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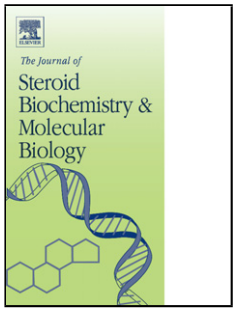

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Twenty years of the G protein-coupled estrogen receptor GPER: historical and personal perspectives

Short title: GPER's first 20 years

Matthias Barton[1], Edward J. Filardo[2], Stephen J. Lolait[3],
Peter Thomas[4], Marcello Maggiolini[5], Eric R. Prossnitz[6]

[1] Molecular Internal Medicine, University of Zürich, 8057 Zürich, Switzerland.
[2] Rhode Island Hospital, Brown University, Providence, RI 02903, USA.
[3] Henry Wellcome Laboratories for Integrative Neuroscience and Endocrinology, School of Clinical Sciences, University of Bristol, Bristol, UK.
[4] Marine Science Institute, University of Texas at Austin, Port Aransas, TX 78373, USA.
[5] Department of Pharmacy, Health and Nutritional Sciences, University of Calabria, Rende, Italy.
[6] Department of Internal Medicine, University of New Mexico Health Sciences Center and University of New Mexico Comprehensive Cancer Center, Albuquerque, NM 87131, USA.

*To whom correspondence should be addressed: barton@access.uzh.ch (M.B.), eprossnitz@unm.edu (E.R.P.).

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Highlights

- Clara Szego reported rapid estrogen signaling over 50 years ago.
- GPR30 cloned in 1992 in a search for novel GPCRs.
- Renamed GPER as estrogen-mediated actions were discovered.
- Therapeutic targeting of GPER in a multitude of chronic diseases.
- Personal accounts on discoveries of GPER biology and functions.

Abstract

Estrogens play a critical role in many aspects of female physiology, particularly reproductive function, but also in pathophysiology, and are associated with protection from numerous diseases in premenopausal women. Steroids and particularly estrogen action have been known for ~90 years, with the first evidence for a receptor for estrogen presented ~50 years ago. The original ancestral steroid receptor, extending back into evolution more than 500 million years, was likely an estrogen receptor, whereas G protein-coupled receptors (GPCRs) trace their origins back into history more than one billion years. The classical estrogen receptors (ERα and ERβ) are ligand-activated transcription factors that confer estrogen sensitivity upon many genes. It was soon apparent that these, or novel receptors may also be responsible for the ‘”rapid”/“non-genomic” membrane-associated effects of estrogen. The identification of an orphan GPCR (GPR30, published in 1996) opened a new field of research with the description in 2000 that GPR30 expression is required for rapid estrogen signaling. In 2005-2006, the field was greatly stimulated by two studies that described the binding of estrogen to GPR30-expressing cell membranes, followed by the identification of a GPR30-selective agonist (that lacked binding and activity towards ERα and ERβ). Renamed GPER (G protein-coupled estrogen receptor) by IUPHAR in 2007, the total number of articles in PubMed related to this receptor recently surpassed 1000. In this article, the authors present personal perspectives on how they became involved in the discovery and/or advancement of GPER research. These

areas include non-genomic effects on vascular tone, receptor cloning, molecular and cellular biology, signal transduction mechanisms and pharmacology of GPER, highlighting the roles of GPER and GPER-selective compounds in diseases such as obesity, diabetes, and cancer and the obligatory role of GPER in propagating cardiovascular aging, arterial hypertension and heart failure through the regulation of Nox expression.

Key words: estrogen, GPCR, history, IUPHAR, non-genomic, pathology, pathophysiology, physiology, Charles-Édouard Brown-Séquard, Rudolf Chrobak, Adolf Butenand, Edward Doisy, Ferdinand Mainzer, Clara Szego, Adolf Windaus

Introduction: Discovery of estrogens and their receptors

Estrogens play critical roles in female biology and specifically reproductive function. They are also important in pathophysiology, playing an important role in breast cancer and protection from numerous diseases in premenopausal women [1-10]. In 1889, the French-Irish-American physiologist Charles-Édouard Brown-Séquard (1817-1894), at the age of 72, injected himself with crushed guinea pig and dog testicles, and noticed a "*renewal of vigour and mental clarity*" [11]. Brown-Séquard also reported in 1889 that a surgeon in Marseilles by the name of Villeneuve had administered a fluid made from guinea pig ovaries to one male and two postmenpausal female patients, one of whom was cured from her postmenopausal symptoms by the treatment. He further reported in the same year that a midwife in Paris had injected herself with the filtered juice of guinea pig ovaries leading to therapeutic "benefit", and that an American medical women named Augusta Brown (apparently not related to his family), a graduate at the University of Paris, had injected more than a dozen aged women with the filtered juice of guinea pig ovaries with good effects in hysteria, various uterine affections, and debility due to age [11]. Around the same time, on November 30, 1889, Rudolf Chrobak (1843-1910) in Vienna orally administered freshly minced bovine ovary tissue to a 47 year-old women, albeit with little effect [12]. By contrast, Ferdinand Mainzer (1871-1943), at the time a medical resident, reported in 1896 on the beneficial effects of oral treatment with "Ovarialsubstanz" (a

concentrated extract made from porcine or bovine ovaries) of ovariectomized or postmenopausal women [13, 14], and likely described the first rapid effects related to estrogen: "*the condition improved immediately after administration of Ovarialsubstanz*" [13]. Similar findings were also reported by Landau [15] and Mond [16].

It was at this time that molecular medicine appeared on the horizon, long before the genetic code was deciphered or medical genetics were known. Already in the early 1890s Canadian physician Sir William Osler (1849-1919) at Johns Hopkins University in Baltimore had identified hereditary patterns in families with coronary artery disease [17], and in 1894 the German pathologist Rudolf Virchow, considered the “father of modern pathology” [18, 19] proposed changing the prevailing dogma in anatomy, pathology and disease development, foreseeing the future of cellular and molecular biology and current modern medicine - concepts that were fully compatible with the observations of early endocrinologists discussed above [13-21]. Virchow presented his revolutionary views in a lecture presented at the XI International Medical Congress in Rome on March 30, 1894, which he ended with the following words: “…*The idea of the ‘sedes morborum’, or, as I have designated it, ‘the anatomical concept’. It is this thought that governs the physiology and pathology of today. Whether it leads one back to the cells, as it does me, or whether another formula is developed, it will surely remain the concept for the future. And this future will mark the beginning of its chronology in the days of Morgagni. Let him be honoured!*.” [22]. Ironically, the journal in which Virchow’s lecture was published (Berliner Medizinische Wochenschrift) merged with Therapeutische Monatsheft in 1922 to become Klinische Wochenschrift, and in 1995 changed its name to the Journal of Molecular Medicine [23].

It is worth noting that the chemical identity of steroids and particularly estrogens has only been known for less than a century, work that is largely owed to the American Edward A. Doisy (1893-1986) and the German Adolf Butenand (1903 to 1995), both of whom, in 1929, simultaneously discovered estrone [24-27]. At the time, Butenand was 26, having obtained his PhD only two years earlier in Göttingen with Adolf Windaus (1876 to 1956), the Nobel Laureate in Chemistry in 1928 for the discovery of vitamin D and sterols [28]. By 1935, Butenand had also identified progesterone and testosterone; Doisy later also isolated 17β-estradiol and estriol [27, 29, 30]. Of note, both discoverers of estrogens also went on to win a Nobel Prize.

Butenand received the Nobel Prize in Chemistry in 1939, and Doisy was awarded the Nobel Prize in Physiology or Medicine in 1943 [27, 30].

The first evidence of a receptor for estrogen was presented by Elwood V. Jensen (1920-2012) in the 1950s [29, 31, 32]; however, it took 30 more years until the gene sequence of the first nuclear estrogen receptor was cloned [33-35]. In 2016, the Hungarian-American biologist Clara M. Szego (**Figure 1**) celebrated her 100th birthday. Fifty years earlier, Szego reported increases in cAMP production following acute exposure to estrogen [36]. In the following years Szego found the uterine cAMP increase to be inhibited by glucocorticoids [37] and β blockers (only if the adrenals were intact) [38], and also purified an estrogen receptor protein, demonstrating inhibition of its function using antibodies [39]. Ironically, it was again Szego, who in 1977 first reported estrogen receptors in plasma membrane fractions [40], which she later partially purified and characterized [41, 42]. In the 1980s, the cDNA sequence of first estrogen receptor (today known as ERα) was determined [33-35], and by the mid 1990s, both the second nuclear estrogen receptor (today known as ERβ) and an orphan receptor designated GPR30 were cloned [43-50]. Initially believed to represent a GPCR for cytokines, the function(s) of GPR30 remained elusive until recently. In the following sections, the individual authors will summarize their personal history and perspectives in this field.

GPER in hiding: rapid effects of estrogen

M. Barton

One of the clinical observations for centuries had been that premenopausal women are largely protected from coronary artery disease and its clinical manifestations [51]. I began my work in the field of coronary artery disease and atherosclerosis in 1990 at the Division of Cardiology at Hannover Medical School in Germany, headed by the Swiss physician Paul R. Lichtlen (1929-2005). In the 1960s, Lichtlen had pioneered coronary angiography in Europe at the University of Zürich and moved to Hannover in 1973 [52]. My studies, supported by Andreas Mügge and the cardiothoracic surgeons, were centered around the question of whether estrogen may exert acute vasodilatory effects on human coronary arteries. Human physiology studies had indirectly suggested the existence of rapid effects of estrogen in peripheral veins [53], but they had not

been demonstrated for human arteries. At the time, the identity of endothelium-derived relaxing factor (EDRF) as nitric oxide (NO) had just been discovered [54-56], and only one estrogen receptor (today known as ERα) had been cloned [33-35]; whether this or additional estrogen receptors were involved in the regulation of vascular tone was not known.

During my studies, which took more than three years to complete, it turned out that within minutes, 17β-estradiol causes relaxation of human coronary arteries (**Figure 2**) of both women and men [57]. Results also showed that a 30-minute exposure to 17β-estradiol indirectly improved vasodilation by enhancing endothelium-dependent relaxation [58]. At the time Lichtlen invited me to participate in my first scientific meeting, a small «International Workshop on Atherosclerosis» that he organized on October 9-10,1992 in Celle, Germany. Participants included many notable physician-scientists such as Gustav V. Born (b. 1921), Christian C. Haudenschild, Philippe D. Henry (1935-2013), Helmut Drexler (1951-2009) Elizabeth G. Nabel, David G. Harrison, as well as Peter Ganz, who at the time was also conducting studies on vascular effects of estrogens in humans, which he published in 1994 [59].

In search of the underlying mechanisms to help explaining the observed rapid vasodilatory response, I came across Clara M. Szego's early studies showing induction of cAMP production following acute estrogen exposure [36]. To investigate whether the observed rapid vascular effects involved any changes in cAMP production, I set up similar experiments human coronary arteries with help from Michaela Kuhn, an expert in cyclic nucleotide biology, demonstrating that within 30 minutes of application - the time required to induce full arterial relaxation - 17β-estradiol increased vascular cAMP content [57], both observations that could neither be explained by genomic mechanisms [60], nor by activation of the endothelial L-arginine/NO/guanylate cyclase pathway. In the following year, Benita Katzenellenbogen, utilizing intact uterine tissue, also reported rapid increases in cyclic AMP content after a 30-minute exposure to 17β-estradiol [61]. Two years later, cloning of a second nuclear estrogen receptor was reported and named ERβ [62, 63], as was GPR30 [44], albeit as an orphan receptor without known ligand at the time.

Meanwhile, following the advice of Paul Lichtlen and with his support, I had moved to Switzerland continuing my research in this area and began working on estrogen receptors [64,

65]. In 2002, a young medical student by the name of Matthias Meyer joined my group and we agreed that he would also study acute effects of estrogen on human vascular tissue. Meyer started his research (for which he later received the Medical Faculty's Best Thesis Award at the University of Zürich) and approached me one day, holding a print-out of an article, asking me whether I had seen the paper. It was a article by Eric Prossnitz's group from the University of New Mexico reporting the identification of GPR30 as an intracellular transmembrane estrogen-binding receptor acting through PI3-kinase, thus identifying it as a third *bona fide* estrogen receptor [66]. I immediately thought back to my own studies from the early 1990s and realized that this receptor might explain part of the acute effects of 17β-estradiol on cyclic AMP observed back then, and perhaps also the vascular relaxation. Meyer proposed to also study GPR30 expression in his experiments. He found that human blood vessels indeed express GPR30, which was sensitive to regulation by estrogen. Meyer reported his observations as unpublished data in April 2006 in his first peer-reviewed article [67]. The full data were published in a research report [68] that I dedicated to Paul Lichtlen, who had died in 2005, the same year we had started our work on GPER.

