Quantitative Comparison of Erythropoietin Receptor Levels in the Epithelial *versus* Endothelial Fractions of Primary Breast Tumors

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Abstract. *Background: Erythropoietin (EPO) was shown to reduce tumor survival in recent trials, however, its mechanisms of action are unclear. Efforts to measure tumor EPO receptor (EPOR) are limited by the promiscuity of EPOR antibodies, and concerns as to whether EPOR mRNA measurements are confounded by heterogeneity of tumor vasculature, a known EPOR source. Materials and Methods: This study compared mRNA levels of EPOR and JAK2 in 11 breast tumor epithelial versus endothelial dissections. Results: In nine tumors EPOR mRNA was 2.6 (1.2-5.7)-fold lower in the epithelial fraction, however, this reduction was less than the reduction of endothelial markers. In two tumors, EPOR mRNA was 2.9 (1.7-4.0)-fold higher in the epithelial fraction. The inter-tumor variation in EPOR levels exceeded the intra-tumor variation between fractions. Similar results were obtained for JAK2. Conclusion: Tumor vasculature is not the sole source of EPOR and JAK2, and tumors can be segregated by EPOR and JAK2 levels for correlative analysis with clinical outcomes.*

Erythropoietin (EPO), secreted primarily by the kidneys, is required for erythropoiesis. EPO receptor (EPOR) levels on erythroid progenitors in the marrow peak at approximately 1,100 homodimers per cell (1). EPOR is expressed at markedly lower levels in non-erythroid tissues, nonetheless, EPO has been linked to angiogenesis, and to cytoprotection in non-erythroid tissues including the brain, heart, and kidney (for review see reference 2). Adverse effects of EPO on patient survival in recent phase III trials led to major revisions in guidelines for EPO use in chemotherapy-induced anemia (3). However, mechanisms for the adverse effects of EPO remain unclear and preclinical studies have produced disparate results (for review see reference 4). At issue is whether expression of EPOR on tumor cells or tumor blood vessels is sufficient to impart biologic effects in response to EPO. Previous studies in diverse carcinomas indicate that *EPOR* and *EPO* expression are not elevated in tumor compared to healthy tissue, suggesting that *EPOR* and *EPO* are not oncogenes (5). However, this does not exclude the possibility that low levels of EPOR in tumors might allow them to respond to EPO. Measuring EPOR levels in the archival tumors of participants in completed and ongoing clinical trials of EPO provides an opportunity to determine whether worse outcomes in patients randomized to EPO occur disproportionately among patients whose tumors express higher EPOR levels.

Efforts to assess EPOR protein in tumors have been confounded by the absence of specific antibodies that can detect the low levels of EPOR present in non-erythroid cells (6, 7). Strikingly, elegant ^{125}I-EPO internalization studies in neuroblastoma cells showed that fewer than 50 EPOR homodimers on the cell surface can inhibit apoptosis in response to EPO (8). Thus, although low levels of EPOR can be functional, there are no reagents to assess EPOR protein in primary tumors by immunohistochemistry. Even a sensitive antibody, recently developed by Amgen, exhibited non-specific staining in negative control cells in this application (9).

In contrast, *EPOR* mRNA can be measured specifically by quantitative RT-PCR. However, three issues confront the use of *EPOR* mRNA: (i) degradation that characterizes RNA extracted from formalin-fixed, paraffin-embedded (FFPE) tumors, (ii) concerns as to whether *EPOR* mRNA correlates with surface protein levels, and (iii) uncertainty as to whether *EPOR* expression in endothelial cells, a

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known source of *EPOR*, confounds the characterization of tumors for their level of *EPOR*, since vasculature is known to be heterogeneous between different regions of the same tumor.

