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14. ABSTRACT
Although aromatase inhibitors (AIs) have been shown to be highly effective in treating estrogen receptor positive (ER+) breast cancer, a significant percentage of patients either do not respond to AIs or become resistant to them. Studies suggest that resistance to AIs involves a switch from dependence on ER signaling to dependence on growth factor-mediated pathways, such as human epidermal growth factor receptor-2 (HER2). The mechanism by which HER2 is involved in AI resistance remains mostly unclear. It is, therefore, important to elucidate the HER2-mediated pathway that contributes to AI resistance, and to identify other relevant factors involved that can be used as biomarkers of AI resistance or targets for therapy. One such factor may include HIF-1, a heterodimeric transcription factor made up of an inducible alpha (α) subunit and a constitutively expressed beta (β) subunit. Unlike the well-studied role of hypoxia-regulated HIF-1α in a variety of cancers, nonhypoxic regulation of HIF-1α and its role in cancer remains largely unclear. Here we have investigated HIF1α in AI resistance . Results indicate that basal nonhypoxic HIF-1α protein expression 1) is higher in AI resistant cells than in their AI-sensitive parental cells, 2) is regulated by HER2, and in turn 3) regulates cancer stem cell markers and cancer stem cell characteristics that may contribute to drug resistance. Lastly, EZN-2968, a specific HIF-1 RNA antagonist currently in phase 1 clinical trials has shown potential in treating aromatase inhibitor resistance.[a1]

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INTRODUCTION

This research aims to understand the factors and molecular mechanisms involved in drug-resistant breast cancer, specifically aromatase inhibitor resistant breast cancer. Although aromatase inhibitors (AIs; i.e., letrozole) have been shown to be highly effective in treating estrogen receptor positive (ER+) breast cancer, a significant percentage of patients either do not respond to AIs or become resistant to them. Previous studies suggest that resistance to AIs involves a switch from dependence on ER signaling to dependence on growth factor-mediated pathways, such as human epidermal growth factor receptor-2 (HER2). The mechanism by which HER2 is involved in AI resistance remains mostly unclear. It is, therefore, important to elucidate the HER2-mediated pathway that contributes to AI resistance, and to identify other relevant factors involved that can be used as biomarkers of AI resistance or targets for therapy. One such factor may include HIF-1, a heterodimeric transcription factor made up of an inducible alpha (α) subunit and a constitutively expressed beta (β) subunit. HIF-1 regulates genes important for cell survival, metabolic adaptation, and angiogenesis. Oxygen (O_2) tension is a well-known regulator of HIF-1α, but other factors independent of O_2 can also regulate it. Thus, the purpose of this study is to determine the potential role of nonhypoxic HIF-1 in aromatase inhibitor resistant breast cancer, and whether it could be used as a diagnostic marker and therapeutic target. In vitro breast cancer cell studies, as well as and in vivo xenograft tumore studies will be conducted to test this hypothesis. In addition, EZN-2968, a specific RNA antagonist that specifically targets HIF-1α and is currently in phase 1 clinical trials will be investigated as a potential therapeutic drug.

BODY

Specific Aim 1: To determine the role of ERα and HER2 in the regulation of nonhypoxic HIF-1α expression and activity in letrozole-resistant breast cancer cells (LTLTCa cells).

Task 1: HER2, ERα, HIF-1α, HIF-1β, and β-actin protein expression in MCF-7Ca, LTLTCa, and MCF-7/HER2 cells under basal, nonhypoxic conditions will be determined by western blot analysis.
Experiments: *Experiments for to accomplish this task were reported in the 2012 annual summary and in the manuscript tentatively accepted for publication in Breast Cancer Research.*

Task 2: Effect of inhibiting ERα and/or HER2 on HIF-1α protein expression in MCF-7/HER2 cells will be determined by western blot analysis
Experiments: *Experiments for to accomplish this task were reported in the 2012 annual summary and in the manuscript tentatively accepted for publication in Breast Cancer Research.*

Task 3: HIF-1α phosphorylation state in LTLTCa cells will be determined via λ-phosphatase treatment of protein followed by western blot analysis.
Experiments: *Experiments for this task were accomplished and explained in 2012 annual summary.*

Task 4: Effect of inhibiting ERα and/or HER2 on HIF-1α phosphorylation state in MCF-7/HER2 cells will be studied via λ-phosphatase treatment of protein followed by western blot analysis.
Experiments*: Experiments for this task were accomplished and explained in 2012 annual summary.*

Specific Aim 2: To define HIF-1α's role in letrozole-resistant cell phenotype and in xenograft growth.

Task 5: Effect of HIF-1α inhibition on LTLTCa cell characteristics in vitro will be investigated

5.a) Dose experiment to determine the most effective, yet non-toxic dose of EZN-2968 that can inhibit HIF-1α protein expression in LTLTCa cells will be done by MTT assay and western blot analysis.
Experiments: *Experiments to accomplish this task for BCRP were completed and reported in the 2012 annual summary and in abstract #95 at the annual AACR meeting (2013).*

5.b) Effect of EZN-2968 on cancer stem cell characteristics of LTLTCa cells will be studied:

5.b.i) Effect of EZN-2968 on side population percentage in LTLTCa cells will be determined by Hoecsht 33342 dye efflux and flow cytometry.
Experiments*: Experiments to accomplish this task were completed and reported in the 2012 annual summary and in abstract #95 at the annual AACR meeting (2013).*

5.b.ii) Effect of EZN-2968 on CD44 and CD24 positivity of LTLTCa cells will be determined by flow cytometry.
Experiments: *Experiments to accomplish this task were completed and reported in the 2012 annual summary and in abstract #95 at the annual AACR meeting (2013).*

5.b.iii) Effect of EZN-2968 on mammosphere formation of LTLTCa cells will be determined.
Experiments: *Experiments to accomplish this task have been completed. It was previously reported in the 2012 annual summary that Qiagen HIF-1α siRNA treatment decreased mammosphere formation in LTLTCa cells. Since then, the effect of EZN-2968 on mammosphere*

*formation was also determined. In agreement with Qiagen HIF-1α siRNA, EZN-2968 also significantly decreased mammosphere formation by ~40% (**Figure 1**).*

5.b.iv) Effect of EZN-2968 on microtentacle scoring in LTLTCa cells will be determined by transfection with vector expressing membrane-localized GFP followed by fluorescence microscopy.

