Breast Cancer RESEARCH

REVIEW

The estrogen-regulated anterior gradient 2 (AGR2) protein in breast cancer: a potential drug target and biomarker

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Abstract

Initially discovered as an estrogen-responsive gene in breast cancer cell lines, anterior gradient 2 (*AGR2*) is a developmentally regulated gene belonging to the protein disulfide isomerase (PDI) gene family. Developmentally, AGR2 is expressed in the mammary gland in an estrogen-dependent manner, and AGR2 knockout and overexpression mouse models indicate that the gene promotes lobuloalveolar development by stimulating cell proliferation. Although AGR2 overexpression alone seems insufficient for breast tumorigenesis in mice, several lines of investigations suggest that AGR2 promotes breast tumorigenesis. Overexpression of AGR2 in several breast cancer cell lines increases cell survival in clonogenic assays and cell proliferation, whereas AGR2 loss of function leads to decreased cell cycle progression and cell death. In addition, AGR2 was shown to promote metastasis of breast epithelial cells in an *in vivo* metastasis assay. As a PDI, AGR2 is thought to be involved in the unfolded protein response that alleviates endoplasmic reticulum stress. Since cancer has to overcome proteotoxic stress due to excess protein production, AGR2 may be one of many pro-survival factors recruited to assist in protein folding or degradation or both. When AGR2 is secreted, it plays a role in cellular adhesion and dissemination of metastatic tumor cells. In breast cancer, AGR2 expression is associated with estrogen receptor (ER)-positive tumors; its overexpression is a predictor of poor prognosis. The *AGR2* gene is directly targeted by ER-alpha, which is preferentially bound in tumors with poor outcome. Whereas aromatase inhibitor therapy decreases AGR2 expression, tamoxifen acts as an agonist of AGR2 expression in ER-positive tumors, perhaps contributing to tamoxifen resistance. AGR2 is also overexpressed in a subset of ER-negative tumors. Furthermore, AGR2 expression is associated with the dissemination of metastatic breast cancer cells and can be used as a marker to identify circulating tumor cells and metastatic cells in sentinel lymph nodes. In conclusion, AGR2 is a promising drug target in breast cancer and may serve as a useful prognostic indicator as well as a marker of breast cancer metastasis.

Introduction

The estrogen receptor (ER) is a key regulator of mammary gland development and breast carcinogenesis, regulating pro-proliferative and pro-survival genes in breast epithelial cells. The human anterior gradient 2 (*AGR2*), one of the many targets of ER, was first discovered as an overexpressed gene in ER-positive breast cancer cell lines [1,2] and has since been shown to be overexpressed in breast cancers and many other adenocarcinomas, including colorectal, esophageal, lung, ovarian, pancreatic, and prostate carcinomas [3-10]. Its overexpression in ER-positive breast cancer is associated with poor prognosis, especially in tumors that escape anti-hormone therapies [11]. AGR2 acts by promoting cell proliferation, cell survival, and metastasis of breast cancer cells [12,13]. As ER activation can generate a large influx of gene transcripts, subsequent translation creates stress on the endoplasmic reticulum that the tumor cell must overcome to survive. A member of the protein disulfide isomerase (PDI) family that localizes to the endoplasmic reticulum [14], AGR2 may assist in protein folding and endoplasmic reticulum-assisted degradation (ERAD) of proteins [15]. The ability of ER to activate the *AGR2* gene may therefore allow the tumor to resist proteotoxic stress and avoid cell death. In addition, AGR2 is a secreted protein [13] and, as such, may promote breast cancer metastases by regulating the adhesion and

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dissemination of tumor cells. In this review, we discuss the effects of AGR2 expression in breast cancer cells, highlighting its role as a survival factor in response to proteotoxic stress, its function in mediating tumor metastasis, and the molecular pathways that it acts through to control cell proliferation. Targeted inhibition of AGR2 may be useful in breast cancer as it may induce cell death in response to proteotoxic stress, prevent tumor metastasis, and slow the rate of tumor growth.

AGR2 and related family members

The mammalian anterior gradient proteins, AGR1, AGR2, and AGR3, belong to a family of PDIs, which function in the endoplasmic reticulum to reduce, oxidize, or isomerize disulfide bonds [16]. In addition to having a role in protein folding, PDIs function as molecular chaperones and in the ERAD protein degradation pathway. Outside of the endoplasmic reticulum, PDIs have been described as secreted proteins that function at the cell surface and the extracellular matrix. The specific functions of the AGR2 and AGR3 family members as PDIs are poorly understood. Anterior gradient genes were originally discovered in *Xenopus laevis* and named for their expression patterns in the anterior region of the dorsal ectoderm during late gastrulation [17]. *Xenopus* anterior gradient-2 (XAG-2), a secreted cement gland-specific protein, plays a role in the specification of the dorsoanterior ectoderm to cement gland and forebrain fates. *AGR2*, the human homologue of XAG-2, was first identified as an upregulated gene in the ER-positive MCF-7 breast carcinoma cell line compared with the ER-negative MDA-MB-231 breast carcinoma cell line [1]. *AGR2* was one of eight genes that correlated with ER expression and responded to estradiol treatment in MCF-7 cells, an observation that was validated in eight additional breast carcinoma cell lines and primary tumor samples [2]. The correlation between AGR2 expression and ER positivity of breast cancer cell lines and the ability of estradiol to induce its expression suggested the possibility that AGR2 mediates estrogenic actions in normal physiology and breast cancer.

AGR3 was discovered in a proteomics screen for proteins associated with the cell membrane of the ER-positive T-47D and MCF-7 breast carcinoma cell lines [18]. The human *AGR2* and *AGR3* genes map to chromosome band 7p21.3 and share 71% sequence identity [3,19]. The two are transcribed from the same DNA strand and are separated by only 60 kb of sequence. Although the contiguity of *AGR2* and *AGR3* would suggest that they are co-regulated, the contrary is observed in several cases: in normal mammary gland development, mouse *Agr2* is preferentially expressed in comparison with *Agr3* during pregnancy, lactation, and involution [20], whereas mouse ovarian cancers express *Agr3*, and not *Agr2*, in ER-negative tumors [21]. In addition, uncoupled expression of AGR2 and AGR3 is observed in multiple prostate cancer cell lines, with preferential expression of AGR2 in most cell lines [22]. However, there are cases in which the two genes are co-expressed, such as MCF-7 cells after treatment with estradiol [23]. The mechanisms underlying differential expression of AGR2 and AGR3 are poorly understood and warrant further investigation.

AGR1 is more commonly known as endoplasmic reticulum protein (ERp) 18/19 because of its functional role as a PDI in the endoplasmic reticulum [24]. Homology analyses revealed high similarity between ERp18/19 and AGR2 and AGR3, providing the previously uncharacterized AGR2 and AGR3 proteins with possible functions as PDIs. Whereas AGR2 and AGR3 have both been linked to breast cancer, AGR1 has not been identified as a breast cancer gene of interest in any bioinformatics study to date. However, it is clear that the role of the AGR1 PDI in normal biological settings provides insights into the possible functions of AGR2 and AGR3 in both normal mammary gland morphogenesis and breast carcinogenesis.