Regarding the first issue, using intact RNA from snap-frozen breast tumors *versus* degraded RNA from FFPE pieces of the same tumor and an optimized quantitative RT-PCR assay, a high concordance was found in *EPOR* mRNA determinations, despite the degradation of FFPE-derived RNA (10). Regarding the second issue, using a semi-quantitative Western blot and a panel of 66 cancer cell lines, Swift *et al*. recently reported a correlation between *EPOR* mRNA and protein in some, but not all, cancer cell lines (11). The eight cell lines containing the highest levels of EPOR protein were among the eleven lines with the highest levels of *EPOR* mRNA. The highest *EPOR* mRNA levels were observed in NCIH661 lung cancer cells, which also contained cell surface EPOR as determined by ^{125}I-EPO binding. A correlation has also been reported between *EPOR* mRNA and surface protein (using a specific antibody) in some, but not all, cancer cell lines (10). The apparent lack of correlation among many cancer lines may arise from differences in post-transcriptional regulation but may also reflect inaccuracies in measuring the low levels of EPOR protein that characterize most non-erythroid cell types. In the present study, to address the third aforementioned issue, laser capture microdissection (LCM) was used to quantitatively assess *EPOR, EPO*, and *JAK2*, an EPOR-associated kinase (for review see reference 12) in epithelial *versus* endothelial fractions of primary breast tumors.

Materials and Methods

Laser capture microdissection. The Institutional Review Board granted permission to access tissue donated by women undergoing breast cancer surgery (Department of Defense grant DAMD 17-02-1-0691). Written informed consent was obtained. Samples were frozen within 20-60 minutes of devascularization. Depending on the frozen tumor size, up to 24×9 μm sections were placed on RNase-free polyethylene naphthalate membranes (Leica Microsystems, Wetzlar, Germany). Slides were stained and dehydrated using HistoGene (Molecular Devices, Sunnyvale, CA, USA). An LMD6000 system (Leica) was used to harvest epithelial *versus* stromal/endothelial fractions. The number of captures per slide varied depending on the size of the tumor sample and the extent to which epithelial cells could be clearly separated from stroma.

Quantitative RT-PCR. RNA extraction and genomic DNA digestion were performed using the Absolutely RNA Nanoprep kit (Stratagene, La Jolla, CA, USA). cDNA synthesis, pre-amplification of cDNA using the Taqman cDNA pre-amplification kit (Applied Biosystems, Foster City, CA, USA), quantitative real time PCR, and relative quantification were as described previously (10). All Taqman probes were designed to detect exon junctions. Error bars in relative quantification values represent the standard error of the mean obtained upon normalization to *HMBS, IPO8,* and *B2M*, which were used based on their stability among 16 candidates previously evaluated in breast cancer using the Genorm algorithm (10, 13).

Results

Epithelial versus stromal/endothelial lineage fractionation. A total of 58 hematoxylin-eosin stained frozen sections were screened to identify tumors with clear separation of epithelial and stromal compartments and 17 of them were selected as being suitable for LCM. Figure 1 delineates representative morphologies of tumor epithelial cell enriched fractions (panels A-F) *versus* depleted fractions (panel G). Eleven tumor samples yielded sufficient RNA and exhibited successful fractionation of all lineage markers. The stage and histopathological features of these tumors are summarized in Table I. Fractionation was monitored by the epithelial markers *KRT7* and *TACSTD1*, the endothelial markers *CDH5* and *PECAM1*, and the stromal marker *VIM*. The epithelial markers *KRT7* and *TACSTD1* were mean (range) 6.8 (1.3-19.2) fold and 7.0 (1.4-21.9) fold higher in the epithelial fraction compared to the stromal/endothelial fraction (Figure 2A, B). The endothelial markers *CDH5* and *PECAM1* were reduced by 12.1 (3.4-35.8)-fold and 10.7 (2.0-24.5)-fold in the epithelial fraction compared to the stromal/endothelial fraction (Figure 2C, D), and the stromal marker *VIM* was reduced by 9.5 (1.2-34.2)-fold (Figure 2E). This successful fractionation provided the opportunity to determine the extent to which *EPOR, JAK2,* and *EPO* mRNA segregates as an epithelial *versus* stromal/endothelial marker in each tumor.