***Experiments:** Experiments to accomplish this task have been started and the final experiments are currently ongoing, in collaboration with the laboratory of project mentor Dr. Stuart Martin (UMB). Previous data indicated that ERα+/HER2-, letorozole-sensitive cells, parental MCF-7Ca cells expressed little or no basal HIF-1α protein and significantly lower microtentacle expression than ER-/HER2+, letrozole-resistant LTLTCa cells (refer to project narrative and 2012 annual summary). As shown in **Figure 2**, upregulation of HIF-1α expression by treatment with 100 µM CoCl2 for 2h, increased the number of MCF-7Ca cells that exhibited microtentacle to levels comparable to MDA MB 436 cells, which served as a positive control cell line. CoCl2 is a hypoxia mimetic that prevents HIF-1α proteosomal degradation. It was used in the microtentacle scoring experiment because it allowed for cells to be handled in room air without risk of O2-dependent degradation of HIF-α. The converse experiment of determining the effect of HIF-1α inhibition (via EZN-2968) on microtentacle scoring in LTLTCa cells is currently on going and will be accomplished within the next month.*

5.b.v) Effect of EZN-2968 on cytoskeletal components of microtentacles (vimentin and detyrosinated tubulin) will also be assessed by immunofluorescence

***Experiments:** Experiments to accomplish this task have been conducted and are ongoing. The effect of EZN-2968 on cytoskeletal components (ex. vimentin) was done by real-time RT-PCR and western blot analysis, which are more easily quantifiable. As shown in **Figure 5**, EZN-2968 significantly decreased vimentin mRNA (by ~50%) and protein compared to negative control EZN-3088. Western blot results for B-actin will be completed within 1 week, but equal total protein was loaded per well. Real-time RT-PCR and western blot analyses for glu-tubulin will be completed within the two months. However, it is expected that EZN-2968 will not have significant effect on glu-tubulin expression. This is based on the fact that glu-tubulin expression was not significantly upregulated in MCF-7Ca or LTLTCa cells incubated under hypoxic conditions (1% O2) vs. nonhypoxic conditions (20% O2) (Figure 2).*

***Additional experiments**: Additional experiments were done to correlate EZN-2968's effects on vimentin expression with its effects on invasion and migration potential. Vimentin is associated with invasion and migration of cancer cells (Satelli et al. 2011). EZN-2968 reduced the number of DAPI-stained LTLTCa cells that migrated through a matrigel transwell within 24 h compared to negative control EZN-3088 (**Figure 7**). In agreement with these results, Qiagen HIF-1α siRNA also reduced invasion and migration of LTLTCa cells through a matrigel transwell compared to negative control siRNA.*

Task 6: Effect of HIF-1α inhibition on LTLTCa cell characteristics in vivo in xenograft models will be investigated.

This task has been delayed to Year 3 of the grant due to a back log of xenograft experiments in the lab and problems in 2012-2013 with infection issues among xenograft mice. A number of xenograft experiments were affected by a contagious skin infection among experimental mice. This led to issues with accurately measuring

xenograft tumors and to a daily subcutaneous and topic antibiotic regimen for all mice. In consultation with project mentor, Dr. Angela Brodie, it was decided that due to issues in other xenograft experiments, it was best to delay the xenograft experiments under Task 6 until Year 3 of the grant when issues had been resolved. They have been resolved now.

*In the meantime, however, frozen comparably-sized MCF-7Ca xenograft tumors collected previously during a xenograft experiment conducted in early 2012 for another project were analyzed. In this xenograft tumor experiment, representative MCF-7Ca xenograft tumors were collected from mice that were either continued to be treated with androstenedione (control) for 8 weeks or were then treated with letrozole for 1-16 weeks. In agreement with previous results (Jelovac et al. 2005), HER2 protein expression was increased with 2-4 weeks of letrozole treatment compared to androstenedione control (**Figure 3**). Interestingly, upregulation of HER2 continued, though inconsistently, through 16 weeks when tumors generally become resistant to letrozole's growth inhibitory effects. HIF-1α expression was also low in androestenedione (control) tumors and increased within 2-4 weeks of letrozole treatment.*

Based on these Western blot results, the proposed xenograft experiments have been modified and streamlined to ensure that task 6 is completed within the time frame of the grant. In the first experiment (EZN-2968 dose response), since changes in HIF-1α expression were observed within 4 weeks of letrozole, treatment with EZN-2968 will begin during week 4 of letrozole treatment (week 10 of experiment) and continued until week 8 of letrozole (week 14 of experiment). While the first experiment is being conducted, experiments 2 (ability of EZN-2968 to prolong letrozole-sensitivity of xenograft tumors) and 3 (ability of EZN-2968 to inhibit growth of letrozole-resistant xenograft tumors) will be started.

***Current Conclusions:** Current findings further support the hypothesis that nonhypoxic HIF-1 is regulated by HER2 in vitro and in vivo. HIF-1α protein expression temporally correlated with upregulation of HER2 in letrozole-treated xenograft tumors. Current findings also provide additional evidence that HIF-1 plays a role in the cancer stem cell phenotype of LTLTCa cells (ex. microtentacle formation and invasion and migration through matrigel transwell). Upregulation of HIF-1α in MCF-7Ca cells increased the number of cells exhibiting microtentacles. Conversely, inhibition of HIF-1α decreased the number of LTLTCa cells that invaded and migrated through matrigel transwell. HIF-1 likely plays a role in these characteristics via regulation of cytoskeletal protein vimentin, as changes in HIF-1α expression led to concomitant changes in vimentin mRNA and protein expression. Vimentin has been previously shown to be involved in both microtentacle formation and invasion and migration of breast cancer cells. Lastly, these current findings also further suggest the potential effectiveness of EZN-2968 in inhibiting letrozle-resistant breast cancer cell characteristics.*

Specific Aim 3: To identify HIF-1 target genes that serve as markers of letrozole resistance. (1-1.5 years)
Although this task was not scheduled to start in Years 1 or 2 of the grant, initial experiments were conducted 1) since task 5.b.iv and 5.b.v were being delayed, and 2) to provide data for a published journal article (Gilani et al. Breast Cancer Res Treatment, 2012).

Task 7: To compare expression between in MCF-7Ca and LTLTCa cells of known HIF-1 targets involved in cancer

7.a) To compare protein expression of known HIF-1 targets in MCF-7Ca and LTLTCa cells by western blot analysis.

***Experiments:** Experiments to accomplish this task have been conducted. Experiments to accomplish this task for BCRP were completed and reported in the 2012 annual summary and in Gilani et al. 2012,*

*Since then, expression of vimentin protein in MCF-7Ca and LTLTCa cells was determined by western blot analysis. Vimentin was significantly increased (2.1-fold, $p<0.05$) in LTLTCa cells vs. MCF-7Ca cells (**Figure 2**). As mRNA expression of the other genes studied (refer to task 9) were either not upregulated in LTLTCa cells vs. MCF-7Ca cells, or were not consistently inhibited by HIF-1α siRNAs (Qiagen and EZN-2968), their protein expression was not analyzed for this task.*

7.b) To compare mRNA expression of known HIF-1 targets in MCF-7Ca and LTLTCa cells by real-time RT-PCR analysis.

Experiments: *Experiments to accomplish this task have been conducted. Experiments to accomplish this task for BCRP were completed and reported in the 2012 annual summary and in the manuscript tentatively accepted for publication in Breast Cancer Research. Since then, RT-PCR analyses of other known HIF-1 target genes in MCF-7Ca vs. LTLTCa cells have been done. As indicated in **Figure 4**, demonstrated significant overexpression of vimentin (1.3-fold±0.09 vs. MCF-7Ca 1.0±0.02), angiopoietin like factor 4 (1.9-fold±0.05 vs. MCF-7Ca 1.0±0.02), endothelin 1 (3.0-fold±0.14 vs. MCF-7Ca 1.0±0.03), fibronectin (35.8-fold±1.74 vs. MCF-7Ca 1.0±0.07), and BCRP (2-fold±0.6 vs. MCF-7Ca 1.0±0.2) mRNA expression in LTLTCa cells compared to MCF-7Ca cells. As HIF-1 is a transcription factor that activates target genes, these upregulated genes were studied further in task 9. Interestingly, CXCR, MMP-2, and Kit ligand mRNA expression were significantly decreased compared to MCF-7Ca cells. These genes were not studied further in this project, but will likely be investigated in the future in a separate project.*

Task 8: To determine whether HIF-1α binds to promoters of genes overexpressed in LTLTCa cells by chromatin immunoprecipitation.