The role of AGR2 in breast cancer

AGR2 expression in breast cancer is an indicator of poor outcome

AGR2 was originally described as an overexpressed gene in breast cancer cell lines and later as an overexpressed gene in breast cancer, especially in association with ER positivity [1-3]. The overexpression in breast cancer is clinically relevant as high levels of AGR2 are associated with poor prognosis in ER-positive breast cancer [11,25,26]. In contrast to these three studies showing correlation of high AGR2 levels with poor prognosis, a single study analyzing a mixed population of ER-positive and ER-negative tumors reported improved overall survival rates of breast cancer patients who express AGR2. These results, however, may be confounded as the reported survival analysis did not factor in ER status, which has a profound impact on the prognosis [9]. It is possible that the increased survival rate of AGR2-expressing tumors may be the result of the improved prognosis conveyed by ER expression. One of the studies associating AGR2 expression with poor prognosis used a cohort of patients that were treated only by mastectomy without adjuvant or hormonal therapy [26], whereas a separate study revealed that tamoxifen treatment has an agonist effect on AGR2 expression that may contribute to treatment failures in ER-positive tumors [11]. These observations indicate that the expression of AGR2 in poor-prognosis tumors may be due to multiple regulatory mechanisms: AGR2 expression can be induced in an ER-dependent manner early in tumorigenesis, leading to an

intrinsic therapeutic resistance, or its expression can be induced in response to anti-hormone treatment, leading to an acquired resistance to therapies. Furthermore, high AGR2 expression is sometimes found in ER-negative breast cancers [3], indicating a different mechanism of upregulation, such as physiological stress [27]. Understanding the regulatory mechanisms that control *AGR2* in these situations may shed light on its role in breast cancer, especially the difficult-to-treat cases that exhibit AGR2 overexpression.

Breast tumors can be classified into the basal-like, Her2, luminal A, luminal B, and normal breast-like subtypes on the basis of their intrinsic gene expression profiles [28]. In three independent gene expression data sets [29-31] that classified a total of 1,169 tumors into their respective subtypes, AGR2 is expressed significantly higher in the ER-positive luminal A and B subtypes compared with the ER-negative basal-like, Her2, and normal-like subtypes (Figure 1a), supporting the previously reported overexpression of AGR2 in ER-positive breast cancer. Interestingly, AGR3 is also expressed significantly higher and with the widest range of expression in the luminal subtypes (Figure 1b).

Estrogen-mediated regulation of AGR2 gene expression

Consistent with the correlation between AGR2 and ER expression in breast cancer cell lines [1] and ER-positive breast cancers [3,13,25] and the expression of AGR2 in response to estrogen treatment in breast cancer cell lines [20], an *in vivo* study in which normal human mammary tissue was transplanted into female nude mice exhibited increased AGR2 expression in response to estradiol [32]. Collectively, these data provide strong evidence that AGR2 is regulated by estradiol under normal and pathophysiological conditions. Furthermore, these data suggest that AGR2 may be an important component of the profound estrogen response in breast tissue.

To determine whether ER directly regulates the *AGR2* gene, Hrstka and colleagues [11] treated MCF-7 cells with estradiol and performed chromatin immunoprecipitation (ChIP) of ER-alpha, demonstrating a twofold increase in ER binding to the *AGR2* promoter upon estradiol treatment. Furthermore, ER-alpha was shown to increase expression from a transiently transfected *AGR2* promoter reporter plasmid. These *in vitro* experiments suggest that ER binds directly to *AGR2* to activate transcription of the gene.

Recent reports defining global ER-binding sites through ChIP-Seq and chromatin interaction analysis by paired-end tag sequencing (ChIA-Pet) further support the targeted binding and transcriptional activation of *AGR2* by ER in cell lines and primary tumor tissue [23,33,34]. Treatment of MCF-7 cells with estradiol induces ER binding in the proximal promoter of *AGR2*, subsequently activating *AGR2* transcription as evidenced by increased *AGR2* gene expression and pervasive RNA polymerase II and histone H3 lysine 4 trimethylation (H3K4me3) marks on the chromatin [23] (Figure 2a). Interestingly, the same patterns of estradiol-induced ER binding and subsequent gene expression are observed for *AGR3*, suggesting that *AGR2* and *AGR3* are both expressed in response to estrogen in MCF-7 cells. In addition, we used published peak calls from ChIP-Seq data collected from good- and poor-outcome ER-positive breast cancers, as well as metastases derived from ER-positive breast cancers [33], to search for ER binding in a 20-kb window around the *AGR2* and *AGR3* genes (Figure 2a). We found binding events surrounding *AGR2* in only one good-outcome tumor, whereas four of the seven poor-outcome tumors and all three metastases exhibited multiple ER-binding events around the *AGR2* gene. Similar binding patterns were observed near the *AGR3* gene. Five ER-positive cell lines were also profiled for ER binding, and all of them exhibited multiple ER-binding sites near *AGR2* and *AGR3* (Figure 2a). Furthermore, a separate study performing ChIP-Seq on MCF-7 cells reveals that both *AGR2* and *AGR3* possess a single ER-binding event surrounding the gene in response to estradiol treatment [34], both of which overlap with binding events in the primary tumors and cell lines reported by Ross-Innes and colleagues [33]. Thus, estrogen-mediated activation of ER directly targets *AGR2* and *AGR3* for active gene transcription, and an increased number of occupied ER-binding sites may correlate with poor prognosis. Finally, studies using ChIA-Pet to identify long-range genome interactions found that ER mediates the formation of a 130-kb loop that includes both *AGR2* and *AGR3* [23] (Figure 2b), suggesting that transcriptional hubs generated by ER-induced looping of DNA control the transcription of both genes in MCF-7 cells treated with estradiol. Collectively, these studies give clear evidence that *AGR2* is a direct target of ER in both cell lines and primary tumor cells from breast cancers.

In prostate cancer cells, both estrogens and androgens stimulate AGR2 expression [22,35], and in this case, the activation for both types of ligands seems to depend on the androgen receptor (AR), which binds the *AGR2* gene [22]. The role of AR in regulating *AGR2* in breast cancer has not been investigated.

