UNIVERSITY OF CALIFORNIA, SAN DIEGO

The Role of uPAR in Inducing a Cancer Stem Cell-like Phenotype in Breast Cancer and Allowing Cells to Develop Endocrine Therapy Resistance

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by

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LIST OF ABBREVIATIONS

ABC transporter, ATP-binding cassette transporters
ALDH1, aldehyde dehydrogenase 1
AML, acute myelogenous leukemia
ATF, amino terminal fragment
DN-FAK, dominant-negative FAK
DN-Rac1, dominant-negative Rac1
DN-Ras, dominant-negative Ras
E2, 17β-estradiol
EGFR, epidermal growth factor receptor
EMT, epithelial-mesenchymal transition
EpCAM, epithelial cell adhesion molecule
ERα, estrogen receptor-α
FPRL1, formyl peptide receptor-like 1
GPI, glycosyl-phosphatidylinositol
GPCR, G-protein coupled receptor
HA, hyaloronic acid
HER-2, epidermal growth factor receptor 2
HIF1α, hypoxia induced factor 1α
IHC, immunohistochemistry
JNK1, c-Jun NH2 terminal kinase
LDLR, low density lipoprotein receptor
LRP1, low density lipoprotein receptor-related protein
MET, mesenchymal-epithelial transition
MMP, matrix metalloproteinase
NO, nitric oxide
PAI, plasminogen activator inhibitor
PI3K, phosphoinositide 3-kinase
PR, progesterone receptor
RTK, receptor tyrosine kinase
SERM, selective estrogen receptor modulator
SFK, Src family kinase
SFM, serum-free medium
TAM, Tamoxifen
TGFβ, Transforming growth factor-β
t-PA, tissue plasminogen activator
VLDLR, very low density lipoprotein receptor
uPA, urokinase-type plasminogen activator
uPAR, urokinase-type plasminogen activator receptor

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"Cell signaling by urokinase-type plasminogen activator receptor induces stem cell-like properties in breast cancer cells". 2010. *Cancer Res.* 70(21):8948-58

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ABSTRACT OF THE DISSERTATION

The Role of uPAR in Inducing a Cancer Stem Cell-like Phenotype in Breast Cancer and Contributing to the Development of Endocrine Therapy Resistance

by

Boryana M. Eastman

Doctor of Philosophy in Biomedical Sciences

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Professor Steven Gonias, Chair

The urokinase receptor (uPAR) is a cell-signaling receptor and a negative prognostic indicator in human breast cancer. uPAR-initiated cell signaling contributes to increased cell proliferation, cell survival, migration, and metastasis. In this work we utilize a number of cell and molecular biology techniques to demonstrate for the first time the possible effects of uPAR on cancer initiation, uPA-independent cell-signaling, and endocrine therapy resistance in breast

cancer. We show that uPAR signaling can induce cancer stem cell (CSC)–like properties in breast cancer cell, either concurrently or independently of epithelial-mesenchymal transition. Over-expression of uPAR in human MDA-MB-468 and MCF-7 breast cancer cells promote the emergence of cells with $CD24^{-}/CD44^{+}$ phenotype, characteristics for cancer stem cells. In addition, uPAR expression further increases the abundance of integrin subunits β1/CD29 and α6/CD49f, which are also associated with the stem cell phenotype. The cancer stem cell properties are also evident *in vivo*, when a small number of uPAR over-expressing cells are capable of forming tumors in mice. We further demonstrated the uPAR can signal independently of uPA to induce ERK activating downstream of both H-Ras and Rac1. The transition in uPAR signaling from uPA-dependent and transient to autonomous and sustained is reminiscent of the transformation in ErbB2/HER-2 signaling observed when this gene is amplified in breast cancer. Vitronectin was essential for this activation, which was sensitive to the EGFR tyrosine kinase inhibitors Erlotinib and Gefitinib. In addition uPAR over-expression conferred MCF-7 cells resistance to the estrogen receptor anatagonist, Tamoxifen. Tamoxifen treatment also induced uPAR expression in MCF-7 cells, suggesting an important role for uPAR in endocrine therapy resistance. These studies suggest that uPAR over-expression has effects on breast cancer initiation and may provide a pathway for escape from breast cancer targeting therapeutics. It indicates that its expression could be useful as a biomarker for breast cancer stem cell or for cells that could be potentially resistant to Tamoxifen treatment.

INTRODUCTION

Breast Cancer Incidence and Prognosis

Cancer is one of the leading causes of death in the United States and worldwide, accounting for more than half a million deaths in the US in 2007 and almost eight million deaths worldwide [1]. Breast cancer alone is the second leading cause of cancer death in women and in 2011 it affected more than 230,000 women in the United States alone [1]. It is estimated that breast cancer carries a 12% lifetime risk, affecting one out of every eight women [2]. Breast cancer is most prevalent in postmenopausal, white, non-hispanic females, but affects women of all ages and racial backgrounds [3]. Certain risk factors have been associated with breast cancer, including but not limited to increasing age, family history, certain genetic mutations (BRCA), hormonal factors (estrogen replacement therapy, age at first pregnancy), and lifestyle factors (alcohol and tobacco consumption, diet, etc.) [1, 3].

Breast cancer prognosis is dependent on the stage of the disease. Localized disease has a very good prognosis and 5-year survival rate of more that 90%, whereas cancers that have spread to distant sites have survival rates of less that 20% [3]. In recent years, aggressive breast cancer screening combined with more effective therapeutics has decreased the mortality rates. More than 50% of women over 40 years have had a mammography screening in the last two years, allowing for the identification of early stage disease and its successful treatment [1, 3]. Nevertheless, more efforts are necessary to identify at risk women who do not

undergo screening as often due to lack of health insurance or other contributing factors. Increased rate of mammography screenings has also raised concerns that screening studies identify *in situ* lesions which would not have progressed to breast cancer, leading to over-treatment and undue suffering in certain patient populations [4].

Breast Cancer Treatment

Due to patient advocacy and advances in research, the management of breast cancer has been improved in the last two decades. Breast cancer treatment depends on the extent of the cancer at the time of diagnosis and can include surgery, radiation, and systemic therapy. In all cases, surgery is required to remove the bulk tumor either through mastectomy (removal of the whole breast) or lumpectomy, in which only the affected tissue is excised [5-6]. In all lumpectomy cases, localized radiation is advised to remove residual disease and prevent reoccurrence in the breast [5]. Although these types of treatments are common in all cancers, targeted therapies are available for some forms of breast cancer. In addition to the major classes of drugs (taxanes, anthracyclines, platinum based therapies and others) targeting rapidly dividing cells, a number of specific targeted and hormonal therapies against breast cancer have been developed [7].

One of the most celebrated targeted therapies up to date is Trastuzumab (Herceptin; Genentech, South San Francisco, CA, USA) [8]. Trastuzumab is a monoclonal antibody targeting the human epidermal growth factor receptor 2

(ErbB2/HER-2/neu) which is over-expressed in 25%-30% of breast cancers [9]. HER-2 over-expression allows cancer cells to grow quickly and aggressively and its expression was associated with worsened prognosis before the use of Trastuzumab [10]. Several mechanisms of action have been proposed for Trastuzumab, including preventing HER-2 receptor dimerization, inducing antibody mediated cytotoxicity, and inhibiting angiogenesis [11]. Its effectiveness has been tested in several large scale clinical trials, indicating that addition of Trastuzamab to standard chemotherapy regiments in HER-2 positive breast cancers significantly improves the response to chemotherapy and significantly decreases the recurrence and death associated with the disease [12].

Hormonal therapy is another line of treatment in breast cancer [3]. Normal breast tissue is estrogen receptor alpha (ERα) positive. Many cancers of the breast are also ERα-positive and are dependent on estrogen for their growth [13-14]. In order to deprive these cells from estrogen and slow down their growth, two different classes of drugs were developed. Aromatase inhibitors target the enzyme aromatase which is involved in estrogen synthesis in the peripheral tissue [14-15]. They are used more heavily in recent years and in post-menopausal women they effectively starve the cancer from any available estrogen, preventing further tumor growth [15].

Selective estrogen receptor modulators (SERMs) are another class of drugs targeting the ER [16]. SERMS mode of action is tissue dependent and ranges from complete antagonist to partial agonist [17-18]. The most commonly used drug of this class is Tamoxifen. Multiple large scale trials have demonstrated

the Tamoxifen has an objective effect in both advanced and early disease and is effective in decreasing breast cancer related mortality [19]. Tamoxifen adjuvant treatment reduces recurrence by nearly 50% and has contributed significantly to the decrease of breast cancer mortality in the past two decades [19].

Competitive Tamoxifen binding to the ER antagonizes estrogen action and induces cell-cycle arrest at G_0/G_1, preventing cancer growth [20-21]. Any remaining agonist activity of Tamoxifen is completely negated by co-repressors binding to the ER-Tamoxifen complex [22]. It has been demonstrated that in addition to its profound cytostatic effects, Tamoxifen also has cytotoxic properties in both ER-positive and ER-negative cells [23-24]. Several different mechanisms have been proposed for the cytotoxic effects of Tamoxifen in both ERα-positive and ERα-negative cells. It has been suggested that Tamoxifen blocks mitochondrial respiration by increasing mitochondrial Ca^{2+} concentration and inducing mitochondrial nitric oxide (NO) sythase [25]. In this way, cytochrome c is released from the mitochondria and the cell undergoes apoptosis [25]. In addition, it has been demonstrated that Tamoxifen can induce apoptosis by activating caspase-3 and c-Jun NH2-terminal Kinase-1 (JNK1) in breast cancer cells [26].

In breast cancer, as it is the case with other epithelial cancers, the main cause of morbidity and mortality is associated with metastasis and cancer recurrence. Even with the heavy use of Herceptin and Tamoxifen, a significant portion of breast cancer recur within five years [19, 27]. The resulting metastatic disease is no longer sensitive to the same therapeutics often due to molecular and

metabolic changes the cancer cells have acquired [19, 28]. To combat resistance, new generations of therapeutics have been developed, such as the more effective SERM - fulvestrant [29-30]. Fulvestrant has been shown to be effective in Tamoxifen resistant disease and is currently in clinical use [30].

The Cancer Stem Cell Hypothesis

For over fifty years, researchers have investigated the causes of cancer metastasis and disease relapse. Many different molecular markers have been identified and in the last two decades a new theory has taken hold. It has been suggested that cancer is a hierarchical disease and only a small population of cells is responsible for the growth and development of the disease [31].

It is hypothesized that cancer stem cells not unlike normal adult stem cells have the potential to self renew and the capacity to differentiate into different cell types recapitulating the tumor phenotype [32]. Cancer stem cells were first identified in malignancies of the hematopoietic system as early as 1963, demonstrating that only 1-4% of murine lymphoma cells can form tumors in recipient animals [33]. Several decades later it was shown that human acute myelogenous leukemia (AML) blasts also have a differential proliferative potential and form colonies in methylcellulose at low frequency [34]. These two types of assays – the limiting dilution transplantation of cells in immuno-compromised hosts and the ability of cells to form colonies in non-permissive substrates such as methylcellulose or soft agar, have become the gold standard in determining the cancer stem cell qualities of different populations of cells [31].

In order to exclude the possibility that cells have differential propagating potential due to extrinsic factors it was necessary to identify specifically a subpopulation of cells that is capable of inducing tumorogenesis. With the development of specific monoclonal antibodies against cell surface antigens, such populations were identified in many hematopoietic malignancies [35]. For example leukemia stem cells are identified by a $CD34^{+}/CD38^{-}/CD90^{-}$/IL-$3R^{+}/CD71^{+}$/HLA-DR-$CD117^{-}$ phenotype [36-39]. This very specific phenotype has allowed to identify specific properties of cancer stem cells and to suggest they could be targeted exclusively.

In addition to identifying markers associated with cancer stem cells, it was also necessary to demonstrate that these markers are important in cancer stem cell functioning. In this way, cancer stem cells would be more susceptible to therapies targeting these specific molecules. Two such markers that were identified in cancer stem cells of different origin are aldehyde dehydrogenase 1 (ALDH1) and ATP-binding cassette transporters (ABC-transporters) [40-41]. Both are also highly expressed in normal stem cells, suggesting their importance in stem cell maintenance [42-43].

ALDH1 is a detoxifying enzyme involved in aldehyde oxidation and has been widely identified in normal progenitor and stem cells [42-44]. It has an essential role in retinoid acid biosynthesis, a process important in early stem cell differentiation and stem cell maintenance [45]. ALDH1 has been identified in a

number of cancer stem cells, suggesting it can be used as a universal cancer stem cell marker that could be targeted preferentially [40, 43, 46].

ABC-transporters are highly conserved family of proteins that facilitate the transport of various substances, including various drugs, through the cell membrane [47-48]. High levels of these transporters allowed tissue stem cells to be identified, since they did not accumulate dyes effectively [49-50]. It is thought that stem cells have high levels of these transporters, so they can efflux substances efficiently and be less sensitive to environmental toxins [41, 50]. However, high ABC-transporter levels are not always associated with stem cell properties [51]. Thus both ALDH1 and ABC-transporters appear to be necessary for the normal functioning of tissue stem cells and could be useful in identifying cancer stem cells more reliably, since their absence would interfere with normal cancer stem cell function.

The nature of the hematopoietic system and its very clear delineation has allowed for its convenient study *ex vivo* and has helped identify somatic adult stem cells and cancer stem cells. The study of normal tissue and solid tumors has been significantly more difficult and only recently somatic stem cells have been identified in variety of tissues, such as the skin, lung, breast, brain and others [52-55]. It has been demonstrated that a single tissue specific adult stem cell could regenerate all the cell types in a tissue without showing signs of differentiation itself [31]. Somatic stem cells in most tissues show very low turn-over rates with

the notable exception of stem cells in the intestines, where turn-over is rapid [56]. Multiple studies have identified many signaling networks that are common among different tissue stem cells, such as Wnt, mTOR, Notch, Yap, and GPCRs [57-58].

The improved understanding of the hierarchy of the different tissues and the fact that only a small subpopulations of cells could recapitulate a tumor, have suggested the existence of cancer stem cells in solid tumors. Cancer stem cells have been identified in variety of cancers including brain, breast, and lung malignancies [59-61]. It has been hypothesized that these cells might arise from progenitor cells that have accumulated mutations and it has been demonstrated that they share many properties with normal stem cells, such as increased drug resistance and self-renewal [31]. In addition they are highly metastatic and have the ability to form a tumor at very low numbers.

Breast Cancer Stem Cells

Two seminal papers published in Nature in 2006 identified adult stem cells in murine mammary tissue [54, 62]. These cells were identified by a $CD49f^{high}/CD29^{high}/CD24^{medium}$ phenotype and a single cell was capable of regenerating the entire mammary gland in mice [54, 62]. Normal mammary stem cells have also been identified in humans, exhibiting a $CD49f^{high}/EpCAM^{low}/MUC1^{-}/ALDH1^{high}$ phenotype [63-64]. It has been determined that mammary stem cells lie in the basal location of the luminal compartment of the mammary gland both in the mouse and human, exhibit basal cell like properties, and generally lack ER-α, progesterone receptor (PR), or HER-2 [63, 65-68].

Nevertheless, there have been reports indicating that some progenitor cells might be ERα and/or PR positive, indicating the still developing view of the normal breast hierarchy [69]. In addition, a recent study has identified that small amount of HER-2 is present on the surface of embryonic mammary stem cells suggesting a possible role for HER-2 in cancer stem cells [70]. In addition over-expression of HER-2 in normal mammary epithelial cells and in variety of breast cancer cell lines increases the proportion of progenitor and cancer stem cells respectively [71]. These facts are noteworthy, since HER-2 positive tumors and basal-like breast cancers, which are ERα, PR, and HER-2 negative (triple negative), have the worst prognosis and are often insensitive to conventional chemotherapy [72].

In breast cancer a variety of cancer stem cell markers have been identified. Al-Hajj and colleagues determined that breast cancer cells that expressed high levels of epithelial cell adhesion molecule (EpCAM) and CD44 and low levels of CD24 acted as cancer stem cells and were capable of regenerating a tumor [59]. In addition, they demonstrated that these cells were capable of giving rise to a heterogeneous tumor, consisting of cells with various levels of CD24 and CD44. The low expression of CD24, a protein expressed solely on human luminal epithelial cells, suggested that breast cancer stem cells, not unlike normal mammary stem cells arise from the basal epithelium [68, 73]. Normal mammary stem cells are also rich in EpCAM and ALDH1, which further suggests that breast cancer stem cells could arise from normal tissue stem cells, which are also EpCAM and ALDH1 positive [63-64].

Nevertheless, cancers could also arise from progenitor or fully differentiated cells. For example, transgenic mice over-expressing Her-2 in the mammary epithelium (MMTV-HER2/neu mice) develop tumors with 50% invasiveness within six months [74]. These tumors appear to be maintained by a population of cancer stem cells which are CD49f and CD61 positive, a phenotype typical of luminal progenitor cells [75]. Therefore, even though the presence of cancer stem cells have been demonstrated in a variety of cancers, the origin of the cancer stem cell varies from a stem cell to a progenitor cell and even to a non-progenitor, terminally differentiated cell [31].

CD44 is another cell surface antigen that has been identified as a marker for breast cancer stem cells [76]. CD44 is a glycoprotein, important in hyaloronic acid (HA) binding, which is expressed in a small population of cancer cells and has been associated with cancer stem cell like qualities [76-77]. It has also been suggested that CD44 might have an important role in cancer stem cells homing and migration, due to its ability to initialize signal transduction pathways that support leading edge adhesion and by its weak binding to CD62 cells on endothelial cells [76, 78]. Additionally, CD44 expression contributes to apoptosis and drug resistance in cancer initiating cells by associating with other proteins on the cell membrane. For example, CD44 initiates a cascade which leads to the upregulation of MDR1 (part of the ABC-transporter family) and allows for the two proteins to be co-expressed [79-80]. The increased expression of MDR1 promotes resistance in the cancer stem cells and renders therapy ineffective. CD44 expression is suppressed by p53 binding to a non-canonical binding site on

the CD44 promoter [81]. In the absence of p53, CD44 contributes even more significantly to the tumor initiating abilities of tumurogenic mammary epithelial cells [81]. CD44 and its ligand HA have also been shown to be important in epithelial-mesenchymal transition (EMT) [82].

Epithelial-Mesenchymal Transition (EMT)

EMT is a complex molecular and cellular program, which requires major changes in the cells architecture and behavior [83-85]. Epithelial cells shed their epithelial qualities, including their apical-basal polarity, cell-cell junctions, and lack of motility, and instead acquire mesenchymal properties, such as increased migration abilities, invasion, and heightened resistance to apoptosis [86]. EMT is very important in embryonic development, where it is essential for proper neural crest, heart valves, and secondary palate formation [83, 87]. In normal development EMT is regulated precisely and is dependent on various microenvironment factors guiding the timing and localization of the process [83]. A number of developmental pathways interact to induce EMT and its opposing process – mesenchymal to epithelial transition (MET). Transforming growth factor-β (TGFβ), Notch, Wnt, and various receptor tyrosine kinases (RTKs) have been shown to have great importance in the induction and maintenance of EMT [88-89]. The same pathways that are necessary for the normal formation of various organs during development are disregulated in cancer and have been shown to be important in cancer development and progression [90].

EMT has been shown to be increasingly important in cancer development and progression allowing cells to escape the initial tumor, intravesate, extravesate and invade distant tissues [84, 88]. EMT is initiated by variety of signals usually released by the stromal cells surrounding normal and neoplastic tissue [86]. TGFβ family members are one of the best described and the most potent inducers of EMT [91]. They can induce EMT in variety of ways. They directly phosphorylate members of the SMAD family and the cell polarity protein PAR6, initiating a signaling cascade involving RhoA. They can also modulating other pathways involved in EMT, such as the Notch or Wnt pathways in a tissue specific manner [83, 86, 92-94]. Other developmental pathways, such as Wnt and Notch signaling could also lead to EMT. These pathways are complex and often involve GSK3β and SNAI1 [83]. They also modulate each other or the TGFβ pathway underlining the complexity and specificity of EMT.

A family of zinc finger transcriptions factors sits in the center of EMT signaling and their expression and activity is almost always modulated by upstream pathways leading to EMT [95]. Snail, Slug, and Twist inhibit the expression of epithelial markers, such as E-cadherin, cytokeratin-18, and Muc-1, and induce expression of proteins associated with the mesenchymal phenotype, such as vimentin and fibronectin [95]. The expression of Twist alone in normal kidney epithelial cells or mammary epithelial cells induces a complete loss of epithelial characteristics [96]. Twist downregulates E-cadherin expression, considered a hallmark of EMT by up-regulating another zinc-finger transcription

factor – SNAI2 [97]. Increased Twist expression also promotes migration in different cell lines and metastasis in mice [96, 98]. This is due to its induction of PDGFRα expression and Src activation contributing to the formation of invadopodia in cancer cells [98].

In addition to heterogeneous signals produced by mesenchymal cells, other environmental factors can also induce EMT. For example, prolonged hypoxia and hypoxia-inducible factor-1α (HIF-1α) expression has been shown to induce EMT both *in vitro* and *in vivo* [99-100]. These processes are still mediated by zinc-finger family members and can involve both Twist and Snail [99-100]. A number of signaling molecules were involved in this process, one of which is the urokinase plasminogen activator – uPAR [99].