Experiments: *Experiments to accomplish this task for BCRP were completed and reported in the 2012 annual summary and in the manuscript tentatively accepted for publication in Breast Cancer Research. Experiments to accomplish this task for vimentin are ongoing and will be completed in 2 weeks. Although previous studies have indicated that vimentin is a HIF-1 target gene (Semenza. 2012), the hypoxia responsive element (HRE) to which HIF-1 binds in order to regulate vimentin gene expresson has not been identified. We have currently, analyzed the vimentin promoter and have identified 2 potential HRE sites, based on sequence (**Figure 5**). Primers have been designed and ordered, and chromatin immunoprecipitation experiments will be conducted on existing promoter DNA samples when they arrive (within the next 2 weeks). As mRNA expression of other genes studied (refer to task 9) were either not upregulated in LTLTCa cells vs. MCF-7Ca cells, or were not consistently inhibited by HIF-1α siRNAs (Qiagen and EZN-2968), their protein expression was not analyzed further for this task. These other genes will likely be studied in the future as part of a separate project investigating possible causes of the discrepancies between Qiagen siRNAs and EZN-2968.*

Task 9: To determine whether EZN-2968 decreases expression of known HIF-1 targets in LTLTCa cells.

Experiments: *Experiments to accomplish this task have been conducted. Experiments to accomplish this task for BCRP were completed and reported in the 2012 annual summary and in abstract #95 at the annual AACR meeting (2013). The effect of HIF-1α inhibition by both Qiagen HIF-1α siRNA and EZN-2968 on mRNA expression of the known HIF-1 target genes found to be upregulated in LTLTCa cells was determined by RT-PCR analysis. As expected, both Qiagen HIF-1α siRNA and EZN-2968 significantly decreased HIF-1α mRNA expression in LTLTCa cells (**Figure 6**). However, among the known HIF-1 target genes identified in task 7.b, only vimentin mRNA expression was decreased by both Qiagen HIF-1α siRNA and EZN-2968. mRNA expression of angiopoietin like factor 4, endothelin 1, and fibronectin were not consistently inhibited along with HIF-1α mRNA (**Figure 6**). Furthermore in some cases, there was an increase in mRNA expression when HIF-1α mRNA expression was reduced. Thus, these genes were not studied further in this project. Investigations into their differential regulation by HIF-1 will be conducted in a separate future project.*

Task 10: To determine importance of identified HIF-1 target genes in letrozole-resistant cells by inhibiting target genes via specific pharmacological inhibitors or siRNA followed by in vtiro cancer cell assays (as specific aim 2)

Experiments: *Experiments to accomplish this task have conducted. Findings regarding the importance of cancer stem cell marker and efflux protein BCRP were explained in the 2012 annual summary and were published (Gilani et al. 2012). Since then, the importance of vimentin in LTLTCa cells was also studied. Vimentin is a cytoskeletal protein overexpressed in a number of epithelial cancers, and is associated with accelerated tumor growth, invasion, and poor prognosis (Satelli et al. 2011). Pertinent to current findings, overexpression of vimentin is associated with highly aggressive and metastatic breast cancer cells and increased microtentacle formation (Whipple et al. 2008; Matrone et al. 2010). Thus, studies have been conducted to determine the role of vimentin in LTLTCa cells. Current results indicate that there is a correlation between HIF-1α and vimentin expression and metastatic potential, as indicated by microtentacle formation and invasion and migration ability. In* ***Figure 2****, increased expression of HIF-1α in less aggressive and metastatic MCF-7Ca cells correlated with 1) at least an 8-fold increase in vimentin mRNA and protein expression. and 2) a ~50% increase in the percentage of cells exhibiting microtentacles. In* ***Figure 7,*** *treatment of LTLTCa cells with either HIF-1α siRNA (by Qiagen or EZN-2968) or vimentin siRNA (Qiagen), inhibited invasion and migration ability as determined by transwell migration through a matrigel and DAPI staining.*

Current Conclusions: *Current findings provide additional evidence of a HER2-nonhypoxic HIF-1 signaling pathway in LTLTCa cells, as HIF-1α siRNA EZN-2968 significantly decreased HIF-1α and BCRP in LTLTCa cells. Other cancer stem cell markers were also affected. Lastly, similar to the effects of HIFα siRNAs, BCRP siRNA decreased cancer stem cell characteristics in LTLTCa cells.*

KEY RESEARCH ACCOMPLISHMENTS

- Evidence is provided that HER2 and HIF-1α expression are temporally correlated in vivo in letrozole-treated tumor xenografts.
- Further evidence is provided that nonhypoxic HIF-1 is involved in regulating cancer stem cell characteristics in letrozole-resistant breast cancer cells.
  - Specific inhibition of HIF-1α by siRNA decreased expression of vimentin, a cytoskeletal protein associated with microtentacle expression and invasion and migration ability.
  - Specific inhibition of HIF-1α by siRNA decreased cancer stem cell characteristics (i.e., mammosphere formation, microtentacle expression, and invasion and migration ability).
- Vimentin is involved in regulating cancer stem cell characteristics in letrozole-resistant breast cancer cells.
- Further evidence is provided for the effectiveness of EZN-2968 in inhibiting HIF-1α expression, expression of HIF-1 target gene vimentin, and letrozole-resistant cancer stem cell characteristics.

REPORTABLE OUTCOMES

Abstracts and Presentations:

- Armina Kazi, Amanda Schech, Saranya Chumsri, Preeti Shah, Gauri Sabnis, Yael Gau, Angela Brodie. Inhibition of non-hypoxic HIF-1 expression in letrozole-resistant breast caner cells reduces their cancer stem cell characteristics. Abstract #95 peer-reviewed and presented at American Association of Cancer Researchers (AACR) 2013 Annual Meeting.

Manuscripts:

- Tentatively Accepted:
  - Armina A Kazi, Rabia A Gilani, Amanda J Schech, Saranya Chumsri, Gauri J Sabnis, Preeti Shah, Olga Goloubeva, Shari Kronsberg and Angela H Brodie. Nonhypoxic regulation and role of hypoxia-inducible factor 1 in aromatase inhibitor resistant breast cancer. Tentatively accepted to Breast Cancer Research.

Awarded:

Loyola University Maryland Faculty Development Grant for Summer of 2013. This summer research grant is based on

Employment:

- Have maintained tenure-track assistant professor position Loyola Univerisity Maryland

New research collaborations that will be used to appy for R15 in 2014.

- Have regularly or periodically met with mentors Dr. Angela Brodie, Dr. Saranya Chumsri, and Dr. Stuart Martin.
- In meeting with project mentor Dr. Stuart Martin (UMB) to discuss research progress, I have started a new research collaboration with his laboratory investigating the role of hypoxia on microtentacle formation in breast cancer cells.
- In meeting with project mentor Dr. Saranya Chumsri (UMMC) to discuss research progress, I have started a new research collaboration with her and the Greenbaum Cancer Center investigating the response of postmenopausal obese and overweight patients with ER+ breast cancer to neoadjuvant aromatase inhibitor therapy. My role in this clinical trial is to analyze HIF-1 and miRNA (particularly miRNAs that are associated with HER2 and HIF-1) profiles in patient samples.