In 2006, I contacted Prossnitz who agreed to share his GPER-specific ligands with us. In addition, I was fortunate to obtain mice lacking the *Gper* gene (generated by Jan Rosenbaum [69]) from Debbie Clegg, who was then at the University of Cincinnati, and was also introduced to Cristina and Eugen Brailoiu, experts in calcium biology at Temple University, and physiologist Tom Resta, at UNM. Together, we published a report in January 2009 showing that GPER acts as an intracellular membrane receptor controlling cell proliferation, that it acutely reduces vascular tone and blood pressure, and that its genetic deletion leads to abdominal obesity [70]. We have since developed a very productive collaboration with Prossnitz and our studies – most of them performed by Meyer with enormous curiosity and persistence – led to the identification and characterization of multiple new, particularly genomic roles of GPER in cardiovascular function and disease which we will discuss elsewhere in this issue [71]. Looking back, without Paul Lichtlen's mentoring, support, and trust in a young colleague, we might have never known…

Cloning of an orphan GPCR named GPR30

S. Lolait

The initial success in cloning G protein-coupled receptors (GPCR) was based on targeted approaches that relied predominantly upon biochemical purification of the receptor protein or on expression cloning where prior receptor sequence knowledge was not required. With the realization that GPCRs had a topography of 7 transmembrane (TM) domains and displayed varying degrees of conservation between and across GPCR subclasses, homology cloning came to the fore. This was facilitated by PCR and augmented by bioinformatics analysis of various genomes.

GPR30 started off as an orphan GPCR with no known endogenous (or exogenous) ligand, one of many isolated by numerous labs in the late 1980's - early 1990's. My first foray into the GPCR that was later to be named GPR30/GPER began in late 1991 whilst working at the National Institutes of Mental Health (Bethesda, MD, USA). My colleague Christer Owman and I used PCR with degenerate primers to TM domains of known GPCRs to isolate a number of novel GPCR sequences from a human B-cell lymphoblast cDNA library. The full-length cDNA clones were isolated by February 1992 (**Figure 3**) - one of these was eventually named CMKRL2 (chemokine related receptor like 2) given its closest homology (approx. 30% overall amino acid identity) to chemokine GPCRs, but it did not respond to a battery of chemokines when expressed in cell lines [44]. GPR30 're-emerged' in our lab a few months later in mouse form when I used a similar PCR-based strategy to isolate a novel GPCR cDNA clone (AtT20#9) from a murine AtT20 pituitary tumor cDNA library. Cell lines expressing the corresponding full-length clone did not respond to the peptide ligands in which I was interested at the time (e.g., corticotropin releasing factor, vasopressin) so the clone went to the back of the freezer. Parenthetically, we were interested in fast steroid responses in the lab around this time - there were numerous futile attempts to elicit rapid elevations in intracellular Ca^{2+} levels in response to estrogen, aldosterone or corticosterone in *Xenopus* oocytes expressing both the Ca^{2+}-sensitive photoprotein aequorin and mRNAs isolated from various tissues and cells. We never tested whether cell lines stably expressing our human or mouse GPR30 clones responded to estrogen.

The cloning of CMKRL2 published in 1996 [44] was closely followed over the next year or so by independent reports of the isolation of the same human orphan GPCR cDNA under various

aliases (e.g., CEPR, LyGPR, FEG-1, GPCR-Br [46-50]) and a rat homologue GPR41 [45]. The GPR30 designation was adopted [46, 50] following on from the consecutive numbering of previous GPCR orphans reported in the literature. The cloning of most of the GPR30s was based on PCR with degenerate primers to highly conserved TM regions of known GPCRs, using libraries constructed from cDNA or genomic DNA (as most GPCR genes are intronless or contain very few introns in their open reading frame) as a template. One exception was a GPR30 cDNA sequence deposited in GenBank as 'DRY12' (accession number U58828) in mid-1996 that resulted from an effort to expression clone a receptor for α1-antitrypsin-protease complexes (David Perlmutter, personal communication, 2016). In addition, GPCR-Br/GPR30 was obtained by differential screening of an MCF7 breast tumor cell cDNA library with cDNA probes from estrogen receptor (ER)-positive and ER-negative tumor cell lines. This was the first clue that GPR30 might be related to estrogen responsiveness [46].

The studies noted above showed that the human GPR30 gene maps to chromosome 7 and lacks introns in its protein-coding portion. Its translated DNA sequence (open reading frame) predicts a 375 amino acid protein that has consensus sites for possible post-translational modification (e.g., glycosylation, phosphorylation) and the structural signature of the Class A Rhodopsin-like GPCR subfamily (e.g., it has a ‘DRY’ amino acid motif at the tail of TM3). It is ubiquitously expressed as a 2-3kb mRNA transcript in brain regions (e.g., hypothalamus and hippocampus) and peripheral tissues such as placenta, prostate, lung, heart, liver and lymphoid tissue, and is particularly abundant in some lymphocyte cell lines (but less so in purified lymphocytes) and vascular endothelial cells subjected to shear stress [49]. Gene expression profiles, useful in the deorphanisation of some GPCRs (e.g., cannabinoid CB1 receptor [72]), especially when performed in an anatomical context at the cellular level, provided little insight into a possible endogenous GPR30 ligand.

I discovered in mid-late 2000, when performing DNA sequence comparisons (NCBI BLAST), that our murine AtT20 and human lymphoblast cDNA clones were species homologues - up until that time I had never compared the two translated protein sequences! To my surprise a study had also reported that GPR30-associated intracellular pathways were activated by estrogen [73]. Given my interest in rapid steroid effects, the irony that I had not tested estrogen on our orphan GPCR clones did not escape me!!

Molecular biology of GPR30 and its rapid effects

E. J. Filardo

To play an integral role in the development of a paradigm-shifting idea built by collegial interaction is truly a rewarding experience. My contribution towards the idea that GPCRs promote pre-genomic signaling by sex steroid hormones was rooted in my postdoctoral work in David Cheresh's lab at Scripps Research Institute. Unbeknownst to me, Eric Prossnitz (also at the early stages of his scientific career) was working at the same time in Charlie Cochrane's group, at his lab bench a mere 10 yards from mine. Although we did not collaborate or interact while at Scripps, both of us were working on structure/function relationships centered upon transmembrane receptors and intracellular signaling. The intersection of our work was not as obvious then as it is now. While my work focused on the molecular basis of bidirectional signaling by integrins [74-76], Eric was working on structure-function relationships of chemoattractant receptors of the GPCR superfamily.

My journey towards GPR30 began with my interest in the work by Keith Yamada that described "rapid", pre-genomic intracellular signaling networks activated by integrin occupancy and aggregation [77, 78], and a body of literature that had linked GPCRs and RTKs to integrin function. At that time, little was known about how estrogen, or other steroid hormones, manifested rapid intracellular signals associated with growth factor-like responses and extracellular matrix interactions, essential cellular responses necessary for tissue homeostasis and cell survival. Offered an opportunity to begin my independent career in 1996 by Dr. Kirby Bland at the Brown University School of Medicine, I became interested in understanding the role of estrogen in promoting epidermal growth factor and integrin activation, and its possible importance to breast cancer pathophysiology. A literature review confirmed for me the difficulty in aligning pre-genomic responses by estrogen with the known structure-function relationships assigned to ER and other members of the nuclear steroid hormone receptor family.

By the late 1990s, many laboratories had implicated GPCRs and/or G proteins in estrogen-mediated activation of cAMP and other second messengers [79]; however, the mechanism that linked estrogen to protein kinase signaling was entirely unclear. Robert Lefkowitz [80], and Axel

Ullrich [81] provided a molecular mechanism by which a single GPCR could trigger, in parallel, second messenger and EGFR-mediated signaling pathways through activation of Gα- and Gβγ-dependent signaling mechanisms. Based on these findings, I became intrigued by the idea that an orphan GPCR, whose known ligand had yet-to-be identified, could serve as a singular receptor mechanism capable of activating second messenger and protein kinase pathways. While earlier investigators evaluating pre-genomic estrogen signaling relied solely on ER-positive cell models, I was keen on including ER-negative breast cancer cell models in our analysis. A colleague of mine at Brown, Raymond Frackelton, an expert in EGFR signaling, was a key contributor to these early experiments and pointed out that human SKBr3 breast cancer cells were negative for ERα, and its recently cloned homologue ERβ, at the level of RT-PCR. I remember my surprise, when Jeffrey Quinn from my lab showed me the results that estrogen was capable of promoting EGFR tyrosine phosphorylation in SKBr3 breast cancer cells as well as ER-positive MCF-7 cells. Yet, we observed that the same response was not detected in ER-negative MDA-MB-231 cells despite the fact that EGF stimulation led to potent EGFR activation in these cells. I must have asked Jeff to repeat these experiments at least a dozen times! We were even more surprised that ER antagonists also triggered EGFR activation in SKBr3 cells and these data suggested to us that the mechanism of action was clearly ER-independent.

GPR30 was isolated as a molecular clone by different laboratories and received its orphan designation because its cognate ligand had yet to be identified. We noticed that it was cloned from breast cancer, cardiovascular tissue, brain and other estrogen-responsive tissues [44, 46-50]. We were particularly interested in the fact that it had been isolated by subtractive cloning from breast cancer lines that displayed differences in their competence for pre-genomic signaling by estrogen [46], causing us to consider the possibility that GPR30 may serve as an estrogen receptor. A phone call to Ron Weigel resulted in the use of his GPR30 molecular clone in marker rescue experiments to test the concept that GPR30 may trigger Gα- and Gβγ-dependent activation of adenylyl cyclase and EGFR tyrosine kinase activity [73, 79]. Interested in determining whether we could measure specific estrogen binding associated with GPR30, I contacted Peter Thomas, a comparative endocrinologist with expertise in steroid membrane receptor binding. We had a nice telephone conversation and were excited about designing experiments that would test the possibility that GPR30 may provide an evolutionarily conserved ER-independent mechanism of estrogen signaling. Through the use of a traditional radioligand

binding assay, Peter and I were able to link estrogen binding activity to GPR30 expression in enriched plasma membrane preparations isolated from tissues derived from both man [82, 83] and fish [84]. Key to the strength of these data was the observation that uncoupling $G\alpha_s$ from GPR30 significantly reduced ligand binding [82, 84]. Studies from the Prossnitz lab using an independent approach showed the association of specific GPR30 binding by fluorescently-labeled estrogen derivatives [66] and that selective synthetic agonists/antagonists discriminate GPR30 from ER [85, 86], helping to further establish the concept that GPR30 is an estrogen receptor, and leading to its description as an estrogen receptor now known by its IUPHAR functional designate as GPER [87].

To date, the most vexing part of understanding the molecular mechanism of GPER has been the observation that its expression is commonly, but not exclusively localized to intracellular membranes in many tissues. Endogenous GPER is expressed on the cell surface in certain tissues, including oocytes, pyrimadal neurons and cortical renal epithelia [88]. GPER is not alone in this regard, as many GPCRs show a predominantly intracellular expression pattern and have been shown to function intracellularly [89], particularly following internalization [90], but also on nuclear, Golgi apparatus and endoplasmic reticulum membranes [91, 92]. Nevertheless, the mechanisms that limit GPCR surface expression at the plasma membrane also represent a primary means to dictate cellular responsiveness of many GPCRs to their cognate ligands, particularly those that are not membrane permeable in contrast to lipids and steroids. For this reason, studies that investigate the molecular mechanisms that determine the subcellular distribution of GPER are the next critical step in understanding its mechanism of action, and may be vital in developing effective pharmacological agents that target this novel estrogen receptor.