The Urokinase Plasminogen Activator Receptor - Structure

The urokinase plasminogen activator receptor (uPAR) is a glycosyl-phosphatidylinositol (GPI) anchored glycoprotein receptor, specific for urokinase (uPA) [101]. It is a single chain peptide consisting of 313 amino acids preceded by a 22 amino acid signaling peptide [102]. Its molecular weight is 37,000 Da, but due to extensive glycosylation the mature protein has a molecular weight of approximately 50,000 to 60,000 Da. uPAR consists of three homologous Ly-6 domains - D1, D2, and D3 [103-104]. It could be cleaved of its GPI tail enzymaticaly or produced through alternative splicing and could be detected in

both cultured cells and *in vivo* as soluble uPAR (suPAR) [101, 105-107]. In addition, the link between the D1 and D2 domain could be cleaved to produce another soluble uPAR fragment consisting of only the D2 and D3 domain [104].

uPAR specifically binds a number of different ligands, notably uPA, its single chain proenzyme, pro-uPA, and the amino-terminal fragment of uPA (ATF) [101]. The major site for uPA binding is the most N-terminal domain (D1), but sites on D2 and D3 may also contribute to the binding efficiency [108-110]. In addition, uPAR binds the provisional extracellular matrix protein vitronectin with the major binding site lying in D2 [111-112].

uPAR Expression in Cancer

In normal tissue uPAR expression is almost undetectable, whereas many primary and metastatic tumors express high quantities of uPAR [113-114]. uPAR deficient mice are normally developing, but exhibit defects in neutrophil recruitment, peritoneal macrophage migration, bone-homeostatis, and wound healing [115-117]. Much of uPAR in the body is expressed in the tumor myoepithelial cells and in tumor associated macrophages [118]. Nevertheless, many single epithelial cells exhibit strong uPAR staining. In addition, uPAR positive cells are present in the bone marrow of cancer patients, as well as in the blood stream as circulating tumor cells, qualities typical of highly invasive and cancer stem cells [119-120]. uPAR gene amplification has also been detected in cancers of the breast and pancreas [120-121]. Activation of the uPA-uPAR system shows a strong correlation with poor prognosis in breast cancer and high

expression of uPA and uPAR are independent predictors of disease outcome [113-114, 122-123].

uPAR Protease Function

Historically, uPAR has been studied solely for its role in extracellular matrix degradation due to its binding to uPA. uPA is a serine protease and together with its homologue tissue plasminogen activator (t-PA) is responsible for thrombolysis [124-125]. When uPA or t-PA are present in the blood stream they activate the plasminogen trapped in blood clots, converting it to plasmin and leading directly to fibrin degradation and clot dissociation. uPA is redundant in development and uPA null mice are normally developing, possibly due to the redundant effects of t-PA [126]. uPA/t-PA double knock-out animals, on the other hand, show extensive thrombotic disorders, similar to those observed in mice deficient in plasminogen [127].

When uPA binds to its receptor, uPAR, it is not degraded or internalized at a significant rate [128-129]. Instead it remains accessible at the surface increasing the amount of plasmin in the surroundings. It activates matrix metalloproteinase (MMPs), mainly MMP-2 and MMP-9 facilitating cell migration [130]. Due to the protease activity of uPA, it has been long hypothesized that high uPAR expression in cancer is associated with the increased migratory potential of these cells [101, 131]. However, even in the absence of uPA, uPAR is capable of inducing metastasis *in vivo,* suggesting a more complex role of uPAR in cellular signaling [132].

The activity of the uPA-uPAR complex is regulated by several members of the serine protease inhibitor (serpin) family – the plasminogen activator inhibitor 1 (PAI-1) and the plasminogen activator inhibitor 2 (PAI-2) [133-134]. In contrast to the uPA-uPAR complex, uPAR-uPA-PAI-1 or uPAR-uPA-PAI-2 complexes are readily recognized and rapidly internalized by the members of the low density lipoprotein receptor (LDLR) family - low density lipoprotein receptor-related protein (LRP1), the very low density lipoprotein receptor (VLDLR), and LRP-2 [135-138]. When the complex is internalized the uPA-PAI-1/PAI-2 complex is degraded in the lysosomes and uPAR is recycled back to the cell surface [138-140].

uPAR Signaling

In the last couple of decades uPAR has been studied more for its role in cellular signaling. uPAR does not have a cytoplasmic or transmembrane domain, but through its interaction with other cell surface molecules in lipid rafts, it activates a number of intracellular pathways, influencing cell migration, survival, and proliferation [101, 114]. uPAR interacts with a number of cell surface molecules - G-protein coupled receptors (GPCR), such as the formyl peptide receptor-like (FPRL1), various integrins such as α5β1 and αvβ3, RTKs such as the epidermal growth factor receptor (EGFR) [101, 141-144]. Much of its signaling is uPA dependent and is mediated by Src family kinases (SFKs), phosphatidyl-inositol 3 (PI3K), and Akt [145]. Such signaling is important for migration (FPRL1, integrins) and proliferation (integrins, EGFR). Additionally,

vitronectin binding to uPAR, independently of uPA activates the Rho family of GTPases, including Rac1, and leads to actin remodeling and increased cell migration [146].

uPAR, EMT and Cancer Stem Cells

Previously our lab and others have demonstrated that increased uPAR expression, either due to uPAR up-regulation during hypoxia or over-expression induces EMT in cultured cells [99, 147]. In MDA-MB-468 cells the induction of uPAR is necessary and sufficient for hypoxia induced EMT [99]. As with all EMT processes a zinc-funger transcription factor (Snail) is involved [99]. uPA-uPAR initiated signaling leads to activation of PI3K, c-Src, and Erk [99]. Inhibition of either of these signaling pathways leads to reversal of the process, culminating with cells reestablishing their epithelial phenotype [148]. Due to the fact that uPAR induces EMT and EMT has been implicated in cancer stem cell development [149], we hypothesized that uPAR might play a role in the induction of cancer stem cell phenotype in breast cancer. Indeed, a previous report has indicated that in small cell lung cancer, a rare population of uPAR positive cells is present and resistant to chemotherapy, a characteristic associated with stem cells [150]. In this population uPAR co-localized with MDR1, a member of the ABC transporter family [150]. In addition, increased amounts of uPAR are also associated with increased levels of another molecule implicated in stem cell maintenance - activated surface β1 integrin [151].

uPAR and Therapy Resistance

Another important issue in cancer treatment is acquired treatment resistance, which is often associated with recurrent disease and metastasis. About 70% of breast cancers express ERα, allowing for their successful treatment with anti-estrogens [13]. Over the course of the disease, breast cancer tumors either lose their ERα expression of become resistant to endocrine therapies [152-153]. Over-expression of the RTKs EGFR and HER-2 contributes to hormone therapy resistance [154-155]. These pathways either lead to phosphorylation of ERα inducing ligand independent signaling, or activate alternative pathways – increasing cell cycle proteins (e.g. p-Myc, cyclin D1) and anti-apoptotic factors (e.g. BCL-2, BCL-XL, Mcl-1) [153, 156]. Since uPAR activates similar signaling pathways, trans-activates EGFR, and has been positively correlated with HER2 expression, we hypothesized that uPAR over-expression could also contribute to therapy resistance [120, 142, 157]. uPAR expression is negatively correlated with ERα expression, however, it is also detectable in ERα positive tumors at lower levels [158]. Additionally, increased expression of the uPAR-uPA-PAI-1 system has been associated with increased Tamoxifen resistance in ERα positive relapsed breast cancer [159]. However, the role of uPAR and its downstream signaling in endocrine resistance of breast cancers has not been established.

Statement of Hypothesis

The ability of uPAR to promote EMT, its high expression in breast cancer, its association with worsened prognosis, metastasis, and Tamoxifen

resistance, led us to hypothesize that uPAR has the ability to promote cancer stem cell like qualities in breast cancer cells. We also hypothesize that uPAR plays a role in the survival or ERα positive cells in the absence of estrogen and that its downstream signaling confers them a survival advantage allowing them to become unresponsive to selective estrogen receptor modulator (SERM) treatment. In the following work we have successfully demonstrated that uPAR has the ability to promote cancer stem cell like qualities in breast cancer cells *in vitro* and *in vivo.* In addition, uPAR can signal independently of uPA and promote cancer cell growth and survival in estrogen independent manner. Lastly, we have shown that TAM challenge induces uPAR expression in breast cancer cells, leading us to conclude that uPAR signaling might be important in developed TAM resistance.

The ability of uPAR to promote cancer stem cell like qualities and its role in the survival of estrogen therapy resistant breast cancer cells will be consistent with the fact that uPAR expression is often associated with worsened prognosis and increased metastatic potential. Understanding the specific roles of uPAR will allow us to design better therapeutics and pair drugs targeting the uPA-uPAR system with other therapeutics, improving their efficacy and increasing patient survival.

CHAPTER 1

ABSTRACT

Signaling by urokinase-type plasminogen activator receptor (uPAR) can cause epithelial-mesenchymal transition (EMT) in cultured breast cancer cells. In this report, we show that uPAR signaling can also induce cancer stem cell (CSC)–like properties. Ectopic over-expression of uPAR in human MDA-MB-468 breast cancer cells promoted the emergence of a CD24−/CD44+ phenotype, characteristic of CSCs, while increasing the cell surface abundance of integrin subunits β1/CD29 and α6/CD49f that represent putative mammary gland stem cell biomarkers. uPAR over-expression increased mammosphere formation in vitro and tumor formation in an immunocompromized severe combined immunodeficient (SCID) mouse model of orthotopic breast cancer. Hypoxic conditions that are known to induce EMT in MDA-MB-468 cells also increased cell surface β1/CD29, mimicking the effects of uPAR over-expression. Antagonizing uPAR effector signaling pathways reversed the increase in cell surface integrin expression. Whereas uPAR over-expression did not induce EMT in MCF-7 breast cancer cells, CSC-like properties were nevertheless still induced along with an increase in tumor initiation and growth in the orthotopic setting in SCID mice. Notably, in MCF-7 cell mammospheres, which display a well-defined acinus-like structure with polarized expression of E-cadherin and β1-integrin, cell

collapse into the central cavity was decreased by uPAR overexpression, suggesting that uPAR signaling may stabilize epithelial morphology. In summary, our findings show that uPAR signaling can induce CSC-like properties in breast cancer cells, either concomitantly with or separately from EMT.

INTRODUCTION

The epithelium of the normal mammary gland includes basal and luminal cells. Stem cells are present principally within the basal layer and responsible for normal mammary gland development and regeneration [54]. Normal mammary gland stem cells (MGSC) express high levels of the integrins β1/CD29 and α6/CD49f [54, 62, 151], which serve not only as MGSC markers but may also play a functional role in stem cell behavior, reflecting their activity in cell adhesion and cell signaling [160-161]. According to the cancer stem cell (CSC) hypothesis, a subset of tumor cells may be responsible for development and progression of leukemia, lymphoma, and solid malignancies [63, 162-163]. CSCs show the capacity for self-renewal and the ability to generate daughter cells, which differentiate into the various morphologies and phenotypes observed in the mature cancer. How to best recognize a CSC within a complex tumor cell population remains a topic of intense investigation; however, in breast cancer, CSCs are frequently identified by a CD44high/CD24low phenotype using flow cytometry [59]. Integrin subunits, which are expressed in normal MGSCs, may also be important in breast CSCs [164-165].

Considerable evidence has emerged, suggesting that the cell signaling and transcription regulatory pathways that induce epithelial-mesenchymal transition (EMT) in carcinoma cells overlap with those activated in CSCs [149, 166]. The urokinase-type plasminogen activator (uPA) receptor (uPAR) induces EMT in cancer cells by activating diverse cell signaling pathways, including the Ras–extracellular signal-regulated kinase (ERK) pathway, the phosphoinositide 3-kinase (PI3K)–Akt pathway, and Rac1 [99, 148]. Because uPAR expression is increased in hypoxia, uPAR-induced EMT may be important in large, poorly vascularized tumors. uPAR-induced EMT is reversible. Strategies that have been successfully used to reverse uPAR-induced cancer cell EMT include reoxygenation, blocking uPA binding to uPAR, and targeting cell signaling pathways downstream of uPAR [148].

uPAR is a glycosyl phosphatidylinositiol-anchored receptor [101]. Its activity in cell signaling requires integrin coreceptors, such as α4β1 and α5β1 [167-169]. Receptor tyrosine kinases, such as the epidermal growth factor receptor, may also be engaged in uPAR-dependent cell signaling [143, 170]. It is well known that many cancers show an increased propensity for metastasis in humans when uPAR expression is high [113]. This may be explained by the effects of uPAR on cell signaling, its ability to promote activation of proteases near the leading edge of cell migration, the function of uPAR as a vitronectin adhesion receptor, or its more recently described role in EMT [101, 171-173]. Although the ability of uPAR to promote protease activation near the cell surface

has been studied most exhaustively, at least in mice, uPAR promotes cancer progression by protease-independent mechanisms [132].

In this study, we show that breast cancer cells acquire robust CSC-like properties when uPAR is over-expressed and uPAR-dependent cell signaling is activated. In MDA-MB-468 cells, acquisition of CSC-like properties is associated with EMT; however, in MCF-7 cells, CSC-like properties develop independently of EMT. The difference may reflect availability of uPA, which is important to activate some cell signaling pathways downstream of uPAR, and other receptor systems, which control the physiology of these cells.

MATERIALS AND METHODS

Reagents

Anti-CD24-FITC (ML5), anti-CD44-phycoerythrin (PE; G44-26), and isotype- matched IgGs were from BD Biosciences. Anti-CD29-FITC (HMβ1-1) and anti-CD49f-PE (GoH3) were from Biolegend. Rat monoclonal antibody that recognizes β1 integrin (AIIB2) was from the Developmental Studies Hybridoma Bank (University of Iowa). Monoclonal antibody that detects activated β1 integrin (HUTS4) was from Millipore. Monoclonal human uPAR-specific antibody (ATN658) and polyclonal human uPAR-specific antisera were provided by Dr. Andrew Mazar. E-cadherin–specific monoclonal antibody HECD-1 and polyclonal α6 integrinspecific antibody were from Abcam. Monoclonal antibody

that recognizes vimentin was from Sigma-Aldrich. Secondary antibodies conjugated with Alexa Fluor 488, Alexa Fluor 569, and Alexa Fluor 647 were from Invitrogen. PI3K inhibitor (LY294002) and mitogen-activated protein kinase (MAPK)/ERK kinase 1 (MEK1) inhibitor (PD098059) were from EMD Biosciences. B27 serum-free supplement was from Invitrogen. Basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) were from R&D Systems. Quantitative PCR (qPCR) reagents, including primers and probes for human uPAR and hypoxanthine phosphoribosyltransferase-1 (HPRT-1), were from Applied Biosystems.Primer sets for β1 integrin were synthesized by Integrated DNA Technologies.

Cell culture

Human uPAR over-expressing MDA-MB-468 cells (468/uPAR) and empty vector–transfected MDA-MB-468 cells (468/EV) are previously described [99]. MCF-7 cells that overexpress human uPAR (MCF-7/uPAR) were prepared by transfecting cells with pcDNA-uPAR using Lipofectamine 2000. After selection for 14 days with 500 μg/mL hygromycin, single cell clones were established and screened for uPAR expression by immunoblot analysis. The cells were maintained in DMEM (HyClone) supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin.

Flow cytometry

Cells (3×10^6) were plated in 10-cm dishes and, in some studies, treated with LY294002 (10 μmol/L) or PD098059 (50 μmol/L) in serum-free medium for 18 hours. Cells were resuspended in fluorescence-activated cell sorting buffer [2% bovine serum albumin (BSA) in PBS] and incubated with anti-CD29-FITC (0.5 μg/106 cells), anti-CD49f-PE (10 μL/test), anti-CD24-FITC (10 μL/test), anti-CD44-PE (10 μL/test), or isotype-matched IgG for 30 minutes on ice. To detect uPAR or activated β1 integrin, cells were incubated with primary antibodies (1 μg/10^6 cells) for 1 hour. Primary antibodies were detected using secondary antibodies conjugated with Alexa Fluor 488 or Alexa Fluor 647. Results were analyzed using a FACSCanto II (BD Biosciences) and FlowJo software.

Biotinylation of cell surface proteins

Cells in monolayer culture (1.5×10^6) were washed three times with ice-cold PBS and then treated with EZ-link sulfo-NHS-SS-biotin (1 mg/mL, Pierce) for 15 minutes on ice. Biotinylation reactions were terminated with 100 mmol/L glycine in PBS. After washing with PBS, cell extracts were prepared in radioimmunoprecipitation assay (RIPA) buffer [20 mmol/L sodium phosphate, 150 mmol/L NaCl (pH 7.4), 1% NP40, 0.1% SDS, and 0.5% deoxycholic acid] with protease inhibitor cocktail (Roche). Biotinylated membrane proteins were precipitated with streptavidin-sepharose (GE Healthcare).Proteins were eluted with SDS sample buffer, resolved by SDS-PAGE, electrotransferred to

polyvinylidene difluoride (PVDF) membranes, and probed with primary antibodies.

Immunoblot analysis

Cell extracts were prepared in RIPA buffer containing complete protease inhibitor cocktail. Protein concentrations were determined by bicinchoninic acid assay (Sigma-Aldrich). Equal amounts of cell extracts were resolved by SDS-PAGE, electrotransferred to PVDF membranes, and probed with primary antibodies.

Mammosphere assay

Cells were trypsinized and mechanically disrupted to obtain single-cell suspensions. The cells were then cultured in mammosphere medium (DMEM/F12, B27, 20 ng/mL bFGF, 20 ng/mL EGF, 100 units/mL penicillin, and 100 μg/mL streptomycin) in ultralow attachment 24-well plates (Corning) at a density of 2,000, 500, or 50 cells/500 μL for 7 to 10 days. Mammospheres were imaged and counted under phasecontrast microscopy. Only mammospheres exceeding 100 μm in diameter were counted. As a second assay to assess mammosphere formation, cultures were established in ultralow attachment 96-well plates at a density of one cell per well. Mammosphere formation was assessed after culturing for 7 to 10 days. This second method assured that mammospheres were formed by a single cell.

Tumor formation assay

Animal experimentation was done in accordance with protocols approved by the University of California San Diego Animal Care Program. Anesthetized 8-week-old C.B-17/lcrCrl-scid-BR mice (Charles River Laboratories) were inoculated in the fourth and contralateral fourth mammary fat pads with 50, 100, or 1,000 468/EV or 468/uPAR cells suspended in 50 μL of Matrigel (Sigma). MCF-7/EV or MCF-7/uPAR cells were also injected into mammary fat pads (1×10^6 cells per injection). Primary tumor growth was monitored every 2 to 3 days. One month after injection of MDA-MB-468 cells or 10 weeks after injection of MCF-7 cells, mice were euthanized and the mammary fat pads were visually inspected for tumor. Tumor formation was confirmed by histologic analysis. Data processing and statistical analysis were performed using GraphPad Prism (GraphPad Software, Inc.) and Microsoft Excel (Microsoft Corporation).

Histology and immunohistochemistry

Formalin-fixed tissue was paraffin-embedded. Serial 4-μm sections were stained with H&E. Immunohistochemistry was performed using the Vantana Discovery XT System (Vantana). Sections were pretreated with citric acid buffer and then incubated with polyclonal antibody specific for human uPAR (1:200) followed by peroxidase-conjugated secondary antibody. Peroxidase activity was imaged using 3,3′-diaminobenzidine. Slides were examined using a Leica DM2500 light microscope. Images were acquired using a Leica DFC420 digital camera and Leica Application Suite software.

qPCR analysis

Total RNA was isolated using the RNeasy kit (Qiagen). cDNA was synthesized using the iScript kit (Bio-Rad). qPCR was performed using an Applied Biosystems instrument and a one-step program: 95°C, 10 minutes; 95°C, 30 seconds; and 60°C, 1 minutes for 40 cycles. HPRT-1 gene expression was measured as a normalizer. Results were analyzed by the relative quantity (ΔΔCt) method. All experiments were performed in triplicate with internal duplicate determinations.

Cell adhesion assays

Medium-binding 96-well plates (Corning) were coated with fibronectin (5 μg/mL) or type I collagen (5 μg/mL) for 18 hours at 4°C and then blocked with 1% (w/v) BSA for 2 hours. Cells were washed and resuspended in serum-free DMEM at a concentration of 0.5×10^6 cells/mL and allowed to adhere for 15 minutes at 37°C. Adherent cells were fixed with 4% formaldehyde and stained with crystal violet. The dye was eluted with 10% acidic acid, and absorbance at 570 nm was determined. Each value represents the mean of 18 replicates in three separate experiments.

Immunofluorescence microscopy

Cells were plated on glass coverslips, fixed in 4% formaldehyde, permeabilized in 0.2% Triton X-100, and incubated with antibodies specific for E-cadherin (1:100) or vimentin (1:400) followed by secondary antibodies

conjugated with Alexa Fluor 488 or Alexa Fluor 594. Preparations were mounted on slides using ProLong Gold with 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen) and examined using a Leica DMIRE2 fluorescence microscope. Images were obtained using a 63× oil immersion objective and a Hamamatsu digital camera with SimplePCI software.

Mammospheres were allowed to form in ultralow attachment T25 flasks. The mammospheres were collected by centrifugation at 200 × g, suspended, fixed in 4% formaldehyde, permeabilized with 0.2% Triton X-100, and incubated with antibodies specific for β1 integrin (AIIB2, 1 μg/100 μL) and E-cadherin. Secondary antibodies were conjugated with Alexa Fluor 488 or Alexa Fluor 594. Control preparations were treated equivalently, except for the omission of primary antibody. Stained mammospheres were mounted on chamber slides using ProLong Gold (Invitrogen) and examined using a spectral confocal microscope (C1-si, Nikon).

