Distinct nuclear receptor expression in stroma adjacent to breast tumors

Kevin C. Knower*[1] and Ashwini L. Chand*[1], Natalie Eriksson[2], Kiyoshi Takagi[3], Yasuhiro Miki[3], Hironobu Sasano[3], Jane E. Visvader[4,5], Geoffrey J. Lindeman[4,6], John W. Funder[1], Peter J. Fuller[1], Evan R. Simpson[1,], Wayne D. Tilley[7], Peter J. Leedman[8], J. Dinny Graham[9], George E. O. Muscat[2], Christine L. Clarke[9] and Colin D. Clyne[1,10]

[1] Prince Henry's Institute, Clayton, Victoria, Australia

[2] Institute for Molecular Biosciences, University of Queensland, Brisbane, Queensland, Australia

[3] Department of Pathology, Tohoku University Graduate School of Medicine, Sendai, Japan

[4] The Walter and Eliza Hall Institute of Medical Research (WEHI), Parkville, Victoria, Australia

[5] Department of Medical Biology, The University of Melbourne, Parkville, Victoria, Australia

[6] Department of Medicine, The University of Melbourne, Parkville, Victoria, Australia

[7] Dame Roma Mitchell Cancer Research Laboratories, Discipline of Medicine, Hanson Institute, University of Adelaide, Adelaide, South Australia, Australia

[8] Laboratory for Cancer Medicine, Centre for Medical Research, Western Australian Institute for Medical Research and School of Medicine and Pharmacology, the University of Western Australia, Perth, Western Australia, Australia

[9] Westmead Millennium Institute, University of Sydney, Westmead, New South Wales, Australia

[10] Department of Molecular Biology and Biochemistry, Monash University, Clayton, Victoria, Australia

* These authors contributed equally to this work

Corresponding author and present address: Dr. Kevin C. Knower; Prince Henry's Institute of Medical Research. PO BOX 5152, Clayton, Victoria. AUSTRALIA, 3168; Email: kevin.knower@princehenrys.org; Phone: +61 3 95943249; FAX: +61 3 95946125

Abstract

The interaction between breast tumor epithelial and stromal cells is vital for initial and recurrent tumor growth. While breast cancer associated stromal cells provide a favourable environment for proliferation and metastasis, the molecular mechanisms contributing to this process are not fully understood. Nuclear receptors (NRs) are intracellular transcription factors that directly regulate gene expression. Little is known about the status of NRs in cancer-associated stroma.

Nuclear Receptor Low Density Taqman Arrays were used to compare the gene expression profiles of all 48 nuclear receptor family members in a collection of primary cultured cancer-associated fibroblasts (CAFs) obtained from estrogen receptor (ER)α positive breast cancers (n=9) and normal breast adipose fibroblasts (NAFs) (n=7). Thirty-three of 48 NRs were expressed in both groups, while 11 NRs were not detected in either. Three NRs (dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1 (*DAX-1*); estrogen-related receptor beta (*ERR-β*); and RAR-related orphan receptor beta (*ROR-β*)) were only detected in NAFs, whilst one NR (liver receptor homolog-1 (*LRH-1*)) was unique to CAFs. Of the NRs co-expressed, four were significantly down-regulated in CAFs compared to NAFs (RAR-related orphan receptor-α (*ROR-α*); Thyroid hormone receptor-β (*TR-β*); vitamin D receptor (*VDR*); and peroxisome proliferator-activated receptor-γ (*PPAR-γ*)). Quantitative immunohistochemistry for LRH-1, TR-β and PPAR-γ proteins in stromal fibroblasts from an independent panel of breast cancers (ER-positive (n=15), ER-negative (n=15), normal (n=14)) positively correlated with mRNA expression profiles.

The differentially expressed NRs identified in tumor stroma are key mediators in aromatase regulation and subsequent estrogen production. Our findings reveal a distinct pattern of NR expression that therefore fits with a sustained and increased local estrogen microenvironment in ER-positive tumors.

NRs in CAFs may provide a new avenue for the development of intratumoral-targeted therapies in breast cancer.

Keywords: nuclear receptors, breast cancer, stroma, aromatase, oestrogen, tumor microenvironment

1. Introduction

Tumor epithelial and stromal cell interaction is vital for initial and recurrent breast cancer growth. The stromal environment consisting of fibroblasts, endothelial and immune cells, plays a critical function not only in normal mammary gland morphogenesis but also provides the ideal microenvironment for tumor growth (reviewed in [1, 2]). Key initiating events in the formation of tumors include the active recruitment, by oncogenic precursor tumor cells, of stromal cell types such as endothelial cells and macrophages, all of which induce the required adaptive changes to the microenvironment [3]. While the stroma provides a scaffold for the breast, it also regulates epithelial cell function through paracrine, physical and hormonal exchanges.

Tumor growth is supported by the adjacent stromal tissue comprising mainly of fibroblasts (termed cancer-associated fibroblasts, CAFs) via production of hormones such as estrogens [4, 5] and inflammatory cytokines such as interleukin 6 (IL-6) and stromal cell-derived factor 1α (CXCL12) [6], growth factors such as Transforming Growth Factor β (TGFβ) [7] and the extracellular matrix remodelling enzymes such as the matrix metalloproteinases- 1 and -7 [8]. CAFs behave similarly to wound repair fibroblasts that increase epithelial growth through the secretion of cytokines, growth and extracellular matrix factors [9, 10]. However, unlike wound-healing fibroblasts, CAFs remain activated not undergoing quiescence or apoptosis, as seen during wound closure [11].

For the majority of breast cancers, tumor growth is initiated in the epithelial compartment and is confined to the ducts (ductal carcinoma *in situ*, DCIS) but as the ductal layer breaks down, invasion of epithelial cells occurs (invasive ductal carcinoma, IDC). During these processes, the stroma through cell-cell and cell-microenvironment interactions regulates proliferation, survival, polarity, differentiation and invasive capacity of epithelial cells [1, 3, 12, 13]. The importance of stroma in tumor growth has been functionally demonstrated in animal models. For example, the targeted over-

expression of matrix metalloproteinase 3/ stromelysin-1 in the stroma promoted mammary tumorigenesis [14] and irradiation of the mammary stroma induced tumor growth more rapidly compared with non-irradiated tissue [15]. Among the stromal cells, the fibroblasts are known to have a prominent role in tumor progression. The understanding of the gene networks and pathways mediated by the neoplastic stroma or CAFs is poorly understood.

Nuclear receptors (NRs) are intracellular transcription factors that directly regulate gene expression in response to endocrine hormones and/or lipophilic molecules. Together, NRs affect a wide variety of functions, including development, cholesterol homeostasis, steroidogenesis, reproductive function and metabolism. They are also critically involved in various cancers, in particular breast cancer [16, 17]. This is highlighted by a recent study that discriminates the prognostic value of NRs in breast cancer and identifies novel, clinically relevant, NR signatures [17]. To date, NR expression in stroma has not been specifically addressed.

At the cellular level, NRs regulate proliferation, differentiation and apoptosis mainly via the transcriptional regulation of target gene expression and as major points of convergence of multiple signal transduction pathways. Many of the NRs act as ligand-inducible transcription factors, responding to endogenous and exogenous hormones. There is a distinct subset of NRs that do not have identifiable ligands and are termed orphan NRs. As such, these orphan NRs provide an important avenue for the discovery of novel interacting compounds, with potential impact on many disease outcomes [18, 19]. NRs such as ERα and PR are currently the most important clinical indicators of breast tumor type and stage, response to treatment and prognosis. In addition, data from ERα and PR knockout mouse models demonstrate the functional significance, in particular, of stromal PR in normal mammary gland development [20, 21].

The NR superfamily comprises of 48 different receptors [22]. Little is known regarding the expression of the majority of these NRs in either normal breast stroma or CAFs. We therefore profiled the expression of these forty-eight NRs in breast CAFs obtained from 9 patients with ERα-positive tumors and compared these to breast adipose fibroblasts (NAFs) derived from normal control

subjects. We identified four NRs to be significantly down-regulated in CAFs compared to NAFs: *ROR-α, TR-β, VDR* and *PPAR-γ*. The NRs *DAX-1*, *ERR-β* and *ROR-β* were only detected in NAFs while *LRH-1* was detected only in CAFs. Protein expression levels for LRH-1, TR-β, and PPAR-γ in stromal fibroblasts was confirmed by immunohistochemistry using a larger independent panel of normal and malignant breast tissues. These results identify novel NR targets in CAFs, suggesting these may play important roles in mediating interactions between epithelial and stromal cells within the tumor microenvironment.

2. Materials and Methods

2.1 Patient Information and cell culture

The patient sample group used for TaqMan Low-Density Arrays comprised of women with ERα positive breast tumors (n=9) (Table 1). Primary CAFs were obtained from breast tissue by either centrifugation or cell sorting methodologies as previously described [23, 24]. Clinical details of the patients, tumor type, source and isolation method are summarized in Table 1. As controls, NAFs (n=7) were either obtained from adjacent nonpathological breast tissues (outlined in Table 1) or from women undergoing reduction mammoplasty. Subcutaneous adipose tissue was obtained from cancer-free women at the time of reduction mammoplasty approved by the Southern Health Human Ethics Research Committee at Prince Henry's Institute. Breast tumor specimens were obtained from either Japanese female patients at Tohoku University Hospital and Tohoku Kosai Hospital (Tohoku University School of Medicine and Tohoku Kosai Hospital approved the research protocols (2004-144, 2005-068, and 2006-042, respectively)) or the Victorian Cancer Biobank (with approval from the Human Research Ethics Committees of The Walter and Eliza Hall Institute of Medical Research and Melbourne Health) (Table 1). The histological grade of each specimen was independently evaluated. Informed consent was obtained from these patients before surgery in each institution. All fibroblast cell lines, irrelevant of their source, were maintained in DMEM/F12 growth media supplemented with 15% fetal calf serum and antibiotics as previously described [25, 26]. Validation of NAF and CAF sample groups was performed through the use of the established CAF cell marker Stromal Derived

Factor 1 (*SDF1*) [27]. Significant increases in *SDF-1* mRNA levels were detected in CAFs compared to NAFs in line with previous findings [27, 28] (Supplementary Figure 1).