EPOR, JAK2, and EPO expression in epithelial versus stromal/endothelial fractions. In 9 out of 11 tumor samples, *EPOR* mRNA was 2.6 (1.2-5.7)-fold lower in the epithelial fraction compared to the stromal/endothelial fraction (Figure 2F). However, depletion of *EPOR* mRNA from epithelial fractions was less than the aforementioned extent of depletion of the endothelial markers *CDH5* and *PECAM1*. *EPOR* depletion from epithelial fractions achieved the same extent as both *CDH5* and *PECAM1* in only one of these nine tumors (tumor 2, compare Figure 2C, D and F). Moreover, in two tumors, *EPOR* was higher in the epithelial fraction by 2.9 (1.7-4.0)-fold (tumors 9 and 11, Figure 2F).

Since the tyrosine kinase JAK2 is required for EPOR signaling in erythroid cells and has been implicated in EPOR signaling in non-erythroid cells (14, 15) co-expression of JAK2 is likely a prerequisite for EPOR signaling in tumor cells. Therefore the types of cells within tumors that express *JAK2* mRNA were surveyed. In 8 out of 11 tumor samples, *JAK2* was 8.3 (1.5-44.6) fold lower in the epithelial fraction compared to the stromal/endothelial fraction (Figure 2G). Excluding tumor 7