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CONCLUSIONS

Prior to this study, AI resistance was associated with increased dependence on growth factors and decreased dependence on ERα. However, the role that such molecular changes play in AI resistance and the mechanism by which they elicit their effects were not known. Results from this study demonstrated that nonhypoxic expression of HIF-1 mediates HER2's effects on letrozole-resistance. Specifically, HER2-activated PI3K/Akt pathway increases HIF-1α protein synthesis in LTLTCa cells. HIF-1α, in turn, upregulates expression of BCRP and vimentin, and contributes to letrozole resistance and stem cell characteristics of LTLTCa cells.

Nonhypoxic regulation of HIF-1 expression and activity in LTLTCa cells is due to HER2-activated PI3K/Akt pathway. This is consistent with findings by others indicating hypoxia independent upregulation of HIF-1α in cancer cells by loss of function of tumor suppressor genes and gain of function of oncogenes (Semenza. Trends Pharmacol Sci. 2012). The oncogene HER2/neu, in particular has been previously associated with nonhypoxic HIF-1 (Laughner et al. Mol Cell Biol. 2001; Li et al. Cancer Research. 2005). Laughner et al. and Li et al have demonstrated that transfection of HER2 into NIH/3T3 cells or activation of HER2 in MCF-7 cells led to activation of the PI3K/Akt pathway, and subsequent increased HIF-1 expression via protein synthesis and HIF-1 transcriptional activity. Our current results provide additional evidence that this HER2-PI3K/Akt pathway-HIF-1 signaling mechanism can occur endogenously in HER2+ cells in vitro and in vivo (xenograft tumors) and has physiological relevance (i.e., regulation of cancer stem cell characteristics) as well as potential clinical implication (i.e., AI resistance).

Inherent upregulation of HIF-1α protein expression under nonhypoxic conditions is another novel finding in AI-resistant breast cancer. There is precedence for associating HIF-1 expression with drug resistance in different cancer cell types, including chronic myeloid leukemia cells (Zhao et al. Oncogene. 2010), gastric cancer cells (Liu et al. Mol Cell Biochem. 2007), non-small cell lung cancer cells (Song et al. Cancer

Chemother Pharmacol. 2006), and even breast cancer cells (Flamant et al. Mol. Cancer 2010). However, these previous cases involved hypoxia-induced, HIF-1α rather than the nonhypoxic HIF-1. Our findings are also consistent with previous clinical evidence that HIF-1α is associated with letrozole resistance. Generali et al demonstrated that increase p-MAPK and HIF-1α protein expression were significant determinants of primary letrozole resistance in breast cancer patients. In contrast, increased ERα and decreased p-MAPK were significant determinants of response to letrozole treatment (Generali et al. J Clin Oncol 2009). The protein expression patterns observed by Generali et al. are similar to what is observed in letrozole-resistant LTLTCa and –sensitive MCF-7Ca cells, respectively (Figures 1 and 6 of 2012 annual summary). Although these clinical findings involve de novo letrozole resistance, they still correlate with, and likely pertain to, our laboratory's results on acquired letrozole resistance. These results combined suggest that HIF-1 is involved in both de novo and acquired AI resistance, and therefore could be therapeutically targeted to prevent and treat resistance to letrozole and the other AIs. Lastly, this study indicates that HIF-1 may contribute to letrozole resistance by mediating HER2's effects on target genes, such as BCRP and vimentin. HIF-1 regulated vimentin, in particular, has now been associated with letrozole-resistant cells and their expression of microtentacles and invasion and migration ability (Figures 2-5 and 6).

Overall, this current study provides further evidence that nonhypoxic HIF-1α is an important factor in letrozole resistant breast cancer cells, particularly their ability to invade and migrate. Based on this, a proposed model of acquired AI-resistance may involve the following scenario: under nonhypoxic conditions, when the breast cancer cell population and tumor size have been reduced by letrozole treatment and prior to significant tumor hypoxia, a switch from ERα- to growth factor (i.e., HER2)-mediated signaling occurs, which leads to increased HIF-1α expression and activation of HIF-1 target genes that contribute to AI resistance. Consequently, inhibition of HIF-1 expression and/or activity would prolong cancer cell sensitivity to AIs and prevent recurrence and metastasis. Indeed, a number anti-cancer drugs in clinical use are also known to inhibit HIF-1 (Semenza. Oncogene. 2010). They include HER2 inhibitor, trastuzumab (Laughner et al. Mol Cell Biol.

2001) and lapatinib. Furthermore, as demonstrated in this study that HIF-1 is regulated mainly via PI3K/Akt pathway, inhibition of downstream affecter of this pathway using mTOR inhibitors such as rapamycin, temsirolimus/CCI-779 and everolimus/RAD-001, can also be considered (Laughner et al. Mol Cell Biol. 2001; Majumder et al., Nat Med. 2004; Faivre et al. Nature Reviews Drug Discovery. 2006; Thomas et al.Nat Med. 2006). Novel to this study, EZN-2968, a specific HIF-1α mRNA inhibitor (siRNa), which has previously been shown to reduce cancer cell viability and xenograft tumor growth and is currently under phase I clinical trial (Greenberger et al. Mol Cancer Ther. 2008), appears to be a promising drug to prevent and treat aromatase inhibitor resistance.

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SUPPORTING DATA: Figure legends and Figures

Figure 1. Effect of EZN-2968 on mammosphere formation and cell proliferation in LTLTCa cells.
A, LTLTCa cells were plated in passage media and then treated with 10 μM EZN-3088 negative control siRNA, or 10 μM EZN-2968 HIF-1α -specific LNA for 48 h. Cells were then collected and resuspended in mammosphere media on low-attachment cell culture wells. Results are expressed as number of mammospheres counted per 1,000 cells plated after 14 days in culture (mean ± SEM, n = 6 samples/group; * vs. vehicle, $p < 0.05$; † vs. -3088 negative control). EZN-2968 was confirmed to decrease BCRP expression (0.35- and 0.15-fold vs. negative control, $p < 0.01$; data not shown).

Figure 2. Expression of cytoskeletal proteins in MCF-7Ca and LTLTCa cells, and effect of HIF-1^{α} upregulation either by hypoxia or CoCl2 on cytoskeletal proteins vimentin and glu-tubulin and on microtentacle formation.
A. MCF-7Ca and LTLTCa cells were plated in their respective passage media and allowed to attach under normal cell culture conditions (20% O2). 24 h prior to protein extraction, cells either remained under normal cell culture conditions (20% O2) or transferred to hypoxia conditions (1% O2). Total protein was extracted and HIF-1^{α}, glu-tubulin, vimentin, and β-actin protein were analyzed by Western blot. Densitometry results are expressed as fold-change compared to MCF-7Ca at 20% O2 after normalization to β-actin (mean ± SEM, n = 6 independent cell samples/group; * vs. MCF-7Ca at 20% O2, $p < 0.01$). B, MCF-7Ca and LTLTCa cells were plated in their respective passage media and allowed to attach under normal cell culture conditions (20% O2). 24 h or 48 h prior to RNA extraction, cells either remained under normal cell culture conditions (20% O2) or transferred to hypoxia conditions (1% O2). Total mRNA was extracted and vimentin and 18S rRNA were analyzed by real-time RT-PCR. Real-time results are expressed as the fold-change in mRNA levels compared with 0 h (cells that remained at 20% O2) after normalization to 18S rRNA (mean ± SEM, n = 6 samples/group). C, MDA MB 436 and MCF-7Ca cells were plated and cultured in their respective passage media under normal cell culture conditions. When cells were 80-90% confluency, they were treated with either vehicle or 100 uM CoCl2 for 2 h prior to microtentacle scoring. Results are expressed as percent of cells counted with microtentacles (i.e., frequency).