For immunohistochemical studies, formalin-fixed paraffin-embedded tissue sections of breast cancer and normal breast tissues were obtained from the Australian Breast Cancer Tissue Bank (abctb.org.au) and the Victorian Cancer BioBank (viccancerbiobank.org.au). Tissue sections of normal breast tissue biopsies were obtained from the Susan G. Komen for the Cure Tissue Bank at the IU Simon Cancer Center. Normal tissues were from women with no known history of breast disease and were collected following reduction mammoplasty or from volunteers who donated normal breast tissue biopsies. Breast cancer cases were primary invasive ductal carcinomas, with known hormone receptor status, tumour grade and age at diagnosis. The characteristics of the patient group are described in Table 2. All tissues were obtained with informed consent from donors, and the use of tissues received approval from the human research ethics committees of the participating institutions.

2.2 RNA isolation and QPCR

Total RNA was isolated using the RNeasy Kit according to manufacturers' instructions (Qiagen Australia), DNAse (Ambion) treated to remove contaminating DNA. Reverse transcription of 1.5 μg of total RNA was performed using random hexamers and SuperScript III reverse transcriptase (Invitrogen). To perform quantitative real time pCR, we used TaqMan Low-Density Array (TLDA) micro-fluidic cards (Applied Biosystems, Catalog no. 4379961) which contain custom designed primers for 48 human NRs and 16 control genes. PCR was performed on the ABI 7900HT Real-Time instrument. Briefly, a total of 100 μl reaction mixture with 50 μl cDNA template (330 ng) and an equal volume of TaqMan® universal master mix (Applied Biosystems) was added to each line of TLDA reservoir. PCR conditions were as follows: 2 min at 50°C, 10 min at 94.5°C and 30 s at 97°C, and 1 min at 59.7°C for 40 cycles. Validation of CAF cells was performed using qRT-PCR primers for SDF-1 Fwd 5'-CTC AAC ACT CCA AAC TGT GCC C and Rev 5'-CTC CAG GTA CTC CTG AAT CCA C; β-actin Fwd 5′-TGC GTG ACA TTG CGT GAC ATT AAG GAG AAG-3′ and Rev 5′-GCT CGT AGC TCT TCT CCA in PCR conditions as described above.

2.3 Data Analysis

Data analysis and normalisation to control gene expression was performed with the ABI StatMiner Software as per manufacturers' instructions and detailed methods previously published [29]. The geNorm software (Applied Biosystems) analysis used to select the most stable endogenous controls for data normalisation. The threshold cycle Ct was automatically given by SDS2.2 software package (Applied Biosystems). A Ct >35 was deemed as transcript not detected. Relative quantities (RQ) were determined using the equation: $RQ = 2^{-\Delta\Delta Ct}$. Data is expressed as mean $\pm$ SD. Wilcoxon Non-Parametric test was utilised for statistical analysis and a $P < 0.05$ was considered significant.

2.4 Immunohistochemistry

Immunoperoxidase staining was performed using anti-LRH-1 (NR5A2) rabbit polyclonal (Sigma-Aldrich, Castle Hill, Australia), anti-PPAR-γ mouse monoclonal (Santa Cruz Biotechnology, CA, USA) and anti-TR-β mouse monoclonal (Thermo-Fisher Scientific, North Ryde, Australia) antibodies. Tissues were first heat treated under pressure in citrate buffer, as described previously [30], to reveal epitopes. Primary antibody incubations were performed at 4°C, overnight, either in phosphate buffered saline (pH7.5) containing 0.5% Triton X-100 (PPAR-γ and TR-β) or using Ultravision polymer reagents (LRH-1) as described by the manufacturer (Thermo-Fisher Scientific). Primary antibody binding was revealed by subsequent incubation 30 minutes at room temperature with biotinylated goat secondary antibodies (Dako Australia, Botany, Australia), then one hour, room temperature incubation with streptavidin-horseradish peroxidase conjugate (Dako Australia), followed by colour development with diaminobenzidine substrate solution (Dako Australia). Stained sections were scanned using a Hamamatsu Nanozoomer digital slide scanner. Staining was scored using a three level intensity score and normalizing to percent positive detection as previously described [31]. Results were independently reviewed by two additional investigators who validated the original scoring result. All three observers were experienced researchers with substantial expertise in breast tissue pathology and immunohistochemistry.

3. Results

To assess the relative expression profiles of NRs in breast stromal cells we utilised Low Density Taqman Arrays that detect all 48 NR family members along with 16 internal control genes. Of the 48 NRs, 11 NRs were not detected in either group, while 36 were expressed in the groups of NAFs compared to 34 in the CAF group (Figure 1, Figure 2 and Table 3). When grouped based on their sub-family classifications, both the NAF and CAFs exhibited similar NR expression profiles for endocrine (11 of 12) and adopted orphan (8 of 11) NRs. In contrast, the expression profile of orphan NRs differed between the two study groups. In the NAF samples, 17 of the total 25 orphan NRs were expressed while in the CAF samples 15 were detected out of the 25 orphan NRs (Table 3). The three orphan NRs that were exclusively detected in NAF samples included *DAX-1*, *ERR-β* and *ROR-β* (Figure 1). Interestingly, the expression of the orphan receptor *LRH-1* was detected exclusively in CAF samples (Figure 1). Eleven NRs were consistently not detected in either group - *CAR*, *ER-β*, *ERR-γ*, *FXR*, *HNF4α*, *PNR*, *ROR-γ*, *RXR-γ*, *SF-1*, *SHP* and *TLX* (Figure 2).

Thirty-three NRs were found to be expressed in both NAF and CAF sample groups (Figure 1). The relative expression of these NRs detected in both groups varied greatly, ranging from the highly expressed *GR*, *COUP-TF2* and *LXR-β* NRs to those at very low levels such as *ER-α* or *HNF4-γ* (Figure 2). Despite this, variance in expression patterns of NRs between patient samples was strikingly low. As described above, we identified four orphan NRs that had a defined expression detected in only NAFs (*DAX-1*, *ERR-β* and *ROR-β*) or CAFs (*LRH-1*). Of the 33 NRs detected in both groups, we identified four NRs that showed significant differences in expression between groups. Significantly down-regulated in CAFs compared to NAFs were the endocrine NRs *TR-β* and *VDR*; the adopted orphan NR *PPAR-γ*; and the orphan NR *ROR-α* (Figure 3).

With the exception of *VDR* and *ROR-α*, mRNA levels of those NRs identified as having a differential expression pattern between normal and tumor stromal cells was relatively low in comparison to the majority of NRs expressed in either group. To confirm mRNA expression profiles correlated to those of protein, quantitative immunohistochemistry was performed on three differentially expressed NRs

identified by Taqman Arrays - LRH-1, TR-β and PPAR-γ (Figure 4). A larger independent panel of IDC breast cancers was used for the analysis (ER-positive (n=15), ER-negative (n=15), normal (n=14)) (Table 2). ER-negative samples were used in this instance to determine if changes in NR expression levels were related to ER-status. Immunohistochemical analysis of the NRs in stromal fibroblasts correlated to those identified at the mRNA level. In comparison to normal tissue, both TR-β and PPAR-γ stromal staining was significantly reduced in ER-positive tumors (Figure 4B). Similarly in ER-negative tumors, both TR-β and PPAR-γ levels were reduced compared to normal; however the difference in staining was only significant for PPAR-γ (Figure 4B). Independent of ER-status, PPAR-γ stromal staining was significantly less in the tumor samples compared to normal; and while close to being statistically significant (p=0.053), TR-β levels were also reduced compared to normal tissue regardless of ER-status. Conversely, LRH-1 stromal staining was significantly elevated in both ER-positive and ER-negative tumors, a process also independent on ER-status (Figure 4B).

In summary, analysis of breast stromal fibroblasts indicates a predominantly (5 out of 8) altered pattern of expression of orphan NRs between normal and tumour. Four of these orphan NRs (*DAX-1*, *ERR-β, ROR-β* and *LRH-1*) are restricted to either sample group. Interestingly, four of thirty-three co-expressed NRs were significantly down-regulated in CAFs; these include *TR-β, VDR, PPAR-γ;* and *ROR-α*. An increase in LRH-1 was observed in CAFs compared to NAFs. This reflected the observation that although LRH-1 was detected in a subset of NAFs in most tissues, the percent positivity of LRH-1 expression in CAFs was markedly higher leading to the statistically significant difference observed. Correlation of the protein levels of LRH-1, TR-β and PPAR-γ in an independent breast cancer sample group shows consistency to NR array data. Collectively, our data suggests that differential expression of these nuclear receptors in ER-positive and ER-negative stroma adjacent to a tumour may be an important facet of tumour progression.