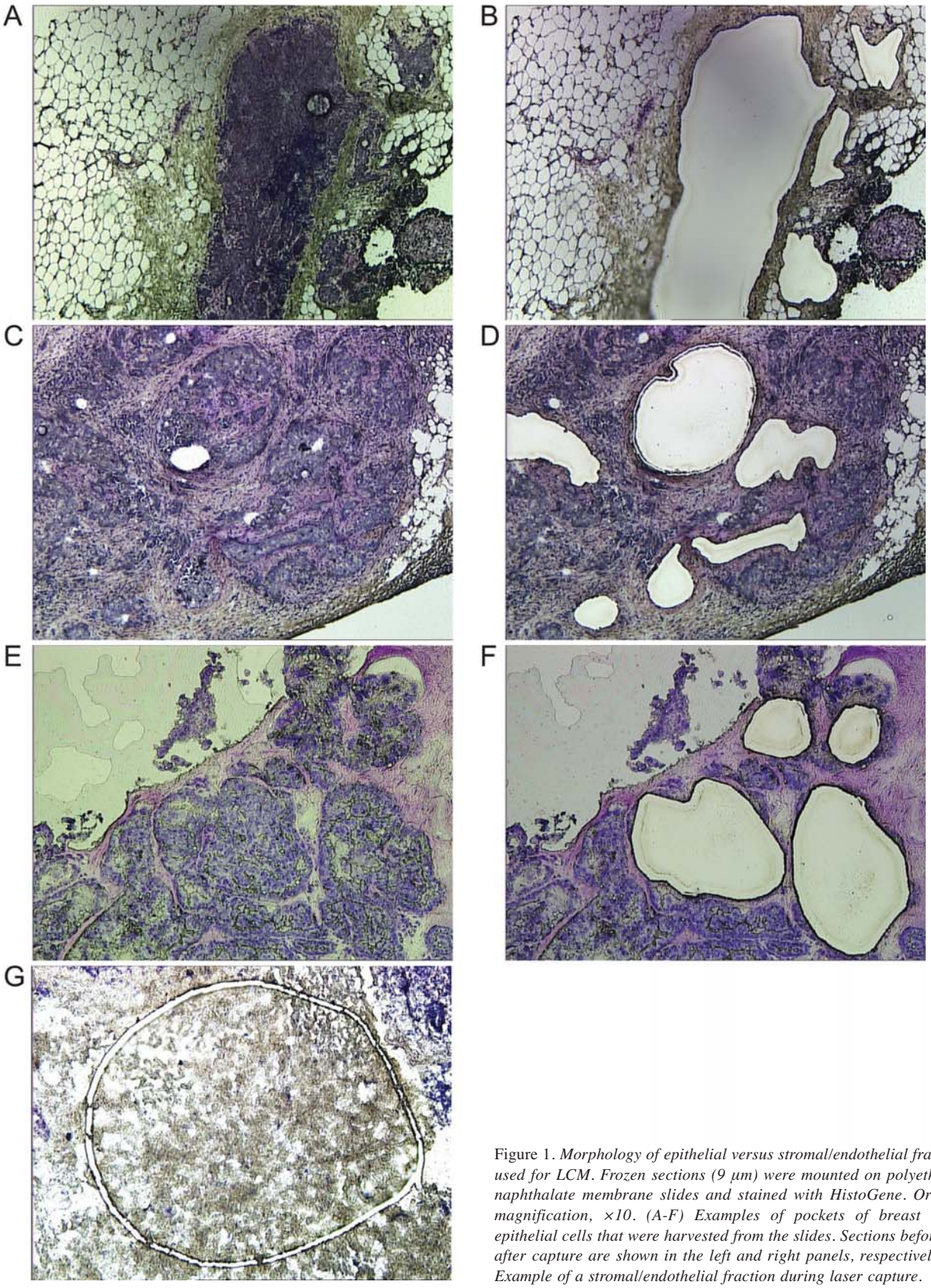

Figure 1. *Morphology of epithelial versus stromal/endothelial fractions used for LCM. Frozen sections (9 μm) were mounted on polyethylene naphthalate membrane slides and stained with HistoGene. Original magnification, ×10. (A-F) Examples of pockets of breast tumor epithelial cells that were harvested from the slides. Sections before and after capture are shown in the left and right panels, respectively. (G) Example of a stromal/endothelial fraction during laser capture.*