AGR2 promotes resistance to tamoxifen treatment in ER-positive breast cancer

Since *AGR2* is a direct target of ER, one would expect ER-positive breast cancers treated with anti-estrogen therapy to exhibit decreased AGR2 levels. This is indeed the case in primary tumors treated with aromatase inhibitors, which reduce the systemic levels of estrogen by preventing the catalysis of androgens into estradiol, whereas the

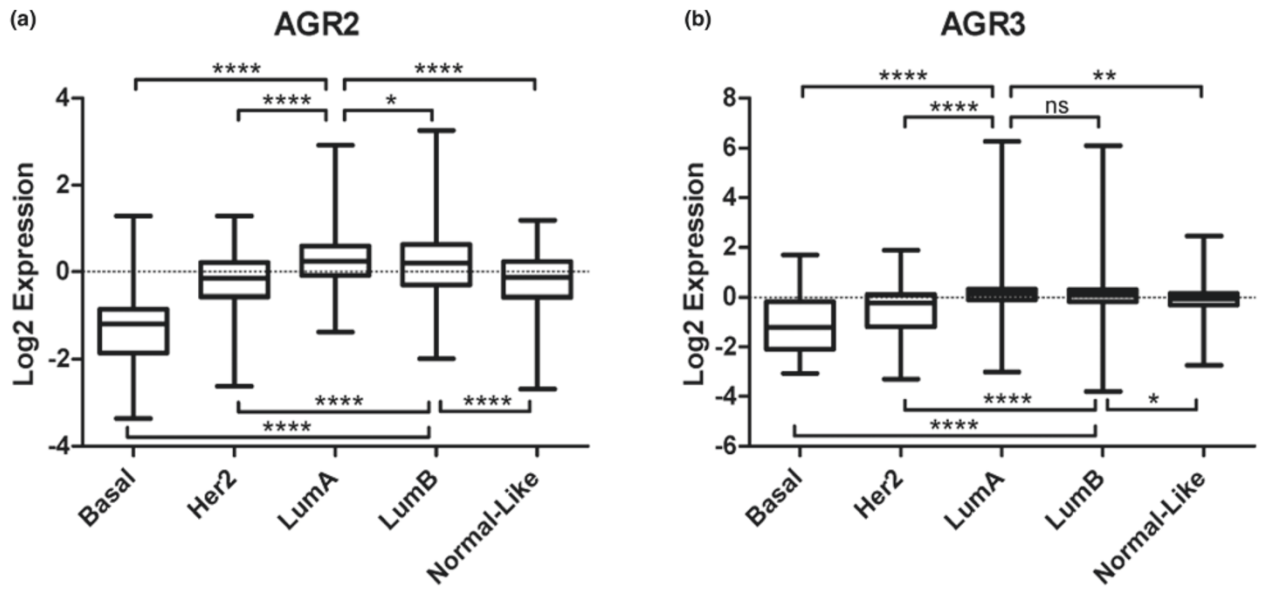

Figure 1. Overexpression of AGR2 and AGR3 in the ER-positive luminal A and B breast cancer subtypes. (a) Primary breast tumor gene expression data from three independent studies [29-31] with a total of 1,169 samples were log2-normalized and mean centered and then combined into one large data set. AGR2 is expressed significantly higher in the ER-positive luminal A (LumA) and B (LumB) subtypes and is lowly expressed in the ER-negative basal-like (Basal), Her2, and normal breast-like (Normal-Like) tumors. **(b)** AGR3 gene expression was analyzed in the same way as AGR2 and similarly was expressed significantly higher in LumA and LumB tumors. **P* <0.05; ***P* <0.01; *****P* <0.0001. AGR2, anterior gradient 2; ER, estrogen receptor; ns, not significant.

opposite is observed when treating the tumor with tamoxifen, which blocks the binding of estradiol to its receptor [11,36]. Interestingly, in MCF-7 cells, tamoxifen treatment induces more robust ER binding to the *AGR2* promoter, and subsequently a greater increase in AGR2 mRNA and protein, than estradiol treatment [11]. Similar results were provided by Hengel and colleagues [37], who searched for proteins induced by tamoxifen in a proteomics screen in MCF-7 cells and reported AGR2 to be the second most induced protein. In addition, ER-positive primary breast tumors treated with tamoxifen have increased AGR2 mRNA transcript levels [11]. In the breast, tamoxifen is classically described as an antagonist of estrogen-mediated signaling through ER, but these data suggest that tamoxifen, which has general agonist effects in the bone and uterus, also acts as an agonist for the *AGR2* gene. Thus, the depletion of estradiol by aromatase inhibitors reduces AGR2 expression, presumably because of decreased overall activity of ER signaling, whereas the inhibition of estradiol binding to its receptor via tamoxifen enhances ER binding to the *AGR2* promoter and subsequently increases gene expression.

Generally, the ER-positive subtype of breast cancer is less aggressive and is associated with good prognosis because of the relatively successful treatment with hormone therapies, such as tamoxifen. However, despite the effectiveness of tamoxifen in the clinical setting, approximately 50% of ER-positive tumors are intrinsically resistant to tamoxifen therapy and approximately 40% of ER-positive early-stage breast cancers that initially respond to endocrine therapy do eventually relapse with a disease resistant to tamoxifen [38,39]. The fact that AGR2 is selectively induced upon tamoxifen treatment could in part explain the intrinsic or acquired resistance (or both) of ER-positive breast cancers leading to decreased overall survival. This is supported by the finding that an increase in AGR2 levels in tamoxifen-resistant ER-positive breast tumors is indicative of poor prognosis [11]. Thus, in the clinical setting, AGR2 may become a useful molecular marker for identifying ER-positive tumors that are unlikely to respond to tamoxifen and require new or additional treatments to circumvent this resistance, such as a combinatorial therapy that includes aromatase inhibitors. Recently, such combinatorial therapy has shown significant improvements in progression-free and overall survival of ER-positive metastatic breast cancers treated with a combination of the aromatase inhibitor anastrozole and the ER inhibitor fulvestrant [40]. As aromatase inhibitors reduce AGR2 expression and fulvestrant has been demonstrated to inhibit ER binding to the *AGR2* promoter in MCF-7 cells, it is possible that AGR2 expression in these tumors has been reduced and contributed to the improved prognosis in these patients.