4. Discussion

Cancer-associated fibroblasts form a heterogeneous population either in response to tumour heterogeneity and/or differences in origin of cell types [32, 33, 34]. This altered stromal environment

that consists of fibroblasts, endothelial and immune cells has significant impact on tumour growth. However what gene networks and pathways contribute to transforming the preneoplastic and cancer-associated fibroblasts are poorly understood. Majority of work characterizing CAFs has focused on alterations to ECM proteins [35], which describe the "activated fibroblast" phenotype [36], secretion of factors such as TGFB and SDF-1 [6] and aromatase production [4, 5]. Our study is the first focused investigation into expression profiles of nuclear receptors in tumor adjacent stroma in ER-positive breast cancers.

Among the similarities, eleven NRs were consistently not detected in either NAFs or CAF samples. Of these, similar results were found for *SHP*, *HNF4α*, *TLX*, *FXR*, *CAR* and *SF-1* in a larger NR profiling study that used whole normal, ER-negative and ER-positive breast tumor tissue [17]. The absence of *PR*, *ROR-γ* and *RXR-γ* expression in the breast stroma had previously not been demonstrated, however the lack of *ER-β* and *ERR-γ* expression is in contrast to previous observations [37, 38, 39].

Eight NRs showed differential mRNA expression profiles in normal and tumor-associated stroma. The expression of *LRH-1* was only detected in CAFs, whereas the NRs *DAX-1*, *ERR-β* and *ROR-β* were detected exclusively in NAFs. Furthermore, we identified four NRs to be significantly down-regulated in CAFs compared to NAFs - *ROR-α*, *TR-β*, *VDR* and *PPAR-γ*. With the exception of *VDR* and *ROR-α*, the identified NRs are expressed at relatively low levels in both sample groups. Despite this, immunohistochemical staining of breast tumour tissue confirmed that protein expression patterns of the NRs LRH-1, TR-β, and PPAR-γ in tumour-associated stroma in ER-positive and ER-negative IDC of the breast matched the mRNA transcript data. While in the NR array data LRH-1 mRNA levels were undetectable in normal fibroblasts, immunohistochemical staining revealed LRH-1 expression in normal breast stroma (Figure 4A). We observed increased LRH-1 immunostaining in tumour-adjacent stroma, consistent with previous reports [40, 41]. Furthermore, we have recently shown discordance between LRH-1 transcriptional variants and protein levels in ER-positive *versus* ER-negative breast cancer cell lines, a mechanism mediated by differences in mRNA stability and

ER-status [42]. While we have not investigated if this discordance holds true for stromal fibroblasts, it is a possibility worth pursuing in the future. The isolation and growth of CAFs and NAFs in culture may also be reflective of this discordance when compared to expression patterns in tissue biopsies.

LRH-1 is well described as playing a prominent role in breast cancer where it is capable of inducing tumor cell proliferation via co-regulation of ER-α target genes, tumour cell migration and invasion [40, 43, 44, 45]. As a consequence, targeting LRH-1 in the breast is an area of intense focus [46, 47]. In support of this, LRH-1 is a key mediator in the activation of the *CYP19A1* gene that encodes the cytochrome P450 enzyme aromatase [40, 48, 49, 50] to increase intratumoral estrogen levels in the tumor stroma [4, 5, 41]. Targeting this process is hypothesized to inhibit estrogen production in a breast specific manner.

We also observed significantly reduced mRNA and protein levels for TR-β and PPAR-γ in tumour-associated stroma when compared with normal stroma. Little is known about the expression patterns of TR-β and PPAR-γ in clinical biopsies, and in the tumor stroma in particular, although both NRs have clear established roles in mammary tumour development and the utility of their ligands as potential breast cancer treatments is of current interest [51]. The effects of a lack of functional TR-β in the development of mammary tumours is evident in an elegant *in vivo* model study where a homozygous loss of function mutation of *Thrb* gene causing a frameshift [52, 53, 54] resulted in increased mammary gland hyperplasia and tumor incidence, in heterozygous PTEN knockout mice [55]. While *in vivo* deletion of *Thrb* gene in the stromal and epithelial compartments may provide further insight into mechanisms affected by TR-β, the study by Guigon and co-workers presents strong functional evidence in support of clinical data where several mutations in *TR-β* gene correlate with increased breast cancer incidence [56].

PPAR-γ has well defined role in adipogenesis involving preadipocyte differentiation into mature adipocytes [57] and its roles in mammary tumorigenesis is also well established. PPARγ delays the *in vivo* progression of 7,12-dimethylbenz[a]anthracene (DMBA)-mediated breast tumours as revealed with PPARγ haploinsufficient mice [58]. In addition, adipocyte-specific PPARγ knockout

mice, tumour incidence is great than that in the control group treated rosiglitazone [59]. Interestingly, rosiglitazone treatment reduced mammary tumour volumes by 50% and this may be via reduction in *BRCA1* expression in mammary stromal adipocytes. Additionally, clinical studies demonstrate an inverse association of PPAR-γ expression with invasive breast cancer [60] and decreased tumor recurrence [61] in patients.

A well-described mechanism via which the tumour-associated stroma contributes to tumour growth is via paracrine estrogen production. The reduced expression of *ROR-α, TR-β, VDR, ERR-β, DAX-1* and *PPAR-γ* and increased *LRH-1* levels in CAFs all have a demonstrated net effect on stromal estrogen synthesis pathway. Adipose tumor stromal cells are the major source of local estrogen in post-menopausal women with ER-positive breast cancer [4, 5]. While LRH-1 has been shown to have a clear role in oestrogen production through the up-regulation of the aromatase gene, via the cancer-associated tissue-specific promoter PII, it is also interesting to note that ROR-α, TR-β, VDR, ERR-β and PPAR-γ, identified in this study with reduced expression, have previously been shown to repress aromatase transcription. We have previously demonstrated that PPAR-γ ligands inhibit PII-derived aromatase expression in breast adipose stromal cells [62]; the TR-β ligand triiodothyronine (T3) is capable of silencing PII-derived aromatase activity in mouse Sertoli cells [63]; calcitriol induces VDR binding and repression of PII-mediated expression and subsequent oestrogen synthesis *in vitro* [64] and *in vivo* [65]; and finally DAX-1 is known to antagonizes aromatase expression in a number of instances [66, 67, 68, 69]. Given the importance of aromatase expression in the breast tumor stroma, our findings reveal a pattern of NR expression that fits with a sustained and increased local estrogen microenvironment. The precise mechanism of the action of these NRs on aromatase in the breast stroma warrants further investigation.

We have identified a distinct set of NRs that may have an important contribution in causing the alterations in the stromal environment in response to a tumour and/or establishing the pre-neoplastic niche for tumour initiation. These NRs have been demonstrated to have roles in mammary development and tumorigenesis. The combined effect of reduced expression of these NRs may result

in significant alterations to the tumour stromal environment via various cellular mechanisms some of which are discussed.

The reduced expression of orphan nuclear receptors ROR-α and related ROR-β, may contribute to breast tumour growth via the regulation of inflammatory pathways. ROR-α is a negative regulator of inflammation, suppressing TNFα-induced expression of COX-2, IL-6 and IL-8 via the inhibition of the NF-κB promoter [70], thereby suppressing tumour metastasis [71]. Moretti *et al* demonstrate that the activation of ROR-α with a selective ligand (CGP 52608) decreases tumor cell proliferation, migration and invasion [72], suggesting that the activation of ROR-α may also be of therapeutic benefit in breast cancers targeting tumor growth [73].

VDR has known roles in the suppression of cancer cell invasion, angiogenesis and metastasis [74, 75, 76]. Indeed VDR has important functions in regulating normal mammary development; in *Vdr* knockout female mice there is more extensive ductal elongation and branching compared to wild-type, increased responsiveness to estrogen and progesterone, represented by increases in ductal epithelial cell proliferation [77]. In the context of our observations, the decrease in VDR expression in the adjacent tissue may thus be a tumor-initiating event whereby the surrounding tissue is more responsive to hormones and for hyperplastic growth of ductal epithelial cells.

The ERRs are highly homologous in structure and functionality [78]. Their primary role is the regulation of cellular metabolism regulation of the glycolytic pathway, the tricarboxylic acid (TCA) cycle and oxidative phosphorylation (reviewed in [79]). This function is particularly relevant in rapidly proliferating cells such as breast tumour cells [80, 81]. While most of these functions are assigned to ERR-α and ERR-γ, specifically ERR-β has been shown to regulate pluripotency in embryonic stem cells and function as a reprogramming factor for the generation of induced pluripotent cells [82, 83, 84]. The loss of expression of ERR-β expression in CAFs may thus signify a distinct change in cell reprogramming.

The orphan nuclear receptor DAX-1 is found to be expressed in NAF but not CAF samples. DAX-1 is unique in that it lacks a DNA binding motif, however, it heterodimerizes with many

members of the NR superfamily to inhibit their activity. Hence a relatively small change in DAX-1 expression may potentially have a profound effect on NR function. Highly expressed in gonadal tissue, knockout of Dax-1 gene in mice results in spermatogenic failure, while in humans abnormalities in DAX-1 genes can lead male-to-female sex reversal [85]. Its roles in cancer have not been fully studied. DAX-1 interacts with the NR5A subfamily of orphan NRs (LRH-1 and SF-1) in several tissues to repress aromatase transcription [66, 67, 68, 69, 86, 87]. Hence its repression may also contribute to the overall increase in aromatase expression in CAFs.