Binding and signal transduction of GPER

P. Thomas

I became interested in nongenomic steroid actions in 1980 when I began investigating the endocrine control of oocyte meiotic maturation in fish. Recent studies in fish and amphibians had indicated that a maturation–inducing steroid (MIS) acts on the oocyte surface through a

membrane receptor to induce meiotic maturation by a transcription-independent mechanism [93, 94]. Reynaldo Patino developed a reliable membrane receptor filtration assay for measurement of MIS receptor binding in our laboratory [95], which proved to be indispensable for our subsequent studies to identify novel steroid receptors. The assay was used to track MIS receptor binding by Yong Zhu during the purification, cloning and characterization of the membrane progestin receptor α (mPRα), which belongs to the progestin adipoQ receptor (PAQR) family, and is the first steroid receptor unrelated to nuclear steroid receptors discovered in vertebrates [33,34,35]. Modifications of the receptor assay were later used in our laboratory by Hakan Berg to track androgen binding during purification, cloning and characterization of the novel membrane androgen receptor, ZIP9, a zinc transporter protein belonging to the SLC39A family [36,37]

Fortunately, the lengthy procedures we used to identify mPRα and ZIP9 were not required to identify the novel membrane estrogen receptor (ER) because a candidate, the orphan GPCR, GPR30, had already been identified by Edward Filardo [73]. He had identified GPR30 as a potential membrane ER based on his pioneering research on estrogen stimulation of adenylyl cyclase/cAMP and EGFR/MAPK signaling in GPR30-positive, nuclear estrogen receptor (ER)-negative breast cancer cells [73, 79, 96]. We gladly accepted Ed's invitation to participate in a collaborative research effort to determine if human GPR30 is a membrane ER. A 17β-estradiol (E2) binding activity with all the characteristics of a membrane ER was identified on SKBR3 cell membranes, which express GPR30 but lack full-length nuclear ERs, and in ER-null HEK293 cells transfected with GPR30, using our well-characterized receptor assay for croaker membrane ER [82, 97]. Competitive binding studies showed that binding to GPR30 is specific for E2, environmental estrogens, and ER antiestrogens (ICI 182,780, tamoxifen), whereas the potent ER agonist, diethylstilbestrol, and other classes of steroids showed no displacement of [^{3}H]-E2 over a wide range of concentrations [82, 98].

At about the same time our initial characterization of GPR30 was published, a paper from Eric Prossnitz's research group employing E2 conjugated to a fluorescent label showed that the E2-conjugate bound the GPR30 with the characteristics of a membrane ER [66]. Confirmation that GPR30 is a membrane ER by the two research groups working independently provided the impetus for further research on the receptor. However, the cellular localization of GPR30 would

remain unresolved as Eric's studies observed GPR30 is expressed predominantly and activated intracellularly [66, 99], in contrast to our results demonstrating it is activated on the cell surface [82, 83].

Interestingly, the ER antiestrogens ICI 182,780 and tamoxifen were found to act as GPER agonists, mimicking the E2-induced increase in cAMP production [82]. The finding that several environmental estrogens, including bisphenol A, nonylphenol, genistein, zearalenone, display relatively high binding affinities for GPER and act as agonists to increase cAMP synthesis [98], provided an early indication that these compounds can disrupt nongenomic estrogen actions through the receptor. Recently, we found that an E2 metabolite, the catecholestrogen 2-hydroxy-E2, binds with high affinity to zebrafish GPER and acts and a GPER antagonist, reversing E2 maintenance of oocyte meiotic arrest in this species [100].

It has been proposed that GPER may also act as an aldosterone receptor based on the observation that GPER expression is involved in aldosterone stimulation of ERK1/2 phosphorylation [101, 102], although direct evidence that GPER binds aldosterone was lacking. In detailed binding experiments, we did not detect any specific [^{3}H]-aldosterone binding to plasma membrane fractions of mouse kidneys that have high GPER expression, or to HEK293 cells expressing recombinant GPER, both of which displayed high binding to [^{3}H]-E2 [103]. Aldosterone and several mineralocorticoid receptor antagonists also did not compete with [^{3}H]-E2 binding to GPER, which suggests that the interactions of aldosterone with GPER do not involve direct binding to the ligand-binding region of the receptor. Consistent with this notion, work by Maggiolini's group has recently shown cross-talk between GPER and MR in mediating the effects of aldosterone [104].

Recent research in our laboratory by Yefei Pang and others has focused on the characteristics and reproductive functions of GPER in fish oocytes. Despite approximately 450 million years of divergent evolution of human and teleost GPERs, their receptor binding characteristics including steroid specificity are remarkably similar [84, 105]. In addition, the human and fish receptors are directly coupled to the same G protein ($G\alpha_s$) and activate the same signaling pathways (adenylyl cyclase/cAMP and EGFR/MAPK) [105, 106]. These comparative studies suggest the membrane ER activity of GPER is its fundamental, conserved primary physiological

function in vertebrates. It is not known, though, whether the reproductive functions of GPER identified in fish are shared by other vertebrates.

Pharmacology and physiology of GPER

E. R. Prossnitz

Having studied G protein-coupled receptor structure/function relationships and signaling pathways in the context of neutrophil activation by chemoattractants since 1989 [107-122], I was immediately intrigued with the idea of a GPCR functioning as an estrogen receptor in 2002 based on the then recent review of GPR30 by Ed Filardo [79]. This work had first been presented to a group of investigators at UNM by Kim Leslie, who was organizing an NCI SPORE application on endometrial cancer. We formed a working group in late 2002 to begin to develop a project to examine the potential contribution of GPR30 to endometrial function and carcinogenesis. Towards that goal, I requested a clone of GPR30 from Ron Weigel (due to his relative geographical proximity), who reported the cloning of GPCR-Br (i.e. GPR30) in 1997 [46]. The original team at UNM included Larry Sklar, with whom I had long been collaborating on chemoattractant GPCR function [123] , as well as the development of high throughput flow cytometry [124], since our days at the Scripps Research Institute, and Tudor Oprea, an expert in cheminformatics, who had recently arrived from AstraZeneca [125, 126]. Larry Sklar soon introduced me to Jeffrey Arterburn at New Mexico State University, a synthetic organic chemist, who by chance was already working of estrogen derivatives for imaging purposes [127, 128]. This group of individuals, along with highly talented post-doctoral fellows, Chetana Revankar in my lab, and Cristian Bologa in the Oprea group, formed the basis of the team that initially explored GPER function in cells [66] and soon screened for GPER-selective ligands [85]. It was Chetana Revankar, who showed me our first image of GPR30-RFP expressed in COS7 cells, which revealed a completely unexpected predominantly intracellularly localized GPER (**Figure 4**), an observation that would later be confirmed in many, but not all, cell types with antibodies detecting endogenous GPER [66], and manually carried out the screening of the top 100 estrogen-like compounds (computationally identified by Tudor Oprea from a library of 10,000 compounds at UNM). The GPER-selective compound, later named G-1, was #92 [85]; one can only wonder how things might have been different had it been #101! In connection with the original Endometrial SPORE application, I was also introduced to Harriet Smith, a

gynecologic oncologist at UNM, with whom I would work to examine the prognostic potential of GPER expression in endometrial and ovarian cancers [129, 130]. It is perhaps ironic that our first publication on GPER, describing E2 binding to GPER and differential signaling by GPER and ERα, was submitted to *Science* in August 2004 [66], within about a week of the paper by Peter Thomas and Ed Filardo, describing similar findings using entirely different approaches [82].

Recognizing that a fundamental question regarding GPER was its physiological role(s) in health and disease states, I realized that we needed to expand our cell-based studies to animal models, an area in which I was not experienced. So it was with great fortune that I discussed our work with my departmental colleague Helen Hathaway, an expert on the development of the mammary gland and murine models of disease [131]. In 2006, I was contacted by and soon (in 2008) met Matthias Barton at a drug discovery meeting in Dubai, beginning a more than 10-year collaboration [70, 132-147]. Various combinations of these individuals (along with superb graduate students, post-docs and technicians) would be central to our studies over the years to demonstrate that 1) GPER-expressing cells exhibited binding of a fluorescent estrogen derivative that colocalized with GPER to intracellular membranes (endoplasmic reticulum and Golgi apparatus) (Arterburn, Sklar, [66]); 2) only cell permeable estrogen derivatives rapidly activate GPER, suggesting intracellular GPER is functionally active (Arterburn, [99]); 3) GPER-selective agonists and antagonists, that do not interact with the classical estrogen receptors, can be identified (Oprea, Sklar, Arterburn, [85, 86, 148, 149]); 4) GPER can be visualized in vivo through the use of novel radio-imaging agents (Arterburn, [150-155]); 5) using murine models, including GPER knockout mice, GPER plays important roles in both breast and endometrial cancer (but not uterine imbibition for example) (Hathaway, [86, 156-158]) as well as in metabolism (Hathaway, [159]) and vascular biology and disease (Barton, [70, 134, 139-142, 145-147, 160, 161]); 6) GPER is prognostic for survival in both endometrial and ovarian cancers (Smith, [129, 130]) and 7) GPER is critical in aging through its regulation of superoxide production via Nox1 (Barton, [162]). Our work to understand the roles of GPER in metabolism [163] and vascular biology [71] and the potential clinical benefits of GPER-selective agonists and antagonists [149] are reviewed elsewhere in this special issue. My progress in this field over the years has not only been made possible by these many individuals, as well as gifted graduate students, technicians and post-docs, but also the co-authors of this article. In

retrospect, I also believe that my background in GPCR molecular pharmacology provided me with a fresh unbiased viewpoint, influenced little by the dogma of nuclear estrogen receptors, that facilitated much of our progress in the field. As a final thought regarding serendipity in science, it was not until working on GPER for some time that I realized, upon searching for new publications by Ed Filardo in Pubmed, that Ed and I had both been post-docs at Scripps in the early 1990's – in fact in neighboring labs!

Transcriptional regulation by GPER

M. Maggiolini

Working in the years 1998-1999 as a visiting professor in Didier Picard's lab at the Université de Genève, Switzerland, I had my first opportunity to provide novel insights into estrogen receptor (ER)-mediated functions in breast cancer [164, 165], and subsequently to demonstrate that certain phytoestrogens may elicit stimulatory effects in this malignancy by activating ERs [166, 167]. Further searching for the multifaceted aspects of estrogen signaling, it was intriguing how I came across GPER. Unexpectedly, ER-negative (but GPER-positive) SKBr3 breast cancer cells displayed relevant biological responses upon estrogen/phytoestyrogen exposure [168]. Initially, it was challenging to understand our results in the context of existing paradigms. However, the initial studies of Filardo's group on GPER helped us to explore new mechanisms, as the silencing of GPER abrogated the effects of the aforementioned compounds [168]. One important aspect of these investigations was that agonist-activated GPER engages the EGFR/MAPK signal transduction pathway resulting in the rapid transcriptional activation of the oncogene c-FOS, which was thereafter used as a molecular sensor of GPER action in other cell contexts such as thyroid and endometrial tumor cells [169, 170]. Working together with Eric Prossnitz (University of New Mexico, USA), we then ascertained that in GPER- and ER-positive ovarian cancer cells, 17β-estradiol and the selective GPER ligand G-1 upregulated several estrogen-responsive genes such as c-FOS, pS2, and cyclins A, D1 and E; in contrast however, progesterone receptor (PR) only responded to E2 [171]. These data, together with the results obtained by silencing experiments and growth assays, suggested that a functional interaction between GPER and ERα may occur when both receptors are expressed in tumor cells [171].

In the framework of my meaningful and long-standing collaboration with Didier Picard, it was fortuitous that a post-doc (D. Prakash Pandey) from his group came to my lab and, working together with my collaborators, defined the gene signature prompted by estrogenic GPER signaling [172]. Interestingly, gene responses included a first tier of transcription factors including SRF, CREB, and members of the ETS family followed by a second wave of transcription factors such as FOS, JUN, CTGF, EGR1, ATF3, C/EBPd and NR4A2 ([172]; see also [173]). These findings allowed us to discover a second critical molecular sensor of GPER action beyond c-*fos*, namely CTGF, as highlighted in several subsequent studies (reviewed in [174]).

Thereafter, we sought to determine the role of GPER in estrogen-mediated function with respect to the main components of the tumor microenvironment, cancer-associated fibroblasts (CAFs), which drive crucial malignant features such as invasion, metastasis and angiogenesis [97-108]. In a recent study [175], we found that ligand-activated GPER generates a feed-forward loop coupling IL1β induction by CAFs to IL1R1 expression by cancer cells, thereby promoting the up-regulation of IL1β/IL1R1 target genes, namely PTGES, COX2, RAGE and ABCG2. As a biological counterpart, the functional liaison between these two cell types triggered migration and invasive features in breast cancer cells, including fibroblastoid cytoarchitecture and F-actin reorganization. These data therefore contributed to the further appreciation of the role elicited by GPER toward pro-tumorigenic inflammatory phenotypes. In addition, we discovered that low oxygen tension, a critical factor in the hypoxic tumor environment, triggers, through HIF-1α, the induction of both GPER and its downstream target CTGF [176]. Similarly, it was remarkable to discover that GPER-mediated signaling up-regulates HIF1α and VEGF, further supporting the involvement of GPER in cancer angiogenesis [177, 178].