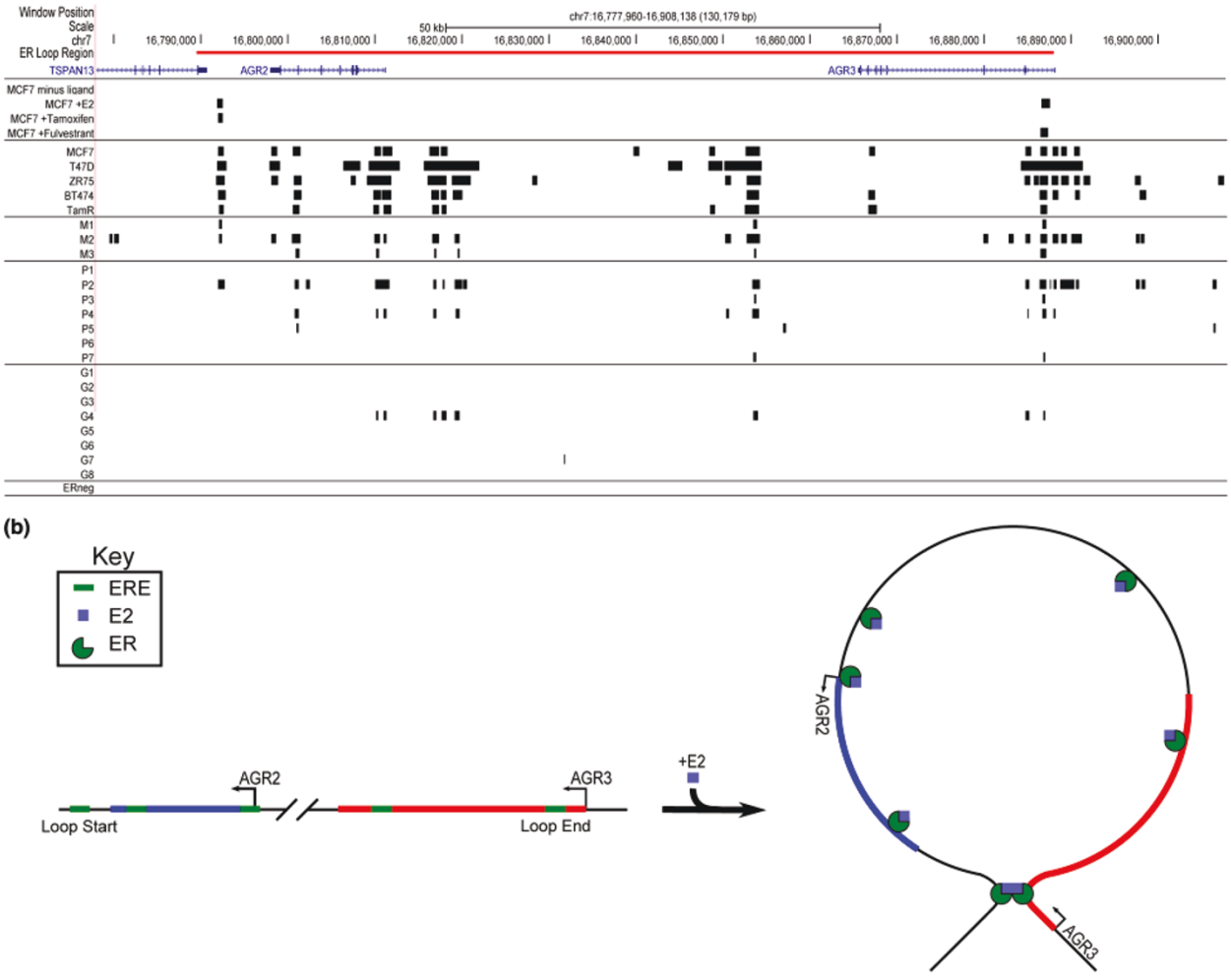

Figure 2. ER-binding and chromatin looping surrounding the *AGR2* and *AGR3* genes. (a) ER-binding sites surrounding the *AGR2* and *AGR3* are displayed for ER-positive cell lines and primary tumors. ChIA-Pet data in MCF-7 cells provided by Fullwood and colleagues [23] define an estradiol-induced loop mediated by ER-binding near *AGR2* and *AGR3* (ER-loop region indicated with red bar). Peaks defined by Welboren and colleagues [34] identify ER-binding upon treatment with estradiol in the proximal promoter of *AGR2* and the first intron of *AGR3* (MCF-7 minus ligand versus MCF-7 +E2). Treatment with tamoxifen alone (MCF-7 +Tamoxifen) induces ER binding to *AGR2* but not *AGR3*, whereas treatment with fulvestrant alone (MCF-7 +Fulvestrant) induces ER binding to *AGR3* but not *AGR2*. Ross-Innes and colleagues [33] profiled ER binding in tamoxifen-responsive (MCF-7, T47D, ZR75) and tamoxifen-resistant (BT474, TamR) cell lines and ER-positive primary breast tumors (M1-3, metastases; P1-7, poor-outcome tumors; G1-8, good-outcome tumors; ERneg, ER-negative tumor). Using the published peak data, we identified ER binding in only one good-prognosis tumor, whereas poor-prognosis and metastatic primary tumors display increased ER binding. Tamoxifen-resistant and -responsive cell lines display pervasive ER binding throughout the region. **(b)** ChIA-PET data from Fullwood and colleagues [23] identify an ER-mediated chromatin loop in MCF-7 cells spanning a 130-kb region that includes the *AGR2* and *AGR3* genes. The loop region, displayed as a red bar in (a), contains multiple putative ER-binding sites indicated by the location of the bound receptors in the loop. AGR2, anterior gradient 2; E2, estradiol; ER, estrogen receptor; ERE, estrogen response element.

The increased expression of AGR2 in ER-positive tumors that become resistant to tamoxifen treatment may be a result of (a) the selection and expansion of an intrinsically resistant subpopulation of cells that exhibit increased binding of ER to *AGR2* or (b) a unique tamoxifen-induced transcriptional program governed by ER that leads to increased binding to the *AGR2* gene or (c) both. In support of the former, differential binding of ER has been described in a cohort of primary ER-positive breast tumors comparing good- and poor-outcome tumors prior to any treatment [33]. In this study, the authors identified a core set of 484 ER-binding sites common to both good- and poor-outcome tumors and 1,791 differentially bound sites (599 and 1,192 unique binding sites in good-outcome and poor-outcome tumors, respectively). Prognostic predictors built from

the genes surrounding these sites are indicative of patient outcome in several independent studies in which gene expression and survival data are available, suggesting that the differential binding regions observed in these tumors could mediate the resistance to anti-cancer therapies, such as tamoxifen. As we noted earlier, these data suggest that *AGR2* is preferentially targeted by ER in the poor-outcome tumors and metastases. However, only four of the seven poor-outcome tumors exhibited binding sites near *AGR2*, indicating plasticity in ER binding even within poor-outcome tumors. As these samples were taken prior to any treatment, it is possible that the other three poor-prognosis tumors represent a population of tumors that become resistant in response to treatment rather than possessing intrinsic therapeutic resistance. To deconstruct the ER-binding patterns that promote intrinsic or induced therapeutic resistance, Ross-Innes and colleagues [33] profiled ER-binding patterns by ChIP-Seq in three tamoxifen-responsive cell lines (MCF-7, ZR75-1, and T-47D) and two tamoxifen-resistant cell lines (BT-474 and TAM-R, a resistant MCF-7 cell line); 6,920 ER-binding events were common to all five cell lines, whereas 8,188 were unique to the tamoxifen-resistant cells and 5,713 were unique to the responsive cells. The authors report that the unique binding events in the tamoxifen-responsive cell lines significantly overlap with those identified in the poor-outcome and metastatic tumors, suggesting that these cell lines more likely resemble poor-outcome tumors. We used the same data to identify ER-binding events surrounding *AGR2* and found consistent binding across all five cell types, irrespective of sensitivity to tamoxifen (Figure 2a). In light of the conclusion that all five cell types resemble poor-outcome tumors, the elevated ER binding around *AGR2* in all of these cells further supports the association between ER-induced *AGR2* expression in primary tumors and poor outcome. In conclusion, we believe that ER-mediated expression of AGR2 could be a hallmark of ER-positive breast carcinomas that can be exploited to identify tumors that will respond poorly to anti-hormone therapy and are poised for dissemination of metastatic tumor cells.