In conclusion, we provide a cell specific NR signature in the cancer microenvironment that may individually or collectively impact tumor proliferation and metastasis. The inter-relationships between NRs, their interactions with co-regulator proteins make them a complex network of transcription factors with wide ranging cellular functions. With this in mind, we suggest that further studies should profile stromal cell characteristics adjacent to the tumor where correlations to steroid receptor status, grade and stage will aid in the development of early prognostic markers.

Figure Legends

Figure 1. Expression of NRs in NAFs and CAFs. Thirty-three NRs are detected in each sample group while DAX-1, ERR-β and ROR-β are uniquely detected in NAFs while LRH-1 is only detected in CAFs.

Figure 2. Expression profiles of NRs in (A) NAFs and (B) CAFs. The levels of NR expression are indicated by the pie charts, and their names are shown in the tables to the right. A ranking of highest to lowest expression is detailed in the lower panel. Normalized NR mRNA-expression levels were defined as Absent if the PCR Ct value was >35, Low if the level was below 1.0 arbitrary units, Moderate if the level was between 1.0 and 10.0, and High if the level was greater than 10.0 arbitrary units.

Figure 3. NR relative expression in NAFs vs CAFs. Normalized NR mRNA-expression levels as grouped in sub-family. Primary cultured CAFs (n=9) and NAFs (n=7). StatMiner Analytical software

was used to perform Non-Parametric (Wilcoxon) tests while geNorm selected the most stable endogenous controls used for normalisation. *p<0.05. Error bars represent standard error of means.

Figure 4. Immunohistochemical staining of LRH-1, TR-β and PPAR-γ in IDC breast cancers. (A) Representative images showing stromal and epithelial cell staining from a panel of ER-positive, ER-negative tumors and normal tissue (Table 2). Scale Bar – 100 μm. (B) Stromal fibroblast staining was scored using a three level intensity score and normalized to percent positive detection. Positive immuno staining is shown in brown while negative control is shown in insert. Student t tests (2 tailed, equal variance for ER-positive/negative vs normal; unequal for cancer vs normal) were performed. ER-positive (n=15), ER-negative (n=15), Normal (n=14). *p<0.05, **p<0.01. Error bars represent standard error of means.

Table 1. The clinicopathological information of breast cancers in which stromal cells were obtained and used for NR arrays. [1] Japanese female patients at Tohoku University Hospital and Tohoku Kosai Hospital, CAFs isolated by collagenase digestion and centrifugation [25]. [2] Obtained from the Victorian Cancer Biobank following cell sorting methodologies [23]. Histological grade was determined according to Robbins et al. and each score represent that of tubular formation, nuclear atypia and mitosis [88]. ER and PR immunoreactivity was evaluated according to Allred et al. [89]. PS: proportional score, IS: Intensity score and TS: total score. HER2 immunoreactivity was evaluated based on the CAP-ASCO guideline [90].

Table 2. The clinicopathological information of breast cancers and normal tissue used for immunohistochemistry. Refer to Materials and Methods for further description.

Table 3. Summary of nuclear receptor expression profiles in NAF and CAF samples groups. Numbers depict amount of nuclear receptors expressed from each sub-classification group. Changes in expression of nuclear receptors (↑ elevated or ↓ decreased) when comparing CAF to NAF sample groups.

Supplementary Figure 1. Supplementary Figure 1. Validation of CAFs using marker SDF-1. RNA isolated from NAFs (n=7) and CAFs (n=9) was used for qRT-PCR of the CAF marker SDF-1. Significant levels of SDF-1 mRNA were detected in CAFs compared to normal. Two-tailed independent t-test *$p<0.05$. Error bars represent standard error of means.

Acknowledgements: This work was supported by the National Health and Medical Research Council of Australia through fellowships to CDC (#338518), JEV (Australia Fellow) and GJL (637307), ERS, PJF and CLC and NHMRC IRIISS; GEOM is a University of Queensland Vice Chancellors research fellow; the Victorian State Government through Victoria Cancer Agency funding of the Victoria Breast Cancer Research Consortium and the Victorian Government's Operational Infrastructure Support Program; the United States Department of Defense fellowship to ALC; a Collaborative Program from the National Breast Cancer Foundation Australia;

Breast cancer and normal tissues were provided by Australian Breast Cancer Tissue Bank (ABCTB), which is supported by the National Health and Medical Research Council of Australia, the Cancer Institute NSW and the National Breast Cancer Foundation; or by the Victorian Cancer BioBank Australia, which is supported by the Victorian Government. ABCTB tissues and samples were made available to researchers on a non-exclusive basis. Normal breast biopsies were obtained from the Susan G. Komen for the Cure Tissue Bank at the IU Simon Cancer Center. We thank contributors to the Susan G. Komen for the Cure Tissue Bank, including Indiana University that collected the samples used in this study, as well as parents and families whose participation and help made this work possible. We would like to thank Drs Elgene Lim and François Vaillant for their assistance in stromal cell collection and Silke Kantimm for assistance with immunohistochemistry. PHI Data Audit #13-08.

Conflicts: The authors declare no conflicts of interest.

References

1. Kalluri, R. and M. Zeisberg, Fibroblasts in cancer, Nat Rev Cancer, 2006. 6(5): p. 392-401.

2. Wiseman, B.S. and Z. Werb, Stromal effects on mammary gland development and breast cancer, Science, 2002. 296(5570): p. 1046-9.

3. Bhowmick, N.A., E.G. Neilson, and H.L. Moses, Stromal fibroblasts in cancer initiation and progression, Nature, 2004. 432(7015): p. 332-7.

4. Zhao, Y., V.R. Agarwal, C.R. Mendelson, and E.R. Simpson, Estrogen biosynthesis proximal to a breast tumor is stimulated by PGE2 via cyclic AMP, leading to activation of promoter II of the CYP19 (aromatase) gene, Endocrinology, 1996. 137(12): p. 5739-42.

5. Simpson, E.R. and Y. Zhao, Estrogen biosynthesis in adipose. Significance in breast cancer development, Ann N Y Acad Sci, 1996. 784: p. 18-26.

6. Allinen, M., R. Beroukhim, L. Cai, C. Brennan, J. Lahti-Domenici, H. Huang, D. Porter, M. Hu, L. Chin, A. Richardson, S. Schnitt, W.R. Sellers, and K. Polyak, Molecular characterization of the tumor microenvironment in breast cancer, Cancer Cell, 2004. 6(1): p. 17-32.

7. Gold, L.I., The role for transforming growth factor-beta (TGF-beta) in human cancer, Crit Rev Oncog, 1999. 10(4): p. 303-60.

8. Egeblad, M. and Z. Werb, New functions for the matrix metalloproteinases in cancer progression, Nat Rev Cancer, 2002. 2(3): p. 161-74.

9. Mueller, M.M. and N.E. Fusenig, Friends or foes - bipolar effects of the tumour stroma in cancer, Nature Reviews Cancer, 2004. 4(11): p. 839-49.

10. Serini, G. and G. Gabbiani, Mechanisms of myofibroblast activity and phenotypic modulation, Experimental Cell Research, 1999. 250(2): p. 273-83.

11. Desmouliere, A., M. Redard, I. Darby, and G. Gabbiani, Apoptosis mediates the decrease in cellularity during the transition between granulation tissue and scar, Am J Pathol, 1995. 146(1): p. 56-66.

12. Mueller, M.M. and N.E. Fusenig, Friends or foes - bipolar effects of the tumour stroma in cancer, Nat Rev Cancer, 2004. 4(11): p. 839-49.

13. Li, H., X. Fan, and J. Houghton, Tumor microenvironment: the role of the tumor stroma in cancer, J Cell Biochem, 2007. 101(4): p. 805-15.

14. Sympson, C.J., M.J. Bissell, and Z. Werb, Mammary gland tumor formation in transgenic mice overexpressing stromelysin-1, Semin Cancer Biol, 1995. 6(3): p. 159-63.

15. Barcellos-Hoff, M.H. and S.A. Ravani, Irradiated mammary gland stroma promotes the expression of tumorigenic potential by unirradiated epithelial cells, Cancer Res, 2000. 60(5): p. 1254-60.

16. Conzen, S.D., Minireview: nuclear receptors and breast cancer, Molecular Endocrinology, 2008. 22(10): p. 2215-28.

17. Muscat, G.E., N.A. Eriksson, K. Byth, S. Loi, D. Graham, S. Jindal, M.J. Davis, C. Clyne, J.W. Funder, E.R. Simpson, M.A. Ragan, E. Kuczek, P.J. Fuller, W.D. Tilley, P.J. Leedman, and C.L. Clarke, Research Resource: Nuclear Receptors as Transcriptome: Discriminant and Prognostic Value in Breast Cancer, Molecular Endocrinology, 2013.

18. Lazarus, K.A., D. Wijayakumara, A.L. Chand, E.R. Simpson, and C.D. Clyne, Therapeutic potential of Liver Receptor Homolog-1 modulators, J Steroid Biochem Mol Biol, 2012. 130(3-5): p. 138-46.

19. Kliewer, S.A., J.M. Lehmann, and T.M. Willson, Orphan nuclear receptors: shifting endocrinology into reverse, Science, 1999. 284(5415): p. 757-60.