Figure 3. Effect of letrozole treatment on protein expression in MCF-7Ca xenograft tumors.
Total protein was extracted and HER2, HIF-1^{α}, and β-actin protein were analyzed by Western blot from tumors obtained from a previously conducted xenograft tumor experiment. In this previous experiment, MCF-7Ca xenografts were grown in female ovariectomized mice. When tumors reached measurable size ~300 mm^3, mice were treated with 100 μg/day androstenedione (A; n=2) or letrozole (100 μg/day androstenedione and 10 μg/d letrozole. At certain timepoints of treatment (1-16 weeks) 2 representative mice from each group were euthanized and tumors were collected and store in -80 C. Shown are representative western blots.

Figure 4. Comparison of mRNA expression in LTLTCa and MCF-7Ca cells.
LTLTCa and parental MCF-7Ca cells were plated and cultured in their respective passage media under normal cell culture (nonhypoxic) conditions. A, Total RNA was extracted and vimentin, angiopoietin-like 4, CXCR, endothelin 1, fibronectin, MMP-2, kit ligand, BCRP, and Twist mRNA, and 18S rRNA were analyzed by real-time RT-PCR analysis. Results are expressed as the fold-change in mRNA levels compared with MCF-7Ca cells after normalization to 18S rRNA (mean ± SEM, n = 6 samples/group; * vs. MCF-7Ca, $p < 0.05$).

Figure 5. Human vimentin promoter (A31892.1). Potential hypoxia response elements (5'-CGTG-3') have been identified in yellow.

Figure 6. Effect of HIF-1α siRNA on mRNA expression in LTLTCa cells.
A, LTLTCa cells were treated with either negative control siRNA (Qiagen or EZN-3088) or HIF-1α siRNA (Qiagen or EZN-2968) for 48 h. Total mRNA was extracted and HIF-1α, vimentin, angiopoietin like factor 4, endothelin 1, and fibronectin mRNA, and 18S rRNA were analyzed by real-time RT-PCR. Real-time results are expressed as the fold-change in mRNA levels compared with negative control after normalization to 18S rRNA (mean ± SEM, n = 4 samples/group). B, LTLTCa cells were treated with either negative control siRNA (EZN-3088) or HIF-1α siRNA (EZN-2968) for 72 h. Total protein was extracted and vimentin protein was analyzed by Western blot analysis.

Figure 7. Effect of HIF-1α inhibition on

LTLTCa cells (n=3 per treatment group) were plated in passage media and treated with either no siRNA (negative and positive control), negative control siRNA (Qiagen or EZN-3088) or HIF-1α siRNA (Qiagen or EZN-2968), or vimentin siRNA (Qiagen) for 48 h after which cells were serum deprived overnight. Cells were then collected and seeded in matrigel transwells containing either serum free media (negative control) or passage media (remaining treatment groups). After 24 h, cells were fixed to membranes and celsl that have invaded and migrated through matrigel transwell were stained with 1:2500 DAPI. Images were taken of each sample and representative blots are shown. Negative control samples were not treated with siRNA and were incubated with serum free media in the transwell prior to imaging. Positive control samples were not treated with siRNA and were incubated with passage media in the transwell prior to imaging.

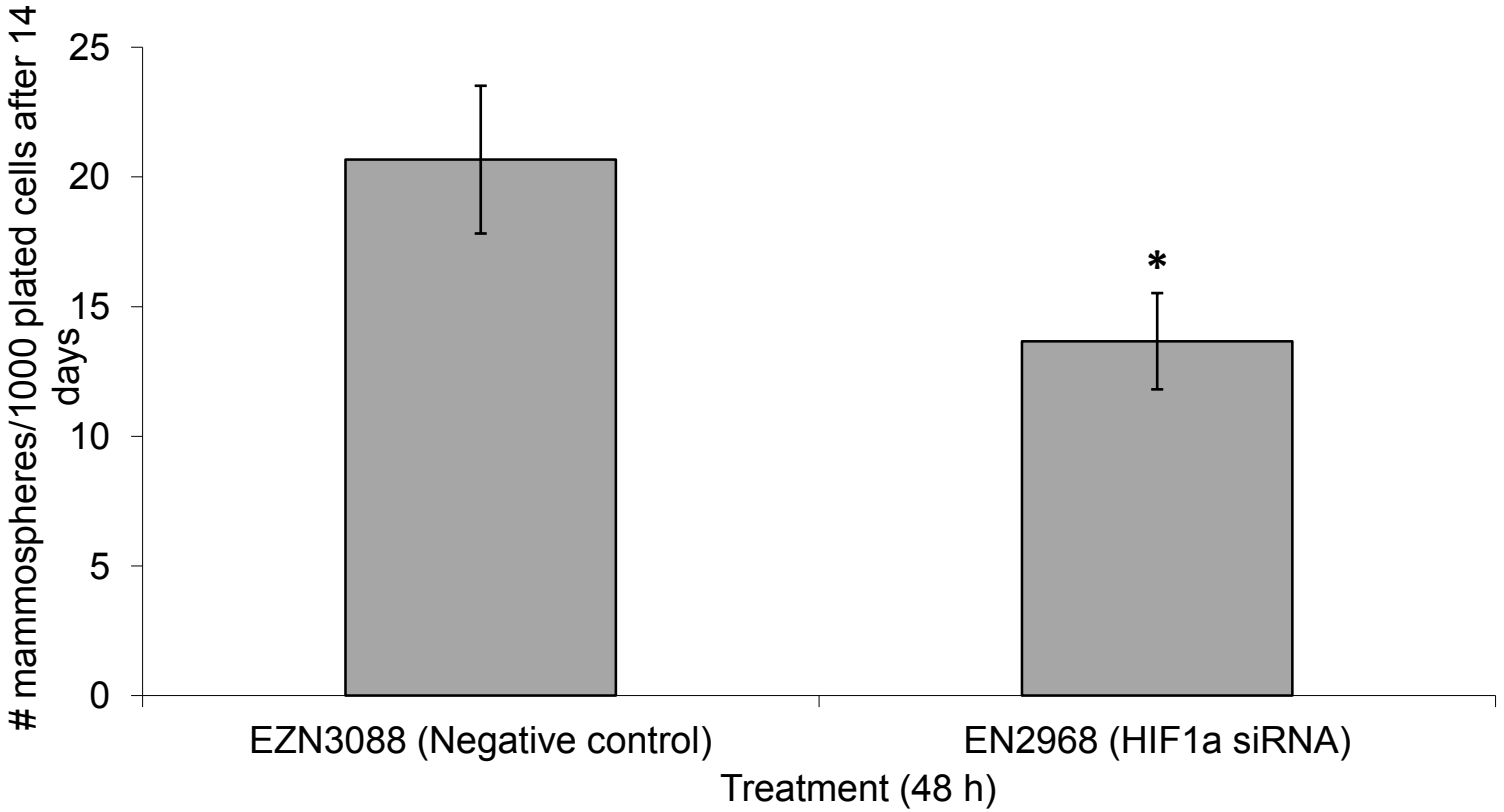
mammospheres/1000 plated cells after 14 days
25
20
15
10
5
0
*
EZN3088 (Negative control)
EN2968 (HIF1a siRNA)
Treatment (48 h)

Figure 1.