AGR2 expression as an indicator of metastatic breast cancer

Detection of metastatic cells in the lymph node or circulating tumor cells (CTCs) in the peripheral blood is strongly associated with poor clinical outcome in breast cancer [41-43]. AGR2 may be a fitting target for detection of metastatic cells as it has been found to promote metastasis in functional experiments and to be expressed in metastatic breast cancer cells in humans. AGR2-induced lung metastases were identified in a rat model overexpressing AGR2 in benign breast tumors, apparently through both blood-borne and lymphatic routes, giving strong *in vivo* evidence that the over-expression of AGR2 alone is sufficient to induce metastases [13]. Consistent with these animal experiments, AGR2 mRNA has been observed in metastatic epithelial cells isolated from the sentinel lymph nodes of patients with breast cancer [41]. In addition, *AGR2* gene expression in blood samples of patients with breast, colorectal, prostate, urothelial, or gastrointestinal cancer is capable of identifying CTCs [42-45]. Thus, the expression of AGR2 in the blood samples of patients with breast cancer or other adenocarcinoma may be a useful indicator of metastatic cancer that will require a more aggressive treatment regimen.

AGR2 mechanisms of action in breast cancer

Despite the thorough characterization of AGR2 expression in normal development and breast cancer and other adenocarcinomas, the function of AGR2 in these tissues remains poorly understood. However, given the functional domains characterized in the AGR2 protein and its localization to both the endoplasmic reticulum and the extracellular membrane, it is likely that AGR2 has a role in relieving endoplasmic reticulum stress, promoting the dissemination of metastatic tumor cells, and stimulating cell survival and proliferation (Figure 3).

AGR2 as a reliever of endoplasmic reticulum stress in breast cancer

Cancer cells must adapt to survive proteotoxic stress created from imbalances in transcriptional output that result in high volumes of translation [46]. High protein translation rates lead to the sequestration of unfolded protein in the endoplasmic reticulum, exerting a burden on the protein-folding and degradation machinery that ultimately creates a toxic cellular environment [47]. This imbalance is counteracted by recruiting protein-folding or proteolytic degradation factors (or both) to relieve this stress, and one example is the chaperone HSP90 that participates in protein folding; HSP90 is expressed in tumor cells in response to excess unfolded protein [48]. Notably, the HSP90 co-chaperone p23 has been reported to induce expression of AGR2 and promote tumor progression, metastases, and drug resistance in breast cancer [49]. Thus, AGR2 may be recruited to the endoplasmic reticulum to assist in the protein-folding machinery to alleviate proteotoxic stress.

As a member of the PDI family, AGR2 aids protein folding and assembly by catalyzing the formation, reduction, and isomerization of disulfide bonds, thereby stabilizing intermediate conformations during protein maturation in the endoplasmic reticulum [15,50]. Its expression, which can be controlled by the unfolded protein response, is involved in the maintenance of

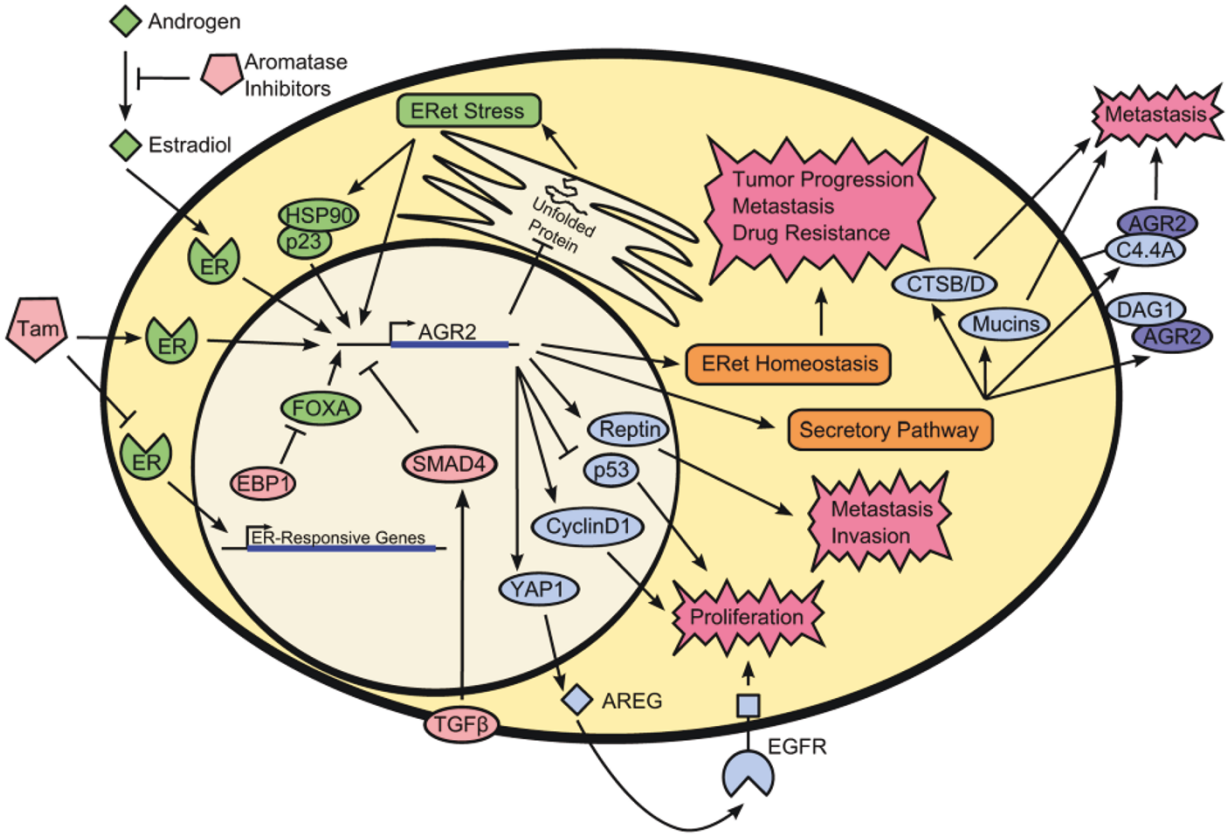

Figure 3. Mechanisms of *AGR2* activation and subsequent cellular effects of AGR2 expression. Green molecules represent activators of *AGR2* expression, and red molecules represent inhibitors. Blue proteins represent targets that AGR2 works through to mediate tumor progression, metastasis, drug resistance, invasion, cell survival, and cell proliferation. AREG, amphiregulin; AGR2, anterior gradient 2; C4.4A, LY6/PLAUR domain containing 3; CTSB/D, cathepsin B and cathepsin D; DAG1, dystroglycan 1; EBP1, ErbB3-binding protein 1; EGFR, epidermal growth factor receptor; ER, estrogen receptor, ERet, endoplasmic reticulum; FOXA, forkhead box family members A1 and A2; HSP90, heat-shock protein 90; Tam, tamoxifen; TGF-β, transforming growth factor-beta; YAP1, yes-associated protein 1.