20. Humphreys, R.C., J. Lydon, B.W. O'Malley, and J.M. Rosen, Mammary gland development is mediated by both stromal and epithelial progesterone receptors, Mol Endocrinol, 1997. 11(6): p. 801-11.

21. Mueller, S.O., J.A. Clark, P.H. Myers, and K.S. Korach, Mammary gland development in adult mice requires epithelial and stromal estrogen receptor alpha, Endocrinology, 2002. 143(6): p. 2357-65.

22. Margolis, R.N., R.M. Evans, and B.W. O'Malley, The Nuclear Receptor Signaling Atlas: development of a functional atlas of nuclear receptors, Molecular Endocrinology, 2005. 19(10): p. 2433-6.
23. Lim, E., F. Vaillant, D. Wu, N.C. Forrest, B. Pal, A.H. Hart, M.L. Asselin-Labat, D.E. Gyorki, T. Ward, A. Partanen, F. Feleppa, L.I. Huschtscha, H.J. Thorne, S.B. Fox, M. Yan, J.D. French, M.A. Brown, G.K. Smyth, J.E. Visvader, and G.J. Lindeman, Aberrant luminal progenitors as the candidate target population for basal tumor development in BRCA1 mutation carriers, Nat Med, 2009. 15(8): p. 907-13.
24. Yamaguchi, Y., H. Takei, K. Suemasu, Y. Kobayashi, M. Kurosumi, N. Harada, and S. Hayashi, Tumor-stromal interaction through the estrogen-signaling pathway in human breast cancer, Cancer Research, 2005. 65(11): p. 4653-62.
25. Miki, Y., T. Suzuki, C. Tazawa, Y. Yamaguchi, K. Kitada, S. Honma, T. Moriya, H. Hirakawa, D.B. Evans, S. Hayashi, N. Ohuchi, and H. Sasano, Aromatase localization in human breast cancer tissues: possible interactions between intratumoral stromal and parenchymal cells, Cancer Research, 2007. 67(8): p. 3945-54.
26. Knower, K.C., S.Q. To, K. Takagi, Y. Miki, H. Sasano, E.R. Simpson, and C.D. Clyne, Melatonin suppresses aromatase expression and activity in breast cancer associated fibroblasts, Breast Cancer Res Treat, 2012. 132(2): p. 765-71.
27. Orimo, A., P.B. Gupta, D.C. Sgroi, F. Arenzana-Seisdedos, T. Delaunay, R. Naeem, V.J. Carey, A.L. Richardson, and R.A. Weinberg, Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion, Cell, 2005. 121(3): p. 335-48.
28. Soon, P.S., E. Kim, C.K. Pon, A.J. Gill, K. Moore, A.J. Spillane, D.E. Benn, and R.C. Baxter, Breast cancer-associated fibroblasts induce epithelial-to-mesenchymal transition in breast cancer cells, Endocr Relat Cancer, 2013. 20(1): p. 1-12.
29. Alexiadis, M., N. Eriksson, S. Jamieson, M. Davis, A.E. Drummond, S. Chu, C.D. Clyne, G.E. Muscat, and P.J. Fuller, Nuclear receptor profiling of ovarian granulosa cell tumors, Horm Cancer, 2011. 2(3): p. 157-69.
30. Mote, P.A., R.L. Balleine, E.M. McGowan, and C.L. Clarke, Colocalization of progesterone receptors A and B by dual immunofluorescent histochemistry in human endometrium during the menstrual cycle, Journal of Clinical Endocrinology & Metabolism, 1999. 84(8): p. 2963-71.
31. Mote, P.A., J.A. Leary, K.A. Avery, K. Sandelin, G. Chenevix-Trench, J.A. Kirk, and C.L. Clarke, Germ-line mutations in BRCA1 or BRCA2 in the normal breast are associated with altered expression of estrogen-responsive proteins and the predominance of progesterone receptor A, Genes Chromosomes Cancer, 2004. 39(3): p. 236-48.
32. Haviv, I., K. Polyak, W. Qiu, M. Hu, and I. Campbell, Origin of carcinoma associated fibroblasts, Cell Cycle, 2009. 8(4): p. 589-95.
33. Zeisberg, E.M., S. Potenta, L. Xie, M. Zeisberg, and R. Kalluri, Discovery of endothelial to mesenchymal transition as a source for carcinoma-associated fibroblasts, Cancer Research, 2007. 67(21): p. 10123-8.
34. Mishra, P.J., P.J. Mishra, R. Humeniuk, D.J. Medina, G. Alexe, J.P. Mesirov, S. Ganesan, J.W. Glod, and D. Banerjee, Carcinoma-associated fibroblast-like differentiation of human mesenchymal stem cells, Cancer Research, 2008. 68(11): p. 4331-9.
35. Bergamaschi, A., E. Tagliabue, T. Sorlie, B. Naume, T. Triulzi, R. Orlandi, H.G. Russnes, J.M. Nesland, R. Tammi, P. Auvinen, V.M. Kosma, S. Menard, and A.L. Borresen-Dale, Extracellular matrix signature identifies breast cancer subgroups with different clinical outcome, J Pathol, 2008. 214(3): p. 357-67.
36. Finak, G., N. Bertos, F. Pepin, S. Sadekova, M. Souleimanova, H. Zhao, H. Chen, G. Omeroglu, S. Meterissian, A. Omeroglu, M. Hallett, and M. Park, Stromal gene expression predicts clinical outcome in breast cancer, Nat Med, 2008. 14(5): p. 518-27.

37. Crandall, D.L., D.E. Busler, T.J. Novak, R.V. Weber, and J.G. Kral, Identification of estrogen receptor beta RNA in human breast and abdominal subcutaneous adipose tissue, Biochem Biophys Res Commun, 1998. 248(3): p. 523-6.
38. Palmieri, C., S. Saji, H. Sakaguchi, G. Cheng, A. Sunters, M.J. O'Hare, M. Warner, J.A. Gustafsson, R.C. Coombes, and E.W. Lam, The expression of oestrogen receptor (ER)-beta and its variants, but not ERalpha, in adult human mammary fibroblasts, J Mol Endocrinol, 2004. 33(1): p. 35-50.
39. Ijichi, N., T. Shigekawa, K. Ikeda, K. Horie-Inoue, T. Fujimura, H. Tsuda, A. Osaki, T. Saeki, and S. Inoue, Estrogen-related receptor gamma modulates cell proliferation and estrogen signaling in breast cancer, J Steroid Biochem Mol Biol, 2011. 123(1-2): p. 1-7.
40. Miki, Y., C.D. Clyne, T. Suzuki, T. Moriya, R. Shibuya, Y. Nakamura, T. Ishida, N. Yabuki, K. Kitada, S. Hayashi, and H. Sasano, Immunolocalization of liver receptor homologue-1 (LRH-1) in human breast carcinoma: possible regulator of insitu steroidogenesis, Cancer Lett, 2006. 244(1): p. 24-33.
41. Zhou, J., T. Suzuki, A. Kovacic, R. Saito, Y. Miki, T. Ishida, T. Moriya, E.R. Simpson, H. Sasano, and C.D. Clyne, Interactions between prostaglandin E(2), liver receptor homologue-1, and aromatase in breast cancer, Cancer Res, 2005. 65(2): p. 657-63.
42. Lazarus, K.A., Z. Zhao, K.C. Knower, S.Q. To, A.L. Chand, and C.D. Clyne, Oestradiol reduces Liver Receptor Homolog-1 mRNA transcript stability in breast cancer cell lines, Biochem Biophys Res Commun, 2013. 438(3): p. 533-9.
43. Chand, A.L., D.D. Wijayakumara, K.C. Knower, K.A. Herridge, T.L. Howard, K.A. Lazarus, and C.D. Clyne, The Orphan Nuclear Receptor LRH-1 and ERalpha Activate GREB1 Expression to Induce Breast Cancer Cell Proliferation, PLoS One, 2012. 7(2): p. e31593.
44. Chand, A.L., K.A. Herridge, E.W. Thompson, and C.D. Clyne, The orphan nuclear receptor LRH-1 promotes breast cancer motility and invasion, Endocr Relat Cancer, 2010. 17(4): p. 965-75.
45. Thiruchelvam, P.T., C.F. Lai, H. Hua, R.S. Thomas, A. Hurtado, W. Hudson, A.R. Bayly, F.J. Kyle, M. Periyasamy, A. Photiou, A.C. Spivey, E.A. Ortlund, R.J. Whitby, J.S. Carroll, R.C. Coombes, L. Buluwela, and S. Ali, The liver receptor homolog-1 regulates estrogen receptor expression in breast cancer cells, Breast Cancer Res Treat, 2011. 127(2): p. 385-96.
46. Rey, J., H. Hu, F. Kyle, C.F. Lai, L. Buluwela, R.C. Coombes, E.A. Ortlund, S. Ali, J.P. Snyder, and A.G. Barrett, Discovery of a New Class of Liver Receptor Homolog-1 (LRH-1) Antagonists: Virtual Screening, Synthesis and Biological Evaluation, ChemMedChem, 2012.
47. Lazarus, K.A., D. Wijayakumara, A.L. Chand, E.R. Simpson, and C.D. Clyne, Therapeutic potential of Liver Receptor Homolog-1 modulators, J Steroid Biochem Mol Biol, 2012.
48. Chand, A.L., K.A. Herridge, T.L. Howard, E.R. Simpson, and C.D. Clyne, Tissue-specific regulation of aromatase promoter II by the orphan nuclear receptor LRH-1 in breast adipose stromal fibroblasts, Steroids, 2011. 76(8): p. 741-4.
49. Clyne, C.D., A. Kovacic, C.J. Speed, J. Zhou, V. Pezzi, and E.R. Simpson, Regulation of aromatase expression by the nuclear receptor LRH-1 in adipose tissue, Mol Cell Endocrinol, 2004. 215(1-2): p. 39-44.
50. Clyne, C.D., C.J. Speed, J. Zhou, and E.R. Simpson, Liver receptor homologue-1 (LRH-1) regulates expression of aromatase in preadipocytes, J Biol Chem, 2002. 277(23): p. 20591-7.
51. Sherman, M.H., M. Downes, and R.M. Evans, Nuclear receptors as modulators of the tumor microenvironment, Cancer Prev Res (Phila), 2012. 5(1): p. 3-10.
52. Kaneshige, M., K. Kaneshige, X. Zhu, A. Dace, L. Garrett, T.A. Carter, R. Kazlauskaite, D.G. Pankratz, A. Wynshaw-Boris, S. Refetoff, B. Weintraub, M.C. Willingham, C. Barlow, and S. Cheng, Mice with a targeted mutation in the thyroid hormone beta receptor gene exhibit impaired growth and resistance to thyroid hormone, Proc Natl Acad Sci U S A, 2000. 97(24): p. 13209-14.
53. Parrilla, R., A.J. Mixson, J.A. McPherson, J.H. McClaskey, and B.D. Weintraub, Characterization of seven novel mutations of the c-erbA beta gene in unrelated kindreds with generalized thyroid hormone resistance. Evidence for two "hot spot" regions of the ligand binding domain, J Clin Invest, 1991. 88(6): p. 2123-30.