As previous studies had attempted to determine whether GPER may be considered a prognostic factor in cancer outcome/survival [179, 180], a better understanding of its regulation was warranted. In this regard, our early as well as more recent studies have provided notable data regarding the potential of diverse growth factors and cognate receptors to modulate GPER, engaging its downstream signaling towards the stimulation of aggressive cancer features [181-188]. Such studies gave us the opportunity to establish a great collaboration with

Antonino Belfiore at the University of Catanzaro, Italy, to examine the molecular signals linking the insulin-like growth factor-I (IGF-I)/IGF-I receptor (IGF-IR) system and GPER, providing further insights on the well-established actions played by the IGF axis in cancer. Over the years, our studies of the actions of estrogen through GPER support the critical roles played by several other members of the GPCR family in cancer development and progression [189]. Last but not least, I must acknowledge the fundamental role played by the many young people who contributed to the aforementioned research topics, some of which are still members of our group, whereas others are working abroad.

Concluding thoughts on future research: the next 20 years

The history of estrogen has been replete with astounding discoveries and advances in medical care. Its critical roles in reproduction/fertility/menopause and breast cancer have resulted in perhaps some of the most transformative clinical advances of our time, namely the induction/prevention of conception (birth control), the alleviation of post-menopausal symptoms and diseases (menopausal hormone therapy), and the treatment of ER-positive breast cancer [27, 190]. Together, modulating the (patho)physiology of estrogen and its receptors has benefitted hundreds of millions of women. Despite our growing knowledge of the mechanisms of estrogen action, much remains to be understood. Although some of the first actions of estrogen to be described would today be defined as rapid or non-genomic, subsequent decades or work focused on the transcriptional or genomic actions of estrogen, before a resurgence of investigations into rapid effects in the 1990s and 2000s. Today, it is clear that the combined rapid and genomic effects of estrogen are critical to its overall function, and that even in the absence of estrogen (i.e. in the unliganded state), "classical" estrogen receptors [191] and GPER [162] exert constitutive (i.e. ligand-independent) functions on physiology and disease [192, 193]. However, these interactions are complex and involve multiple receptor types, both soluble and membrane associated. The development of selective ligands that either activate or inhibit individual receptors not only further our basic understanding of estrogen biology but may also represent important clinical opportunities for drug development [142, 146, 194, 195]. In the next 20 years, much remains to be elucidated regarding the actions of GPER and the possibility of diagnostics and therapeutics targeting the pathways that are regulated by this receptor.

In Memoriam: Oliver Smithies

As the authors were finalizing this manuscript, they learned of the death of Oliver Smithies (1925-2017) on January 10 [196, 197]. Smithies, like Mario Capecchi and Sir Martin Evans, had pioneered the generation of transgenic animals using embryonic stem cells [198, 199], for which they received the 2007 Nobel Prize in Physiology or Medicine [200]. The technology introduced by Smithies was essential for studying the role of GPER in physiology and disease, as it was used to generate the first GPER-deficient mouse in 2007 [69, 70, 131, 162, 201].

Competing interests

M.B. and E.R.P. are inventors on a U.S. patent application for the therapeutic use of compounds targeting GPER, E.R.P. is an inventor on U.S. patent Nos. 7,875,721 and 8,487,100 for GPER-selective ligands and imaging agents.

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References

1. Stuenkel, C. A. (2017) Menopause, hormone therapy and diabetes. Climacteric, 1-13.
2. Almeida, M., Laurent, M. R., Dubois, V., Claessens, F., O'Brien, C. A., Bouillon, R., Vanderschueren, D., Manolagas, S. C. (2017) Estrogens and androgens in skeletal physiology and pathophysiology. Physiol. Rev. 97, 135-187.
3. Lobo, R. A., Pickar, J. H., Stevenson, J. C., Mack, W. J., Hodis, H. N. (2016) Back to the future: Hormone replacement therapy as part of a prevention strategy for women at the onset of menopause. Atherosclerosis 254, 282-290.
4. Lobo, R. A., Davis, S. R., De Villiers, T. J., Gompel, A., Henderson, V. W., Hodis, H. N., Lumsden, M. A., Mack, W. J., Shapiro, S., Baber, R. J. (2014) Prevention of diseases after menopause. Climacteric 17, 540-56.
5. Li, R., Cui, J., Shen, Y. (2014) Brain sex matters: estrogen in cognition and Alzheimer's disease. Mol. Cell. Endocrinol. 389, 13-21.
6. Hage, F. G. and Oparil, S. (2013) Ovarian hormones and vascular disease. Curr. Opin. Cardiol. 28, 411-6.
7. Oparil, S. and Miller, A. P. (2005) Gender and blood pressure. J. Clin. Hypertens. (Greenwich) 7, 300-9.
8. Calhoun, D. A. and Oparil, S. (1998) The sexual dimorphism of high blood pressure. Cardiol. Rev. 6, 356-363.
9. Oparil, S. (1999) Arthur C. Corcoran Memorial Lecture. Hormones and vasoprotection. Hypertension 33, 170-6.
10. Xing, D., Nozell, S., Chen, Y. F., Hage, F., Oparil, S. (2009) Estrogen and mechanisms of vascular protection. Arterioscler. Thromb. Vasc. Biol. 29, 289-95.
11. Corner, G. W. (1965) The early history of the oestrogenic hormones - The Sir Henry Dale Lecture for 1964. J. Endocrinol. 31, III-XVII.
12. Schneider, H. P. (2002) Geburtshilfe und Frauenheilkunde: Sicht eines deutschen Herausgebers. Zentralbl. Gynakol. 124, 79-83.
13. Mainzer, F. (1896) Vorschlag zur Behandlung der Ausfallserscheinungen nach Castration. Dtsch. Med. Wochenschr. 22, 188.
14. Mainzer, F. (1896) Zur Behandlung amenorrhoischer und klimakterischer Frauen mit Ovarialsubstanz. Dtsch Med Wohenschr 25, 393-396.
15. Landau, M. (1896) Zur Behandlung von Beschwerden der natürlichen und antizipierten Klimax mit Eierstocksubstanz. Berl Klin Wochenschr, 557.
16. Mond, R. (1896) Kurze Mittheilung über die Behandlung der Beschwerden bei natürlicher oder durch Operation veranlaßter Amenorrhoe mit Eierstocksconserven (Ovariin Merck). Münch Med Wschr 14, 314-316.
17. Barton, M. (2013) Prevention and endothelial therapy of coronary artery disease. Curr. Opin. Pharmacol. 13, 226-41.
18. Rather, L. J. (1957) Rudolph Virchow and scientific medicine. AMA Arch. Intern. Med. 100, 1007-14.
19. Cameron, R. (1958) A symposium in honour of the centenary of Virchow's cellular pathology (1858-1958): 1858. J. Clin. Pathol. 11, 463-72.
20. Boyd, S. (1897) On oophorectomy in the treatment of cancer. Br. Med. J. 2, 890-896.
21. Fosbery, W. H. S. (1897) Severe climacteric flushings successfully treated by ovarian extract. The British Medical Journal 1, 1039.

22. Virchow, R. (1894) Morgagni und der anatomische Gedanke. Rede, gehalten am 30. März auf dem XI. internationalen medicinischen Congress zu Rom. Berl Klin Wochenschr 31, 345-350.
23. Ganten, D. (1995) The Journal of Molecular Medicine: tradition, continuity, and renaissance. J. Mol. Med. (Berl.) 73, 1-3.
24. Allen, E. and Doisy, E. A. (1983) Landmark article Sept 8, 1923. An ovarian hormone. Preliminary report on its localization, extraction and partial purification, and action in test animals. By Edgar Allen and Edward A. Doisy. JAMA 250, 2681-3.
25. Butenandt, A. (1929) Über „Progynon", ein krystallisiertes weibliches Sexualhormon. Die Naturwissenschaften 17, 879.
26. Doisy, E. A., Veler, C. D., Thayer, S. (1930) The preparation of the crystalline ovarian hormone from the urine of pregnant women. J. Biol. Chem. 86, 499-509.
27. Simpson, E. and Santen, R. J. (2015) Celebrating 75 years of oestradiol. J. Mol. Endocrinol. 55, T1-20.
28. Wolf, G. (2004) The discovery of vitamin D: the contribution of Adolf Windaus. J. Nutr. 134, 1299-302.
29. O'Malley, B. W. and Khan, S. (2013) Elwood V. Jensen (1920-2012): father of the nuclear receptors. Proc. Natl. Acad. Sci. U. S. A. 110, 3707-8.
30. Notelovitz, M. (1999) Discovery and identification of estrogen. Menopause 6, 2-3.
31. Jensen, E. V., Jacobson, H. I., Walf, A. A., Frye, C. A. (2010) Estrogen action: a historic perspective on the implications of considering alternative approaches. Physiol. Behav. 99, 151-62.
32. Jensen, E. (2012) A conversation with Elwood Jensen. Interview by David D. Moore. Annu. Rev. Physiol. 74, 1-11.
33. Walter, P., Green, S., Greene, G., Krust, A., Bornert, J. M., Jeltsch, J. M., Staub, A., Jensen, E., Scrace, G., Waterfield, M., et al. (1985) Cloning of the human estrogen receptor cDNA. Proc. Natl. Acad. Sci. U. S. A. 82, 7889-93.
34. Greene, G. L., Gilna, P., Waterfield, M., Baker, A., Hort, Y., Shine, J. (1986) Sequence and expression of human estrogen receptor complementary DNA. Science 231, 1150-4.
35. Green, S., Walter, P., Greene, G., Krust, A., Goffin, C., Jensen, E., Scrace, G., Waterfield, M., Chambon, P. (1986) Cloning of the human oestrogen receptor cDNA. J. Steroid Biochem. 24, 77-83.
36. Szego, C. M. and Davis, J. S. (1967) Adenosine 3',5'-monophosphate in rat uterus: acute elevation by estrogen. Proc. Natl. Acad. Sci. U. S. A. 58, 1711-8.
37. Szego, C. M. and Davis, J. S. (1969) Inhibition of estrogen-induced cyclic AMP elevation in rat uterus. II: By glucocorticoids. Life Sci. 8, 1109-16.
38. Szego, C. M. and Davis, J. S. (1969) Inhibition of estrogen-induced elevation of cyclic 3',5'-adenosine monophosphate in rat uterus. I. By beta-adrenergic receptor-blocking drugs. Mol. Pharmacol. 5, 470-80.
39. Soloff, M. S. and Szego, C. M. (1969) Purification of estradiol receptor from rat uterus and blockade of its estrogen-binding function by specific antibody. Biochem. Biophys. Res. Commun. 34, 141-7.
40. Pietras, R. J. and Szego, C. M. (1977) Specific binding sites for oestrogen at the outer surfaces of isolated endometrial cells. Nature 265, 69-72.