endoplasmic reticulum homeostasis by acting as a checkpoint in the endoplasmic reticulum quality control system and by redirecting misfolded proteins to the ERAD machinery [14,15]. AGR2 contains a putative endoplasmic reticulum retention motif and has been demonstrated to indirectly associate with endoplasmic reticulum membrane-bound ribosomes via nascent protein chains, presumably acting through a CXXS thioredoxin-like domain motif that may assist in disulfide bond formation and isomerization. Although thioredoxin activity of AGR2 has yet to be demonstrated, it has been shown to enhance endoplasmic reticulum folding capacity in cancer cells, allowing them to cope with acute endoplasmic reticulum stress [15]. It has also been shown to be important for the homeostasis of intestinal cells by relieving endoplasmic reticulum stress [51]. AGR2 likely plays a similar role in normal mammary development as it is most highly expressed during late pregnancy and lactation [20], a period during which high volumes of milk protein are produced in the secretory alveolar buds that may benefit from the assistance of additional protein-folding and secretory factors such as AGR2 to cope with the increased protein load. In addition, milk protein expression at the mRNA level is downregulated in a mammary-specific *Agr2* knockout mouse model and upregulated in a mammary-specific *Agr2* overexpression mouse model [20], suggesting that AGR2 may play a role as a gatekeeper at both the protein and mRNA level to regulate the total protein load a cell can withstand without entering proteotoxic-induced cell death pathways. Tumor cells may overexpress AGR2 to promote cell survival by allowing the cell to withstand excess protein production associated with transformed cells. Major histocompatibility complex-1 (MHC-1) molecules, important for tumor surveillance, are potential targets of AGR2 as other PDIs have been shown to play a role in their folding and antigen loading [52,53]. In this respect, it is of interest that AGR2 has been used

as an immunotherapy target in experimental colorectal cancer [54].

A proposed method of general cancer treatment is to induce proteotoxic stress through the inhibition of protein-folding and protein degradation machinery. Targeted inhibition of AGR2, or any other protein involved in either the protein-folding or degradation pathways, would sensitize the tumor cell to proteotoxic stress and suppress tumorigenesis. The anti-cancer drug geldanamycin induces proteotoxic stress by inhibiting HSP90 through its required ATPase domain and could possibly inhibit the expression of AGR2 as it has been shown to be a downstream target of the HSP90 co-chaperone p23 [49]. Thus, the use of geldanamycin or the development of other proteotoxic stress-inducing anti-cancer drugs may be useful in a combinatorial therapy with anti-estrogens to treat ER-positive tumors that overexpress AGR2.

Mechanistic roles of AGR2 in promoting breast cancer metastasis

The AGR2 protein contains a canonical cleavable N-terminal signal peptide targeting it to the secretory pathway and has been biochemically demonstrated to be localized to secretory endosome-like organelles and at the extracellular surface [18,55]. Several secreted proteins, including the metastasis-associated GPI-anchored C4.4A protein and the extracellular domain of alpha-dystroglycan (DAG-1) [3,51,56], have been found in *in vitro* experiments to directly interact with AGR2, suggesting potential mechanisms for AGR2 in promoting tumor metastasis [13] through the regulation of receptor adhesion and interaction with extracellular matrix. Although these functions have not been validated at the molecular level, they are supported by work on other cancers in which AGR2, acting as a cell surface antigen, is involved in the dissemination of pancreatic tumor cells through the activation of cathepsins B and D [55]. Together, these data suggest that, in addition to being active within the endoplasmic reticulum, AGR2 is a secreted protein that interacts with the cell surface to modulate adhesion and promote dissemination of tumor cells.

AGR2 may also promote the production of mucins (MUCs), a family of secreted and transmembrane proteins that provide a protective mucous barrier to epithelial cells and, when overexpressed in breast cancer, participate in cell signaling to promote proliferation, invasion, and metastasis [57,58]. MUC1, overexpressed in most breast cancers, is an ER-responsive gene [59] that also interacts with ER to stabilize the protein and enhance binding to DNA [60]. AGR2 has been demonstrated to be an essential regulator of intestinal MUC2 [51,56], the airway epithelial MUC5AC and MUC5B [61], and pancreatic MUC1 production important for tumor initiation and progression [14]. Although studies on the role of AGR2 in promoting MUC expression are lacking, it is plausible that AGR2 could promote breast cancer metastasis through the regulation of MUCs.

Further evidence that AGR2 promotes the dissemination of tumor cells lies within the nucleus. At the transcriptional level, ErbB3-binding protein 1 (EBP1) suppresses the invasive ability of prostate cancer cells by inhibiting FOXA1- and FOXA2-mediated expression of AGR2 and thereby decreasing the metastatic behavior of these cells [62]. At the protein level, AGR2 binds in the nucleus to Reptin [63], which has been reported to modulate the invasive activity of cancer cells with metastatic potential [64]. As a member of the highly conserved RuvBl1/2 superfamily, Reptin contains two ATP-binding motifs, and loss or gain of ATP binding at these sites results in altered AGR2-binding properties. AGR2 uses a divergent peptide substrate-binding loop to bind to Reptin, and this interaction site could be a target for inhibiting the AGR2-Reptin complex, which would be highly relevant as Reptin can form protein-protein interactions with many proteins implicated in cancer, including Myc, Tip60, APPL1, Pontin, and telomerase holoenzyme complexes [65-68]. Therefore, the validation of Reptin as an AGR2-binding protein gives rise to a potentially novel signaling complex involved in pro-metastatic cancer development [63].

AGR2 as a regulator of cell proliferation

Several pieces of evidence suggest that AGR2 is a regulator of cell proliferation, although the molecular mechanisms by which it acts on cell proliferation are poorly understood. In the context of normal mammary gland morphogenesis, deletion of AGR2 in the mouse mammary gland leads to reduced lobuloalveolar development during late stages of pregnancy, whereas forced AGR2 expression leads to precocious lobuloalveolar development [20]. Functionally, AGR2 was demonstrated to regulate cell proliferation in these models, as the *Agr2*-null and *Agr2*-overexpressing mice exhibited reduced and increased cell proliferation, respectively. In addition, transient AGR2 knockdown in MCF-7 cells significantly reduced the number of estradiol-induced proliferating cells, implicating AGR2 as an estrogen-responsive regulator of proliferation in breast cancer cells. Understanding the connection between ER-mediated *AGR2* expression and normal cell proliferation could give novel insights into the effects of AGR2 overexpression and subsequent cell proliferation in ER-positive breast cancer.

Recent work suggests that AGR2 is incorporated in several pathways that promote tumor cell growth and survival. In lung and esophageal adenocarcinoma cell lines, AGR2 induces the expression of the epidermal

growth factor receptor (EGFR) ligand amphiregulin (AREG) via the Hippo signaling pathway co-activator YAP1 and subsequently stimulates EGFR signaling and proliferation [69]. In addition, AGR2 promotes cell cycle progression and cell survival in ER-positive breast cancer cell lines via multiple cancer signaling pathways, mainly ER, cyclin D1, pSrc, c-Myc, and survivin [12]. Furthermore, Aryl hydrocarbon receptor (AhR) signaling activated by genotoxicants induces the expression of AGR2, ultimately leading to tumor progression by inhibiting DNA-damage response through p53 activity [70]. In Barrett's esophagus, AGR2 is overexpressed and was demonstrated to repress p53 activity by inhibiting phosphorylation of the protein in response to DNA damage, thereby promoting cell survival [71]. As such, therapeutically targeting the AGR2 pathway may prove to be beneficial to attenuate cell proliferation and induce p53-dependent apoptosis.