54. Meier, C.A., B.M. Dickstein, K. Ashizawa, J.H. McClaskey, P. Muchmore, S.C. Ransom, J.B. Menke, E.H. Hao, S.J. Usala, B.B. Bercu, and et al., Variable transcriptional activity and ligand binding of mutant beta 1 3,5,3'-triiodothyronine receptors from four families with generalized resistance to thyroid hormone, Mol Endocrinol, 1992. 6(2): p. 248-58.
55. Guigon, C.J., D.W. Kim, M.C. Willingham, and S.Y. Cheng, Mutation of thyroid hormone receptor-beta in mice predisposes to the development of mammary tumors, Oncogene, 2011. 30(30): p. 3381-90.
56. Silva, J.M., G. Dominguez, J.M. Gonzalez-Sancho, J.M. Garcia, J. Silva, C. Garcia-Andrade, A. Navarro, A. Munoz, and F. Bonilla, Expression of thyroid hormone receptor/erbA genes is altered in human breast cancer, Oncogene, 2002. 21(27): p. 4307-16.
57. Hamza, M.S., S. Pott, V.B. Vega, J.S. Thomsen, G.S. Kandhadayar, P.W. Ng, K.P. Chiu, S. Pettersson, C.L. Wei, Y. Ruan, and E.T. Liu, De-novo identification of PPARgamma/RXR binding sites and direct targets during adipogenesis, PLoS One, 2009. 4(3): p. e4907.
58. Tikoo, K., P. Kumar, and J. Gupta, Rosiglitazone synergizes anticancer activity of cisplatin and reduces its nephrotoxicity in 7, 12-dimethyl benz{a}anthracene (DMBA) induced breast cancer rats, Bmc Cancer, 2009. 9: p. 107.
59. Skelhorne-Gross, G., A.L. Reid, A.J. Apostoli, M.A. Di Lena, R.E. Rubino, N.T. Peterson, M. Schneider, S.K. SenGupta, F.J. Gonzalez, and C.J. Nicol, Stromal adipocyte PPARgamma protects against breast tumorigenesis, Carcinogenesis, 2012. 33(7): p. 1412-20.
60. Papadaki, I., E. Mylona, I. Giannopoulou, S. Markaki, A. Keramopoulos, and L. Nakopoulou, PPARgamma expression in breast cancer: clinical value and correlation with ERbeta, Histopathology, 2005. 46(1): p. 37-42.
61. Jiang, W.G., A. Douglas-Jones, and R.E. Mansel, Expression of peroxisome-proliferator activated receptor-gamma (PPARgamma) and the PPARgamma co-activator, PGC-1, in human breast cancer correlates with clinical outcomes, Int J Cancer, 2003. 106(5): p. 752-7.
62. Rubin, G.L., Y. Zhao, A.M. Kalus, and E.R. Simpson, Peroxisome proliferator-activated receptor gamma ligands inhibit estrogen biosynthesis in human breast adipose tissue: possible implications for breast cancer therapy, Cancer Research, 2000. 60(6): p. 1604-8.
63. Catalano, S., V. Pezzi, A. Chimento, C. Giordano, A. Carpino, M. Young, M.J. McPhaul, and S. Ando, Triiodothyronine decreases the activity of the proximal promoter (PII) of the aromatase gene in the mouse Sertoli cell line, TM4, Molecular Endocrinology, 2003. 17(5): p. 923-34.
64. Krishnan, A.V., S. Swami, L. Peng, J. Wang, J. Moreno, and D. Feldman, Tissue-selective regulation of aromatase expression by calcitriol: implications for breast cancer therapy, Endocrinology, 2010. 151(1): p. 32-42.
65. Swami, S., A.V. Krishnan, J.Y. Wang, K. Jensen, L. Peng, M.A. Albertelli, and D. Feldman, Inhibitory effects of calcitriol on the growth of MCF-7 breast cancer xenografts in nude mice: selective modulation of aromatase expression in vivo, Horm Cancer, 2011. 2(3): p. 190-202.
66. Wang, Z.J., B. Jeffs, M. Ito, J.C. Achermann, R.N. Yu, D.B. Hales, and J.L. Jameson, Aromatase (Cyp19) expression is up-regulated by targeted disruption of Dax1, Proc Natl Acad Sci U S A, 2001. 98(14): p. 7988-93.
67. Gurates, B., S. Sebastian, S. Yang, J. Zhou, M. Tamura, Z. Fang, T. Suzuki, H. Sasano, and S.E. Bulun, WT1 and DAX-1 inhibit aromatase P450 expression in human endometrial and endometriotic stromal cells, J Clin Endocrinol Metab, 2002. 87(9): p. 4369-77.
68. Gurates, B., A. Amsterdam, M. Tamura, S. Yang, J. Zhou, Z. Fang, S. Amin, S. Sebastian, and S.E. Bulun, WT1 and DAX-1 regulate SF-1-mediated human P450arom gene expression in gonadal cells, Mol Cell Endocrinol, 2003. 208(1-2): p. 61-75.
69. Lanzino, M., P. Maris, R. Sirianni, I. Barone, I. Casaburi, A. Chimento, C. Giordano, C. Morelli, D. Sisci, P. Rizza, D. Bonofiglio, S. Catalano, and S. Ando, DAX-1, as an androgen-target gene, inhibits aromatase expression: a novel mechanism blocking estrogen-dependent breast cancer cell proliferation, Cell Death Dis, 2013. 4: p. e724.