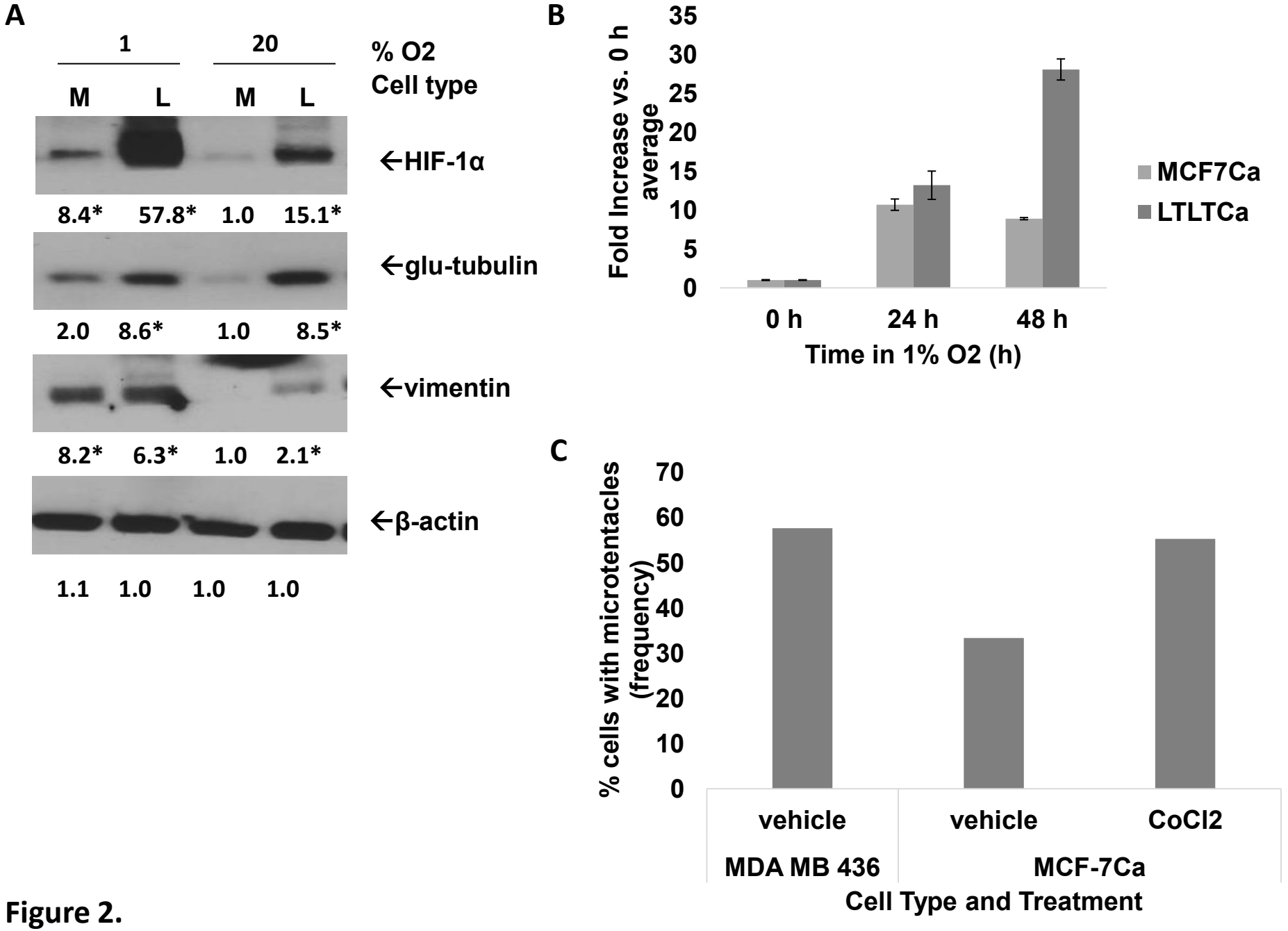
A
1
20
% O2
Cell type
M
L
M
L
←HIF-1α
8.4*
57.8*
1.0
15.1*
←glu-tubulin
2.0
8.6*
1.0
8.5*
←vimentin
8.2*
6.3*
1.0
2.1*
←β-actin
1.1
1.0
1.0
1.0
B
Fold Increase vs. 0 h average
35
30
25
20
15
10
5
0
0 h
24 h
48 h
Time in 1% O2 (h)
MCF7Ca
LTLTCa
C
% cells with microtentacles (frequency)
70
60
50
40
30
20
10
0
vehicle
vehicle
CoCl2
MDA MB 436
MCF-7Ca
Cell Type and Treatment

Figure 2.

A

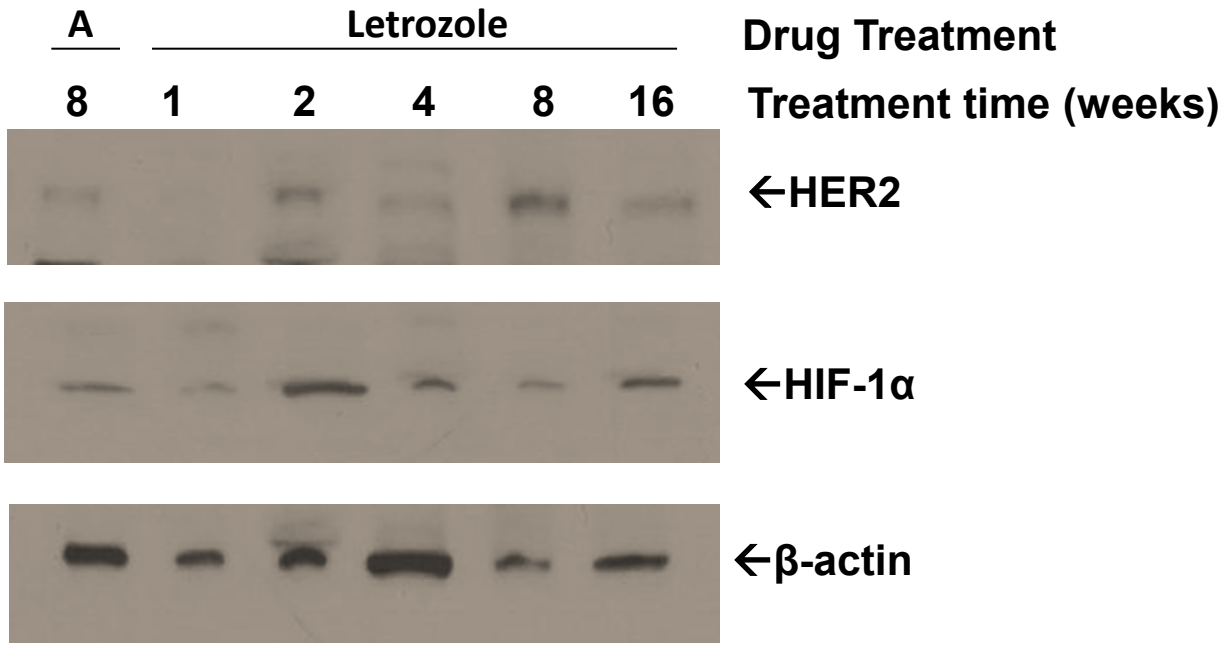
A
Letrozole
Drug Treatment
8
1
2
4
8
16
Treatment time (weeks)
←HER2
←HIF-1α
←β-actin

Figure 3.