Further evidence that AGR2 participates in cell proliferation is provided by its targeted regulation by transforming growth factor-beta (TGF-β) signaling, which governs multiple aspects of cell behavior, including proliferation, apoptosis, differentiation, and migration. In normal epithelial cells and early-stage breast carcinogenesis, TGF-β acts as an inhibitor to cell proliferation; however, in advanced-stage tumors, it promotes cell survival, motility, and invasiveness [72]. In pancreatic ductal adenocarcinoma, *AGR2* gene expression is suppressed by TGF-β in a SMAD4-dependent manner [14]. Mutations to SMAD4 lead to the overexpression of AGR2 and consequently tumor progression. Thus, AGR2 could be a contributor to the TGF-β switch from cytostatic effects to promotion of malignancy. Collectively, these data provide strong evidence for AGR2 regulation of cell proliferation in cancer through multiple signaling pathways.

Conclusions

AGR2 plays an important role in the development and progression of breast cancer and several other adenocarcinomas. Its expression, stimulated by ER signaling and endoplasmic reticulum stress, correlates with poor outcome in patients with breast cancer. It acts by promoting tumor metastasis, cell survival, cell proliferation, and resistance to anti-hormone therapies. Mechanistically, AGR2 assists in protein folding to maintain endoplasmic reticulum homeostasis, interacts with the extracellular matrix through its location on the cell surface, and is thought to promote cell proliferation through several signaling pathways. Further investigation into these mechanistic functions is necessary to elucidate the cellular networks through which AGR2 regulates tumor progression and metastasis. The recent generation of AGR2 inducible knockout and overexpression mouse models provides opportunities to study the mechanisms of AGR2 in breast cancer and other adenocarcinomas [20]. Many questions regarding the functional role of AGR2 in normal mammary gland development and breast cancer remain, such as whether AGR2 is a key mediator of ER-mediated cell proliferation. The mechanism for heterogeneous AGR2 expression in ER-positive breast cancer, in addition to how tamoxifen acts as an agonist to AGR2 expression, needs to be explored. Furthermore, there is a need to determine how important PDI function is for the biological effects of AGR2 in the mammary gland and breast cancer, and if AGR2 functions primarily as a PDI, its key enzymatic targets need to be defined. If AGR2 is a key survival factor, allowing ER-positive breast cancer cells to overcome endoplasmic reticulum stress, it may become an effective therapeutic target in breast cancer. Further studies into the development of drugs targeting AGR2 and the pathways that it mediates may lead to useful treatment options for patients with difficult-to-treat tamoxifen-resistant and metastatic tumors. A therapy directly targeting AGR2 would also prove beneficial to the treatment of the many other adenocarcinomas that are dependent on the function of AGR2. However, it would be necessary to determine the relevance of AGR3 in these tumors, as it may be able to compensate for the loss of AGR2. Future drugs may have to target both AGR2 and AGR3 to be effective. In addition to being a treatment target, AGR2 is a marker of poor prognosis and can be used to identify CTCs and breast cancer metastasis.

Abbreviations

AGR2, anterior gradient 2; ChIP, chromatin immunoprecipitation; CTC, circulating tumor cell; EGFR, epidermal growth factor receptor; ER, estrogen receptor; ERP, endoplasmic reticulum protein; MUC, mucin; PDI, protein disulfide isomerase; TGF-β, transforming growth factor-beta.

Competing interests

The authors declare that they have no competing interests.