70. Delerive, P., D. Monte, G. Dubois, F. Trottein, J. Fruchart-Najib, J. Mariani, J.C. Fruchart, and B. Staels, The orphan nuclear receptor ROR alpha is a negative regulator of the inflammatory response, EMBO Rep, 2001. 2(1): p. 42-8.
71. Xiong, G., C. Wang, B.M. Evers, B.P. Zhou, and R. Xu, RORalpha suppresses breast tumor invasion by inducing SEMA3F expression, Cancer Research, 2012. 72(7): p. 1728-39.
72. Moretti, R.M., M. Montagnani Marelli, M. Motta, and P. Limonta, Role of the orphan nuclear receptor ROR alpha in the control of the metastatic behavior of androgen-independent prostate cancer cells, Oncol Rep, 2002. 9(5): p. 1139-43.
73. Jetten, A.M. and E. Ueda, Retinoid-related orphan receptors (RORs): roles in cell survival, differentiation and disease, Cell Death Differ, 2002. 9(11): p. 1167-71.
74. Beer, T.M. and A. Myrthue, Calcitriol in cancer treatment: from the lab to the clinic, Mol Cancer Ther, 2004. 3(3): p. 373-81.
75. Colston, K.W. and C.M. Hansen, Mechanisms implicated in the growth regulatory effects of vitamin D in breast cancer, Endocr Relat Cancer, 2002. 9(1): p. 45-59.
76. Welsh, J., Vitamin D and prevention of breast cancer, Acta Pharmacol Sin, 2007. 28(9): p. 1373-82.
77. Zinser, G., K. Packman, and J. Welsh, Vitamin D(3) receptor ablation alters mammary gland morphogenesis, Development, 2002. 129(13): p. 3067-76.
78. Horard, B. and J.M. Vanacker, Estrogen receptor-related receptors: orphan receptors desperately seeking a ligand, J Mol Endocrinol, 2003. 31(3): p. 349-57.
79. Deblois, G. and V. Giguere, Oestrogen-related receptors in breast cancer: control of cellular metabolism and beyond, Nat Rev Cancer, 2013. 13(1): p. 27-36.
80. Suzuki, T., Y. Miki, T. Moriya, N. Shimada, T. Ishida, H. Hirakawa, N. Ohuchi, and H. Sasano, Estrogen-related receptor alpha in human breast carcinoma as a potent prognostic factor, Cancer Res, 2004. 64(13): p. 4670-6.
81. Chang, C.Y., D. Kazmin, J.S. Jasper, R. Kunder, W.J. Zuercher, and D.P. McDonnell, The metabolic regulator ERRalpha, a downstream target of HER2/IGF-1R, as a therapeutic target in breast cancer, Cancer Cell, 2011. 20(4): p. 500-10.
82. Tremblay, G.B., T. Kunath, D. Bergeron, L. Lapointe, C. Champigny, J.A. Bader, J. Rossant, and V. Giguere, Diethylstilbestrol regulates trophoblast stem cell differentiation as a ligand of orphan nuclear receptor ERR beta, Genes Dev, 2001. 15(7): p. 833-8.
83. Chen, J. and J. Nathans, Estrogen-related receptor beta/NR3B2 controls epithelial cell fate and endolymph production by the stria vascularis, Dev Cell, 2007. 13(3): p. 325-37.
84. Feng, B., J. Jiang, P. Kraus, J.H. Ng, J.C. Heng, Y.S. Chan, L.P. Yaw, W. Zhang, Y.H. Loh, J. Han, V.B. Vega, V. Cacheux-Rataboul, B. Lim, T. Lufkin, and H.H. Ng, Reprogramming of fibroblasts into induced pluripotent stem cells with orphan nuclear receptor Esrrb, Nat Cell Biol, 2009. 11(2): p. 197-203.
85. Ludbrook, L.M., P. Bernard, S. Bagheri-Fam, J. Ryan, R. Sekido, D. Wilhelm, R. Lovell-Badge, and V.R. Harley, Excess DAX1 Leads to XY Ovotesticular Disorder of Sex Development (DSD) in Mice by Inhibiting Steroidogenic Factor-1 (SF1) Activation of the Testis Enhancer of SRY-box-9 (Sox9), Endocrinology, 2012. 153(4): p. 1948-58.
86. Sablin, E.P., A. Woods, I.N. Krylova, P. Hwang, H.A. Ingraham, and R.J. Fletterick, The structure of corepressor Dax-1 bound to its target nuclear receptor LRH-1, Proc Natl Acad Sci U S A, 2008. 105(47): p. 18390-5.
87. Suzuki, T., M. Kasahara, H. Yoshioka, K. Morohashi, and K. Umesono, LXXLL-related motifs in Dax-1 have target specificity for the orphan nuclear receptors Ad4BP/SF-1 and LRH-1, Mol Cell Biol, 2003. 23(1): p. 238-49.
88. Robbins, P., S. Pinder, N. de Klerk, H. Dawkins, J. Harvey, G. Sterrett, I. Ellis, and C. Elston, Histological grading of breast carcinomas: a study of interobserver agreement, Hum Pathol, 1995. 26(8): p. 873-9.
89. Allred, D.C., J.M. Harvey, M. Berardo, and G.M. Clark, Prognostic and predictive factors in breast cancer by immunohistochemical analysis, Mod Pathol, 1998. 11(2): p. 155-68.

90. Wolff, A.C., M.E. Hammond, J.N. Schwartz, K.L. Hagerty, D.C. Allred, R.J. Cote, M. Dowsett, P.L. Fitzgibbons, W.M. Hanna, A. Langer, L.M. McShane, S. Paik, M.D. Pegram, E.A. Perez, M.F. Press, A. Rhodes, C. Sturgeon, S.E. Taube, R. Tubbs, G.H. Vance, M. van de Vijver, T.M. Wheeler, and D.F. Hayes, American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer, Arch Pathol Lab Med, 2007. 131(1): p. 18-43.

Table 1. Clinicopathological information on breast cancer sample group

Patient ID	Age (years)	Receptor Status	Tumor Grade	Tumor Type
1[1]	47	ER+, PR+, HER2-	Grade II	IDC
2[1]	76	ER+, PR+, HER2-	Grade II	IDC
3[1]	69	ER+, PR+, HER2-	Grade II	IDC
4[1]	74	ER+, PR+, HER2-	Grade I	IDC
5[1]	48	ER+, PR-, HER2+	Grade II	IDC
6[2]	60	ER+, PR+, HER2+	Grade II	IDC
7[2]	70	ER+, PR+, HER2-	Grade II	IDC
8[2]	66	ER+, PR-, HER2-	Grade II	IDC
9[2]	66	ER+, PR+, HER2-	Grade II	DCIS

Table 2. Clinicopathological information on normal and malignant breast samples

Clinical feature	Cohort details
Tumour cohorts	
ER-positive (n)	15
Grade I (n)	1
Grade II (n)	10
Grade III (n)	4
Age, mean (range) years	65 (36-90)
ER-negative (n)	15
Grade II (n)	4
Grade III (n)	11
Age, mean (range) years	60 (27-85)
Normal breast cohorts (n)	14
Pre-menopausal (n)	11
Reduction mammoplasty (n)	7
Breast biopsy (n)	4
Age, mean (range) years	38 (22-46)
Post-menopausal (n)	3
Breast biopsy (n)	3
Age, mean (range) years	57 (55-58)

716 Table 3. Summary of nuclear receptor expression profiles in NAF and CAF samples groups.

717

	Nuclear Receptor Expression Profile		
Sub-classification	**NAFs**	**CAFs**	**Relative change in CAFs vs NAFs**
Endocrine receptors (n=12)	11	11	↓ TR-β
			↓ VDR
Orphan receptors (n=25)	17	15	↓ DAX-1
			↓ ERR-β
			↓ ROR-α
			↓ ROR-β
			↑ LRH-1
Adopted orphan receptors (n=11)	8	8	↓ PPAR-γ
Total (n=48)	36	34	

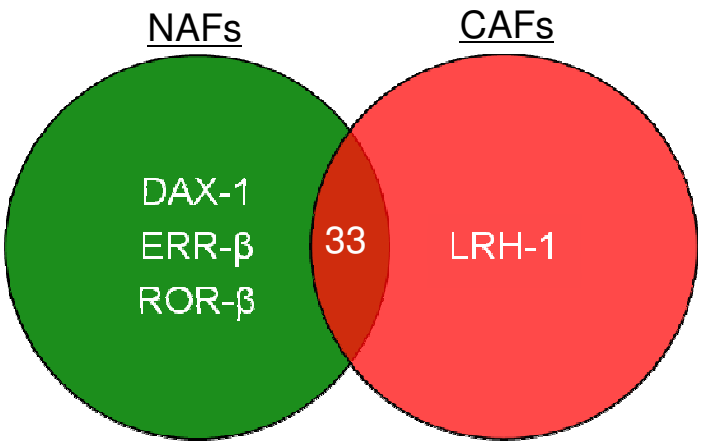
718

719

720

721

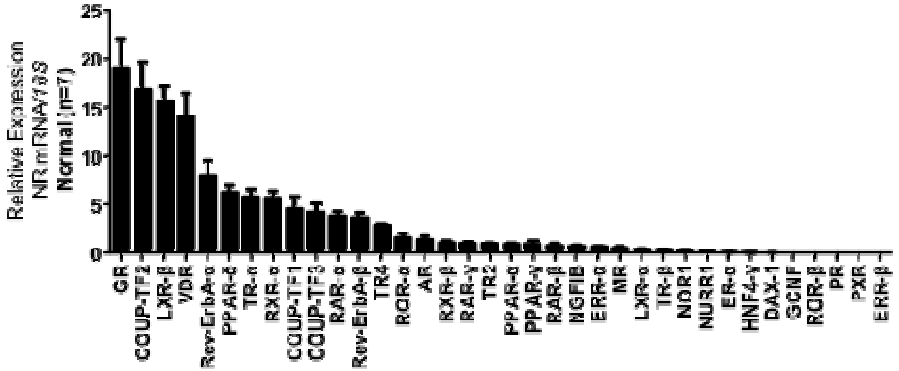
722


NAFs
CAFs
DAX-1
ERR-β
ROR-β
33
LRH-1

A

NAFs

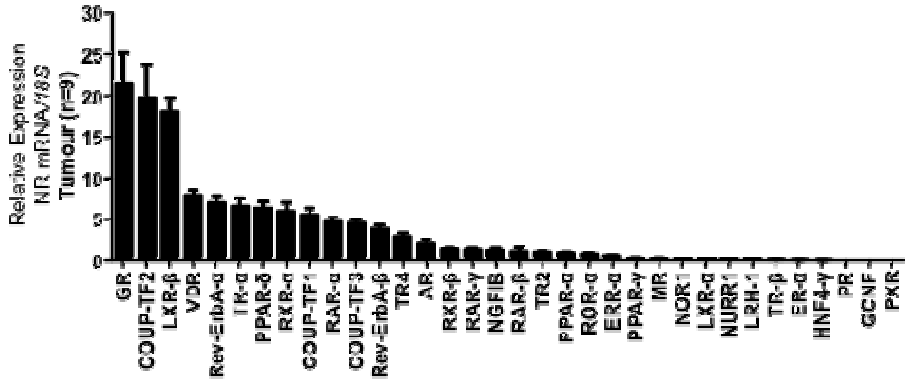
Absent		Low				Moderate		High
CAR	ERR-γ	DAX-1	ER-α	ERR-α	ERR-β	AR	COUP-TF1	GR
ER-β	FXR	GCNF	HNF4-γ	LXR-α	MR	COUP-TF3	PPAR-δ	COUP-TF2
HNF4-α	LRH-1	NGFIB	NURR1	NOR1	PR	RAR-α	RAR-γ	LXR-β
PNR	ROR-γ	PXR	PPAR-α	PPAR-γ	RAR-β	Rev-ErbA-α	Rev-ErbA-β	VDR
RXR-γ	SF-1	ROR-β	TR-β	TR2		ROR-α	RXR-α	
SHP	TLX					RXR-β	TR-α	
						TR4		



