunity, whereas the majority of them were repressed in ERαKO mice. Moreover, induction of transcripts involved in cell differentiation, Ca^{2+} response, cytoskeleton, protein biosynthesis and secretion, glycolysis and oxidative phosphorylation were seen only in ERαKO mice. Interestingly, Snx5 and two no-match tags were inversely regulated by E_2 in WT and ERαKO mice. Thus, the E_2/ERα signalling seems

to be attributed to WT mice. These results reinforce the opposing actions of the signalling type, and constitute useful information to understand the cellular mechanisms of E_2-mediated gene regulations in tissues *in vivo*. The ERGs found in the present study contribute to ameliorate the comprehension of the specific ERs action, which is required to be characterized in order to target specific agonists or antagonists in the treatment of cancer or other pathologies associated with the E_2 action via ERs types.

Conflict of interest

Authors declare no conflict of interest that would prejudice its impartiality.

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