RESULTS

uPAR over-expressing MDA-MB-468 cells acquire CSC-like properties

Hypoxia increases uPAR expression, activates uPAR-dependent cell signaling, and induces EMT in MDA-MB-468 breast cancer cells [99]. Equivalent changes in cell phenotype are observed when uPAR is overexpressed. Because others have suggested that the cell signaling pathways responsible for EMT in cancer cells overlap with those activated in CSCs [149], we tested whether uPAR

overexpression in MDA-MB-468 cells induces CSC-like properties. Figure 1-1A shows that the abundance of cell surface uPAR was substantially increased in uPAR-overexpressing (468/uPAR) cells. Although the level of CD44 was already high in control (468/EV) cells, a subpopulation of the cells shifted to a higher level of CD44 expression when uPAR was over-expressed. CD24 was significantly decreased in the majority of the 468/uPAR cells (Fig. 1-1B).

Next, we examined whether uPAR over-expression alters the abundance of cell surface integrins that have been described as MGSC biomarkers. Total β1/CD29 was substantially increased at the cell surface in 468/uPAR cells, as was activated β1, as determined with antibody HUTS4. α6/CD49f was also increased, although the increase observed by flow cytometry was less pronounced than that observed for β1/CD29 (Fig. 1-1C). To confirm that cell surface α6/CD49f is regulated in 468/uPAR cells, we biotinylated cell surface proteins for isolation by affinity precipitation. Figure 1-1D shows that cell surface α6/CD49f was increased in 468/uPAR cells, whereas the total level of α6/CD49f was unchanged.

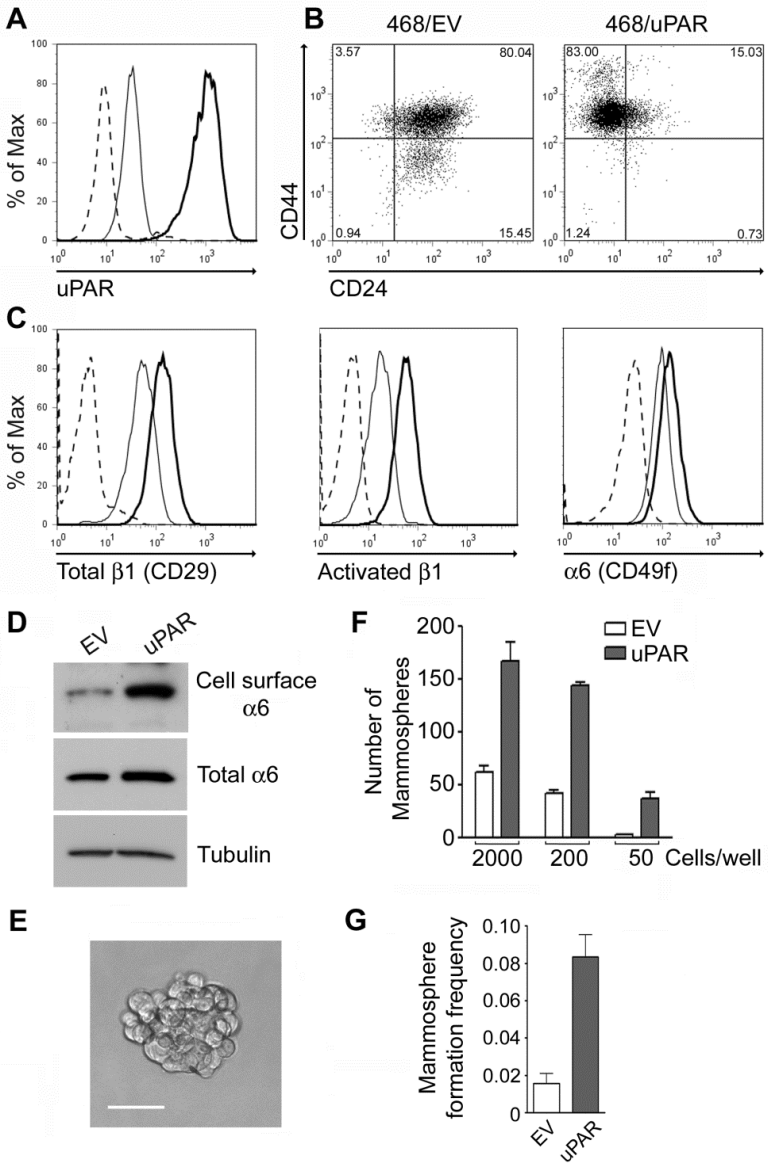

Figure 1-1. uPAR over-expression in MDA-MB-468 cells induces CSC-like properties. *A*, flow cytometry to detect uPAR in 468/uPAR (heavy curve) and 468/EV (light curve) cells. *B*, CD24 and CD44 were determined in 468/uPAR and 468/EV cells. *C*, total β1/CD29, activated β1, and α6/CD49f were determined in 468/uPAR (heavy curve) and 468/EV (light curve) cells. The isotype-matched IgG is shown with a dashed line. *D*, α6 integrin and tubulin in affinity precipitates and whole-cell extracts from 468/EV and 468/uPAR cells were determined. *E*, representative mammosphere formed by 468/uPAR cells. Bar, 50 μm. *F*, 468/uPAR and 468/EV cells were introduced into suspension culture at the indicated densities. Mammospheres were counted. The bar graph shows one of three independent experiments, which generated similar results (mean ± SEM). *G*, single-cell cultures were maintained for 7 to 10 d before determining the frequency of mammosphere formation (n = 4, mean ± SEM).

468/uPAR and 468/EV cells formed mammospheres in suspension culture, providing evidence that cells with self renewal capacity were present (Fig. 1-1E). The mammospheres did not show a well-organized structure compared with those formed by MCF-7 cells (see below). Nevertheless, the number of mammospheres formed by 468/uPAR cells was significantly increased ($P < 0.01$) compared with the number formed by 468/EV cells (Fig. 1-1F). The ability of uPAR over-expression to promote mammosphere formation was shown in different studies in which suspension cultures were established at varied density. To confirm that mammospheres formed from a single cell, single-cell cultures of 468/uPAR cells and 468/EV cells were established in ultralow attachment 96-well plates. Figure 1G shows that the percentage of cultures that generated a mammosphere was significantly increased when uPAR was over-expressed ($n = 4$, $P < 0.01$).

To test whether uPAR over-expression promotes tumor initiation by MDA-MB-468 cells in vivo, we performed limiting dilution assays, injecting 1,000, 100, or 50 cells into mammary fat pads in severe combined immunodeficient (SCID) mice. As shown in Table 1-1, when 1,000 or 100 cells were injected, 468/uPAR and 468/EV cells formed tumors equivalently. However, when 50 cells were injected, 468/uPAR cells formed tumors at a significantly increased rate ($P < 0.05$, Chi square test; $p=0.13$, Fisher exact test). The results of our flow cytometry experiments, mammosphere assays, and limiting dilution studies support a model in which uPAR over-expression engenders CSC-like properties in MDA-MB-468 cells.

Table 1-1. Frequency of tumor formation by 468/EV and 468/uPAR cells when injected into mammary fat pads in limiting dilution.

Number of Cells/ Injection	468/EV	468/uPAR	*P* value
1000	4/4	4/4	
100	4/4	4/4	
50	2/8	6/8	$p<0.05$

Statistical analysis = χ^2

uPAR-initiated cell signaling regulates the subcellular distribution of β1 integrin

Because MDA-MB-468 cells express uPA endogenously, overexpression of uPAR results in activation of PI3K and ERK/MAPK [99]. uPAR also physically associates with integrins in the plasma membrane [167]. Either of these nonmutually exclusive mechanisms could be responsible for the increase in cell surface β1 integrin observed in 468/uPAR cells. To test whether cell signaling is involved, 468/uPAR cells were treated with the MEK inhibitor, PD098059 (50 μmol/L), or with the PI3K inhibitor, LY294002 (10 μmol/L). In three separate experiments, both reagents significantly decreased the level of cell surface β1/CD29, α6/CD49f, and activated β1/CD29, as determined with antibody HUTS4 (Fig. 1-2).

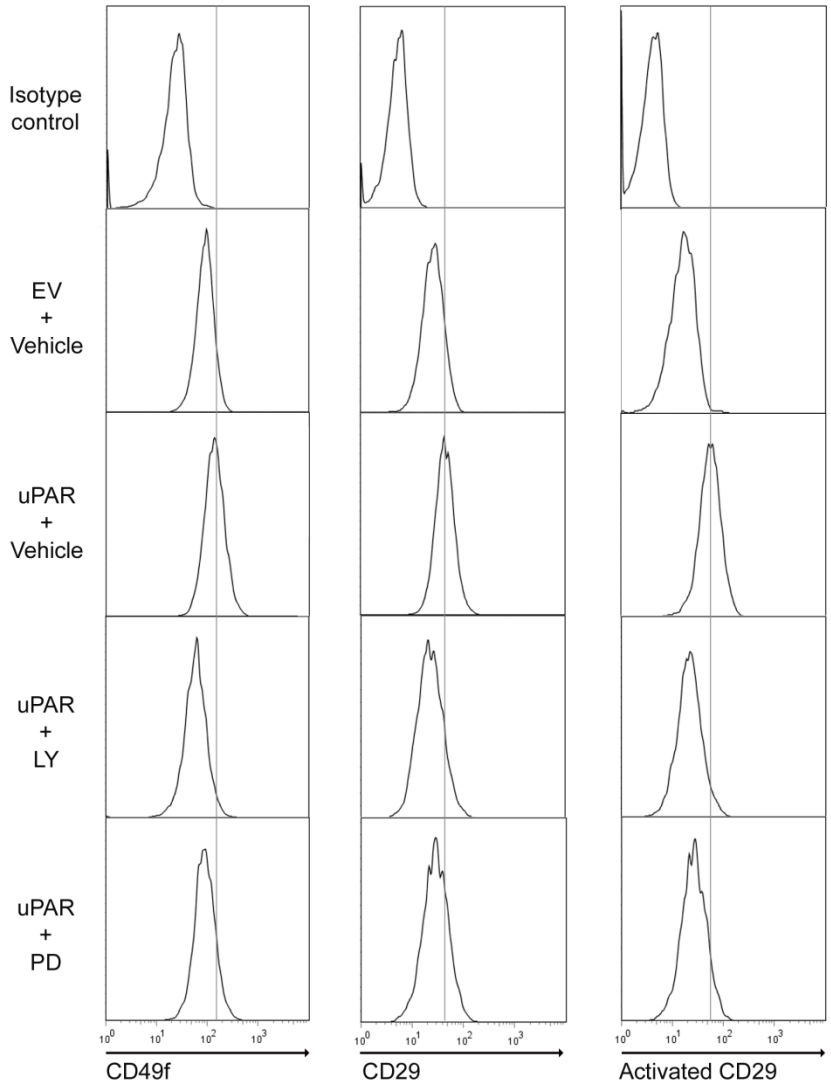

Figure 1-2. Cell surface integrin expression in 468/uPAR cells is inhibited by antagonizing PI3K or MEK. 468/EV and 468/uPAR cells were treated with LY294002 (10 μmol/L), PD098059 (50 μmol/L), or vehicle for 18 h. β1/CD29, activated β1, and α6/CD49f were determined by flow cytometry. The results are representative of three independent experiments.

As a second model system to study cells in which increased uPAR expression drives EMT, we exposed MDA-MB-468 cells to 1.0% O2 for 48 hours. Figure 1-3A shows that uPAR mRNA expression increased 3-fold, confirming our previous results [99]. β1 integrin subunit mRNA was unchanged. The cell surface abundance of β1 integrin was examined by biotinylation and affinity precipitation. Figure 1-3B shows that cell surface β1 integrin was substantially increased in MDA-MB-468 cells that were exposed to hypoxia. β1 integrin in whole-cell extracts distributed into two bands: the mature form found at the cell surface, which is more slowly migrating, and the more rapidly migrating immature form. Although the total level of β1 integrin (sum of two bands) did not change significantly in hypoxia, the distribution of β1 integrin between the two bands shifted so that more mature β1 integrin was present. This result confirms our affinity precipitation data, showing that hypoxia increases the abundance of cell surface β1 integrin.

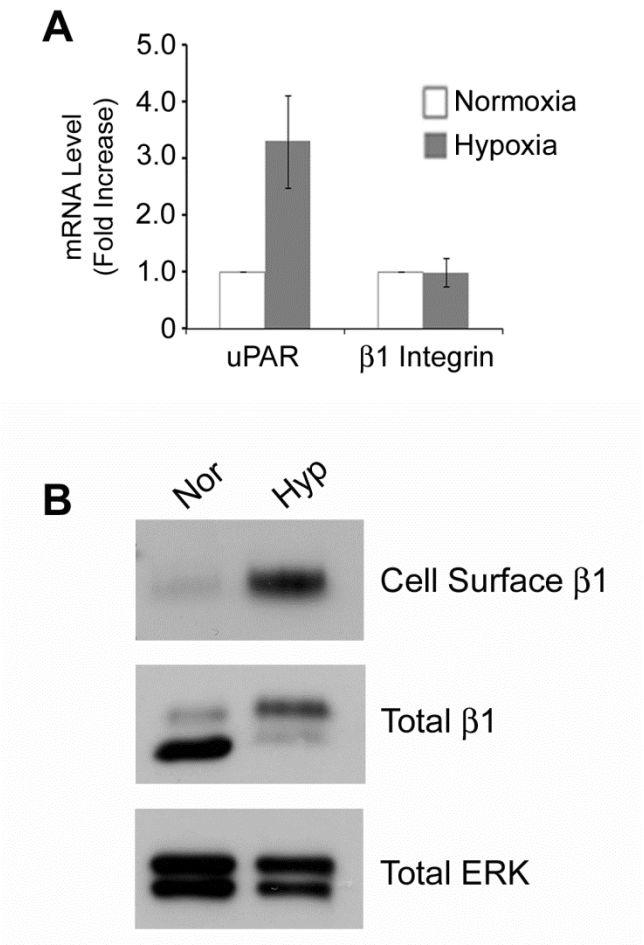

Figure 1-3. Hypoxia increases the abundance of cell surface β1 integrin in MDA-MB-468 cells. *A*, MDA-MB-468 cells were cultured in 21% O2 (open columns) or 1.0% O2 (closed columns) for 48 h. uPAR and β1 integrin mRNA were determined by qPCR and standardized against the levels in cells cultured in 21% O2 (mean ± SEM; n = 3). *B*, MDA-MB-468 cells were cultured in 21% or 1.0% O2 for 48 h. Affinity precipitates and whole-cell extracts were analyzed to detect β1 integrin and, as a control, total ERK/MAPK.

uPAR overexpression induces CSC-like properties independently of EMT in MCF-7 breast cancer cells

uPAR over-expression does not induce EMT uniformly in all cancer cells [99]. The degree to which the cancer cells express uPA endogenously and the function of other receptors that regulate cancer cell physiology may be involved. MCF-7 cells are estrogen receptor–positive breast cancer cells that express low levels of uPAR and undetectable uPA [172]. We over-expressed uPAR in MCF-7 cells and isolated two single-cell clones (C4 and C5). uPAR over-expression was confirmed by flow cytometry (Fig. 1-4A). Neither of the uPAR-over-expressing MCF-7 cell clones showed signs of EMT. Figure 1-4B shows the results of experiments performed with C5 cells, which expressed the highest level of cell surface uPAR. Compared with cells that were transfected with empty vector (MCF-7/EV), C5 MCF-7/uPAR cells showed an unchanged level of E-cadherin and no vimentin. By contrast, 468/uPAR cells showed a substantial decrease in E-cadherin and high levels of vimentin, as previously shown [99]. These results were confirmed by immunoflorescence microscopy (Fig. 1-4C). Note the robust vimentin immunopositivity in uPAR-overexpressing MDA-MB-468 cells. uPAR-overexpressing MCF-7 cells retained E-cadherin at cell-cell junctions, whereas vimentin was not detected.

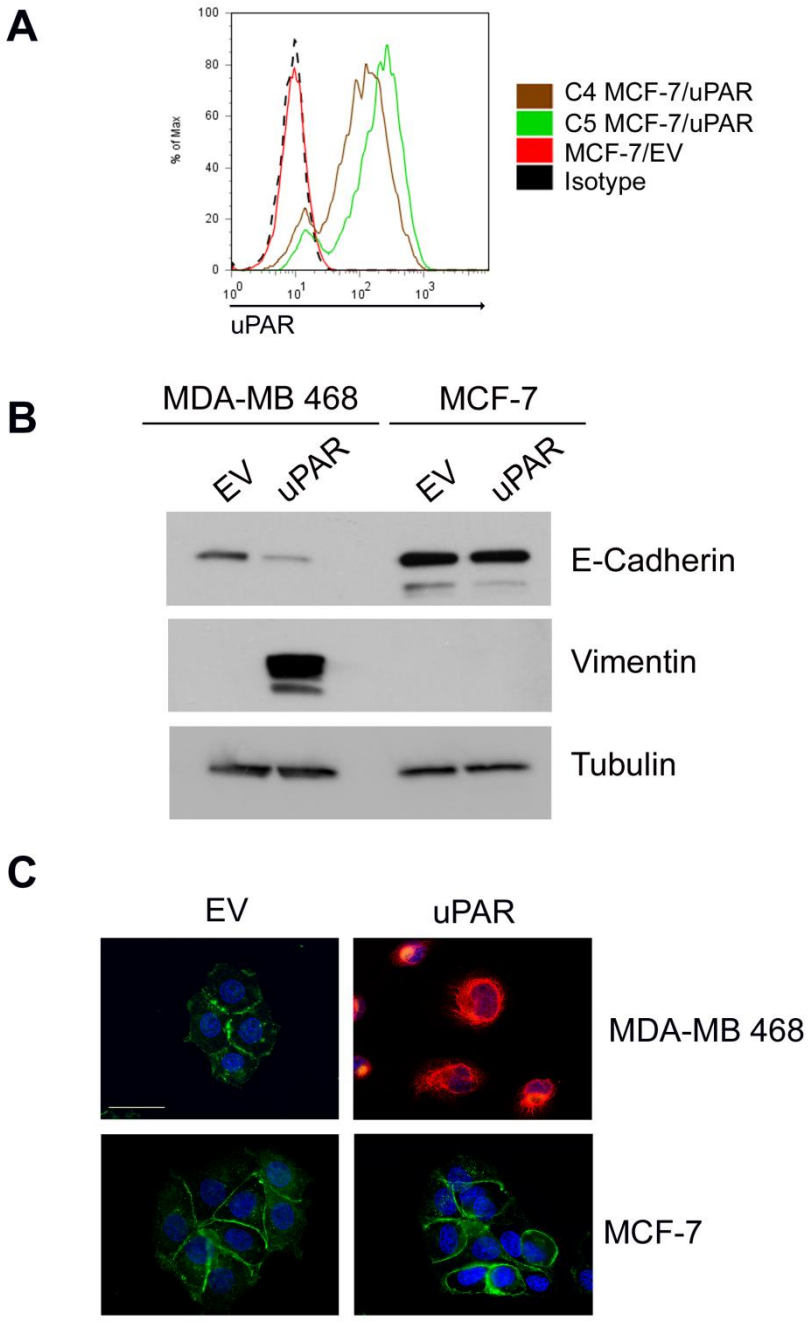

Figure 1-4. uPAR over-expression does not induce EMT in MCF-7 cells. *A*, flow cytometry to detect uPAR in C4 MCF-7/uPAR, C5 MCF-7/uPAR, and MCF-7/EV cells. *B*, cell extracts from 468/EV, 468/uPAR, MCF-7/EV, and C5 MCF-7/uPAR cells were subjected to immunoblot analysis to detect E-cadherin, vimentin, and tubulin. *C*, 468/EV, 468/uPAR, MCF-7/EV, and MCF-7/uPAR cells were immunostained to detect E-cadherin (green), vimentin (red), and DAPI (blue). The representative photomicrographs show all channels imaged simultaneously. Bar, 30 μm.

To determine whether uPAR overexpression induces CSC-like properties in MCF-7 cells, C4 and C5 MCF-7/uPAR cells were analyzed by flow cytometry (Fig. 1-5A). CD24 expression was decreased in the majority of the C4 and C5 MCF-7/uPAR cells compared with MCF-7/EV cells. Again, as is frequently the case in cancer cell lines, CD44 was already expressed at high levels in the control cells; uPAR overexpression slightly decreased CD44 in the C5 clone. However, both β1/CD29 and α6/CD49f were increased in the C4 and C5 MCF-7/uPAR cells.

Because the increase in cell surface integrin expression induced by uPAR overexpression in MCF-7 cells was less robust than that observed in 468/uPAR cells, we performed cell adhesion assays to a test whether integrin function was regulated. Adhesion of C5 MCF-7/uPAR cells and control MCF-7/EV cells to the β1 integrin ligands, type 1 collagen and fibronectin, was compared. Figure 1-5B shows that adhesion of C5 cells to both substrata was significantly increased ($P < 0.01$).

Mammosphere assays were performed to compare the self renewal activity of C5 MCF-7/uPAR and MCF-7/EV cells. Mammospheres formed by MCF-7 cells showed substantial differentiation into spheroid-like structures (Fig. 1-5C). Regularly contoured external borders and partially hollow central cavities were observed. As shown in Fig. 1-5D, uPAR overexpression in MCF-7 cells increased the frequency of mammosphere formation 4-fold to 5-fold irrespective of the number of cells that were introduced into each well ($P < 0.05$ at each density). In experiments with single-cell cultures, uPAR over-expression increased

mammosphere formation 3-fold ($P < 0.05$), confirming the results shown in Fig. 1-5D.