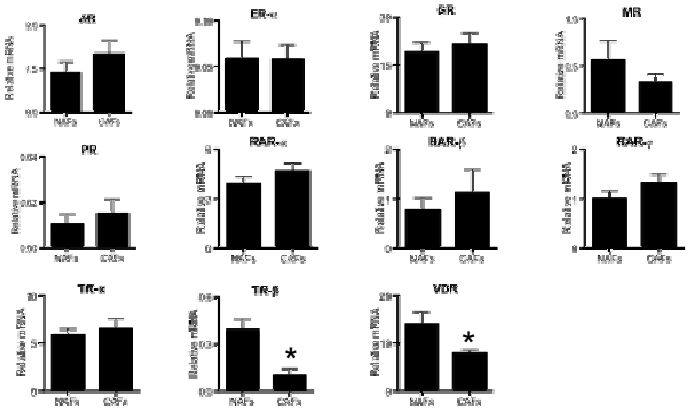
B

CAFs

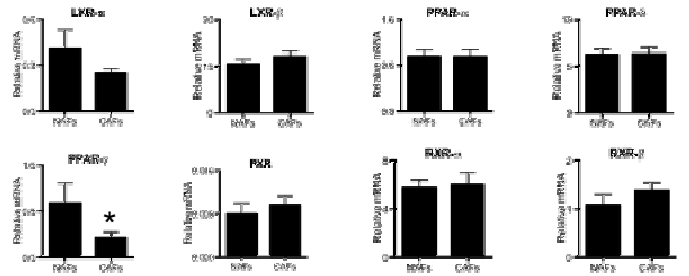
Absent		Low				Moderate		High
CAR	DAX-1	ERR-α	ER-α	GCNF	HNF4-γ	AR	COUP-TF1	COUP-TF2
ER-β	ERR-γ	LXR-α	LRH-1	MR	NURR1	COUP-TF3	NGFIB	GR
ERR-β	FXR	NOR1	PPAR-α	PPAR-γ	PXR	PPAR-δ	Rev-ErbA-α	LXR-β
HNF4-α	PNR	PR	ROR-α	TR-β		Rev-ErbA-β	RAR-β	
ROR-β	ROR-γ					RAR-α	RAR-γ	
RXR-γ	SF-1					RXR-α	RXR-β	
SHP	TLX					TR2	TR4	
						TR-α	VDR	



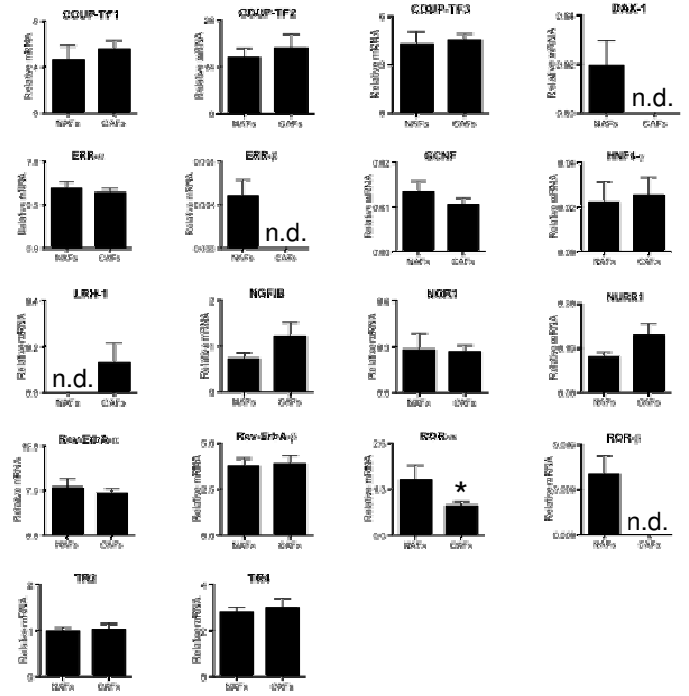
Endocrine Receptors

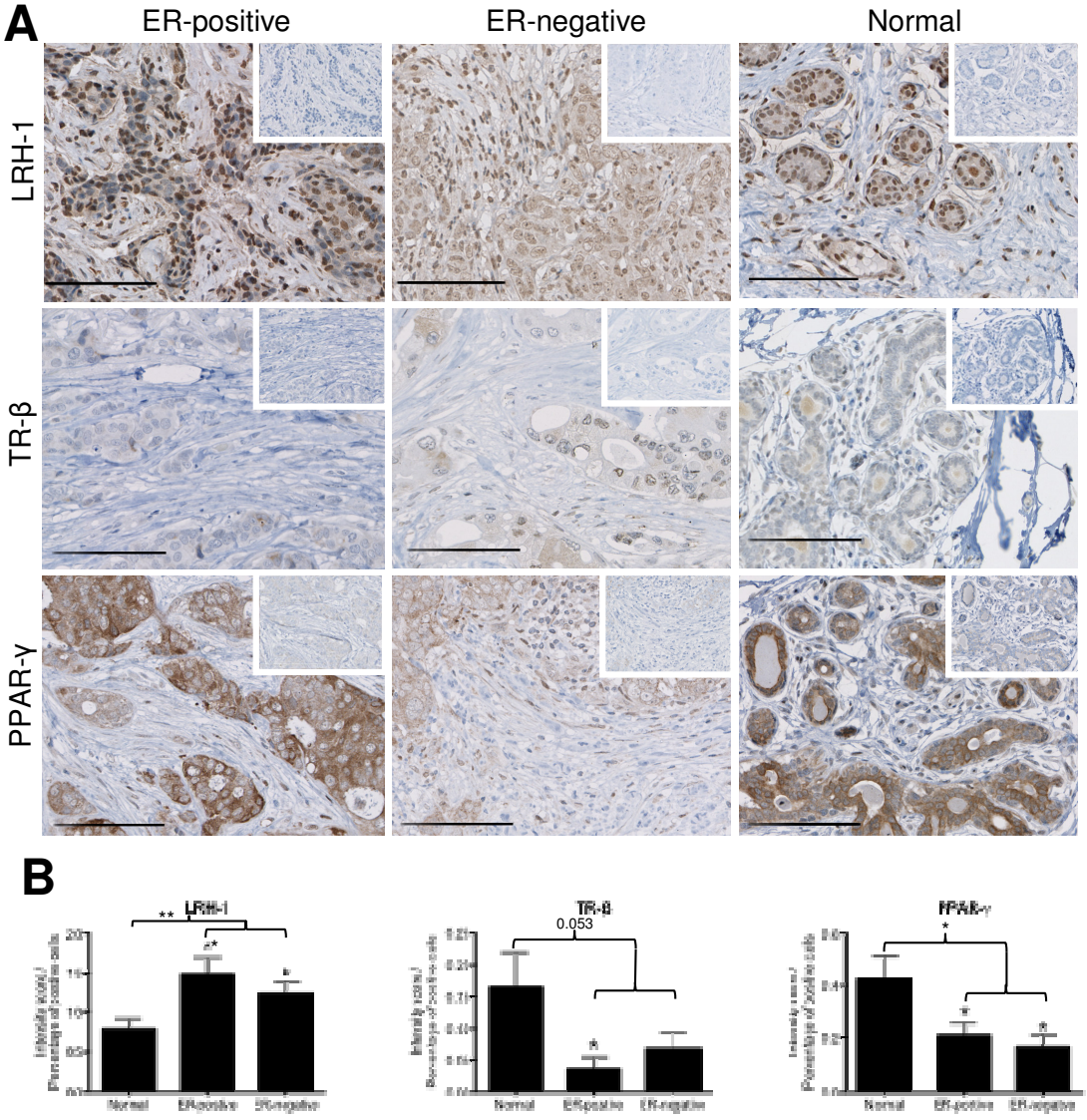


Adopted Orphan Receptors



Orphan Receptors




A
ER-positive
ER-negative
Normal
LRH-1
TR-β
PPAR-γ

B
LRH-1
TR-β
0.053
PPAR-γ
Normal
ER-positive
ER-negative

Author/s:
Knower, KC; Chand, AL; Eriksson, N; Takagi, K; Miki, Y; Sasano, H; Visvader, JE; Lindeman, GJ; Funder, JW; Fuller, PJ; Simpson, ER; Tilley, WD; Leedman, PJ; Graham, JD; Muscat, GEO; Clarke, CL; Clyne, CD

Title:
Distinct nuclear receptor expression in stroma adjacent to breast tumors

Date:
2013-11-01

Citation:
Knower, K. C., Chand, A. L., Eriksson, N., Takagi, K., Miki, Y., Sasano, H., Visvader, J. E., Lindeman, G. J., Funder, J. W., Fuller, P. J., Simpson, E. R., Tilley, W. D., Leedman, P. J., Graham, J. D., Muscat, G. E. O., Clarke, C. L. & Clyne, C. D. (2013). Distinct nuclear receptor expression in stroma adjacent to breast tumors. BREAST CANCER RESEARCH AND TREATMENT, 142 (1), pp.211-223. https://doi.org/10.1007/s10549-013-2716-6.