41. Pietras, R. J. and Szego, C. M. (1980) Partial purification and characterization of oestrogen receptors in subfractions of hepatocyte plasma membranes. Biochem. J. 191, 743-60.
42. Pietras, R. J. and Szego, C. M. (1979) Estrogen receptors in uterine plasma membrane. J. Steroid Biochem. 11, 1471-83.
43. McCoy, R. L. and Permutter, D. H. (1996) Cloning of novel IL8-related receptors from human hepatic tissue. EMBL/GenBank/DDBJ databases.
44. Owman, C., Blay, P., Nilsson, C., Lolait, S. J. (1996) Cloning of human cDNA encoding a novel heptahelix receptor expressed in Burkitt's lymphoma and widely distributed in brain and peripheral tissues. Biochem. Biophys. Res. Commun. 228, 285-92.
45. Bonini, J. A., Anderson, S. M., Steiner, D. F. (1997) Molecular cloning and tissue expression of a novel orphan G protein-coupled receptor from rat lung. Biochem. Biophys. Res. Commun. 234, 190-3.
46. Carmeci, C., Thompson, D. A., Ring, H. Z., Francke, U., Weigel, R. J. (1997) Identification of a gene (GPR30) with homology to the G-protein-coupled receptor superfamily associated with estrogen receptor expression in breast cancer. Genomics 45, 607-17.
47. Feng, Y. and Gregor, P. (1997) Cloning of a novel member of the G protein-coupled receptor family related to peptide receptors. Biochem. Biophys. Res. Commun. 231, 651-4.
48. Kvingedal, A. M. and Smeland, E. B. (1997) A novel putative G-protein-coupled receptor expressed in lung, heart and lymphoid tissue. FEBS Lett. 407, 59-62.
49. Takada, Y., Kato, C., Kondo, S., Korenaga, R., Ando, J. (1997) Cloning of cDNAs encoding G protein-coupled receptor expressed in human endothelial cells exposed to fluid shear stress. Biochem. Biophys. Res. Commun. 240, 737-41.
50. O'Dowd, B. F., Nguyen, T., Marchese, A., Cheng, R., Lynch, K. R., Heng, H. H., Kolakowski Jr, L. F., George, S. R. (1998) Discovery of three novel G-protein-coupled receptor genes. Genomics 47, 310-3.
51. Heberden, W. (1772) Some account of a disorder of the breast. Medical Transactions of The Royal College of Physicians of London 2, 59-72.
52. Barton, M., Grüntzig, J., Husmann, M., Rösch, J. (2014) Balloon angioplasty - the legacy of Andreas Grüntzig, M.D. (1939-1985). Front Cardiovasc Med 1, 15.
53. Reynolds, S. R. and Foster, F. I. (1939) Peripheral vascular action of estrogen in the human male. J. Clin. Invest. 18, 649-55.
54. Furchgott, R. F. and Zawadzki, J. V. (1980) The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature 288, 373-6.
55. Mitka, M. (1998) 1998 NObel Prize winners are announced: three discoverers of nitric oxide activity. JAMA 280, 1648.
56. Ignarro, L. J., Buga, G. M., Wood, K. S., Byrns, R. E., Chaudhuri, G. (1987) Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. Proc. Natl. Acad. Sci. U. S. A. 84, 9265-9.
57. Mügge, A., Riedel, M., Barton, M., Kuhn, M., Lichtlen, P. R. (1993) Endothelium independent relaxation of human coronary arteries by 17β-oestradiol in vitro. Cardiovasc. Res. 27, 1939-42.

58. Barton, M., Cremer, J., Mügge, A. (1998) 17β-estradiol acutely improves endothelium-dependent relaxation to bradykinin in isolated human coronary arteries. Eur. J. Pharmacol. 362, 73-6.
59. Lieberman, E. H., Gerhard, M. D., Uehata, A., Walsh, B. W., Selwyn, A. P., Ganz, P., Yeung, A. C., Creager, M. A. (1994) Estrogen improves endothelium-dependent, flow-mediated vasodilation in postmenopausal women. Ann. Intern. Med. 121, 936-41.
60. Barton, M. (1994) Akute Wirkungen von 17β-Oestradiol auf den Tonus menschlicher Arterien in vitro. Doctor of Medicine (D.M.) Dissertation, Hannover Medical School, Hannover, Germany, 1-79.
61. Aronica, S. M., Kraus, W. L., Katzenellenbogen, B. S. (1994) Estrogen action via the cAMP signaling pathway: stimulation of adenylate cyclase and cAMP-regulated gene transcription. Proc. Natl. Acad. Sci. U. S. A. 91, 8517-21.
62. Kuiper, G. G., Enmark, E., Pelto-Huikko, M., Nilsson, S., Gustafsson, J. A. (1996) Cloning of a novel receptor expressed in rat prostate and ovary. Proc. Natl. Acad. Sci. U. S. A. 93, 5925-30.
63. Mosselman, S., Polman, J., Dijkema, R. (1996) ER beta: identification and characterization of a novel human estrogen receptor. FEBS Lett. 392, 49-53.
64. Heigl, A., Lachat, M., Lattmann, T., Luscher, T., Barton, M. (2002) Acute effects of 17β-oestradiol on functional activity of endothelin-converting enzymes in human arteries and veins. Clin. Sci. (Lond.) 103 Suppl 48, 438S-441S.
65. Traupe, T., Stettler, C. D., Li, H., Haas, E., Bhattacharya, I., Minotti, R., Barton, M. (2007) Distinct roles of estrogen receptors a and b mediating acute vasodilation of epicardial coronary arteries. Hypertension 49, 1364-70.
66. Revankar, C. M., Cimino, D. F., Sklar, L. A., Arterburn, J. B., Prossnitz, E. R. (2005) A transmembrane intracellular estrogen receptor mediates rapid cell signaling. Science 307, 1625-30.
67. Meyer, M. R., Haas, E., Barton, M. (2006) Gender differences of cardiovascular disease: new perspectives for estrogen receptor signaling. Hypertension 47, 1019-26.
68. Haas, E., Meyer, M. R., Schurr, U., Bhattacharya, I., Minotti, R., Nguyen, H. H., Heigl, A., Lachat, M., Genoni, M., Barton, M. (2007) Differential effects of 17β-estradiol on function and expression of estrogen receptor a, estrogen receptor b, and GPR30 in arteries and veins of patients with atherosclerosis. Hypertension 49, 1358-63.
69. Wang, C., Dehghani, B., Magrisso, I. J., Rick, E. A., Bonhomme, E., Cody, D. B., Elenich, L. A., Subramanian, S., Murphy, S. J., Kelly, M. J., Rosenbaum, J. S., Vandenbark, A. A., Offner, H. (2008) GPR30 contributes to estrogen-induced thymic atrophy. Mol. Endocrinol. 22, 636-48.
70. Haas, E., Bhattacharya, I., Brailoiu, E., Damjanovic, M., Brailoiu, G. C., Gao, X., Mueller-Guerre, L., Marjon, N. A., Gut, A., Minotti, R., Meyer, M. R., Amann, K., Ammann, E., Perez-Dominguez, A., Genoni, M., Clegg, D. J., Dun, N. J., Resta, T. C., Prossnitz, E. R., Barton, M. (2009) Regulatory role of G protein-coupled estrogen receptor for vascular function and obesity. Circ. Res. 104, 288-91.
71. Meyer, M. R. and Barton, M. (2017) GPER blockers as Nox downregulators: a new drug class to target chronic non-communicable diseases. J. Steroid Biochem. Mol. Biol. this issue.

72. Matsuda, L. A., Lolait, S. J., Brownstein, M. J., Young, A. C., Bonner, T. I. (1990) Structure of a cannabinoid receptor and functional expression of the cloned cDNA. Nature 346, 561-4.
73. Filardo, E. J., Quinn, J. A., Bland, K. I., Frackelton, A. R., Jr. (2000) Estrogen-induced activation of Erk-1 and Erk-2 requires the G protein-coupled receptor homolog, GPR30, and occurs via trans-activation of the epidermal growth factor receptor through release of HB-EGF. Mol. Endocrinol. 14, 1649-60.
74. Filardo, E. J., Deming, S. L., Cheresh, D. A. (1996) Regulation of cell migration by the integrin beta subunit ectodomain. J. Cell Sci. 109 (Pt 6), 1615-22.
75. Filardo, E. J., Brooks, P. C., Deming, S. L., Damsky, C., Cheresh, D. A. (1995) Requirement of the NPXY motif in the integrin beta 3 subunit cytoplasmic tail for melanoma cell migration in vitro and in vivo. J. Cell Biol. 130, 441-50.
76. Filardo, E. J. and Cheresh, D. A. (1994) A beta turn in the cytoplasmic tail of the integrin alpha v subunit influences conformation and ligand binding of alpha v beta 3. J. Biol. Chem. 269, 4641-7.
77. Miyamoto, S., Teramoto, H., Coso, O. A., Gutkind, J. S., Burbelo, P. D., Akiyama, S. K., Yamada, K. M. (1995) Integrin function: molecular hierarchies of cytoskeletal and signaling molecules. J. Cell Biol. 131, 791-805.
78. Miyamoto, S., Akiyama, S. K., Yamada, K. M. (1995) Synergistic roles for receptor occupancy and aggregation in integrin transmembrane function. Science 267, 883-5.
79. Filardo, E. J. (2002) Epidermal growth factor receptor (EGFR) transactivation by estrogen via the G-protein-coupled receptor, GPR30: a novel signaling pathway with potential significance for breast cancer. J. Steroid Biochem. Mol. Biol. 80, 231-8.
80. Luttrell, L. M., Daaka, Y., Lefkowitz, R. J. (1999) Regulation of tyrosine kinase cascades by G-protein-coupled receptors. Curr. Opin. Cell Biol. 11, 177-83.
81. Prenzel, N., Zwick, E., Daub, H., Leserer, M., Abraham, R., Wallasch, C., Ullrich, A. (1999) EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF. Nature 402, 884-8.
82. Thomas, P., Pang, Y., Filardo, E. J., Dong, J. (2005) Identity of an estrogen membrane receptor coupled to a G protein in human breast cancer cells. Endocrinology 146, 624-32.
83. Filardo, E., Quinn, J., Pang, Y., Graeber, C., Shaw, S., Dong, J., Thomas, P. (2007) Activation of the novel estrogen receptor, GPR30, at the plasma membrane. Endocrinology 148, 3236-3245.
84. Thomas, P., Alyea, R., Pang, Y., Peyton, C., Dong, J., Berg, A. H. (2010) Conserved estrogen binding and signaling functions of the G protein-coupled estrogen receptor 1 (GPER) in mammals and fish. Steroids 75, 595-602.
85. Bologa, C. G., Revankar, C. M., Young, S. M., Edwards, B. S., Arterburn, J. B., Kiselyov, A. S., Parker, M. A., Tkachenko, S. E., Savchuck, N. P., Sklar, L. A., Oprea, T. I., Prossnitz, E. R. (2006) Virtual and biomolecular screening converge on a selective agonist for GPR30. Nat. Chem. Biol. 2, 207-12.
86. Dennis, M. K., Burai, R., Ramesh, C., Petrie, W. K., Alcon, S. N., Nayak, T. K., Bologa, C. G., Leitao, A., Brailoiu, E., Deliu, E., Dun, N. J., Sklar, L. A., Hathaway, H. J., Arterburn, J. B., Oprea, T. I., Prossnitz, E. R. (2009) In vivo effects of a GPR30 antagonist. Nat. Chem. Biol. 5, 421-7.

87. Prossnitz, E. R. and Arterburn, J. B. (2015) International Union of Basic and Clinical Pharmacology. XCVII. G protein-coupled estrogen receptor and its pharmacologic modulators. Pharmacol. Rev. 67, 505-40.
88. Gaudet, H. M., Cheng, S. B., Christensen, E. M., Filardo, E. J. (2015) The G-protein coupled estrogen receptor, GPER: The inside and inside-out story. Mol. Cell. Endocrinol.
89. Marrache, A. M., Gobeil, F., Zhu, T., Chemtob, S. (2005) Intracellular signaling of lipid mediators via cognate nuclear G protein-coupled receptors. Endothelium 12, 63-72.
90. Tsvetanova, N. G., Irannejad, R., von Zastrow, M. (2015) G protein-coupled receptor (GPCR) signaling via heterotrimeric G proteins from endosomes. J. Biol. Chem. 290, 6689-96.
91. Boivin, B., Vaniotis, G., Allen, B. G., Hebert, T. E. (2008) G protein-coupled receptors in and on the cell nucleus: a new signaling paradigm? J. Recept. Signal Transduct. Res. 28, 15-28.
92. Campden, R., Audet, N., Hebert, T. E. (2015) Nuclear G protein signaling: new tricks for old dogs. J. Cardiovasc. Pharmacol. 65, 110-22.
93. Godeau, J. F., Schorderet-Slatkine, S., Hubert, P., Baulieu, E. E. (1978) Induction of maturation in Xenopus laevis oocytes by a steroid linked to a polymer. Proc. Natl. Acad. Sci. U. S. A. 75, 2353-7.
94. Jalabert, B. (1976) In vitro oocyte maturation and ovulation in rainbow trout (Salmo gairdneri), northern pike (Esox lucius), and goldfish (Carassius auratus). J Fish Res Board.Can 33, 974–988.
95. Patino, R. and Thomas, P. (1990) Characterization of membrane receptor activity for 17 alpha, 20 beta, 21-trihydroxy-4-pregnen-3-one in ovaries of spotted seatrout (Cynoscion nebulosus). Gen. Comp. Endocrinol. 78, 204-17.
96. Filardo, E. J., Quinn, J. A., Frackelton, A. R., Jr., Bland, K. I. (2002) Estrogen action via the G protein-coupled receptor, GPR30: stimulation of adenylyl cyclase and cAMP-mediated attenuation of the epidermal growth factor receptor-to-MAPK signaling axis. Mol. Endocrinol. 16, 70-84.
97. Loomis, A. K. and Thomas, P. (2000) Effects of estrogens and xenoestrogens on androgen production by Atlantic croaker testes in vitro: evidence for a nongenomic action mediated by an estrogen membrane receptor. Biol. Reprod. 62, 995-1004.
98. Thomas, P. and Dong, J. (2006) Binding and activation of the seven-transmembrane estrogen receptor GPR30 by environmental estrogens: a potential novel mechanism of endocrine disruption. J. Steroid Biochem. Mol. Biol. 102, 175-9.
99. Revankar, C. M., Mitchell, H. D., Field, A. S., Burai, R., Corona, C., Ramesh, C., Sklar, L. A., Arterburn, J. B., Prossnitz, E. R. (2007) Synthetic estrogen derivatives demonstrate the functionality of intracellular GPR30. ACS Chem. Biol. 2, 536-44.
100. Chourasia, T. K., Pang, Y., Thomas, P. (2015) The catecholestrogen, 2-hydroxyestradiol-17beta, acts as a G protein-coupled estrogen receptor 1 (GPER/GPR30) antagonist to promote the resumption of meiosis in zebrafish oocytes. Biol. Reprod. 92, 69, 1-13.