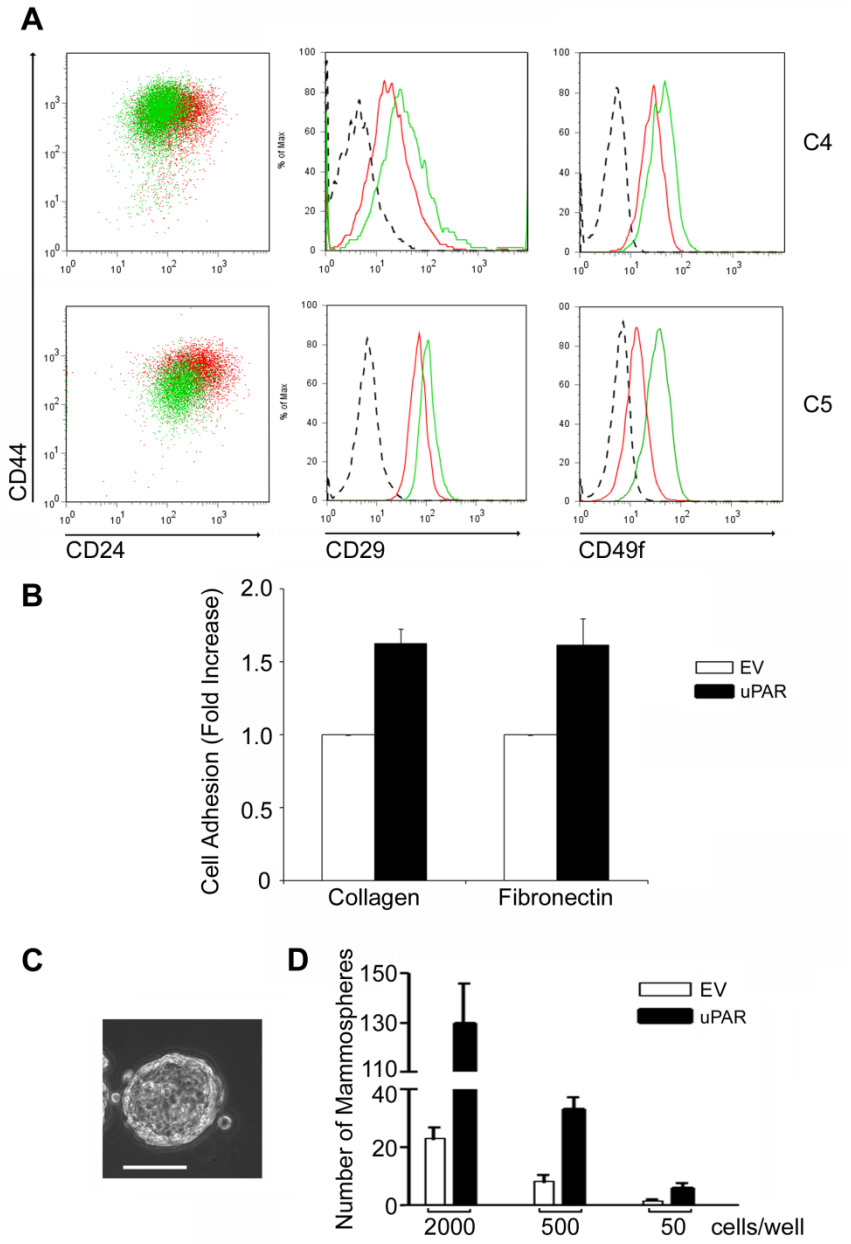

Figure 1-5. uPAR over-expression induces CSC-like properties in MCF-7 cells. *A*, CD24, CD44, β1/CD29, and α6/CD49f were determined in MCF-7/EV cells, C4 MCF-7/uPAR cells, and C5 MCF-7/uPAR cells by flow cytometry (red, MCF-7/EV; green, MCF-7/uPAR; dashed line, isotype control). *B*, MCF-7/EV and C5 MCF-7/uPAR cells were seeded in collagen- or fibronectin-coated plates. Cell adhesion is expressed as the fold increase relative to that observed with EV cells (mean ± SEM, n = 3). *C*, representative mammosphere formed by C5 MCF-7/uPAR cells. Bar, 100 μm. *D*, mammosphere formation by C5 MCF-7/uPAR and MCF-7/EV cells was determined after seeding wells with the indicated number of cells. The bar graph represents one of three independent experiments, which generated similar results (n = 4 in each study, mean ± SEM).

Nonmalignant mammary epithelial cells, such as MCF10A cells, form polarized, acinus-like spheroids in three-dimensional culture, recapitulating the grandular structure of normal mammary glands [174]. In the acinus-like structures, β1 integrin is localized basally and E-cadherin to cell junctions. Figure 6A shows that, in mammospheres formed by MCF-7 cells, β1 integrin polarized principally to the inner surface of the central cavity. Some β1 integrin was also detected at cell junctions, reminiscent of normal mammary gland structure in vivo [151, 175]. E-cadherin localized to cell-cell junctions and also, to some extent, to the inner surface of the central cavities, co-localizing with β1 integrin. In the control MCF-7/EV cells, the inner cavities of most mammospheres were partially collapsed by cells growing inward. By contrast, in mammospheres formed by uPAR over-expressing MCF-7 cells, collapse of cells into the central cavity was much less frequent. As a result, these mammospheres more rigorously approximated a normal mammary gland acinus-like structure. The distribution of β1 integrin and E-cadherin in mammospheres formed by C5 MCF-7/uPAR cells was similar to that observed in MCF-7/EV cells. These studies confirm that, in MCF-7 cells, CSC-like properties are induced by uPAR overexpression independently of signs of EMT.

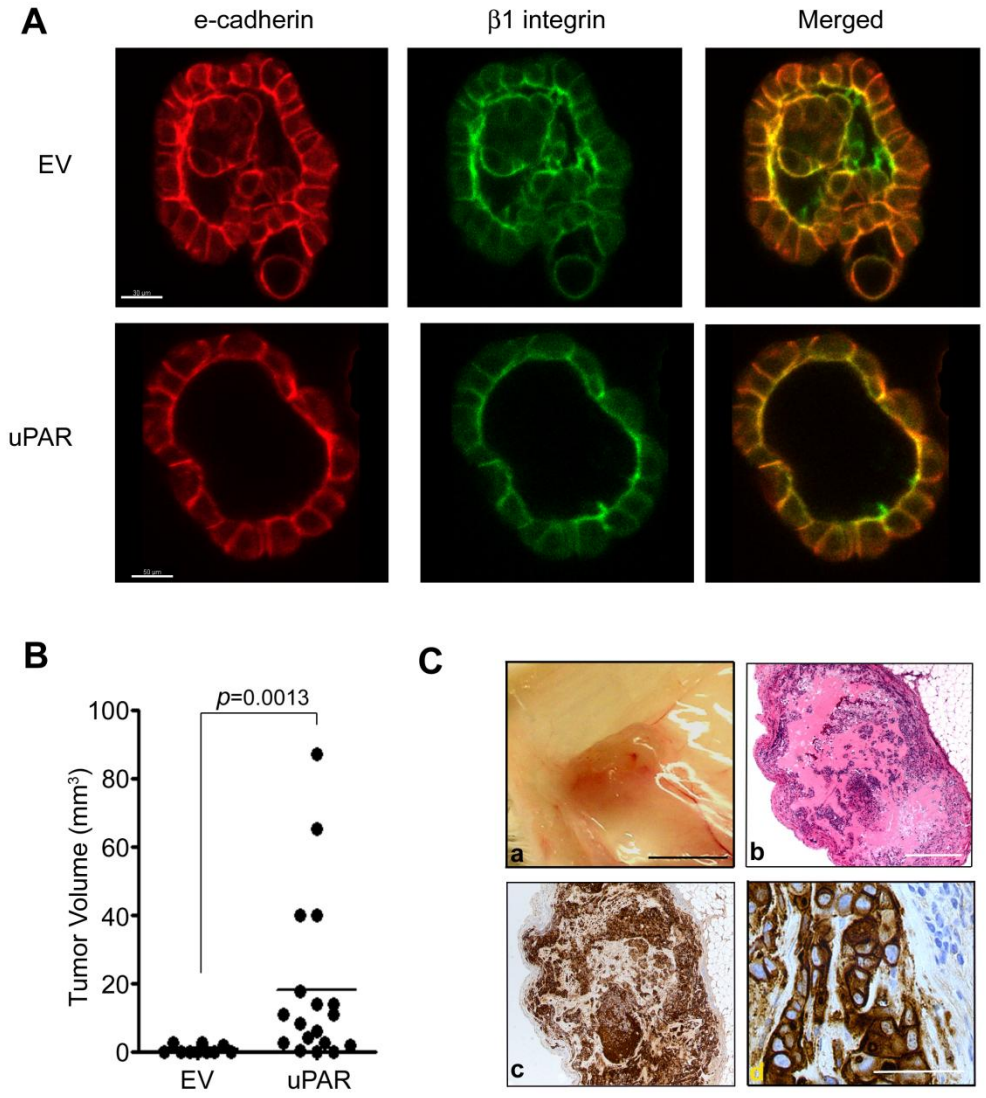

Figure 1-6. MCF-7/uPAR cells form well-differentiated mammospheres and initiate tumor formation at an increased frequency *in vivo*. *A*, mammospheres formed by MCF-7/EV and C5 MCF-7/uPAR cells were immunostained to detect E-cadherin (red) and β1 integrin (green). Single confocal optical sections (1 μm) are shown. Bar, 30 μm for MCF-7/EV cells and 50 μm for C5 MCF-7/uPAR cells. *B*, SCID mice were injected with C5 MCF-7/uPAR or MCF-7/EV cells. Tumor volume was determined after surgical resection (median, P = 0.0013, Mann-Whitney rank sum test). *C*, images of tumors formed by MCF-7/uPAR cells include (a) the gross tumor (bar, 0.5 mm), (b) microscopic section of a representative tumor (bar, 500 μm), (c) immunohistochemistry to detect human uPAR (magnification, 5×), and (d) higher magnification image of immunohistochemistry to detect human uPAR. Notice blue-counterstained nuclei of nonmalignant mouse cells infiltrating the tumor in the top right-hand corner (magnification, 40×; bar, 50 μm).

uPAR overexpression in MCF-7 cells promotes tumor initiation in vivo

In the absence of estrogen supplementation, orthotopic tumor formation by MCF-7 cells in SCID mice is limited even when a large number of cells is injected [176]. As shown in Table 1-2, uPAR over-expression significantly increased the frequency of tumor formation from equivalently sized inoculums ($P < 0.05$, Chi square test; $p=0.06$, Fisher exact test). Tumors formed by uPAR-over-expressing cells grew much larger in the time frame of the study (Fig. 6B); the mean volume of the tumor was increased >20-fold. The malignant cells within the tumors remained robustly uPAR-immunopositive in vivo, as determined by immunehistochemistry (Fig. 6C). Nonmalignant mouse cells that infiltrated the tumor were immunonegative for uPAR, as determined with our human uPAR-specific antibody.

Table 1-2. Frequency of tumor formation by MCF7/EV and MCF7/uPAR cells when injected into mammary fat pads.

Cell type	Frequency	*P* value
MCF7/EV	5/10	0.0228
MCF7/uPAR	16/18	

Statistical analysis = χ^2

DISCUSSION

In diverse human malignancies, high levels of uPAR expression are associated with an increased propensity for cancer progression and metastasis [113, 177]. Studies analyzing uPAR at the cellular and molecular level have revealed multiple candidate mechanisms. The ability of uPAR to accelerate a number of extracellular reactions, including conversion of the zymogen form of uPA into the active two-chain form (tcuPA; [178]) and plasminogen activation by tcuPA [179], promotes proteolysis and remodeling of the extracellular matrix (ECM). Because uPAR polarizes to the leading edge of cellular migration [180], increased ECM remodeling may facilitate cancer cell migration and invasion through tissue boundaries.

uPAR may also promote cancer progression by its effects on cell adhesion and cell signaling. The importance of this mechanism is supported by recent mouse model experiments [132]. In addition to uPA, uPAR functions as a receptor for the ECM protein vitronectin [173]. Binding of uPA or vitronectin to uPAR activates distinct cell signaling pathways, which collectively support cell migration and survival [132, 146, 157, 171-172, 181]. Understanding how uPAR-dependent cell signaling regulates cancer cell physiology is an important goal. In some cancer cell lines, increased uPAR expression and activation of uPAR-dependent cell signaling in hypoxia induce EMT [99]. Our evidence suggests that the full continuum of uPAR initiated cell signaling pathways, including those activated downstream of uPA and vitronectin, may be necessary for EMT [99,

148]. However, the factors that control whether uPAR induces EMT remain incompletely understood.

In this study, we showed that uPAR over-expression does not induce EMT in MCF-7 cells. In fact, the polarized epithelial morphology of these cells may be strengthened by uPAR, because collapse of cells into central cavities of mammospheres was decreased. Whether EMT occurs in human cancer in vivo and thus represents a relevant pathway affecting cancer progression remains unsettled [182]. Hypoxia induced EMT, under the control of uPAR, is reversible [148]. Thus, the fact that metastases in organs such as the lungs frequently show well-defined epithelial morphology does not preclude that EMT occurred as a step in the metastasis cascade.

In a cell type that undergoes uPAR-induced EMT (MDA-MB-468) and in a cell type that does not (MCF-7), uPAR overexpression engendered cells with biomarkers and properties of CSCs. In MDA-MB-468 cells, uPAR overexpression significantly increased the likelihood of tumor initiation by a small number of cancer cells in vivo. uPAR overexpression was associated with a CD44high/CD24low phenotype, increased cell surface β1/CD29 and α6/CD49f, and a significant increase in mammosphere formation frequency. Thus, in this cell type, the correlation between EMT and CSC-like properties was upheld. In MCF-7 cells, CSC-like properties associated with uPAR overexpression included decreased CD24 expression, an increase in the cell surface abundance of β1/CD29 and α6/CD49f and an increased frequency of mammosphere formation. Although the requirement for estrogen supplementation in vivo precluded typical serial

dilution studies with MCF-7 cells, we did show that uPAR over-expression significantly increases the frequency of tumor initiation and tumor growth in SCID mice. We previously showed that uPAR-induced EMT is reversible [148]. Because the signaling pathways downstream of uPAR that are responsible for EMT and CSC-like properties may be at least partially overlapping, it is possible that uPAR-induced CSC-like properties may also be dynamic and reversible.

MDA-MB-468 cell mammospheres showed poorly defined structure consistent with the loss of epithelial morphology and EMT. By contrast, mammospheres formed by MCF-7 cells showed a highly ordered and polarized structure with β1 integrin localized principally to a single surface and E-cadherin at cell-cell junctions. Interestingly, the β1 integrin subunit localized mainly to the internal surface of the mammosphere, opposite the location in a normal mammary acinus or duct. MCF-7 cells are frequently thought to have arisen from luminal epithelium [183]. The increase in expression of cell surface β1 integrin, which accompanies uPAR overexpression, may indicate a transformation to a more basal cell phenotype.

In response to uPAR overexpression or hypoxia, β1 integrin in MDA-MB-468 cells relocated to the cell surface from intracellular pools. It is well established that uPAR associates with integrins in the plasma membrane [167-168, 184-186] and that integrins function as co-receptors in uPAR-initiated cell signaling [167, 171, 186-187]. Receptors other than uPAR, which activate similar signal transduction pathways, may also cause relocation of β1 integrin to the cell

surface [188]. This process may provide a positive feedback loop by which uPAR-initiated cell signaling is strengthened.

When the cell surface abundance of uPAR was increased, the level of activated β1 integrin was increased, as determined with antibody HUTS4. Integrins exist in conformations that favor ligand binding or not; fluctuation between these conformational states may be controlled by cell signaling pathways [189], many of which are controlled downstream of uPAR [171, 190]. Thus, it is not surprising that HUTS4 reactivity in uPAR-over-expressing MDA-MB-468 cells was decreased by inhibitors of PI3K and ERK/MAPK activation. However, these results do not rule out the possibility that uPAR also regulates integrin conformation by cis-interactions within the plasma membrane. Regulation of integrin activation by uPAR represents another possible positive feedback pathway by which uPAR and integrins may cooperate to control cancer cell physiology.

For the most part, uPAR has been studied as a gene product that controls invasion and metastasis of existing cancers. The function of uPAR as a gene product involved in generating CSC-like properties refocuses attention on this receptor to earlier steps in cancer development. In normal human adults, uPAR is sparsely expressed in tissues and organs [113]. Thus, uPAR could serve as a target for cancer diagnostics development or therapeutics aimed at early stages of cancer.

Chapter 1, in full, is adaptation from material that appears in *Cancer Research* 70(21):8948-58. The dissertation author was co-first author together

with Dr. Minji Jo. In addition, the paper was authored by Drue Webb, Shinako Takimoto, Dr. Kontstantine Stoletov, Dr. Richard Klemke, and Dr. Steven Gonias.

CHAPTER 2

ABSTRACT

Binding of urokinase-type plasminogen activator (uPA) to its receptor, uPAR, in estrogen receptor-α (ERα) expressing breast cancer cells, transiently activates ERK downstream of FAK, Src family kinases, and H-Ras. Herein, we show that when uPAR is over-expressed, in two separate ERα-positive breast cancer cell lines, ERK activation occurs autonomously of uPA and is sustained. Autonomous ERK activation by uPAR requires H-Ras and Rac1. A mutated form of uPAR, which does not bind vitronectin (uPAR-W32A), failed to induce autonomous ERK activation. Expression of human uPAR or mouse uPAR but not uPAR-W32A in MCF-7 cells provided a selection advantage when these cells were deprived of estrogen in cell culture for two weeks. Similarly, MCF-7 cells that express mouse uPAR formed xenografts in SCID mice that survived and increased in volume in the absence of estrogen supplementation, probably reflecting the pro-survival activity of phospho-ERK. Autonomous uPAR signaling to ERK was sensitive to the EGFR tyrosine kinase inhibitors, Erlotinib and Gefitinib. The transition in uPAR signaling from uPA-dependent and transient to autonomous and sustained is reminiscent of the transformation in ErbB2/ HER2 signaling observed when this gene is amplified in breast cancer. uPAR over-expression may provide a pathway for escape of breast cancer cells from ERα-targeting therapeutics.

INTRODUCTION

Estrogen receptor-α (ERα) is expressed in up to 75% of all cases of adenocarcinoma of the breast [191-192]. These tumors are frequently treated with agents that inhibit ERα activation, down-regulate ERα, or reduce estrogen synthesis [14, 16]. Unfortunately, in many cases, tumor cells acquire molecular changes that allow resistance to anti-estrogens. The resulting cancers are frequently aggressive and rapidly progressing. Previously described receptors that may become activated and allow escape from ERα-targeting drugs include the EGF receptor (EGFR), ErbB2/HER2, and Insulin-like Growth Factor Receptor-1 (IGF1R) [192-195]. In addition to its function as a transcription factor, ERα activates Src family kinases (SFKs), ERK, and phosphatidylinositol 3-kinase (PI3K) [196]. Thus, it is reasonable to hypothesize that other receptors, which activate the same cell-signaling pathways, may offset the requirement for estrogen in breast cancer cells.

The urokinase receptor (uPAR) gene may be amplified in cancer of the breast and pancreas [120-121]. In diverse solid tumors, uPAR expression is correlated with disease progression [113, 177, 197-199]. Although uPAR plays a pivotal role in activation of protease cascades at the cell surface, important processes in cancer have been linked to uPAR-initiated cell-signaling, including cell migration, survival, release from states of dormancy, epithelial-mesenchymal transition (EMT), and cancer stem cell-like behavior [99, 101, 171, 200-201]. In mice, uPAR promotes cancer metastasis independently of urokinase-type plas-

minogen activator (uPA), implicating pathways other than or in addition to protease activation [132].

uPAR is GPI-anchored and thus, utilizes a system of co-receptors and transactivation pathways to trigger cell-signaling [101]. uPAR-initiated cell-signaling is regulated by two ligands, uPA and vitronectin, which interact with distinct binding sites [112, 173, 202-204]. Some signaling pathways activated downstream of uPAR, such as that leading to Rac1, do not require uPA [146, 181, 205]. Whether uPA is necessary for uPAR-dependent ERK activation is less clear. There is evidence that ERK may be activated in the absence of uPA; however, uPA produced endogenously by the cells may be involved [143, 200, 204]. It is extremely important to understand the mechanism by which uPAR activates ERK because in cancer cells, phospho-ERK is a potent cell-survival factor [206]. In ERα-expressing MCF-7 breast cancer cells, uPAR-dependent ERK activation is strictly dependent on uPA [145, 172, 187]. This pathway also requires focal adhesion kinase (FAK) and SFKs [145, 171, 187, 200]. EGFR transactivation may be involved [143, 170].

When expressed at high levels, receptor tyrosine kinases (RTKs) may signal independently of ligands. One example involves ErbB2/HER2, which dimerizes and signals in the absence of ligand in breast cancer cells [207-208]. In this study, we show that over-expression of uPAR in two distinct ERα-expressing breast cancer cell types transforms the uPAR signaling mechanism so that ERK is activated autonomously of uPA. Autonomous uPAR signaling to ERK occurs downstream of H-Ras and Rac1, unlike uPA-induced signaling, which occurs

downstream of H-Ras alone [145, 172, 187]. Activation of autonomous uPAR signaling provides a selection advantage for ERα-expressing breast cancer cells when estrogen is unavailable *in vitro* and *in vivo*. Because uPAR-initiated cell-signaling has been reported to involve receptor dimerization or oligomerization [209-210], we hypothesize that the transformation in uPAR signaling mechanism reported here is similar to that previously reported for ErbB2/HER2 [207].