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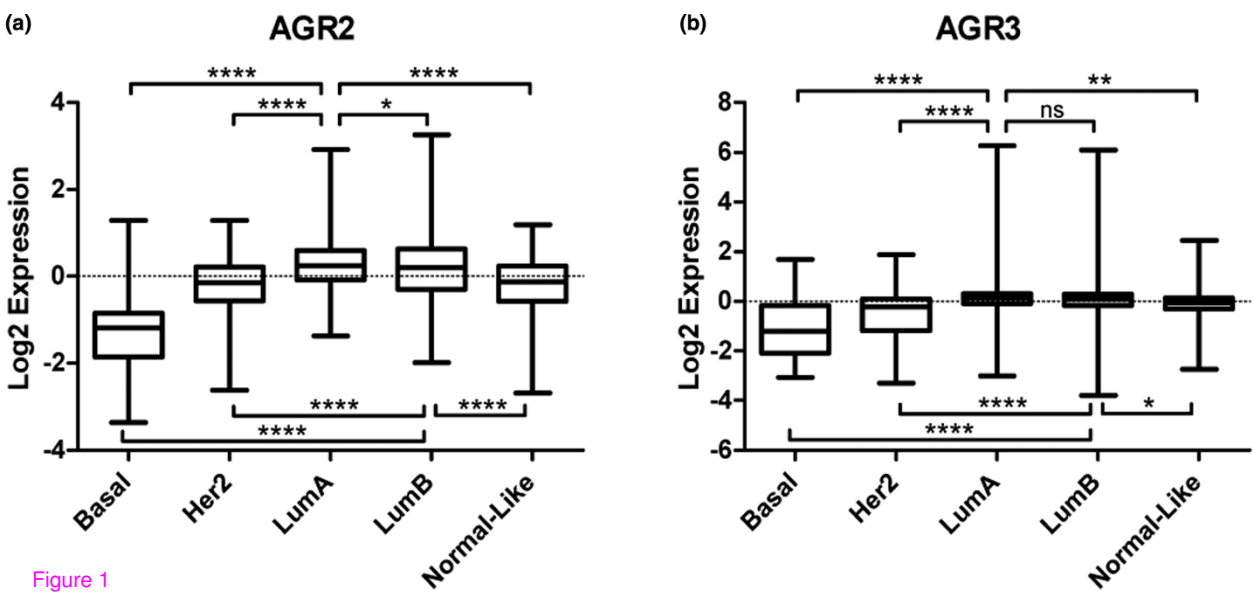
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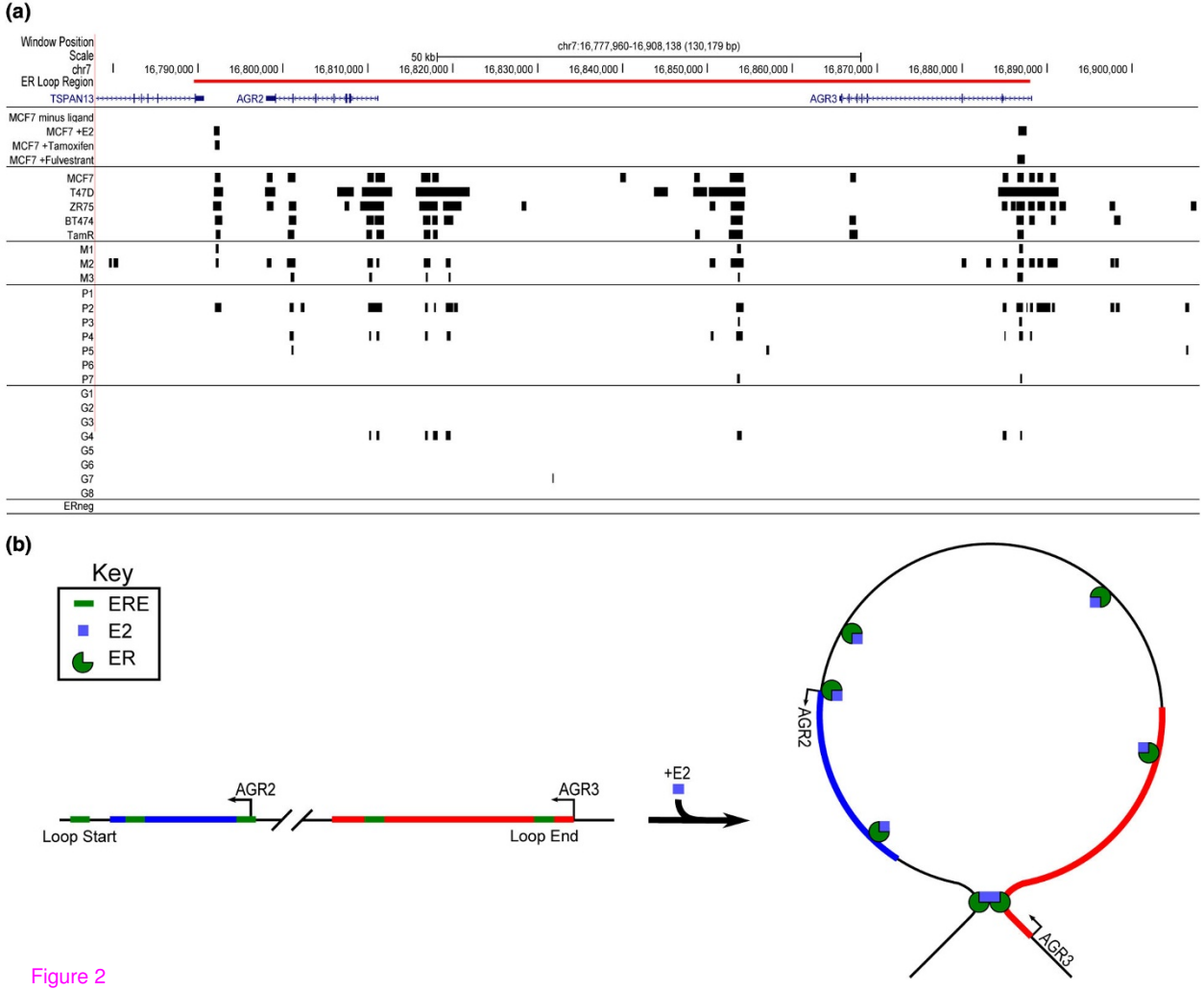
Figure 1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AGR2
AGR3
MCF7 minus liqand
MCF7 +E2
MCF7 +Tamoxifen
MCF7 +Fulvestrant
MCF7
T47D
ZR75
BT474
TamR
M1
M2
M3
P1
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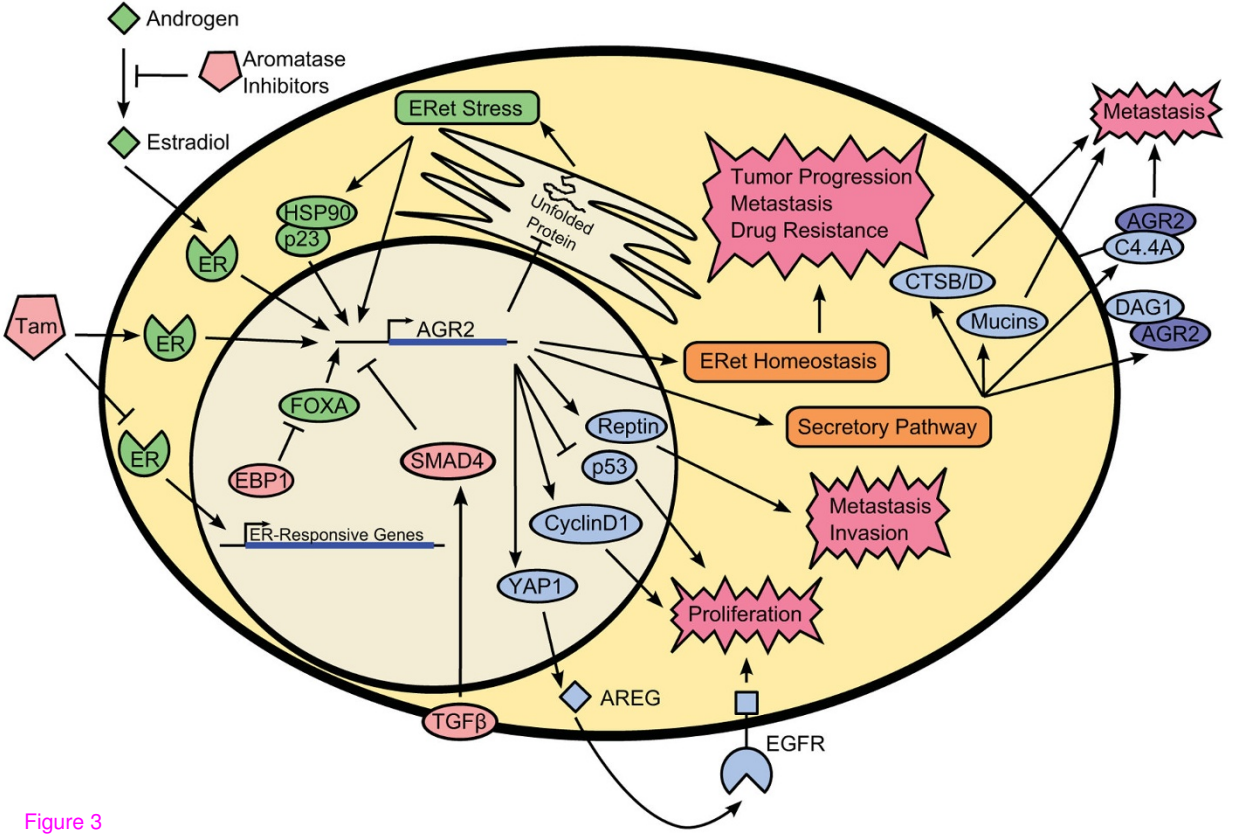
Figure 2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YAP1
TGFβ
AREG
EGFR
Tumor Progression
Metastasis
Drug Resistance
ERet Homeostasis
Secretory Pathway
Metastasis
Invasion
Proliferation
CTSB/D
Mucins
Metastasis
AGR2
C4.4A
DAG1
AGR2

Figure 3