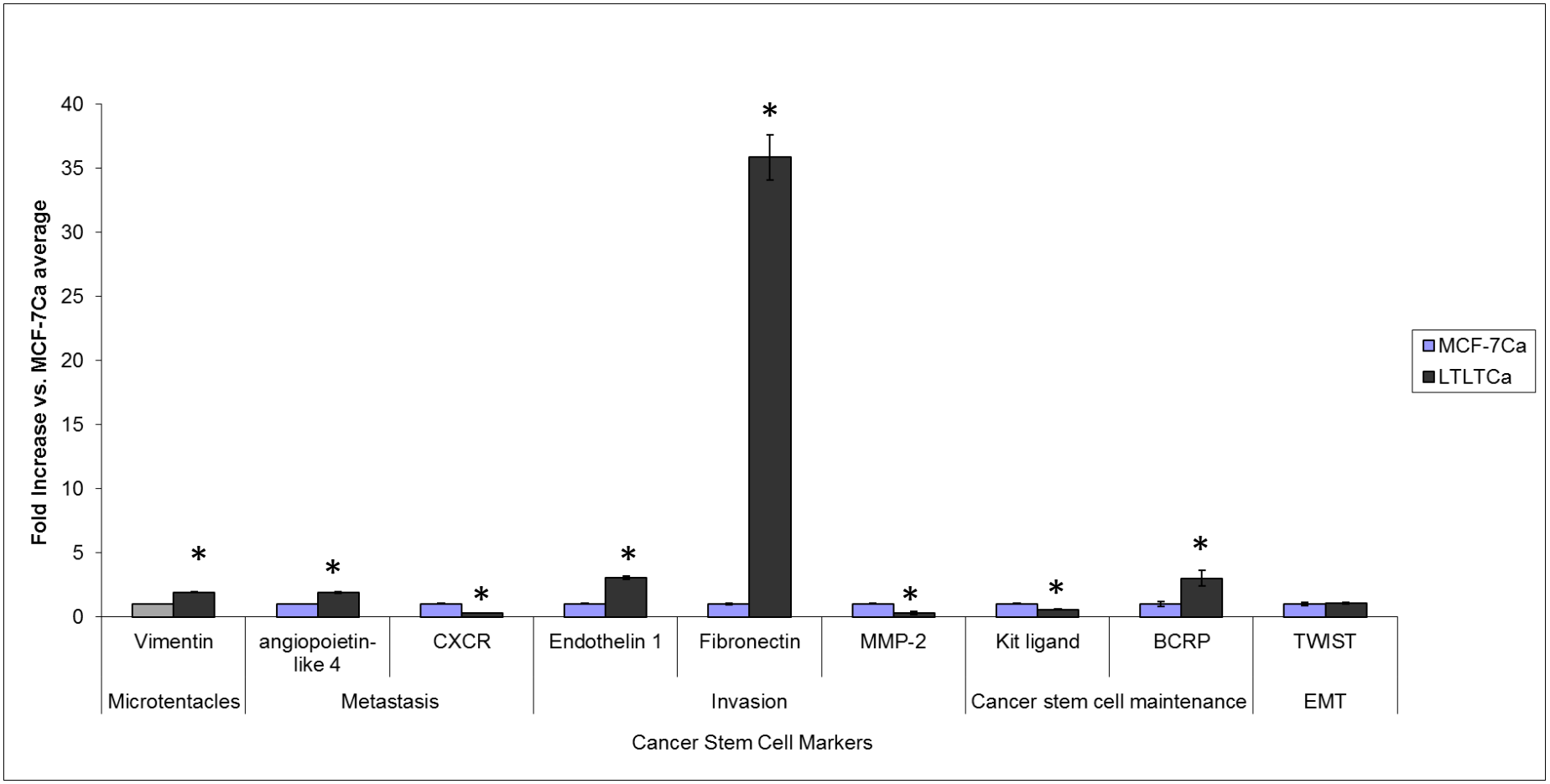
Fold Increase vs. MCF-7Ca average
40
35
30
25
20
15
10
5
0
MCF-7Ca
LTLTCa
Vimentin
angiopoietin-like 4
CXCR
Endothelin 1
Fibronectin
MMP-2
Kit ligand
BCRP
TWIST
Microtentacles
Metastasis
Invasion
Cancer stem cell maintenance
EMT
Cancer Stem Cell Markers

Figure 4.