MATERIALS AND METHODS

Reagents

17β-estradiol (E2) was from Sigma-Aldrich (St. Louis, MO). The PDGF receptor kinase-selective inhibitor, Tyrphostin AG1296, and the SFK inhibitor, PP2, were from EMD Biosciences (Gibbstown, NJ). Erlotinib and Gefitinib were from LC Laboratories (Woburn, MA). Expression constructs encoding dominant-negative Rac1 (DN-Rac1/Rac1-S17N), dominant-negative H-Ras (DN-H-Ras/Ras-S17N), dominant-negative focal adhesion kinase (FAK) (DN-FAK/FAK-Y397F), wild-type FAK, and hemagglutinin (HA)-tagged ERK1 are previously described [145, 181, 187, 211]. Expression constructs encoding human uPAR and mouse uPAR also are previously described [132, 142]**.** The full-length human uPAR cDNA in pCDNA3.1 was mutated at a single base-pair to generate uPAR-W32A, using the Quick-Change Site-Directed Mutagenesis Kit (Agilent Tech-nologies, Santa Clara, CA). Human uPAR-specific antibody was from Molecular

Diagnostics (Stamford, CT). Allophycocyanin (APC)-conjugated human uPAR-specific antibody and isotype-matched control antibody for flow cytometry were from eBioscience (San Diego, CA). Mouse uPAR-specific antiserum was generously provided by Dr. Andrew Mazar (Northwestern University, Evanston, IL). Phospho-ERK-specific antibody was from Cell Signaling Technologies (Danvers, MA). Rac/Cdc42 assay reagent (PAK1-PBD), which includes residues 67-150 of p21-activated kinase fused to glutathione-*S*-transferase and coupled to glutathione-Sepharose, was from Millipore (Billerica, MA), as was the antibody that detects total ERK. Rac1-specific antibody was from BD Biosciences (Franklin Lakes, NJ). Horseradish peroxidase-conjugated antibodies specific for mouse or rabbit IgG were from GE Healthcare (Piscataway, NJ). qPCR reagents, including primers and probes, were from Applied Biosystems (Foster City, CA).

Cell Culture

Low passage MCF-7 and MDA-MB-231 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Hyclone) supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/ml), and streptomycin (100 μg/ml). MDA-MB 361 cells (ATCC) were maintained in RPMI-1640 medium supplemented with 10% FBS, penicillin (100 units/ml), and streptomycin (100 μg/ml). MCF-7 cells were transfected to express mouse uPAR using lipofectamine 2000 (Invitrogen), selected in hygromycin (0.4 mg/ml), and single-cell cloned. MCF-7 cells that over-express human uPAR are previously described [201].

uPAR-specific siRNA smart pool and siCONTROL non-targeting control (NTC) siRNA pool were obtained from Dharmacon. siRNA transfection was performed with Lipofectamine 2000 in serum-free medium (SFM). siRNAs were introduced twice, at 24 and 72 h. Mouse uPAR mRNA expression was determined by qPCR and immunoblot analysis.

Real-time qPCR

Total RNA was isolated from cells in culture using the NucleoSpin RNA II kit (Machery-Nagel). cDNA was synthesized using the iScript cDNA synthesis kit (BioRad). qPCR was performed using a StepOnePlus instrument (Applied Biosystems) and a one-step program: 95° C, 20s; 95° C, 1s; and 60° C, 20s for 40 cycles. HPRT-1 gene expression was measured as a normalizer. Results were analyzed by the relative quantity (ΔΔCt) method. Experiments were performed in duplicate with internal triplicate determinations.

Analysis of Cell-signaling

Cell extracts were prepared in radioimmune precipitation assay (RIPA) buffer (20 mM sodium phosphate, 150 mM NaCl, pH 7.4, 1% Triton, 0.1% SDS, 0.5% sodium deoxycholate) containing complete protease inhibitor mixture (Roche Applied Science) and 1 mM sodium orthovanadate. Protein concentrations were determined by bicinchoninic acid assay (Sigma-Aldrich). Equal amounts of cell extract were subjected to SDS-PAGE, electrotransferred to PVDF

membranes, and probed with primary antibodies to detect phospho-ERK and total ERK.

In experiments in which cells were transfected with constructs encoding wild-type FAK, DN-FAK, DN-Rac1, or DN-H-Ras, the construct encoding HA-ERK1 was introduced simultaneously using Lipofectamine 2000 or FugeneHD (Roche), so that ERK activation could be monitored exclusively in the transfected cells [145, 187]. HA-ERK1 also was introduced in transient transfection studies with the construct encoding mouse uPAR, human uPAR, or uPAR-W32A. Cell extracts were prepared and HA-ERK1 was immunoprecipitated from equal amounts of cell extracts (500 μg) using HA antibody-conjugated agarose beads (Sigma-Aldrich). Immunoprecipitates were subjected to immunoblot analysis and probed with antibodies specific for phospho-ERK and total ERK.

GTP-loaded Rac1 was determined by affinity-precipitation using PAK1-PBD, which recognizes only the GTP-bound forms of Rac1 and Cdc42, as previously described [132, 181]. Mouse uPAR-expressing and control MCF-7 cells were cultured in 10 cm plates for 16 h. Cultures were washed with ice-cold PBS and extracted in the supplied buffer supplemented with protease inhibitor cocktail and 1 mM sodium orthovanadate. The extracts were incubated with 15 μg of PAK1-PBD reagent for 45 min at 4°C. The glutathione-Sepharose was washed four times and then treated with SDS sample buffer to dissociate the PAK1-PBD and associated proteins. Immunoblot analysis was performed to detect Rac1. Samples of each cell extract also were subjected to immunoblot analysis before incubation with PAK1-PBD to determine total Rac1. Immuno-

blots were digitized and quantified using Quantity One 1-D Analysis Software (BioRad).

Flow Cytometry

Cells (3 x 10^5) were plated in 6-well plates and co-transfected to express wild-type uPAR or uPAR-W32A. Cells were co-transfected with pEGFP to express green fluorescent protein (GFP). After 24 h, cells were suspended 2% bovine serum albumin (BSA) in PBS and then incubated with APC-conjugated uPAR-specific antibody (0.5 μg/1 x 10^5 cells) or isotype-matched IgG for 60 min at 4º C. Flow cytometry was performed using a FACSCanto II flow cytometer (BD Biosciences). Results were analyzed with FlowJo software.

Orthotopic Xenografts

Animal experiments were performed in accordance with protocols approved by the University of California San Diego Animal Care Program. Anesthetized 8-week-old C.B-17/lcrCrl-scid-BR mice (Charles River Laboratories) were inoculated bilaterally in the fourth mammary fat pad with M3, M4 or control MCF-7 cells (1 x 10^6), which were transfected with empty vector (EV), suspended in 50 μl of Matrigel (Sigma). Primary tumor growth was monitored weekly. Ten weeks after tumor cell injection, the mice were euthanized and the mammary fat pads were visually inspected for tumor. Tumor formation was

confirmed by histological analysis. Xenograft tumor volumes (vol) were calculated using the formula: Vol = $(4/3)\pi$ x ((largest radius + smaller radius)/2)^3. Data processing and statistical analysis were performed using GraphPad Prism (GraphPad Software, Inc.) and Microsoft Excel (Microsoft Corporation).

Histology and Immunohistochemistry Analysis of Mouse Tissues

Formalin-fixed tissue was paraffin-embedded. Serial 4 μm sections were stained with hematoxylin and eosin. Immunohistochemistry (IHC) was performed using the Vantana Discovery® XT System (Vantana). Sections were pretreated with citric acid buffer and then incubated with polyclonal antibody specific for mouse uPAR (1:200) or phospho-ERK followed by peroxidase-conjugated secondary antibody. Peroxidase activity was imaged using 3,3'-diaminobenzidine. Slides were examined using a Leica DM2500 light microscope. Images were acquired using Leica DFC420 digital camera and Leica Application Suite software.

RESULTS

uPAR over-expression induces uPA-independent ERK activation

MCF-7 breast cancer cells express low levels of uPAR and undetectable levels of uPA [172]. ERK activation downstream of uPAR is entirely dependent

on exogenously-added uPA [172, 187]. To test whether the level of uPAR expression affects the mechanism by which uPAR activates ERK, we over-expressed human uPAR in MCF-7 cells and derived two cloned cell lines (H1 and H5). Fig. 2-1A shows that uPAR expression was substantially increased in the H1 and H5 cells. The level of phospho-ERK, observed in the absence of added uPA, also was increased in H1 and H5 cells, compared with the level observed in control (EV) cells that were transfected with empty vector.

To confirm that the increase in phospho-ERK was not an artifact resulting from single-cell cloning, we examined MCF-7 cells that were transiently trans-fected to over-express human uPAR. The cells were co-transfected to express HA-tagged ERK1, to permit analysis of ERK phosphorylation selectively in the transfected cells. Fig. 2-1B shows that HA-ERK1 activation was increased by uPAR over-expression, in the absence of exogenously added uPA.

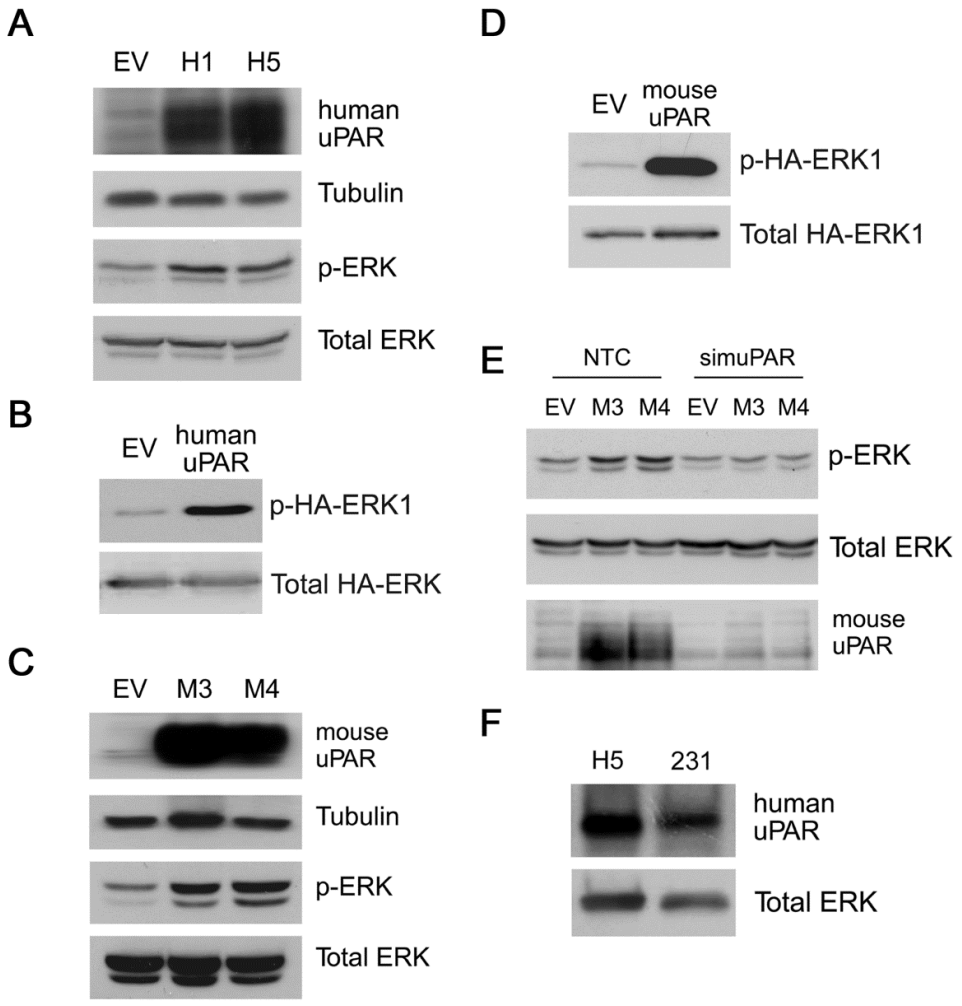

Figure 2-1. High levels of uPAR expression induce autonomous signaling to ERK. *A*, Cell extracts from H1, H5 and control/EV MCF-7 cells were subjected to immunoblot analysis to detect human uPAR, phospho-ERK (p-ERK), total ERK, and tubulin as a loading control. *B*, MCF-7 cells were transiently co-transfected to express human uPAR or empty vector (EV) and HA-ERK1. Cell extracts were immuneprecipitated with HA-specific antibody and subjected to immunoblot analysis to detect phospho-ERK and total ERK. *C*, Cell extracts from M3, M4, and control/EV MCF-7 cells were subjected to immunoblot analysis to detect mouse uPAR, phospho-ERK, total ERK, and tubulin. *D*, MCF-7 cells were transiently co-transfected to express HA-ERK1 and mouse uPAR or control vector (EV). Cell extracts were immunoprecipitated with HA-specific antibody and subjected to immunoblot analysis to detect phospho-ERK and total ERK. *E*, Mouse uPAR was silenced in M3, M4, and control/EV MCF-7 cells. Cell extracts were subjected to immunoblot analysis to detect mouse uPAR, phospho-ERK (pERK) and total ERK. *F*, Cell extracts from MDA-MB-231 cells and H5 cells were subjected to immunoblot analysis to detect human uPAR and total ERK.

In control qPCR and immunoblotting experiments, we confirmed that H1 and H5 cells do not express uPA, like the parental MCF-7 cells (Fig. 2-2). Thus, our results suggested that uPAR over-expression in MCF-7 cells induces ERK activation autonomously of uPA. To further test this hypothesis, we transfected MCF-7 cells to express mouse uPAR. uPA-binding to uPAR is highly species-specific [132, 212-213], precluding ligation of mouse uPAR by trace levels of human uPA, which may have been produced by the MCF-7 cells. As shown in Fig. 2-1C, ERK was activated, in the absence of exogenously added uPA, in two cloned cell lines that express mouse uPAR (M3 and M4). MCF-7 cells that were transiently transfected to express mouse uPAR and HA-ERK1 also demonstrated increased HA-ERK1 activation, in the absence of exogenously added uPA (Fig. 2-1D).

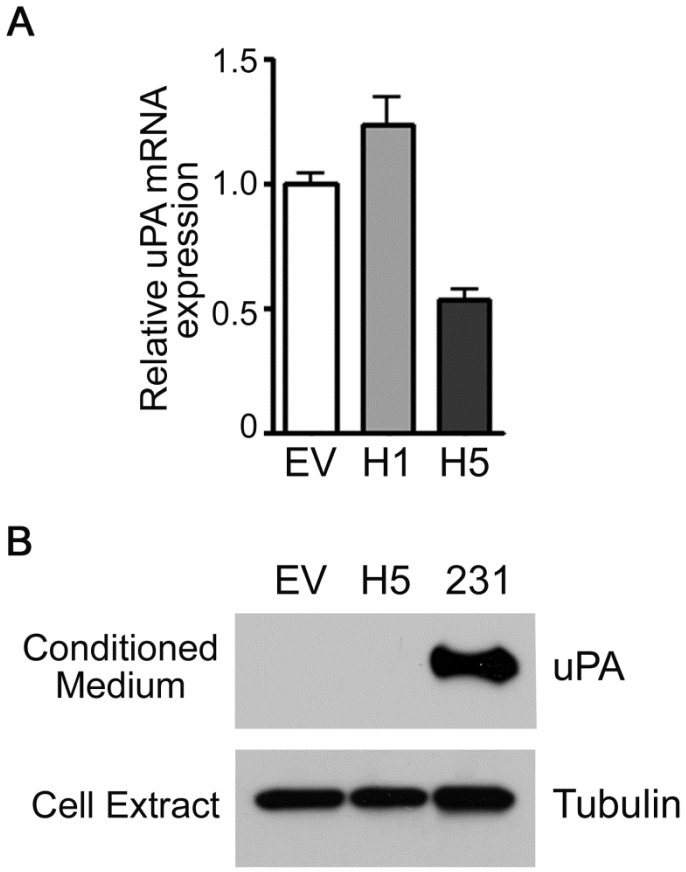

Figure 2-2. uPAR over-expression does not increase uPA expression in MCF-7 cells. *A*, Relative uPA mRNA expression was determined in the human uPAR over-expressing H1 and H5 cells, as well as in control EV cells (mean ± SEM, n=3). *B*, Conditioned SFM was collected from cultures of EV, H5, and MDA-MB-231 cells at equivalent confluency and concentrated 10 times. MDA-MB-231 cells, which express high levels of uPA serve as a control. Cell extracts were subjected to immunoblot analysis to detect Tubulin.

To confirm that the increase in ERK activation, observed when uPAR was over-expressed, was due to uPAR, we silenced uPAR gene expression in M3 and M4 cells. The extent of silencing was nearly complete, as determined by qPCR (Supplementary Fig. 2-3) and by immunoblot analysis (Fig. 2-1E). Phospho-ERK was decreased to the level observed in control MCF-7 cells when mouse uPAR expression was silenced with siRNA.

To estimate the extent of uPAR over-expression in our transfected cell lines, we compared the abundance of uPAR in H5 cells and wild-type MDA-MB-231 breast cancer cells. MDA-MB 231 cells are highly aggressive cancer cells that metastasize readily in animal model systems [214-215]. uPAR signaling in MDA-MB-231 cells occurs independently of exogenously-added uPA [200]. By immunoblot analysis and densitometry, the level of uPAR in H5 cells was only 25% higher than that detected in MDA-MB-231 cells (Fig. 2-1F). Thus, the transformation in uPAR signaling mechanism, observed in transfected MCF-7 cells, reflects a level of uPAR that may be found naturally in breast cancer cells, especially when uPAR gene amplification occurs [120-121].

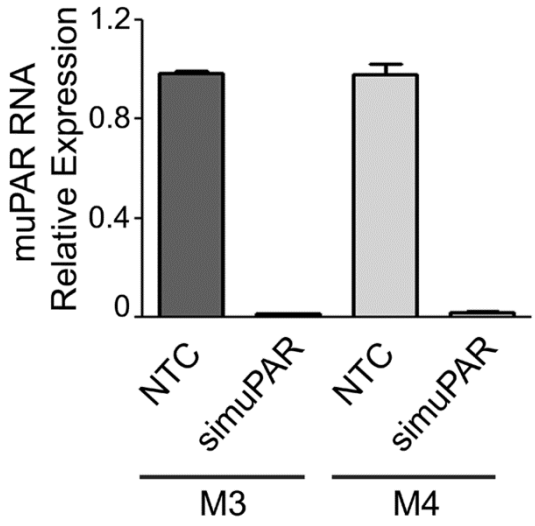

Figure 2-3. Mouse uPAR is silenced in mouse uPAR over-expressing cells. Mouse uPAR was silenced in M3, M4, and control/EV MCF-7 cells. Relative mouse uPAR mRNA expression was determined by qPCR (mean ± SEM, n=3).

uPAR regulates ERK activation only in the absence of E2

In the studies presented thus far, cells were cultured in SFM for 18 h before analyzing ERK activation. Limited ERα activation was possible due to phenol red in the medium [216]. In Fig. 2-4A, mouse uPAR-expressing and control MCF-7 cells were cultured for 18 h in SFM, in the presence or absence of E2 (20 nM). Although ERK activation was substantially increased in M3 and M4 cells in the absence of E2, the difference was neutralized by E2 supplementation. These results suggest that uPAR may control ERK activation in ERα-positive breast cancer cells, principally when E2 is absent or when drugs that inhibit the E2-ERα signaling system are introduced.

To test whether uPAR over-expression activates ERK autonomously of uPA in a second ERα-positive breast cancer cell line, we studied MDA-MB-361 cells. These cells express more uPAR mRNA than MCF-7 cells but less than MDA-MB 468 cells (Fig. 2-4B). When MDA-MB 361 cells were transiently transfected to express mouse uPAR, the basal level of phospho-HA-ERK1 was increased (Fig. 2-4C). MDA-MB-361 cells express low levels of uPA (results not shown); however, because this is a human cell line, uPA that is produced endogenously should not bind significantly to mouse uPAR [132, 212-213].