101. Feldman, R. D. and Gros, R. (2011) Unraveling the mechanisms underlying the rapid vascular effects of steroids: sorting out the receptors and the pathways. Br. J. Pharmacol. 163, 1163-9.
102. Funder, J. W. (2011) GPR30, mineralocorticoid receptors, and the rapid vascular effects of aldosterone. Hypertension 57, 370-2.
103. Cheng, S. B., Dong, J., Pang, Y., LaRocca, J., Hixon, M., Thomas, P., Filardo, E. J. (2014) Anatomical location and redistribution of G protein-coupled estrogen receptor-1 during the estrus cycle in mouse kidney and specific binding to estrogens but not aldosterone. Mol. Cell. Endocrinol. 382, 950-9.
104. Rigiracciolo, D. C., Scarpelli, A., Lappano, R., Pisano, A., Santolla, M. F., Avino, S., De Marco, P., Bussolati, B., Maggiolini, M., De Francesco, E. M. (2016) GPER is involved in the stimulatory effects of aldosterone in breast cancer cells and breast tumor-derived endothelial cells. Oncotarget 7, 94-111.
105. Pang, Y., Dong, J., Thomas, P. (2008) Estrogen signaling characteristics of Atlantic croaker G protein-coupled receptor 30 (GPR30) and evidence it is involved in maintenance of oocyte meiotic arrest. Endocrinology 149, 3410-26.
106. Peyton, C. and Thomas, P. (2011) Involvement of epidermal growth factor receptor signaling in estrogen inhibition of oocyte maturation mediated through the G protein-coupled estrogen receptor (Gper) in zebrafish (Danio rerio). Biol. Reprod. 85, 42-50.
107. Prossnitz, E. R. and Ye, R. D. (1997) The N-formyl peptide receptor: a model for the study of chemoattractant receptor structure and function. Pharmacol. Ther. 74, 73-102.
108. Key, T. A., Bennett, T. A., Foutz, T. D., Gurevich, V. V., Sklar, L. A., Prossnitz, E. R. (2001) Regulation of formyl peptide receptor agonist affinity by reconstitution with arrestins and heterotrimeric G proteins. J. Biol. Chem. 276, 49204-12.
109. Potter, R. M., Key, T. A., Gurevich, V. V., Sklar, L. A., Prossnitz, E. R. (2002) Arrestin variants display differential binding characteristics for the phosphorylated N-formyl peptide receptor carboxyl terminus. J. Biol. Chem. 277, 8970-8.
110. Gilbert, T. L., Bennett, T. A., Maestas, D. C., Cimino, D. F., Prossnitz, E. R. (2001) Internalization of the human N-formyl peptide and C5a chemoattractant receptors occurs via clathrin-independent mechanisms. Biochemistry 40, 3467-75.
111. Prossnitz, E. R., Kim, C. M., Benovic, J. L., Ye, R. D. (1995) Phosphorylation of the N-formyl peptide receptor carboxyl terminus by the G protein-coupled receptor kinase, GRK2. J. Biol. Chem. 270, 1130-7.
112. Prossnitz, E. R., Schreiber, R. E., Bokoch, G. M., Ye, R. D. (1995) Binding of low affinity N-formyl peptide receptors to G protein. Characterization of a novel inactive receptor intermediate. J. Biol. Chem. 270, 10686-94.
113. Prossnitz, E. R. (1997) Desensitization of N-formylpeptide receptor-mediated activation is dependent upon receptor phosphorylation. J. Biol. Chem. 272, 15213-9.
114. Gilbert, T. L., Prossnitz, E. R., Sklar, L. A. (1999) The uncoupled state of the human formyl peptide receptor. J. Recept. Signal Transduct. Res. 19, 327-40.
115. Maestes, D. C., Potter, R. M., Prossnitz, E. R. (1999) Differential phosphorylation paradigms dictate desensitization and internalization of the N-formyl peptide receptor. J. Biol. Chem. 274, 29791-5.

116. Prossnitz, E. R., Gilbert, T. L., Chiang, S., Campbell, J. J., Qin, S., Newman, W., Sklar, L. A., Ye, R. D. (1999) Multiple activation steps of the N-formyl peptide receptor. Biochemistry 38, 2240-7.
117. Bennett, T. A., Maestas, D. C., Prossnitz, E. R. (2000) Arrestin binding to the G protein-coupled N-formyl peptide receptor is regulated by the conserved "DRY" sequence. J. Biol. Chem. 275, 24590-4.
118. Bennett, T. A., Foutz, T. D., Gurevich, V. V., Sklar, L. A., Prossnitz, E. R. (2001) Partial phosphorylation of the N-formyl peptide receptor inhibits G protein association independent of arrestin binding. J. Biol. Chem. 276, 49195-203.
119. Vines, C. M., Xue, M., Maestas, D. C., Cimino, D. F., Prossnitz, E. R. (2002) Regulation of N-formyl peptide-mediated degranulation by receptor phosphorylation. J. Immunol. 169, 6760-6.
120. Key, T. A., Foutz, T. D., Gurevich, V. V., Sklar, L. A., Prossnitz, E. R. (2003) N-formyl peptide receptor phosphorylation domains differentially regulate arrestin and agonist affinity. J. Biol. Chem. 278, 4041-7.
121. Shi, M., Bennett, T. A., Cimino, D. F., Maestas, D. C., Foutz, T. D., Gurevich, V. V., Sklar, L. A., Prossnitz, E. R. (2003) Functional capabilities of an N-formyl peptide receptor-G(alpha)(i)(2) fusion protein: assemblies with G proteins and arrestins. Biochemistry 42, 7283-93.
122. Vines, C. M., Revankar, C. M., Maestas, D. C., LaRusch, L. L., Cimino, D. F., Kohout, T. A., Lefkowitz, R. J., Prossnitz, E. R. (2003) N-formyl peptide receptors internalize but do not recycle in the absence of arrestins. J. Biol. Chem. 278, 41581-41584.
123. Sklar, L. A., Edwards, B. S., Graves, S. W., Nolan, J. P., Prossnitz, E. R. (2002) Flow cytometric analysis of ligand-receptor interactions and molecular assemblies. Annu. Rev. Biophys. Biomol. Struct. 31, 97-119.
124. Edwards, B. S., Oprea, T., Prossnitz, E. R., Sklar, L. A. (2004) Flow cytometry for high-throughput, high-content screening. Curr. Opin. Chem. Biol. 8, 392-8.
125. Olsson, T. and Oprea, T. I. (2001) Cheminformatics: a tool for decision-makers in drug discovery. Curr Opin Drug Discov Devel 4, 308-13.
126. Oprea, T. I. and Gottfries, J. (2001) Chemography: the art of navigating in chemical space. J Comb Chem 3, 157-66.
127. Arterburn, J. B., Rao, K. V., Perry, M. C. (2000) Novel 17α-ethynylestradiol derivatives: Sonogashira couplings using unprotected phenylhydrazines. Tetrahedron Lett. 41, 839-842.
128. Arterburn, J. B., Corona, C., Rao, K. V., Carlson, K. E., Katzenellenbogen, J. A. (2003) Synthesis of 17α-substituted estradiol-pyridin-2-yl hydrazine conjugates as effective ligands for labeling with Alberto's complex fac-[Re(OH2)3(CO)3]+ in water. J. Org. Chem. 68, 7063-70.
129. Smith, H. O., Leslie, K. K., Singh, M., Qualls, C. R., Revankar, C. M., Joste, N. E., Prossnitz, E. R. (2007) GPR30: a novel indicator of poor survival for endometrial carcinoma. Am. J. Obstet. Gynecol. 196, 386.e1-11.
130. Smith, H. O., Arias-Pulido, H., Kuo, D. Y., Howard, T., Qualls, C. R., Lee, S. J., Verschraegen, C. F., Hathaway, H. J., Joste, N. E., Prossnitz, E. R. (2009) GPR30 predicts poor survival for ovarian cancer. Gynecol. Oncol. 114, 465-71.
131. Prossnitz, E. R. and Hathaway, H. J. (2015) What have we learned about GPER function in physiology and disease from knockout mice? J. Steroid Biochem. Mol. Biol. 153, 114-26.

132. Meyer, M. R., Haas, E., Prossnitz, E. R., Barton, M. (2009) Non-genomic regulation of vascular cell function and growth by estrogen. Mol. Cell. Endocrinol. 308, 9-16.
133. Prossnitz, E. R. and Barton, M. (2009) Signaling, physiological functions and clinical relevance of the G protein-coupled estrogen receptor, GPER Prostaglandins Other Lipid Mediat. 89, 89-97.
134. Meyer, M. R., Baretella, O., Prossnitz, E. R., Barton, M. (2010) Dilation of epicardial coronary arteries by the G protein-coupled estrogen receptor agonists G-1 and ICI182,780. Pharmacology 86, 58-64.
135. Barton, M., Meyer, M. R., Prossnitz, E. R. (2011) Estrogen-independent activation of estrogen receptors. Hypertension 57, 1056-7.
136. Meyer, M. R., Clegg, D. J., Prossnitz, E. R., Barton, M. (2011) Obesity, insulin resistance and diabetes: sex differences and role of oestrogen receptors. Acta Physiol (Oxf) 203, 259-69.
137. Meyer, M. R., Prossnitz, E. R., Barton, M. (2011) The G protein-coupled estrogen receptor GPER/GPR30 as a regulator of cardiovascular function. Vascul. Pharmacol. 55, 17-25.
138. Prossnitz, E. R. and Barton, M. (2011) The G-protein-coupled estrogen receptor GPER in health and disease. Nat. Rev. Endocrinol. 7, 715-26.
139. Meyer, M. R., Amann, K., Field, A. S., Hu, C., Hathaway, H. J., Kanagy, N. L., Walker, M. K., Barton, M., Prossnitz, E. R. (2012) Deletion of G protein-coupled estrogen receptor increases endothelial vasoconstriction. Hypertension 59, 507-512.
140. Meyer, M. R., Field, A. S., Kanagy, N. L., Barton, M., Prossnitz, E. R. (2012) GPER regulates endothelin-dependent vascular tone and intracellular calcium. Life Sci. 91, 623-7.
141. Meyer, M. R., Fredette, N. C., Barton, M., Prossnitz, E. R. (2013) Regulation of vascular smooth muscle tone by adipose-derived contracting factor. PLoS One 8, e79245.
142. Meyer, M. R., Fredette, N. C., Howard, T. A., Hu, C., Ramesh, C., Daniel, C., Amann, K., Arterburn, J. B., Barton, M., Prossnitz, E. R. (2014) G protein-coupled estrogen receptor protects from atherosclerosis. Sci. Rep. 4, 7564.
143. Prossnitz, E. R. and Barton, M. (2014) Estrogen biology: New Insights into GPER function and clinical opportunities. Mol. Cell. Endocrinol. 389, 71-83.
144. Barton, M. and Prossnitz, E. R. (2015) Emerging roles of GPER in diabetes and atherosclerosis. Trends Endocrinol. Metab. 26, 185-192.
145. Meyer, M. R., Fredette, N. C., Barton, M., Prossnitz, E. R. (2015) G protein-coupled estrogen receptor inhibits vascular prostanoid production and activity. J. Endocrinol. 227, 61-9.
146. Meyer, M. R., Fredette, N. C., Daniel, C., Sharma, G., Amann, K., Arterburn, J. B., Barton, M., Prossnitz, E. R. (2016) Obligatory Role for GPER in cardiovascular aging and disease. Sci Signaling 9, ra105.
147. Meyer, M. R., Fredette, N. C., Sharma, G., Barton, M., Prossnitz, E. R. (2016) GPER is required for the age-dependent upregulation of the myocardial endothelin system. Life Sci. 159, 61-5.
148. Dennis, M. K., Field, A. S., Burai, R., Ramesh, C., Petrie, W. K., Bologa, C. G., Oprea, T. I., Yamaguchi, Y., Hayashi, S., Sklar, L. A., Hathaway, H. J., Arterburn, J. B., Prossnitz, E. R. (2011) Identification of a GPER/GPR30 antagonist with