1 ctgcagttaa tccttttcag tacaccataa atctaaatac tctcaaaaaa acctgtgcct
61 tttcaattgc tactaaatca cgagaagact gatttacata gtctcctttt atctcccttg
121 gcgggtaagt actcagctct gctcgttact aatattgaaa caacagccct tgaattgagt
181 gatttcccta gaaaggttaa ggtgaccgaa tctgaacact ccctccatgt cttggacacg
241 aagtttttt tctgcgtaga cagttttatc ccctcatccc aaggtcaatt gcacgaattc
301 ttttggaaaa cagaacctat ggcatttccc agacaaatca ccgtgaaccc tgtactgtgc
361 attgctgtcc taaaattaac acataaatct attgccgcca aagattctgt catttgtgtt
421 acataattgc ctttcatttg aactcattaa tcaaattggg gtttttaagc aacacctaat
481 taattcttta actggctcat attaaccttt aatgacttcc accagggtaa aaaccactga
541 tcactgagtt ctattttgaa actacggacg tcgagtttcc tctttcaccc agaattttca
601 gatcttgttt aaaaagttgg gtgtggtttc atgggggggag ggggaagagc gagaggagac
661 cagagggacg ggggcgggga ctctgcaaga aaaaccttcc cggtgcaatc gtgatctggg
721 aggcccacgt atggcgcctc tccaaaggct gcagaagttt cttgctaaca aaaagtccgc
781 acattcgagc aaagacaggc tttagcgagt tattaaaaac ttaggggcgc tcttgtcccc
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961 taaaccgcta cgagccctca atcggcggga cagcagggcg cggtgagtca ccgccggtga
1021 ctaagcgacc ccacccctct ccctcgggct ttcctctgcc accgccgtct cgcaactccc
1081 gccgtccgaa gctggactga gcccgttagg tcgatggaca gaggcgcggg ccggagcagc
1141 ccccctttcc aagcgggcgg cgcgcgaggc tgcggcgagg cctgagccct gcgttcctgc
1201 gctgtgcgcg ccccccaccc cgcgttccaa tctcaggcgc tctttgtttc tttctccgcg
1261 acttcagatc tgagggattc cttactcttt cctcttcccg ctcctttgcc cgcgggtctc
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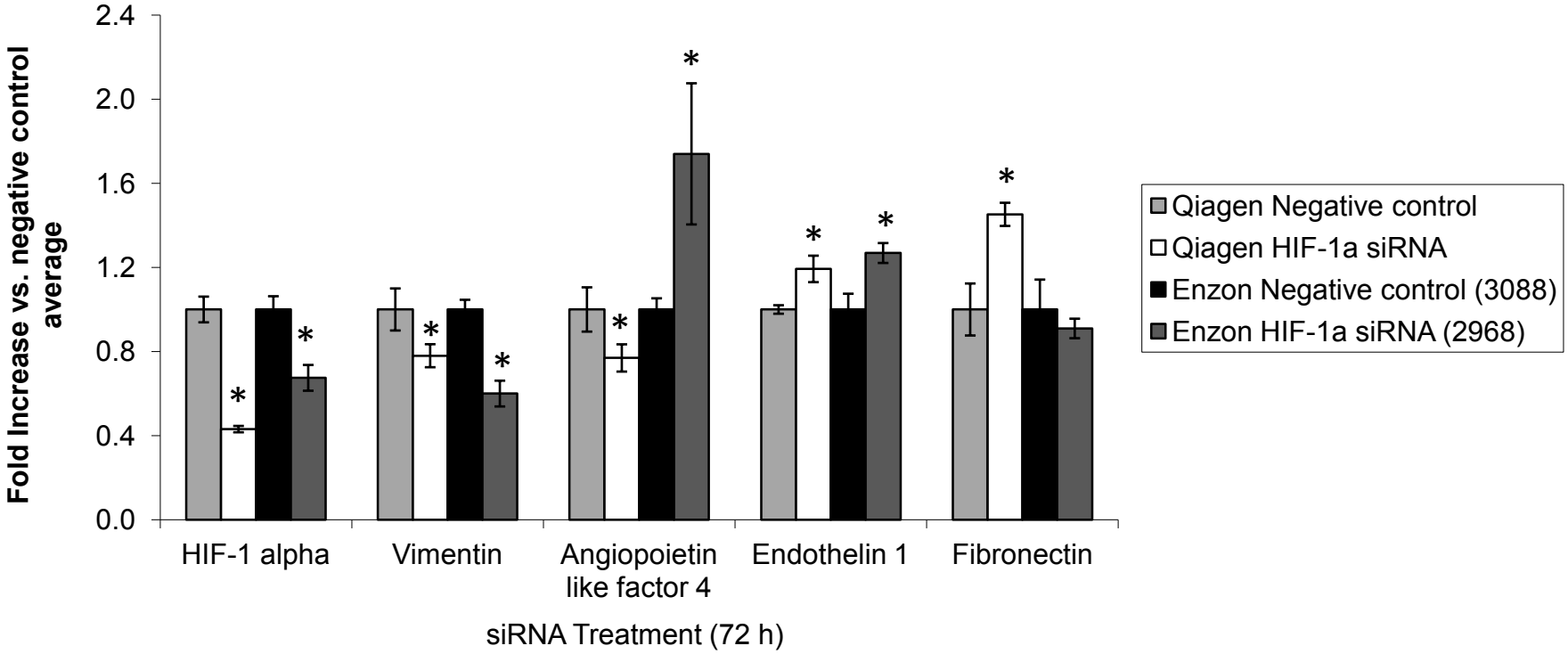
1441 tttttcagca ccccagggtg agcccagctc agactatcat ccggaaagcc cccaaaagtc
1501 ccagcccagc gctgaagtaa cgggaccatg cccagtccca cgccccggag caggaaggct
1561 cgaggcgccc ccaccccacc cgcccaccct ccccgcttct cgctaggtcc cgattggctg
1621 gggcgctccg cggctgggat ggcagtggga ggggaccctc tttcctaacg gggttataaa
1681 aacagcgccc tcggcggggt ccagtcctct gccactctcg ctccgaggtc cccgcgccag
1741 agacgcagcc gcgctcccac cacccacacc caccgcgccc tcgttcgcct cttctccggg
1801 agccagtccg cgccaccgcc

Figure 5.

A


Fold Increase vs. negative control average
2.4
2.0
1.6
1.2
0.8
0.4
0.0
HIF-1 alpha
Vimentin
Angiopoietin like factor 4
Endothelin 1
Fibronectin
siRNA Treatment (72 h)
Qiagen Negative control
Qiagen HIF-1a siRNA
Enzon Negative control (3088)
Enzon HIF-1a siRNA (2968)

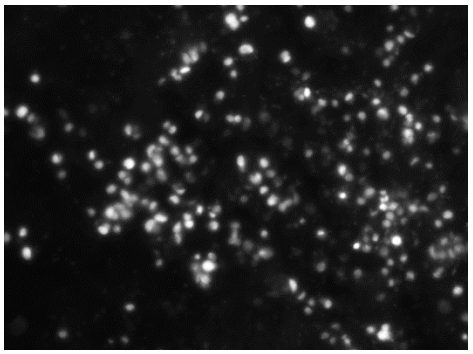
B


E3088
E2968
←vimentin

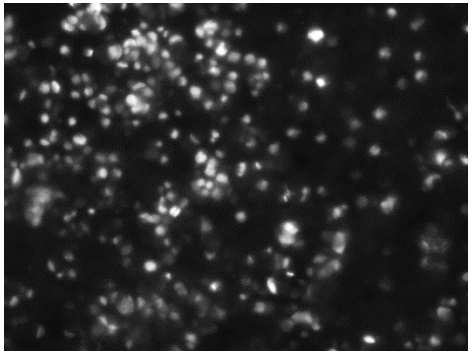
Figure 6.

Negative control

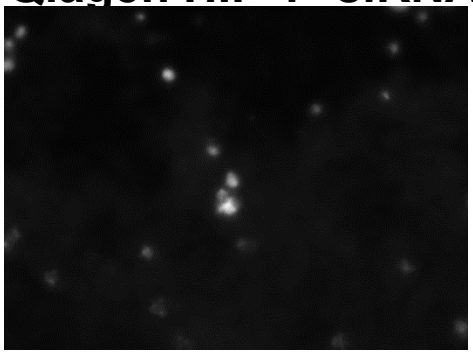
Positive control



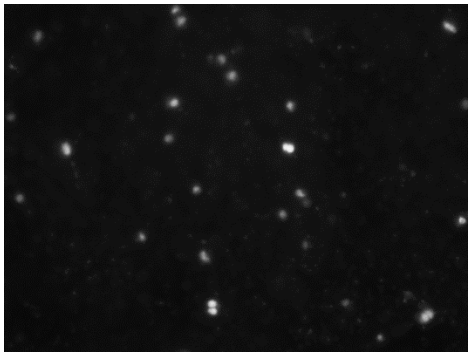
Qiagen negative control



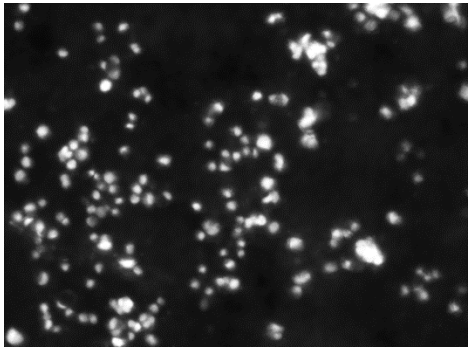
Qiagen HIF-1α siRNA



Qiagen vimentin siRNA



EZN-3088



EZN-2968

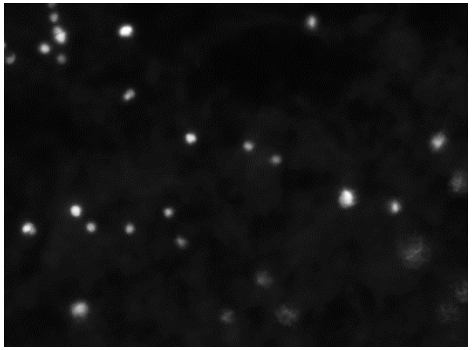


Figure 7.