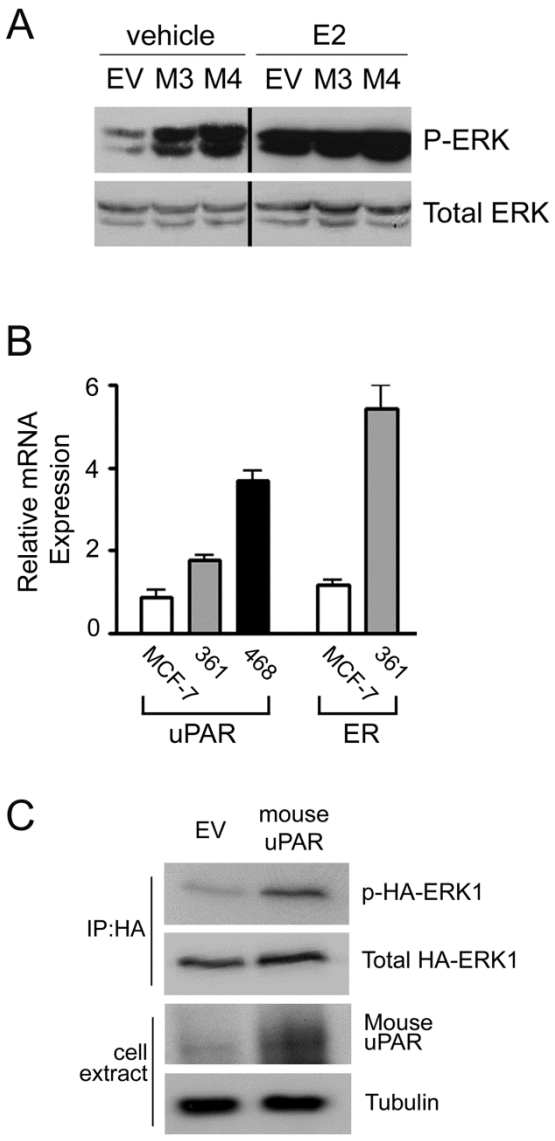

Figure 2-4.**Autonomous uPAR signaling in the presence of E2 and in a second model system.** *A*, M3, M4, and EV/control MCF-7 cells were cultured in SFM for 18 h and treated with 20 nM E2 or vehicle in SFM for an additional 18 h. Cell extracts were subjected to immunoblot analysis to detect phospho-ERK (p-ERK) and total ERK. *B*, Relative mRNA expression was determined for uPAR and ERα in MCF-7 cells, MDA-MB-361 cells, and MDA-MB-468 cells (mean ± SEM, n=3). *C*, MDA-MB-361 cells were transiently co-transfected to express HA-ERK1 and mouse uPAR or control vector (EV). Cell extracts were immunoprecipitated with HA-specific antibody and subjected to immunoblot analysis to detect phospho-ERK (p-ERK) and total ERK. Total cell extracts were subjected to immunoblot analysis to detect mouse uPAR and tubulin.

Common factors in the pathway by which uPAR activates ERK, autonomously and in response to uPA

When cells are cultured in serum-containing medium, vitronectin is the major protein that coats tissue-culture plastic [217]. A single mutation in the structure of uPAR (W32A) blocks the interaction of uPAR with vitronectin [204]. To assess whether sustained ERK activation, resulting from uPAR over-expression, requires uPAR-binding to vitronectin, MCF-7 cells were transfected to express uPAR-W32A or wild-type uPAR and GFP. Flow cytometry studies were performed to detect cell-surface uPAR in the GFP-gated population. Fig. 2-5A shows that the level of cell-surface uPAR was similar in cells that expressed wild-type uPAR or uPAR-W32A.

Next, MCF-7 cells were transfected to express wild-type uPAR or uPAR-W32A and HA-ERK1. Fig. 2-5B shows that HA-ERK1 was phosphorylated in cells that express wild-type uPAR but not in cells that express uPAR-W32A. Thus, association of uPAR with vitronectin appears to be necessary for autonomous uPAR signaling to ERK.

SFKs and FAK are required for uPA-induced ERK activation in MCF-7 cells [145, 187]. SFKs also have been implicated in the uPA-independent pathway by which vitronectin-binding to uPAR leads to Rac1 activation [146, 205]. To test the role of FAK in autonomous uPAR signaling to ERK, we transfected M3 cells and control EV cells to express DN-FAK or wild-type FAK. Cells were co-transfected to express HA-ERK1. The level of phospho-HA-ERK1 was ap-

proximately equivalent in control EV cells that were transfected with wild-type FAK or DN-FAK (Fig. 2-5C). By contrast, in M3 cells, DN-FAK decreased the level of phospho-HA-ERK1, suggesting that FAK is necessary for autonomous ERK activation in these cells. In control experiments, we confirmed that wild-type FAK and DN-FAK were expressed at similar levels in both EV and M3 cells. With both constructs, the transfection efficiency was higher in M3 cells; however, the HA-ERK1 co-transfection procedure corrects for differences in transfection efficiency.

The SFK-selective pharmacological inhibitor, PP2, also substantially decreased phospho-ERK in M3 and M4 cells, suggesting an important role for SFKs in autonomous uPAR signaling to ERK (Fig. 2-5D). As a control, we examined the PDGF receptor-selective tyrosine kinase inhibitor (TKI), AG1296. ERK phosphorylation in M3 or M4 cells was unchanged by AG1296.

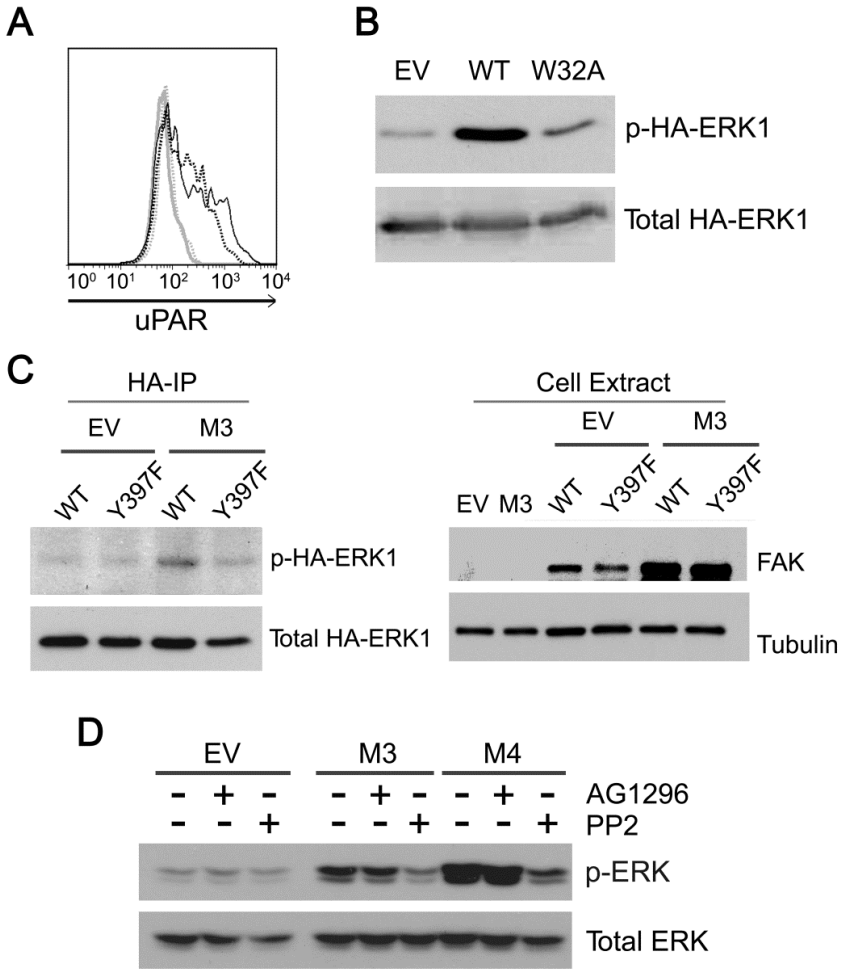

Figure 2-5. **Molecular mechanisms of autonomous uPAR signaling to ERK.** *A*, MCF-7 cells were transiently co-transfected to express GFP and wild-type uPAR (dark solid tracing), uPAR-W32A (dark broken tracing), or control vector (light solid tracing). Cell-surface uPAR expression was determined by flow cytometry. The isotype control is shown with the light broken tracing. *B*, MCF-7 cells were transiently co-transfected to express HA-ERK1 and wild-type uPAR, uPAR-W32A or control vector (EV). Cell extracts were immunoprecipitated with HA-specific antibody and subjected to immunoblot analysis for phospho-ERK (p-ERK) and total ERK. *C*, M3 and control EV cells were transiently co-transfected to express HA-ERK1 and DN-FAK or wild-type FAK. Extracts were immunoprecipitated with HA-specific antibody and subjected to immunoblot analysis. Representative total cellular extracts of transiently transfected M3 and control EV cells, as well as non-transfected controls were subjected to immonoblot analysis to detect FAK and Tubulin. *D*, M3, M4, and EV/control MCF-7cells were treated with the SFK inhibitor (PP2, 1 μM), the PDGF receptor inhibitor (AG1296, 10 μM), or vehicle for 18 h in SFM. Extracts were analyzed for phospho-ERK and total ERK.

Rac1 and H-Ras cooperate in autonomous uPAR signaling to ERK

uPAR over-expression activates Rac1 in multiple cell types [132, 146, 181, 205]. Fig. 2-6A shows that the level of GTP-loaded Rac1 was increased in both M3 and M4 cells, as anticipated. To test whether the increase in phospho-ERK occurs downstream of activated Rac1 in mouse uPAR-expressing MCF-7 cells, M3, M4, and EV cells were transfected to express DN-Rac1 [181] and HA-ERK1. DN-Rac1 did not significantly change the level of phospho-HA-ERK1 in EV cells (Fig. 2-6B). By contrast, in both M3 and M4 cells, DN-Rac1 decreased phospho-HA-ERK1, albeit incompletely. To confirm that DN-Rac1 was expressed in M3, M4 and EV cells, we performed immunoblotting experiments to detect total Rac1 and DN-Rac1, which is GFP-tagged. DN-Rac1 was detected in all three cell lines.

To study the role of H-Ras in autonomous uPAR signaling to ERK, cells were co-transfected to express DN-H-Ras and HA-ERK1. DN-H-Ras is previously described [211]. In control EV cells, DN-H-Ras did not significantly affect phospho-HA-ERK1. By contrast, DN-H-Ras decreased phospho-HA-ERK1 in both M3 and M4 cells (Fig. 2-6C). Like DN-Rac1, the effects of DN-H-Ras were incomplete. These results support a model in which autonomous uPAR signaling to ERK reflects the activity of two separate pathways, involving H-Ras and Rac1, which converge at the level of ERK. Unfortunately, we were unable to simultaneously express DN-Rac1 and DN-H-Ras in MCF-7 cells, due to the decreased viability of dually transfected cells.

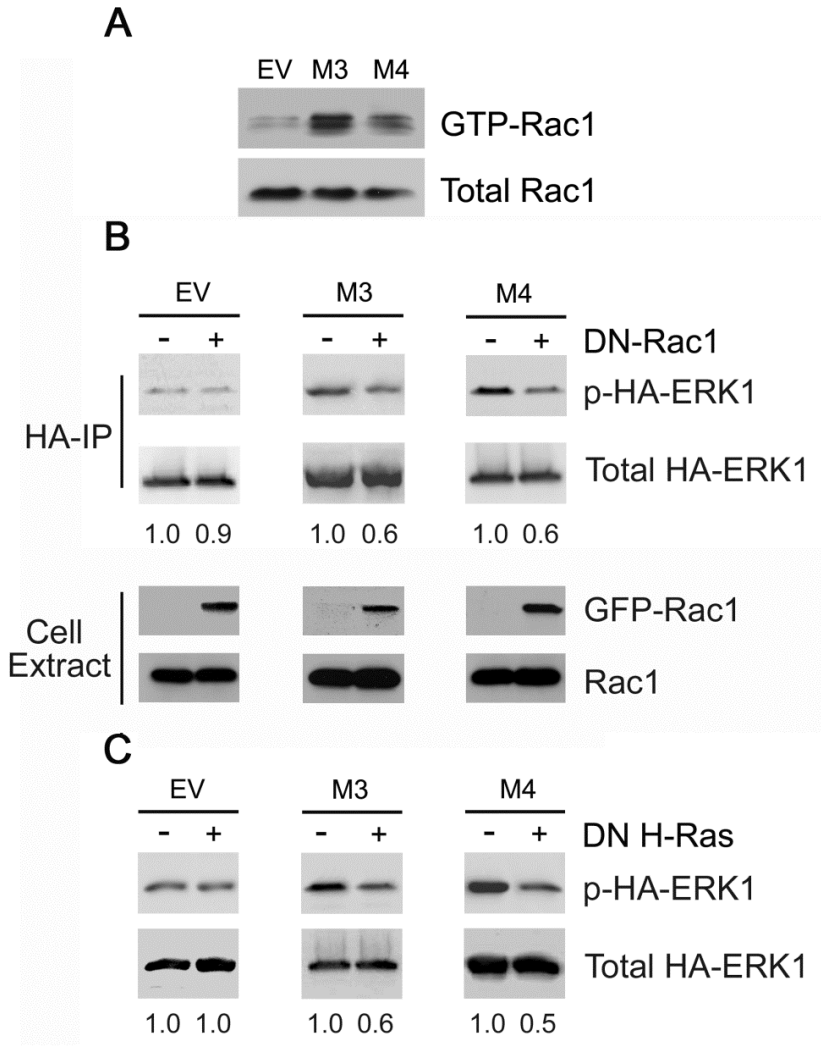

Figure 2-6. **Rac1 and H-Ras cooperate to induce autonomous uPAR signaling to ERK.** *A*, M3, M4, and EV/control MCF-7 cells extracts were affinity precipitated with PAK-1 PBD and subjected to immunoblot analysis to detect GTP-bound Rac1. The original cell extracts were also subjected to immunoblot analysis to determine total Rac1 as a loading control. *B*, M3, M4, and EV MCF-7 cells were transiently co-transfected to express HA-ERK1 and DN-Rac1 or control vector. Extracts were immunoprecipitated with HA-specific antibody and subjected to immunoblot analysis to detect phosphor-ERK (p-ERK) and total ERK. Representative total cellular extracts were subjected to immunoblot analysis to detect endogenous Rac1 and GFP-tagged DN-Rac1. *C*, M3, M4, and EV MCF-7 cells were transiently co-transfected to express HA-ERK1 and DN-H-Ras or control vector. Extracts were immunoprecipitated with HA-specific antibody and subjected to immunoblot analysis to detect phospho-ERK and total ERK. Ratios of phospho-HA-ERK1 to total-HA-ERK1 were determined by densitometry and are reported under the immunoblots.

uPAR over-expression provides a selection advantage for MCF-7 cells in the absence of E2 in vitro and in vivo

To test whether uPAR expression provides a survival advantage for ERα-positive cells when E2 is not available, we transfected MCF-7 cells to express human uPAR or mouse uPAR [201]. Instead of selecting the cells with antibiotics, cultures were maintained in medium that was supplemented with charcoal-treated serum (CTS) and E2 (20 nM) or vehicle for 2 weeks. As shown in Fig. 2-7A, cells that were E2-deprived demonstrated mouse uPAR mRNA levels that were increased about 4-fold, on average, compared with E2-treated cells ($p<0.05$, Student's t-test). In cells that were transfected to over-express human uPAR, E2 deprivation increased uPAR mRNA levels 2.5-fold ($p<0.05$). We interpret these results to indicate that, in the absence of E2, uPAR over-expression provides a growth/survival advantage and cells which express higher levels of uPAR are selectively recovered.

We performed the equivalent experiment with cells that were transfected to express uPAR-W32A because this form of uPAR does not support cell-signaling. uPAR-W32A failed to provide a selection advantage when cells were deprived of E2 for 2 weeks (Fig. 2-7A). In additional control experiments, E2 deprivation did not significantly affect uPAR mRNA expression when MCF-7 cells were transfected with empty vector.

MCF-7 cells typically form tumors in SCID mice only when estrogen supplementation is provided [176, 194, 216]. We previously demonstrated that human uPAR over-expression in MCF-7 cells significantly increases the

frequency of tumor formation and growth in the absence of estrogen supplementation [201]. The mechanism was not determined. In new studies, we inoculated mouse uPAR-expressing and control MCF-7 cells into mammary fat pads in SCID mice (10^6 cells/injection). Tumor formation and volume were assessed at 10 weeks.

M3 and M4 cells formed tumors that were significantly increased in volume ($p<0.05$), compared with the tumors formed by control cells (Fig. 2-7B). Representative H&E-stained sections of recovered tumors are shown in Fig. 2-7C. Mouse uPAR-expressing cells tended to invade outside the Matrigel capsule, as anticipated. In IHC studies, mouse uPAR was clearly detected in both M3 and M4 cells *in vivo*. The cancer cells in tumors formed by EV cells were mouse uPAR-immunonegative, as anticipated. We also performed IHC studies to detect phospho-ERK *in vivo* in tumors formed by EV, M3, and M4 cells. Foci of robustly phospho-ERK-positive cancer cells were abundant in tumors formed by M3 and M4 cells. Tumors formed by control EV cells were phospho-ERK negative at the level of sensitivity of the antibody. These results confirm that the increase in ERK phosphorylation, observed in M3 and M4 cells *in vitro*, is retained *in vivo* and may be responsible for the increase in tumor volume observed in the absence of E2 supplementation.

By staining adjacent sections from individual tumors, we showed that mouse uPAR- immunopositive cells were frequently but not uniformly immunopositive for phospho-ERK (Fig. 2-7D). Factors that may have influenced

whether cells were phospho-ERK-immunopositive include cell cycle phase and the cellular microenvironment.

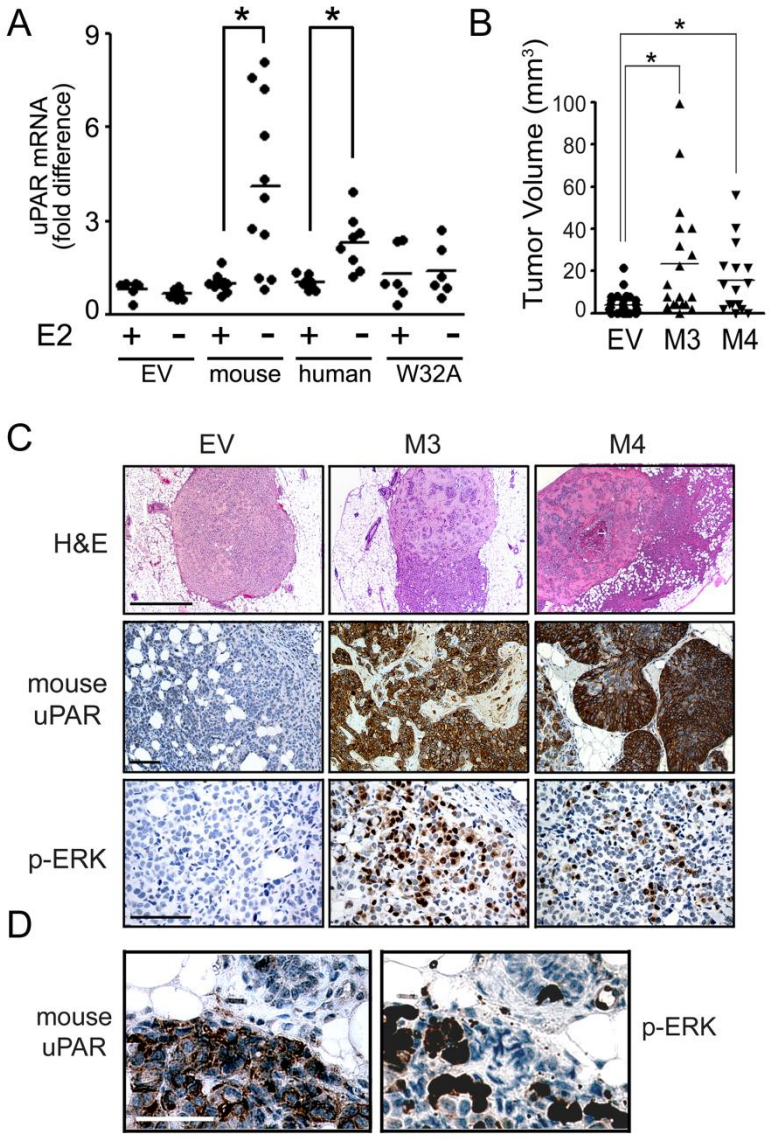

Figure 2-7. **E2 deficiency selects for uPAR-expressing MCF-7 cells *in vitro* and in orthotopic xenografts *in vivo*.** *A*, MCF-7 cells were transfected to express mouse uPAR, human uPAR, uPAR-W32A, or control vector and cultured in the presence or absence of 20 nM E2 for two weeks in phenol red-free DMEM supplemented with CTS. Relative uPAR mRNA expression was determined by qPCR (*, $p<0.05$, Student's t-test). *B*, SCID mice were injected with M3, M4 or control/EV MCF-7 cells. Tumor volume was determined after surgical resection (mean, *, $p<0.05$, Mann-Whitney rank sum test). *C*, Images of tumors formed by M3, M4, and EV cells include representative H&E-stained sections of the tumors (first row; 5x, bar, 100 μm), IHC to detect mouse uPAR (second row; 20x, bar 10 μm) and phospho-ERK (third row; 40x, bar 10 μm). *D*, Adjacent sections of a representative tumor were stained to detect mouse uPAR and phospho-ERK (40x, bar, 10 μm).

EGFR TKIs block autonomous uPAR signaling to ERK

EGFR-specific TKIs are efficacious in the treatment of a number of cancers in which EGFR gene amplification and/or mutations are prevalent [218-221]. Recent studies suggest that EGFR TKIs may be useful in the treatment of breast cancer [222-224]. Because EGFR co-receptor activity has been implicated in the pathway by which uPA-binding to uPAR activates ERK [142-143, 170], we studied the effects of two EGFR TKIs on ERK activation in uPAR over-expressing MCF-7 cells.

M3, M4, and EV cells were treated with Erlotinib or Gefitinib for 24 h. Fig. 2-8A shows that both TKIs almost entirely neutralized the increase in ERK activation in M3 cells. In M4 cells, the decrease in ERK activation was less complete, but still substantial. To further test the activity of EGFR TKIs in uPAR over-expressing MCF-7 cells, we applied a transient transfection strategy. MCF-7 cells were transfected to express mouse uPAR and HA-ERK1 and treated with Erlotinib or Gefitinib for 24 h. Fig 2-8B shows that the EGFR TKIs blocked the increase in HA-ERK1 associated with transient mouse uPAR expression.