improved estrogen receptor counterselectivity. J. Steroid Biochem. Mol. Biol. 127, 358-66.
149. Prossnitz, E. R. (2017) GPER modulators: opporunity Nox and the heels of a class Akt. J Ster Biochem Mol Biol, this issue (in press).
150. Ramesh, C., Bryant, B., Nayak, T., Revankar, C. M., Anderson, T., Carlson, K. E., Katzenellenbogen, J. A., Sklar, L. A., Norenberg, J. P., Prossnitz, E. R., Arterburn, J. B. (2006) Linkage effects on binding affinity and activation of GPR30 and estrogen receptors ERalpha/beta with tridentate pyridin-2-yl hydrazine tricarbonyl-Re/(99m)Tc(I) chelates. J. Am. Chem. Soc. 128, 14476-7.
151. Nayak, T. K., Hathaway, H. J., Ramesh, C., Arterburn, J. B., Dai, D., Sklar, L. A., Norenberg, J. P., Prossnitz, E. R. (2008) Preclinical development of a neutral, estrogen receptor-targeted, tridentate 99mTc(I)-estradiol-pyridin-2-yl hydrazine derivative for imaging of breast and endometrial cancers. J. Nucl. Med. 49, 978-86.
152. Nayak, T. K., Dennis, M. K., Ramesh, C., Burai, R., Atcher, R. W., Sklar, L. A., Norenberg, J. P., Hathaway, H. J., Arterburn, J. B., Prossnitz, E. R. (2010) Influence of charge on cell permeability and tumor imaging of GPR30-targeted 111In-labeled nonsteroidal imaging agents. ACS Chem. Biol. 5, 681-90.
153. Ramesh, C., Nayak, T. K., Burai, R., Dennis, M. K., Hathaway, H. J., Sklar, L. A., Prossnitz, E. R., Arterburn, J. B. (2010) Synthesis and characterization of iodinated tetrahydroquinolines targeting the G protein-coupled estrogen receptor GPR30. J. Med. Chem. 53, 1004-14.
154. Burai, R., Ramesh, C., Nayak, T. K., Dennis, M. K., Bryant, B. K., Prossnitz, E. R., Arterburn, J. B. (2012) Synthesis and characterization of tricarbonyl-Re/Tc(I) chelate probes targeting the G protein-coupled estrogen receptor GPER/GPR30. PLoS One 7, e46861.
155. Nayak, T. K., Ramesh, C., Hathaway, H. J., Norenberg, J. P., Arterburn, J. B., Prossnitz, E. R. (2014) GPER-targeted, 99mTc-labeled, nonsteroidal ligands demonstrate selective tumor imaging and in vivo estrogen binding. Mol. Cancer Res. 12, 1635-43.
156. Petrie, W. K., Dennis, M. K., Hu, C., Dai, D., Arterburn, J. B., Smith, H. O., Hathaway, H. J., Prossnitz, E. R. (2013) G protein-coupled estrogen receptor-selective ligands modulate endometrial tumor growth. Obstet. Gynecol. Int. 2013, 472720.
157. Marjon, N. A., Hu, C., Hathaway, H. J., Prossnitz, E. R. (2014) G protein-coupled estrogen receptor (GPER) regulates mammary tumorigenesis and metastasis. Mol. Cancer Res. 12, 1644-1654.
158. Scaling, A. L., Prossnitz, E. R., Hathaway, H. J. (2014) GPER mediates estrogen-induced signaling and proliferation in human breast epithelial cells and normal and malignant breast. Horm. Cancer 5, 146-60.
159. Sharma, G., Hu, C., Brigman, J. L., Zhu, G., Hathaway, H. J., Prossnitz, E. R. (2013) GPER deficiency in male mice results in insulin resistance, dyslipidemia, and a proinflammatory state. Endocrinology 154, 4136-45.
160. Meyer, M. R., Barton, M., Prossnitz, E. R. (2014) Functional heterogeneity of NADPH oxidase-mediated contractions to endothelin with vascular aging. Life Sci. 118, 226-31.

161. Meyer, M. R., Fredette, N. C., Barton, M., Prossnitz, E. R. (2014) Endothelin-1 but not angiotensin II contributes to functional aging in murine carotid arteries. Life Sci.
162. Meyer, M. R., Fredette, N. C., Daniel, C., Sharma, G., Amann, K., Arterburn, J. B., Barton, M., Prossnitz, E. R. (2016) Obligatory role for GPER in cardiovascular aging and disease. Sci Signal 9, ra105.
163. Sharma, G., Mauvais-Jarvis, F., Prossnitz, E. R. (2017) Roles of G protein-coupled estrogen receptor GPER in metabolic regulation. J. Steroid Biochem. Mol. Biol., (in press, this issue).
164. Maggiolini, M., Carpino, A., Bonofiglio, D., Pezzi, V., Rago, V., Marsico, S., Picard, D., Ando, S. (2001) The direct proliferative stimulus of dehydroepiandrosterone on MCF7 breast cancer cells is potentiated by overexpression of aromatase. Mol. Cell. Endocrinol. 184, 163-71.
165. Maggiolini, M., Donze, O., Jeannin, E., Ando, S., Picard, D. (1999) Adrenal androgens stimulate the proliferation of breast cancer cells as direct activators of estrogen receptor alpha. Cancer Res. 59, 4864-9.
166. Maggiolini, M., Statti, G., Vivacqua, A., Gabriele, S., Rago, V., Loizzo, M., Menichini, F., Amdo, S. (2002) Estrogenic and antiproliferative activities of isoliquiritigenin in MCF7 breast cancer cells. J. Steroid Biochem. Mol. Biol. 82, 315-22.
167. Maggiolini, M., Bonofiglio, D., Marsico, S., Panno, M. L., Cenni, B., Picard, D., Ando, S. (2001) Estrogen receptor alpha mediates the proliferative but not the cytotoxic dose-dependent effects of two major phytoestrogens on human breast cancer cells. Mol. Pharmacol. 60, 595-602.
168. Maggiolini, M., Vivacqua, A., Fasanella, G., Recchia, A. G., Sisci, D., Pezzi, V., Montanaro, D., Musti, A. M., Picard, D., Ando, S. (2004) The G protein-coupled receptor GPR30 mediates c-fos up-regulation by 17beta-estradiol and phytoestrogens in breast cancer cells. J. Biol. Chem. 279, 27008-27016.
169. Vivacqua, A., Bonofiglio, D., Albanito, L., Madeo, A., Rago, V., Carpino, A., Musti, A. M., Picard, D., Ando, S., Maggiolini, M. (2006) 17beta-estradiol, genistein, and 4-hydroxytamoxifen induce the proliferation of thyroid cancer cells through the G protein-coupled receptor GPR30. Mol. Pharmacol. 70, 1414-23.
170. Vivacqua, A., Bonofiglio, D., Recchia, A. G., Musti, A. M., Picard, D., Ando, S., Maggiolini, M. (2006) The G protein-coupled receptor GPR30 mediates the proliferative effects induced by 17b-estradiol and hydroxytamoxifen in endometrial cancer cells. Mol. Endocrinol. 20, 631-46.
171. Albanito, L., Madeo, A., Lappano, R., Vivacqua, A., Rago, V., Carpino, A., Oprea, T. I., Prossnitz, E. R., Musti, A. M., Ando, S., Maggiolini, M. (2007) G protein-coupled receptor 30 (GPR30) mediates gene expression changes and growth response to 17beta-estradiol and selective GPR30 ligand G-1 in ovarian cancer cells. Cancer Res. 67, 1859-66.
172. Pandey, D. P., Lappano, R., Albanito, L., Madeo, A., Maggiolini, M., Picard, D. (2009) Estrogenic GPR30 signalling induces proliferation and migration of breast cancer cells through CTGF. EMBO J. 28, 523-532.
173. Maggiolini, M. and Picard, D. (2010) The unfolding stories of GPR30, a new membrane-bound estrogen receptor. J. Endocrinol. 204, 105-14.
174. Lappano, R., Pisano, A., Maggiolini, M. (2014) GPER function in breast cancer: an overview. Front. Endocrinol. (Lausanne) 5, 66.

175. De Marco, P., Lappano, R., De Francesco, E. M., Cirillo, F., Pupo, M., Avino, S., Vivacqua, A., Abonante, S., Picard, D., Maggiolini, M. (2016) GPER signalling in both cancer-associated fibroblasts and breast cancer cells mediates a feedforward IL1beta/IL1R1 response. Sci. Rep. 6, 24354.
176. Recchia, A. G., De Francesco, E. M., Vivacqua, A., Sisci, D., Panno, M. L., Ando, S., Maggiolini, M. (2011) The G protein-coupled receptor 30 is up-regulated by hypoxia inducible factor-1a (HIF-1a) in breast cancer cells and cardiomyocytes. J. Biol. Chem. 286, 10773-82.
177. De Francesco, E. M., Lappano, R., Santolla, M. F., Marsico, S., Caruso, A., Maggiolini, M. (2013) HIF-1alpha/GPER signaling mediates the expression of VEGF induced by hypoxia in breast cancer associated fibroblasts (CAFs). Breast Cancer Res. 15, R64.
178. De Francesco, E. M., Pellegrino, M., Santolla, M. F., Lappano, R., Ricchio, E., Abonante, S., Maggiolini, M. (2014) GPER mediates activation of HIF1alpha/VEGF signaling by estrogens. Cancer Res. 74, 4053-64.
179. Fahlen, M., Zhang, H., Lofgren, L., Masironi, B., VON Schoultz, E., VON Schoultz, B., Sahlin, L. (2016) Expression of estrogen receptors in relation to hormone levels and the Nottingham Prognostic Index. Anticancer Res. 36, 2839-47.
180. Jacenik, D., Cygankiewicz, A. I., Krajewska, W. M. (2016) The G protein-coupled estrogen receptor as a modulator of neoplastic transformation. Mol. Cell. Endocrinol. 429, 10-8.
181. Albanito, L., Sisci, D., Aquila, S., Brunelli, E., Vivacqua, A., Madeo, A., Lappano, R., Pandey, D. P., Picard, D., Mauro, L., Ando, S., Maggiolini, M. (2008) Epidermal growth factor induces G protein-coupled receptor 30 expression in estrogen receptor-negative breast cancer cells. Endocrinology 149, 3799-808.
182. De Marco, P., Bartella, V., Vivacqua, A., Lappano, R., Santolla, M. F., Morcavallo, A., Pezzi, V., Belfiore, A., Maggiolini, M. (2013) Insulin-like growth factor-I regulates GPER expression and function in cancer cells. Oncogene 32, 678-88.
183. Vivacqua, A., Lappano, R., De Marco, P., Sisci, D., Aquila, S., De Amicis, F., Fuqua, S. A., Ando, S., Maggiolini, M. (2009) G protein-coupled receptor 30 expression is up-regulated by EGF and TGF alpha in estrogen receptor alpha-positive cancer cells. Mol. Endocrinol. 23, 1815-26.
184. Lappano, R., De Marco, P., De Francesco, E. M., Chimento, A., Pezzi, V., Maggiolini, M. (2013) Cross-talk between GPER and growth factor signaling. J. Steroid Biochem. Mol. Biol. 137, 50-6.
185. De Marco, P., Romeo, E., Vivacqua, A., Malaguarnera, R., Abonante, S., Romeo, F., Pezzi, V., Belfiore, A., Maggiolini, M. (2014) GPER is regulated by insulin in cancer cells and cancer-associated fibroblasts (CAFs). Endocr. Relat. Cancer 21, 739-53.
186. De Marco, P., Cirillo, F., Vivacqua, A., Malaguarnera, R., Belfiore, A., Maggiolini, M. (2015) Novel aspects concerning the functional cross-talk between the Insulin/IGF-I system and estrogen signaling in cancer cells. Front. Endocrinol. (Lausanne) 6, 30.
187. Avino, S., De Marco, P., Cirillo, F., Santolla, M. F., De Francesco, E. M., Perri, M. G., Rigiracciolo, D., Dolce, V., Belfiore, A., Maggiolini, M., Lappano, R.,