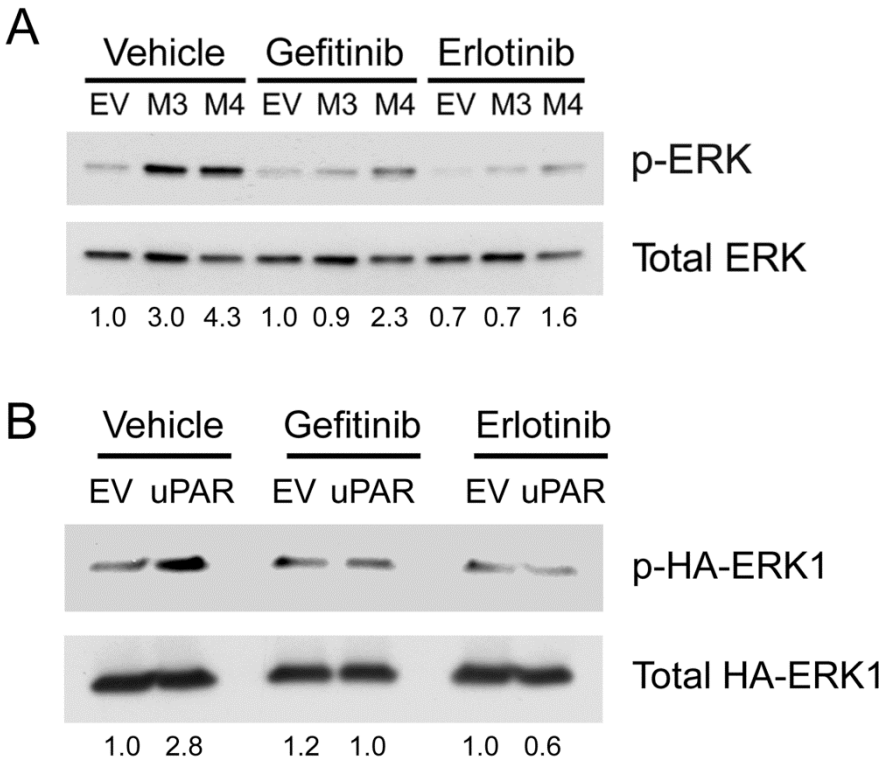

Figure 2-8. EGFR-specific TKIs block autonomous uPAR signaling in MCF-7 cells. *A*, M3, M4, and EV cells were treated with the EGFR inhibitors, Gefitinib (1 μM) and Erlotinib (1 μM), or with vehicle for 24 h in SFM. Extracts were analyzed for phospho-ERK and total ERK. *B*, MCF-7 cells were transiently co-transfected to express mouse uPAR or empty vector (EV) and HA-ERK1 and treated with Gefitinib (1 μM), Erlotinib (1 μM), or with vehicle for 24 h in SFM. Cell extracts were immunoprecipitated with HA-specific antibody and subjected to immunoblot analysis to detect phospho-ERK and total ERK. Ratios of phospho-ERK to total ERK or phospho-HA-ERK1 to total-HA-ERK1 were determined by densitometry and are reported under the immunoblots.

DISCUSSION

The mechanism by which uPAR triggers cell-signaling remains incompletely understood. Co-receptors, including integrins and FPRL1, and transactivation pathways involving RTKs have been implicated [101]. The role of ligand-binding to uPAR in cell-signaling also is incompletely understood. The pathway that leads to activation of Rac1, downstream of p130Cas and DOCK180, is strictly dependent on uPAR-binding to vitronectin [146, 205]. Although the literature regarding activation of the H-Ras-ERK pathway is less clear, in MCF-7 cells, ERK activation requires uPA-binding to uPAR [145, 187].

In this study, we have shown that increased uPAR expression transforms the mechanism of ERK activation downstream of uPAR so that it occurs autonomously of uPA and is sustained. Given the central role of ERK in important cellular processes, including proliferation, survival, and cell migration [206], autonomous uPAR signaling to ERK has substantial potential to impact breast cancer cell physiology. The transition in uPAR-dependent cell-signaling from ligand (uPA)-dependent to -independent is analogous to the paradigm observed with ErbB2/HER2 in breast cancer cells [207]. The genes for ErbB2/HER2 and uPAR may be amplified in the same human breast cancer cells [120].

Unlike uPA-initiated ERK activation, which is entirely dependent on H-Ras [187], autonomous uPAR signaling to ERK apparently occurs downstream of Rac1 and H-Ras. Pathways by which Rac1 promote ERK activation are

previously reported. For example, p21-activated kinase promotes ERK activation by increasing association of MEK1 with ERK [225].

One mechanism accounting for the transient nature of ERK activation, in uPA-treated cells, is MEK-dependent SOS phosphorylation, which promotes SOS dissociation from Grb2 and Shc [187, 226]. It is also possible that uPA-binding to uPAR triggers pulsatile cell-signaling events and that uPAR recycling is necessary for sustained cell-signaling [227]. Autonomous uPAR signaling to ERK was observed in cells that were cultured in SFM for 18 h. Thus, the level of activated ERK detected represents a steady-state. Compared with transient ERK activation, sustained ERK activation, observed with uPAR over-expression, is more likely to impact processes such as gene transcription and cell growth [227-231].

It has been proposed that uPAR-initiated cell-signaling requires uPAR dimerization or oligomerization [209-210]. If this model is correct, an explanation may be presented for the transformation in uPAR signaling mechanism observed here. When uPAR is present at low abundance, uPA is needed to promote uPAR dimerization or oligomerization [232]. However, with increased uPAR expression, oligomerization of uPAR in the absence of uPA should be favored, triggering autonomous signaling.

uPA and its inhibitor, PAI-1, have been implicated in development of resistance to the anti-estrogen drug, Tamoxifen, in breast cancer patients [159]. This is important because uPA and PAI-1 form a complex, which still binds to uPAR and induces sustained ERK activation unlike free uPA, which induces

transient ERK activation [227]. As a result, uPA-PAI-1 complex may selectively promote cancer cell survival [227]. Autonomous uPAR signaling provides a selection advantage for MCF-7 cells, in the absence of uPA and PAI-1, *in vitro* and *in vivo*. When MCF-7 cells were transfected to express mouse or human uPAR and cultured in E2-deficient or -replete medium (no prior antibiotic selection), E2 deficiency selected for cells with higher levels of uPAR. These results may be explained by the ability of uPAR to promote ERK activation when E2 is absent.

The xenografting experiments performed in this study utilized MCF-7 cells that express mouse uPAR. The improvement in survival and growth of the MCF-7 cell orthotopic xenografts was similar to that previously observed when MCF-7 cells were transfected to over-express human uPAR [201]. Unlike mouse uPAR, human uPAR cannot bind uPA that is produced by non-malignant cells in the tumor microenvironment. Thus, the previously reported growth advantage of human uPAR over-expressing MCF-7 cells *in vivo* was unexplained. From the studies reported here, we now understand that uPA is not required for activation of the H-Ras-ERK pathway in human uPAR-over-expressing MCF-7 cells. Furthermore, for the first time in this study, we have demonstrated that the ability of uPAR to activate ERK in breast cancer cells is retained when the cells are implanted in mammary fat pads *in vivo* in mice. Autonomous uPAR signaling to ERK occurs in the microenvironment of a tumor.

To determine whether the level of uPAR expression in our transfected MCF-7 cells was substantially higher than what may be encountered in wild-type

cells, we compared H5 cells and MDA-MB-231 cells. The uPAR protein level was only 25% higher in the H5 cells. We therefore conclude that the transformation in uPAR signaling mechanism described here may occur in breast cancer cells without genetic modification. Autonomous uPAR signaling may provide a pathway for breast cancer cell survival when estrogen is absent or in patients that are treated with drugs that antagonize ERα [192-195]. uPAR is expressed at increased levels in hypoxia, which gradually develops as tumors enlarge [99]. Thus, intrinsic to the process of tumor growth may be a pathway for increased uPAR expression. uPAR gene amplification also may increase uPAR expression to a level that is sufficient for autonomous signaling to ERK.

Autonomous uPAR signaling was apparently dependent on EGFR co-receptor activity because the TKIs, Erlotinib and Gefitinib, inhibited ERK activation. Although EGFR co-receptor function has been observed in cells that are treated with uPA, the ability of uPA to induce ERK activation is not strictly dependent on the EGFR because responses are detected in EGFR-deficient cells [143, 170]. EGFR and uPAR also collaborate to promote activation of the mitogenic transcription factor, STAT5b [142, 170, 233]. Although EGFR inhibitors are not routinely used in breast cancer therapy, new studies suggest that these TKIs may be effective in cancers that relapse after treatment of ERα antagonists, such as Tamoxifen [222, 224]. By inhibiting autonomous uPAR signaling, EGFR TKIs may counteract the pro-survival advantage imparted by uPAR in ERα-positive cells, under estrogen deprivation conditions. Furthermore, the ability of Erlotinib and Gefitinib to inhibit autonomous uPAR signaling may

explain why these drugs show efficacy in some patients with Tamoxifen-resistant breast cancer.

From these studies, we propose a model for uPAR signaling to ERK in which the uPAR concentration in the plasma membrane is critical. As the uPAR expression level increases, for example with increasing tumor hypoxia, a trans-formation in the mechanism of uPAR signaling may occur, triggering autonomous and sustained cell-signaling to ERK in the absence of uPA. Rac1, H-Ras, and the EGFR cooperate to induce these changes. Further work will be required to determine the effects of uPAR over-expression on other signaling pathways known to be activated downstream of uPAR.

CONCLUSIONS

We demonstrate that the requirement for uPA to initiate cell-signaling downstream of uPAR, in ERα-expressing breast cancer cells, depends on the uPAR expression level. At high expression levels, uPAR signals autonomously to ERK and this pathway provides a selection advantage for breast cancer cells in the absence of estrogen. Autonomous uPAR signaling to ERK occurs down-stream of H-Ras and Rac1, unlike uPA-initiated cell-signaling, which occurs downstream of H-Ras alone. The EGFR-selective TKIs, Erlotinib and Gefitinib, inhibit autonomous uPAR signaling.

Chapter 2, in full, is adaptation from material that appears in *Cellular Signaling* 24(9):1847-55. The dissertation author was the primary investigator and author of this paper. The paper is co-authored with Dr. Minji Jo, Drue Webb, Shinako Takimoto and Dr. Steven Gonias.

CHAPTER 3

ABSTRACT

The urokinase receptor (uPAR) is a cell-signaling receptor and a negative prognostic indicator in human breast cancer. In this study, we show that culturing estrogen receptor-α (ERα) positive MCF-7 cells in the presence of the anti-estrogen, Tamoxifen (TAM) for a short time selects for cells with increased uPAR expression, but no increased EGFR or HER2 expression. In addition, MCF-7 cells in which uPAR was over-expressed, demonstrated increased proliferation and decreased cell death when treated with TAM. This transformation provides a pathway for development of TAM resistance in ERα positive breast cancers.

INTRODUCTION

Estrogen receptor-α (ERα) is expressed in up to 75% of all cases of adenocarcinoma of the breast [13, 191-192]. These tumors are typically dependent on estrogen for growth and survival and thus, may be treated with anti-estrogens, which target estrogen receptor-α (ERα), or with aromatase inhibitors, which disrupt estrogen synthesis [14, 16]. The anti-estrogen, 4-OH Tamoxifen (TAM), is an effective adjuvant therapy for ERα-positive breast cancers [234]. Unfortunately, about a third of women treated with TAM relapse within 15 years [234]. Multiple mechanisms have been described to explain acquired TAM resis

tance [156], including *de novo* loss of ERα [152] and activation of receptor tyrosine kinases (RTKs) such as the epidermal growth factor receptor (EGFR) and ErbB2/HER-2 [153]. RTKs signal to cell cycle proteins (c-Myc, cyclin D1) and anti-apoptotic factors (BCL-2, BCL-XL, Mcl-1), promoting breast cancer cell survival and proliferation in the presence of TAM [153, 156].

The urokinase-type plasminogen activator receptor (uPAR) is a glycosylphosphatidyl inositol-anchored membrane protein that assembles into a multiprotein receptor complex with cell-signaling activity [101, 235-236]. uPAR-dependent cell-signaling is triggered by binding of either of two major ligands, uPA or vitronectin [146, 172-173]. The binding sites for uPA and vitronectin are distinct and the cell-signaling pathways activated downstream of uPA and vitronectin may be at least partially distinct as well [112, 143, 202-203]. In ERα positive breast cancer cells, uPA-binding to uPAR activates ERK, downstream of focal adhesion kinase (FAK), Src family kinases (SFKs), and H-Ras [145, 171, 187, 200]. Vitronectin-binding to uPAR activates Rac1, downstream of p130Cas and DOCK180 [146, 205].

uPAR gene amplification has been reported in cancer of the breast and pancreas [120-121]. In many forms of cancer, including breast cancer, uPAR expression is correlated with disease progression and associated with a negative prognosis [113, 177, 197-199]. uPAR-dependent cell-signaling has been implicated in breast cancer cell migration, survival, release from states of dormancy, epithelial-mesenchymal transition (EMT), and stem cell-like behavior [99,

101, 171, 184, 200-201, 237]. Because uPAR expression in healthy tissues is limited, newly developed drugs that target uPAR may be expected to have limited toxicity [113, 238]; however, currently, uPAR-targeting therapeutics are not generally available.

uPAR expression in human breast cancer is inversely correlated with ERα expression [158]. uPA and its inhibitor, plasminogen activator inhibitor-1 (PAI-1), have been identified as predictors of TAM resistance in relapsed breast cancer [159]. Although PAI-1 blocks uPA protease activity, uPA-PAI complex still binds to uPAR and induces sustained ERK activation, which promotes cancer cell survival [157, 227].

In this study, our goal was to determine whether uPAR gene amplification may facilitate development of TAM resistance in ERα positive breast cancer cells. When cells are selected with TAM *in vitro*, uPAR expression increases independently of EGFR or HER-2 expression. In addition, cells over-expressing uPAR also exhibit increased survival when treated with TAM.

MATERIALS AND METHODS

Reagents

TAM and MTT reagent were from Sigma-Aldrich (St. Louis, MO). Phenol-red free DMEM media was from Invitrogen (Grand Island, NY).

Cell Culture

Low passage MCF-7 cells were maintained in DMEM (Hyclone, Rockford, IL) supplemented with 10% fetal bovine serum (FBS). MCF-7 cells that over-express mouse uPAR, M3 and M4, are previously described [239]. Low passage MCF-7 cells were cultured in phenol red free DMEM (Invitrogen) either supplemented with TAM or vehicle control.

Real-time qPCR

Total RNA was isolated from cells in culture using the NucleoSpin RNA II kit (Macherey-Nagel, Bethlehem, PA). cDNA was synthesized using the iScript cDNA synthesis kit (BioRad, Hercules, CA). qPCR was performed using a StepOnePlus instrument (Applied Biosystems). Results were analyzed by the relative quantity method, using HPRT-1 as normalizer.

Statistics

Statistics were performed using GraphPad Prism software (La Jolla, CA). Linear regressions were plotted using inbuilt tools. In addition, Pearson correlation coefficients were calculated using the Correlation tool.

BrdU incorporation

BrdU incorporation assays were performed using the FITC BrdU flow kit (BD Biosciences). Cells were prepared according to manufacturer's instructions.

BrdU incorporation was determined using a FACSCanto II flow cytometer (BD Biosciences) and analyzed with FlowJo Software. Statistical analysis was performed by GraphPad Prism (GraphPad Software, Inc., La Jolla, CA).

Cell Viability Determination

$2x10^5$ cells were plated in 6 well plates and treated with 5uM TAM for 6 days in complete media. MTT reagent was added to a final concentration of 5ng/ml and cells were incubated for 4 hours at 37C. The reagent was developed in acidified isoproponal (0.01M HCl, 0.05% SDS in isoproponol) for 5 minutes and absorbance was read at 570nM. Background readings were performed at 690nM.

RESULTS

TAM treatment selects for increased uPAR expression in MCF-7 breast cancer cells

Long-term culturing of MCF-7 cells in the presence of anti-estrogens selects for cells that express increased levels of EGFR or HER-2, providing a model of changes that may induce anti-estrogen resistance *in vivo* [154-155]. We cultured MCF-7 cells in phenol red-free DMEM supplemented with 5 µM TAM and charcoal-treated serum (CTS) for 2 weeks to determine whether uPAR may be regulated similarly to EGFR and HER-2. Fig. 3-1A shows that uPAR expression increased 2-fold or more in 26% of the TAM-treated cultures, compared with control cultures which demonstrated minimal variability in uPAR

expression ($p<0.001$, Fisher exact test). uPA mRNA expression was also increased in eight Tamoxifen treated cultures compared to vehicle treated controls ($p<0.01$, Fisher exact test). The absolute increase in uPAR and uPA was different from TAM-treated culture to culture. EGFR and HER-2 mRNA showed minimal variability in both the vehicle and TAM-treated cultures indicating that a longer treatment period is necessary in order to induce increased RTK expression (Fig 3-1 C-D).

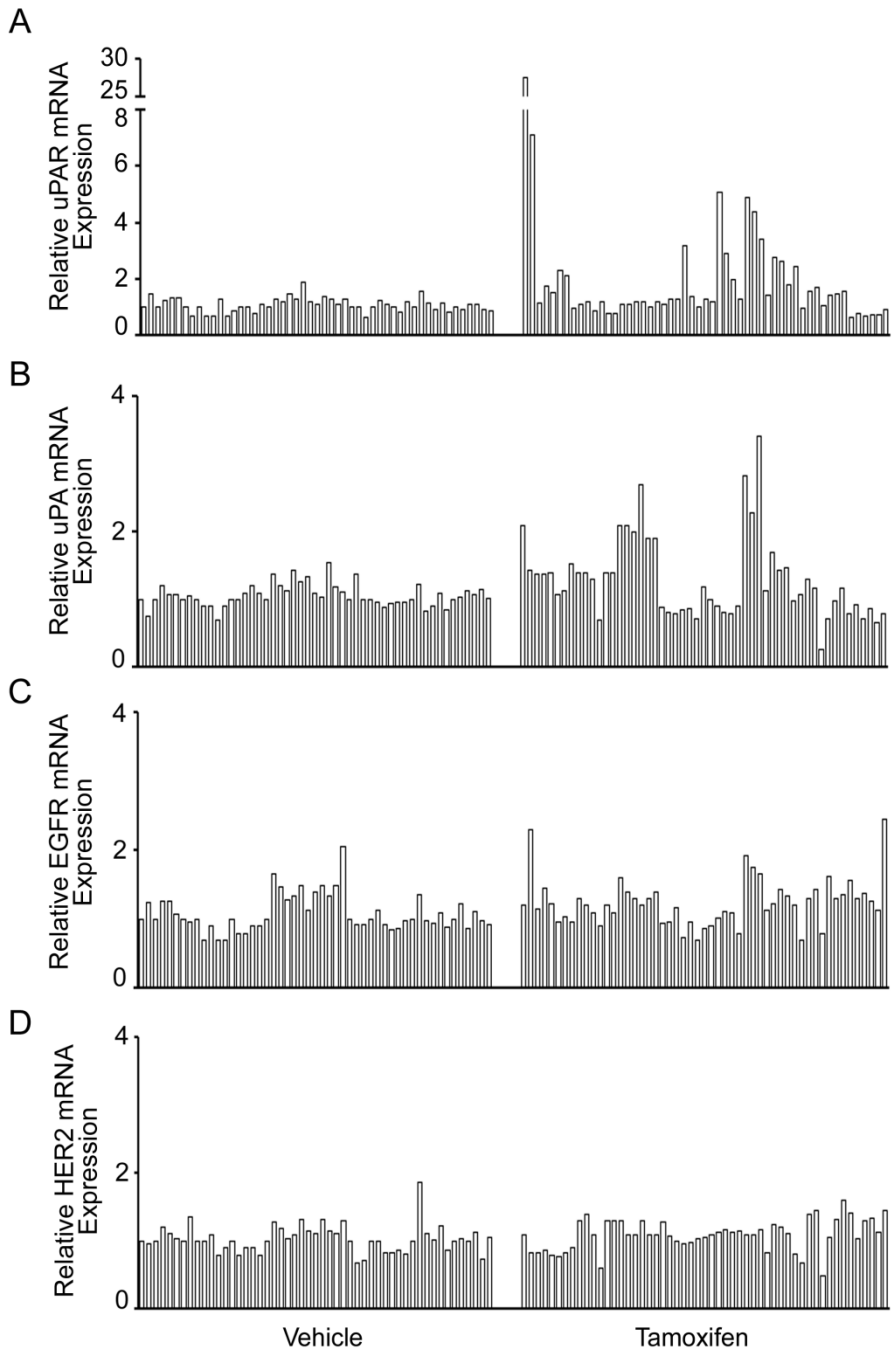

Figure 3-1. TAM treatment selects for increased uPAR expression in MCF-7 cells. MCF-7 cells were treated with 5 μM TAM or with vehicle for two weeks in phenol red-free DMEM supplemented with CTS. *A.* Relative mRNA expression was determined by qPCR for uPAR. *B.* Relative mRNA expression was determined by qPCR for uPA. *C.* Relative mRNA expression was determined by qPCR for EGFR. *D.* Relative mRNA expression was determined by qPCR for HER-2. Each bar depicts a single culture.