Vivacqua, A. (2016) Stimulatory actions of IGF-I are mediated by IGF-IR cross-talk with GPER and DDR1 in mesothelioma and lung cancer cells. Oncotarget.
188. Bartella, V., De Francesco, E. M., Perri, M. G., Curcio, R., Dolce, V., Maggiolini, M., Vivacqua, A. (2016) The G protein estrogen receptor (GPER) is regulated by endothelin-1 mediated signaling in cancer cells. Cell. Signal. 28, 61-71.
189. Lappano, R. and Maggiolini, M. (2011) G protein-coupled receptors: novel targets for drug discovery in cancer. Nat Rev Drug Discov 10, 47-60.
190. Jordan, V. C. (2011) Four decades of discovery in breast cancer research and treatment--an interview with V. Craig Jordan. Interview by Marc Poirot. Int. J. Dev. Biol. 55, 703-12.
191. Lu, Q., Schnitzler, G. R., Vallaster, C. S., Ueda, K., Erdkamp, S., Briggs, C. E., Iyer, L. K., Jaffe, I. Z., Karas, R. H. (2016) Unliganded estrogen receptor alpha regulates vascular cell function and gene expression. Mol. Cell. Endocrinol. 442, 12-23.
192. Bond, R. A. and Ijzerman, A. P. (2006) Recent developments in constitutive receptor activity and inverse agonism, and their potential for GPCR drug discovery. Trends Pharmacol. Sci. 27, 92-6.
193. Seifert, R. and Wenzel-Seifert, K. (2002) Constitutive activity of G-protein-coupled receptors: cause of disease and common property of wild-type receptors. Naunyn Schmiedebergs Arch. Pharmacol. 366, 381-416.
194. Paterni, I., Granchi, C., Katzenellenbogen, J. A., Minutolo, F. (2014) Estrogen receptors alpha (ERalpha) and beta (ERbeta): subtype-selective ligands and clinical potential. Steroids 90, 13-29.
195. Madak-Erdogan, Z., Kim, S. H., Gong, P., Zhao, Y. C., Zhang, H., Chambliss, K. L., Carlson, K. E., Mayne, C. G., Shaul, P. W., Korach, K. S., Katzenellenbogen, J. A., Katzenellenbogen, B. S. (2016) Design of pathway preferential estrogens that provide beneficial metabolic and vascular effects without stimulating reproductive tissues. Sci Signal 9, ra53.
196. Sancar, A. (2017) Oliver Smithies (1925-2017). Science 355, 695.
197. Kucherlapati, R. (2017) Oliver Smithies (1925-2017). Nature 542, 166.
198. Smithies, O. and Neill, U. S. (2015) A conversation with Oliver Smithies. J. Clin. Invest. 125, 3997-8.
199. Smithies, O. and Coffman, T. (2015) A conversation with Oliver Smithies. Annu. Rev. Physiol. 77, 1-11.
200. Vogel, G. (2007) Nobel Prizes. A knockout award in medicine. Science 318, 178-9.
201. Ford, J., Hajibeigi, A., Long, M., Hahner, L., Gore, C., Hsieh, J. T., Clegg, D., Zerwekh, J., Oz, O. K. (2011) GPR30 deficiency causes increased bone mass, mineralization, and growth plate proliferative activity in male mice. J. Bone Miner. Res. 26, 298-307.
202. Mügge, A., Riedel, M., Barton, M., Kuhn, M., Lichtlen, P. R. (1993) Estrogen dilates human coronary arteries in vitro by an increase in cyclic GMP and cyclic AMP smooth muscle content. Eur. Heart J. 14 (Abstract Suppl.), 16.
203. Yokomizo, T., Izumi, T., Chang, K., Takuwa, Y., Shimizu, T. (1997) A G-protein-coupled receptor for leukotriene B4 that mediates chemotaxis. Nature 387, 620-4.

204. Campbell, R. E., Tour, O., Palmer, A. E., Steinbach, P. A., Baird, G. S., Zacharias, D. A., Tsien, R. Y. (2002) A monomeric red fluorescent protein. Proc. Natl. Acad. Sci. U. S. A. 99, 7877-82.
205. Cheng, S. B., Graeber, C. T., Quinn, J. A., Filardo, E. J. (2011) Retrograde transport of the transmembrane estrogen receptor, G protein-coupled receptor 30 (GPR30/GPER) from the plasma membrane towards the nucleus. Steroids 76, 892-6.
206. Cheng, S. B., Quinn, J. A., Graeber, C. T., Filardo, E. J. (2011) Down-modulation of the G-protein-coupled estrogen receptor, GPER, from the cell surface occurs via a trans-Golgi-proteasome pathway. J. Biol. Chem. 286, 22441-55.

Figure Legends

Figure 1. Biologist Clara M. Szego, PhD. Szego was born in Budapest, Hungary, on March 23, 1916. She was a pioneer in the study of estrogen biology (beginning in the 1940s) and in the rapid responses to estrogen (in the 1960s and 1970s), making seminal contributions in both fields. In 1956, Dr. Szego became a Guggenheim fellow while working at the University of California, Los Angeles, and in 1957, she was honored as one of ten *Women of the Year* by the Los Angeles Times. The photograph was taken in 1957 and provided by the Los Angeles Times.

Figure 1

Figure 2. Rapid non-genomic effects of estrogen in a human coronary artery ring (Barton). Shown on the left is an organ bath allowing *ex vivo* study of vascular tone of an intact human coronary artery (4 mm in diameter) obtained during cardiac transplantation (suspended between to steel hooks in physiological salt solution at 37°C). The right panel shows an original tracing of the first ever recorded response to 17β-estradiol of a human coronary artery obtained from a female patient. The cardiac transplantation and experiment took place on May 1, 1992. The left anterior descending (LAD) artery was obtained from a 22 year-old women requiring a heart transplant after developing dilated cardiomyopathy (DCM) and severe heart failure as a

complication of a viral infection. Upper panel: Coronary artery tone was recorded after exposure to a contractile substance (prostaglandin F_2). Once a stable plateau was reached, a single concentration of the non-selective estrogen receptor agonist 17β-estradiol (E2, 3 µmol/L) was added to the bath, causing immediate and complete relaxation of the pre-contracted artery. By contrast, the solvent control ethanol (ETOH, lower panel) had only marginal effects on arterial tone. Within 30 min, E2 also increased coronary artery cAMP content [57]. At the time of the experiment, only a single ER protein was known; neither ER nor GPER had been cloned, and it was later shown that the rapid dilator response [70] and cAMP increase [58, 202] involve GPER. Left panel: original organ chamber showing a human coronary artery suspended from a Hugo Sachs force transducer; photograph taken by the author (M.B.) at the Division of Cardiology of Hannover Medical School in 1991. Right panel reproduced from reference [60].

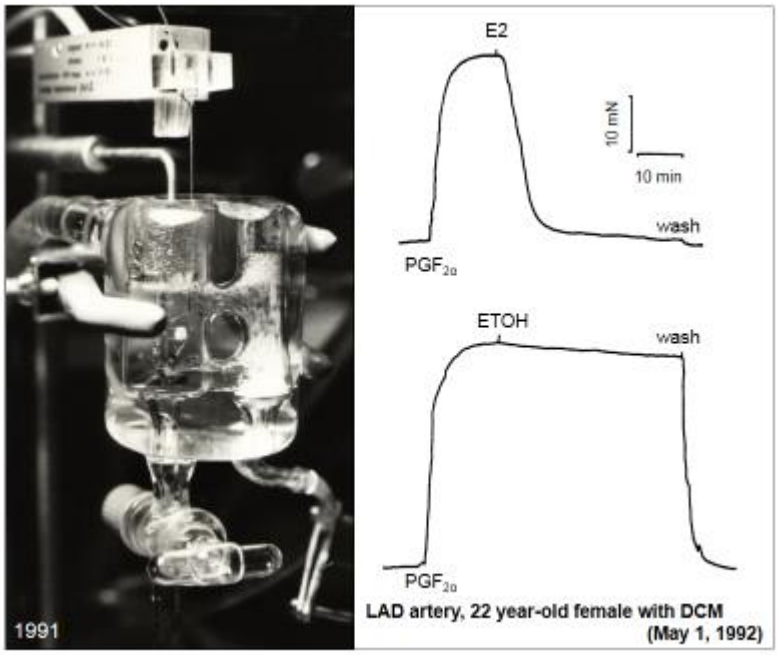

Figure 2

Figure 3. The first cloning of a cDNA for a receptor that would later be called GPR30 and eventually GPER (Lolait). The cloning of the human GPR30 (GPER) cDNA was first published in 1996 [44] as detailed in the text. In fact, this cDNA (designated clone 47-2) was isolated over 4 years earlier, as evidenced in a note from the first (Christer Owman) to senior (Stephen Lolait) author indicating that the clone was isolated prior to mid-Feb 1992. The note details the DNA concentrations of sets of oligonucleotides (48 base pairs) specific for a number of cDNA clones encoding some orphan GPCRs isolated in the lab around the same time. The GPER clone 47-2 and oligonucleotides Lym 1 and Lym 2 (in red) are detailed in the publication.

Clone 21-9 encoded a GPCR that was later deorphanised by others as the leukotriene B4 receptor (LTB4R) [203].

Figure 3

Figure 4. The first image of GPR30 expressed in COS7 cells (Prossnitz). In order to localize the site(s) of GPR30 expression, the cDNA for GPR30 was fused in frame at the 3' (C-terminal) end to GFP (green fluorescent protein) or mRFP (monomeric red fluorescent protein [204], shown in red) and subcloned in pcDNA3. COS7 cells were transfected with the plasmid construct and imaged by confocal microscopy, using TO-PRO-3 as a nuclear stain (blue) as in [66]. This first image to test the new plasmid construct came as a great surprise as GPCRs (see localization of β2-adrenergic receptor in Fig. 1A of [66]) are usually expressed predominantly at the plasma membrane (with some expression often detectable in the Golgi apparatus, as a result of accumulation during processing and transport to the plasma membrane). On the contrary, however, the fluorescent protein fusions of GPR30 showed a predominantly intracellular localization, being expressed in the endoplasmic reticulum and Golgi apparatus with essentially no detectable receptor at the plasma membrane. Although at first we suspected the C-terminal fusion of fluorescent proteins might be impeding processing and transport, staining of endogenously expressed GPR30 with newly generated antibodies would reveal a similar expression pattern. Overtime, it would become clear that although most cells exhibit significant localization of GPER intracellularly, GPER does traffic to the plasma membrane (to varying extents, likely dependent on cell type) [82, 83, 105], from where it is

constitutively internalized (in an apparently ligand-independent manner) leading to retrograde transport to intracellular membrane compartments, including the endoplasmic reticulum [88, 205, 206]. It is perhaps not entirely surprising that GPER can function intracellularly [99], as estrogen is freely membrane permeable and must “find its way” to the nucleus to activate the classical estrogen receptors ERα and ERβ.

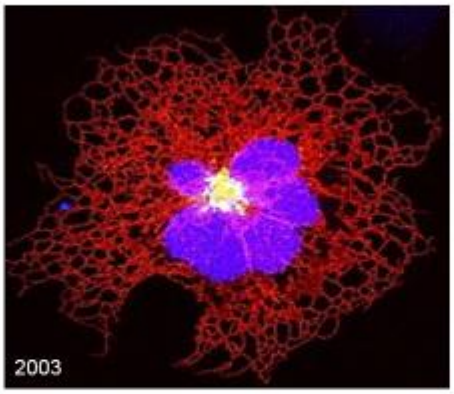

Figure 4