Since a significant number of cultures had increased uPAR and uPA mRNA expression in the TAM treated cultures, we performed correlation studies, in order to establish whether their expression was associated. The uPAR expression was weakly correlated with the expression of uPA (Pearson r=0.2726), but was independent of EGFR and HER-2 expression (Fig. 3-2B). These results indicate that uPAR and uPA could be co-regulated in response to Tamoxifen treatment and initiate either uPA-dependent or –independent uPAR signaling in MCF-7 cells.

The culture which exhibited the highest increase in uPAR mRNA also had increased uPA expression. This same culture also showed a 4-fold increase in the AP-1 transcription factor component, FosB, which is known to regulate expression of genes in the uPA/uPAR system (Fig. 3-3) [240-241]. This could offer a potential mechanism by which uPAR mRNA expression is increased or maintained in the presence of Tamoxifen.

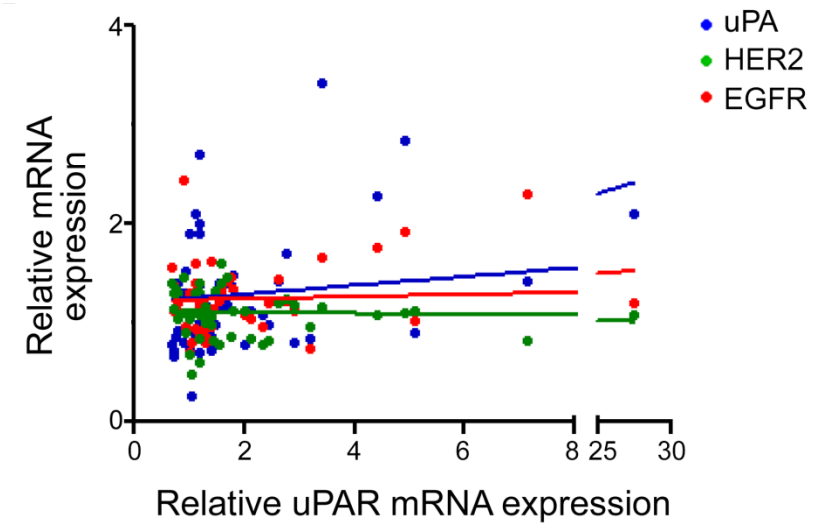

Figure 3-2. uPAR expression is weakly correlated to uPA expression in TAM treated cultures. MCF-7 cells were treated with 5 µM TAM or with vehicle for two weeks in phenol red-free DMEM supplemented with CTS. The level of uPA, EGFR, and HER-2 expression is plotted against the expression of uPAR in 53 TAM-treated cultures. Each dot depicts a single culture.

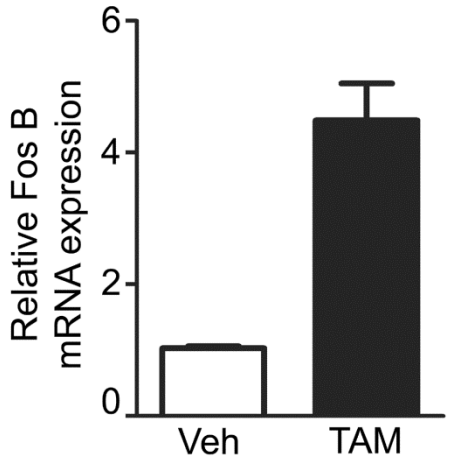

Figure 3-3. FosB expression is increased in TAM treated cultures with high uPAR expression. MCF-7 cells were treated with 5 μM TAM or with vehicle for two weeks in phenol red-free DMEM supplemented with CTS. Relative FosB mRNA expression was determined by qPCR. The expression of FosB was compared between the TAM-treated culture with the highest uPAR mRNA expression (black bar) and a control culture (white bar).

uPAR over-expression promotes TAM resistance and increased cell growth in vitro

In order to further study the role of uPAR on TAM resistance, separate from other signaling molecules, we utilized MCF-7 cells that over-express mouse uPAR and do not express uPA – M3 and M4 cells [239]. M3 cells, M4 cells, and cells transfected with empty vector (EV cells) were treated with 5 μM TAM for six days. The number of viable EV cells decreased by about 40%. Cell death was significantly decreased ($p<0.05$) in cultures of M3 and M4 cells (Fig. 3-4A). In addition mouse uPAR expression significantly increased MCF-7 cell proliferation. In the studies shown in Fig. 3-4B, cells were transferred to SFM for 2 h and then, pulse-treated with bromodeoxyuridine (BrdU) for 2 h. Equivalent results were also obtained when cells were deprived of E2 for up to three weeks before performing experiments.

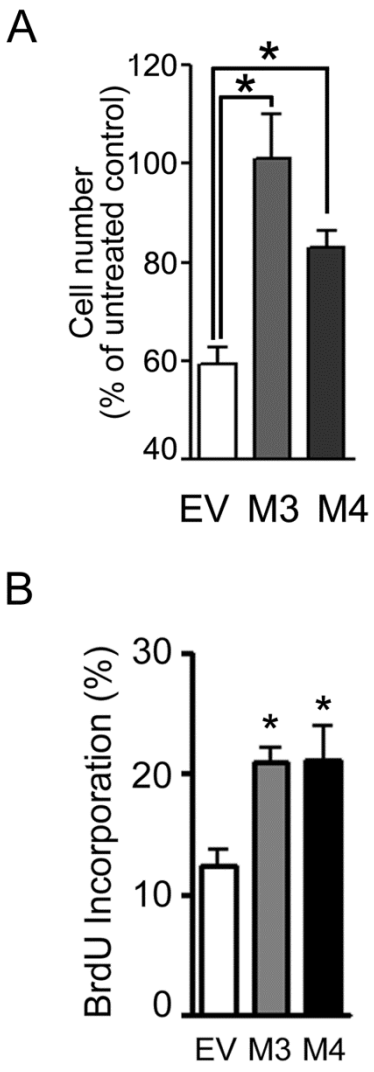

Figure 3-4. uPAR over-expression promotes TAM resistance and increased cell growth *in vitro.* *A.* M3, M4, and EV cells were treated with 5 μM TAM in complete media for 6 days. The decrease in cell number was determined by MTT assay (mean ± SEM, n=3; *,$p<0.05$, Student's t-test). *B.* BrdU incorporation by M3, M4, and EV cells in SFM was determined after exposure to BrdU for 2 h (mean ± SEM, *, $p<0.05$, Student's t-test).

CONCLUSIONS

High levels of certain RTKs such as EGFR and HER-2 contribute to Tamoxifen resistance in MCF-7 cells [154-155]. Increased levels of EGFR and HER-2 have also been implicated in TAM resistance *in vivo* [153]. In this study we demonstrated that increased uPAR expression also promotes MCF-7 cell survival when cells are treated with TAM.

We began this study by performing *in vitro* experiments, which were previously used to identify EGFR and HER-2 as receptors that facilitate resistance to TAM. TAM treatment selected for MCF-7 cells with increased uPAR and uPA expression. The expression of uPAR and uPA was weakly correlated and was independent of the expression of other RTKs such as EGFR or HER-2. This suggests that increased expression and activity of the uPA-uPAR system alone may overcome both the cytostatic and the cytotoxic effects of TAM [25-26, 242]. These results also suggest that even short-term treatment of patients with TAM may select for tumor cells with high levels of uPAR expression which may later contribute to therapy escape.

We have previously documented the pro-survival effect of uPAR signaling in breast cancer cells both in the presence and absence of its ligand uPA [200, 239]. In this work, we also demonstrated that over-expression of mouse uPAR alone, in the absence of endogenous uPA improves the survival of MCF-7 cells treated with TAM. This could be due to the combined effect of the increased proliferation of these cells in addition to their decreased sensitivity to the effects of TAM.

The MCF-7 cells selected by the TAM-treatment are likely to have higher tumorigenic and metastatic potential, similar to cells over-expressing uPAR exogenously [132, 201]. Their increased uPA expression would provide further advantage, activating numerous signaling pathways leading to increased proliferation, survival and migration.

GENERAL CONCLUSIONS

Conclusions

In this thesis work we demonstrated that the GPI-anchored protein uPAR plays an important role in inducing cancer stem cell like qualities in breast cancer cells. This was evident not only by the redistribution of cancer stem cell specific cellular membrane markers, but also by the ability of uPAR over-expressing cells to proliferate and form mammospheres in anchorage independent conditions. Furthermore uPAR over-expressing cells were capable of forming tumors at lower seeding numbers (MDA-MB-468 cells) and with higher incidence (both MDA-MB-468 cells and MCF-7 cells). Interestingly, although uPAR over-expression induces EMT in MDA-MB-468 cells, a process previously implicated in breast cancer stem cell appearance, it does not cause MCF-7 cells to undergo EMT. Nevertheless, both cell lines acquire cancer stem cell like properties when uPAR is over-expressed, indicating that EMT is not necessary for the induction of cancer stem cell qualities. The ability of cells to undergo EMT when uPAR is over-expressed could be dependent on the presence of its ligand uPA. In the absence of uPA, uPAR activates only some of the pathways that are initiated when uPA is abundant [99, 200, 236, 239]. For example, PI3K activation is crucial for the induction of EMT in MDA-MD-468 cells, but this pathway is not activated in the uPAR over-expressing MCF-7 cells, possibly due to the lack of endogenous uPA [99]. Nevertheless, the ERK activation alone was sufficient to induce cancer stem cell like qualities in these cells [239].

The fact that uPAR plays a role in the induction of cancer stem cell like qualities indicates that uPAR might play a much more important role in cancer initiation and development. Although uPAR-initiated signaling has been well documented in recent decades, it is still thought that uPAR's major role is in cancer metastasis and advanced disease [113, 132]. This work suggests that uPAR expression on a small population of breast cancer cells could have consequences that extend further than previously thought. Many advanced cancers have high uPAR expression that could possibly be capturing a later stage of tumor development, in which the cellular population with cancer stem cell like properties has expanded dramatically.

In this work we also demonstrated that uPAR can initiate signaling in the absence of its ligand uPA. Previously, it has been thought that uPA is crucial for uPAR-initiated signaling, inducing conformation changes in uPAR and facilitating its interaction with other membrane partners, such as vitronectin [243]. ERK and Akt activation leading to increased cell survival and proliferation have been exclusively linked to presence of endogenous or exogenous uPA, both in ER-positive and ER-negative cells [172, 200]. The fact that when expressed at high levels, uPAR can also signal independently is reminiscent of how RTKs such as HER-2 could signal in a ligand-independent fashion when expressed at sufficiently high levels. Nevertheless, uPAR alone cannot recapitulate all aspects of uPA-uPAR signaling. For example, uPAR alone activates ERK, but not Akt signaling, in contrast to uPA-uPAR-initiated signaling which activates both [99].

This could be due to further conformational changes in uPAR upon uPA binding or signaling through other membrane partners in addition to vitronectin.

The pro-survival effects of uPAR-initiated signaling could be observed in the absence of uPA, emphasizing the mitogenic effects of ERK signaling. The proliferation and pro-survival effects of uPAR ligand independent signaling could be observed *in vitro* (increased BrdU incorporation, decreased sensitivity to TAM) and *in vivo* (cells forming larger tumors at increased frequency). In wild type MCF-7 cells, increased ERK signaling could be only observed in the presence of exogenous uPA, indicating that the low endogenous uPAR expression is not sufficient to induce ERK activation [172]. When uPAR is over-expressed at levels similar to those in other breast cancer cell lines, such as MDA-MB-231 cells, a significant increase in ERK phosphorylation is observed. Interestingly, in MDA-MB-231 cells, the uPAR expression alone is not sufficient for ERK activation, since neutralization of uPA with uPA specific antibody leads to decreased ERK activation and decreased proliferation [200]. One possible explanation for this discrepancy is that breast cancer cell lines with high expression of uPAR are completely dependent on uPA-uPAR signaling and the neutralization of either of the components of the system leads to immediate negative effects. On the other hand it is possible that there are other fundamental differences between these two cells lines arising from their different origin (basal-MDA-MB-231 vs. luminal-MCF-7) and their ER and PR status [183].

The third part of this thesis work focused on the role of uPAR expression and signaling on the development of TAM resistance in breast cancer and in particular in MCF-7 cells. Previous reports have indicated that several RTKs such as EGFR and HER-2 contribute to TAM resistance in MCF-7 cells, as well as in patients [154-155, 244]. Since uPAR-initiated signaling activates some of the same downstream pathways, we hypothesized that uPAR could also contribute to therapy resistance. We chose to treat MCF-7 cells with higher concentration of TAM over shorter period of time hoping to mimic the toxic amounts of TAM breast cancer cells are exposed to during treatment. In this short time frame we observed an increase in uPAR and uPA expression in a substantial percentage of the treated cultures, although their expression was not correlated. In contrast, we did not see an increase in the expression of HER-2 or EGFR expression. This finding suggested that uPAR expression and uPAR signaling could be providing a first line of defense against the cytostatic and cytotoxic effects of TAM. This is consistent with our previous findings that uPAR could induce cancer stem cell like properties in breast cancer cells, rendering them less sensitive to a variety of therapeutics [41]. The increase in uPAR expression in the TAM treated cells could either be due to increased uPAR mRNA expression or stabilization, or alternatively to recovering cells with higher uPAR levels that are naturally occurring in the cellular population. Although we did not provide a definitive answer to this question, in at least one of the cultures with high uPAR and uPA expression, we also observed an increase in the AP-1 component FosB. The increased expression of components of the AP-1 transcription factor complex

could lead to increased AP-1 activity, which promotes the expression of both uPA and uPAR [240-241, 245]. This finding could provide some insight into the mechanism by which uPAR expression is up-regulated, although further work is necessary.

In addition to being induced by TAM treatment, uPAR expression was also protective against TAM challenge. Cells that over-express uPAR were less sensitive to the effects of TAM and were able to survive better in the absence of estrogen. These findings support the hypothesis that uPAR plays a functional role in the survival of TAM treated cells and the increase of uPAR expression in the TAM-treated MCF-7 cells is not an experimental artifact. These two sets of experiments provide a solid base and warrant further investigation into the role of uPAR in the development of TAM resistance, not only in MCF-7 cells, but also in other cell lines and in patients.

Future Directions

Based on the findings of this thesis work, I have identified a few potential lines of future work which could contribute greatly to the uPAR research field and to the clinical management of cancer patients in general.

I. Characterization of the role of uPAR in epithelial cancer stem cells of different origins

Cancer stem cells, their properties and identification have started to occupy a more central role in our understanding of cancer development and recurrence. Identification of markers associated with stem cell like properties have become paramount in our ability to target them effectively. Identifying uPAR as a cancer stem cell marker in breast cancer not only expands our understanding of the various functions of uPAR, but also improves our understanding of cancer stem cell biology. In addition to playing a role in cancer stem cells of the breast, it has also been previously demonstrated that uPAR plays a role in the maintenance of small cell lung cancer stem cells [150]. That prompts the question whether uPAR might have a broader role in epithelial cancer stem cells, especially in cancers of the pancreas, where uPAR gene amplification has been observed [121].

Once a role for uPAR in inducing cancer stem cell like qualities in other epithelial cancers has been identified, it would be interesting to further investigate the effects of uPAR over-expression *in vivo*. For example, uPAR conditional over-expression in different mouse epithelial tissues would give us a better understanding of the role of uPAR in spontaneous cancers. I hypothesize that if uPAR indeed had oncogene properties, we would be able to observe an increase in spontaneous cancers in these transgenic animals. If this was not the case, these animals could be crossed with other transgenic mice allowing us to observe a

more noticeable increase in cancer incidence, cancer metastasis, or an increased pool of cancer stem cells. Since EGFR has been identified as an important modulator of uPAR signaling in a variety of cancers, the MMTV-EGFR over-expressing transgenic mouse would be an ideal first model organism in which to test the role of uPAR over-expression [246].

In addition to delineating the role of uPAR in cancer stem cells of various origins, it would also be interesting to use uPAR as a biomarker to establish whether a certain therapeutic is targeting the cancer stem cell pool. The expression of uPAR could easily be determined by immunohistochemistry in biopsy samples pre- and post- treatment and the percentage of cells expressing uPAR could be compared. In the samples treated with therapeutics targeting cancer stem cells, we would expect to see a sizeable decrease in the percentage of uPAR expressing cells.

II. Potential ways to target uPAR positive cancer stem cells

In order to capitalize on the role of uPAR in cancer stem cells, it is important to identify ways of treating these cells specifically. In this work and previously we have described several pathways downstream of uPAR that could be initiated in the presence or absence of uPA. Neutralizing these pathways results in reversal of the uPAR induced phenotype [148, 201]. However, most of these molecules are central to a number of different pathways and their systemic

neutralization would be detrimental to patients. The fact that uPAR binding to vitronectin is important to at least some aspects of uPAR-initiated signaling identifies a potential therapeutic niche. It could be possible to neutralize uPAR signaling by preventing its binding to vitronectin. Since uPAR binding to vitronectin is dependent on the tryptophan at position 32, it is possible to design a small peptide which disrupts the interaction between uPAR and vitronectin specifically [204]. This peptide could be modeled after the uPAR-binding amino acid sequence of vitronectin. A similar approach has been previously utilized to design peptides which prevent the binding of various integrins to vitronectin [247]. Although these peptides are successful in neutralizing uPAR signaling, their effect is cell specific, depending on the integrins expressed in the targeted cell population. The effects of a peptide neutralizing the binding of uPAR to vitronectin and implicitly to many different integrins would be more dominant and have an effect in a variety of cells. Although, it is difficult to deliver peptides systemically, novel methods utilizing various techniques are currently in development [248-249]. In addition, such peptides could be initially delivered intratumorally to evaluate their *in vivo* potential.

III. Role of uPAR expression in TAM therapy resistance

Another finding of this work is the ability of uPAR to protect cells against the cytotoxic and cytostatic effects of TAM. Following TAM-treatment, resistant

cell lines were established. It would be interesting to determine whether these cell lines remain TAM resistant after challenge withdrawal or they revert back to their initial phenotype. My hypothesis is that the uPAR expressing cells would maintain their increased uPAR expression, or be able to rapidly up-regulate it in the event of a repeat TAM challenge. Furthermore, it would be also important to verify that the increased uPA and uPAR mRNA expression translates to increased protein levels.

In addition to establishing the uPA and uPAR levels in these cell lines, we would also need to fully characterize their *in vitro* and *in vivo* properties. I hypothesize, that similarly to MCF-7 cells over-expressing uPAR exogenously, these cells would have increased proliferation, migration, and survival after TAM challenge. Despite the lack of EGFR increase, these cells might also exhibit increased EGFR transactivation due to the increased uPAR expression. *In vivo*, I expect these cells to form more tumors in mice in the absence of estrogen, similarly to human uPAR over-expressing MCF-7 cells [201, 239]. These cells might also form substantially larger tumors, since they are conditioned to grow in estrogen-deficient environment.

However, the most interesting questions about therapy resistance can only be answered *in vivo* – in mice or in patients. One way to answer the question whether uPAR contributes to therapy resistance would be to establish MCF-7 cell tumors in the presence of estrogen in mice and then withdraw the estrogen and treat these animals with TAM systemically. If uPAR contributes to therapy

resistance and is induced by TAM treatment, we would be able to recover uPAR-positive tumor cells from these animals. A second way to establish whether uPAR plays a role in therapy resistance in mice is to establish tumors with either control or uPAR over-expressing MCF-7 cells in the presence of estrogen pellets. Once the tumors have reached a certain size, the estrogen pellets could be removed and TAM treatment could start. I hypothesize that the control tumors would decrease in size at a higher rate that the tumors established with the uPAR over-expressing MCF-7 cells. These experiments would give us a more definite answer about the role of uPAR in TAM resistance in breast cancer *in vivo*. Furthermore, we could also utilize other ER-positive breast cancer cell lines, in order to summarize our findings over a larger set of cells.

Since we demonstrated that EGFR is important in uPA-independent uPAR-initiated cell signaling, we could also investigate the effects of EGFR targeting drugs on uPAR-induced TAM resistance. We could establish tumors as previously described and treat them simultaneously with TAM and EGFR inhibitors. It is likely that the combined effect of TAM and EGFR inhibitors would prevent the induction of uPAR expression in MCF-7 cells. It is also expected that the tumors established with uPAR over-expressing MCF-7 cells would be sensitive to EGFR inhibitors and would decrease in size similarly to the tumors established with the control MCF-7 cells. If successful, the results of these experiments could easily be clinically translated, identifying breast cancer tumors with high uPAR expression as good candidates for EGFR targeting therapies.

We could further verify our findings in cancer biopsies and in patients. A few studies have already indicated a possible link between expression of members of the uPAR system and TAM resistance [250]. We could further these studies, by systematically reviewing biopsies from patients who have relapsed after TAM treatment and comparing them to their pre-treatment diagnostic biopsies. I hypothesize that uPAR would be expressed in a substantial number of biopsies recovered from patients with relapsed disease. Furthermore, I hypothesize that patients who have high uPAR expression levels in their pre-treatment biopsies would be more likely to relapse after TAM treatment, regardless of their initial ER status.

Summary

The present work expands greatly on the role of uPAR in breast cancer, introducing new roles for uPAR-initiated signaling. In addition, it presents exciting new opportunities in which uPAR expression could be used in the clinic as a biomarker for EGFR sensitive breast cancer cell populations or as a predictor for TAM resistance and cancer stem cell like qualities. Further experimental and clinical work focusing on these observations would be exciting and could potentially contribute to better understanding of breast and other uPAR-expressing cancers and could lead to more patient customized cancer therapy.

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