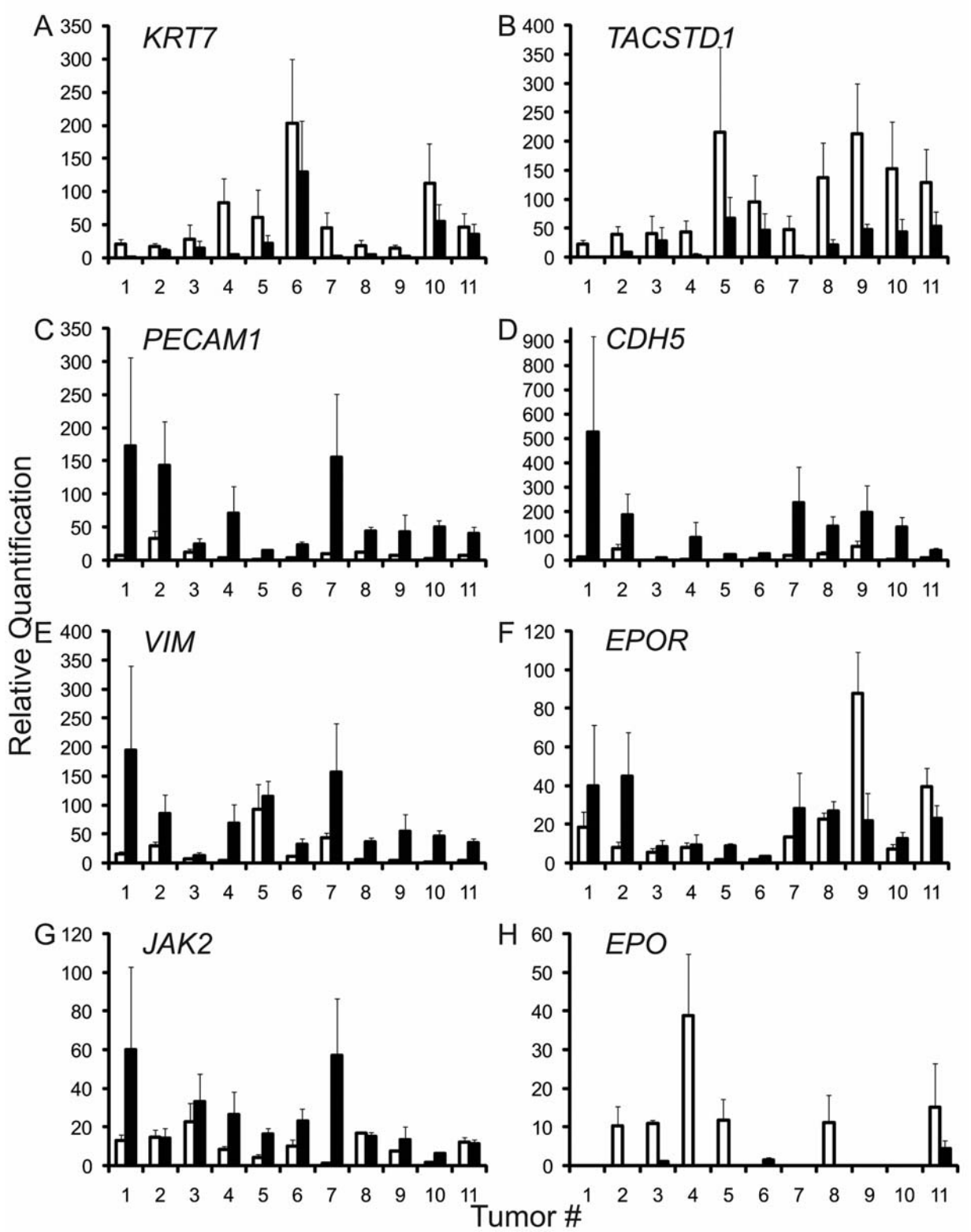

Figure 2. *mRNA levels in epithelial versus stromal/endothelial fractions. Relative quantification values (y-axis) were normalized to an average of three endogenous control genes (B2M, HMBS, and IPO8). For each tumor (x-axis), the expression level of each marker is shown in the epithelial fraction (open bars) and the stromal/endothelial fraction (closed bars). For each gene, the lowest expressing fraction was assigned a value of 1. Error bars represent the standard error of the mean values obtained upon normalization to three control genes.*

Table I. *Summary of clinical characteristics, lineage fractionation, and EPOR/JAK2/EPO mRNA expression*.*

#	Age (years)	ER	PR	Her2	Stage	Histology	*KRT7*	*TACSTD1*	*CDH5*	*PECAM1*	*VIM*	*EPOR*	*JAK2*	*EPO*
1	37	+	+	NA	I	Colloid/ Mucinous	Epi 18.5-fold	Epi 21.9-fold	S/VE 35.8-fold	S/VE 24.5-fold	S/VE 12.6-fold	S/VE 2.1-fold	S/VE 4.7-fold	UD
2	56	+	+	NA	IIIC	Lobular	Epi 1.5-fold	Epi 4.2-fold	S/VE 3.8-fold	S/VE 4.4-fold	S/VE 3.0-fold	S/VE 5.7-fold	Epi 1.0-fold	UD
3	46	+	+	+	IIIA	Ductal	Epi 2.0-fold	Epi 1.4-fold	S/VE 3.9-fold	S/VE 2.0-fold	S/VE 1.7-fold	S/VE 1.6-fold	S/VE 1.5-fold	Epi 9.8-fold
4	46	–	–	+	IIA	Ductal	Epi 19.2-fold	Epi 10.2-fold	S/VE 17.5-fold	S/VE 17.3-fold	S/VE 17.1-fold	S/VE 1.2-fold	S/VE 3.1-fold	UD
5	56	–	–	NA	IIB	Ductal	Epi 2.8-fold	Epi 3.2-fold	S/VE 19.7-fold	S/VE 11.6-fold	S/VE 1.2-fold	S/VE 5.5-fold	S/VE 3.9-fold	UD
6	64	–	–	+	IIB	Ductal	Epi 1.6-fold	Epi 2.0-fold	S/VE 3.9-fold	S/VE 6.0-fold	S/VE 3.0-fold	S/VE 2.2-fold	S/VE 2.2-fold	UD
7	42	+	+	NA	IIIA	Ductal	Epi 16.4-fold	Epi 17.8-fold	S/VE 10.6-fold	S/VE 15.4-fold	S/VE 3.7-fold	S/VE 2.1-fold	S/VE 44.6-fold	UD
8	48	+	+	NA	I	Ductal	Epi 4.4-fold	Epi 6.4-fold	S/VE 5.2-fold	S/VE 3.5-fold	S/VE 6.8-fold	S/VE 1.2-fold	Epi 1.1-fold	UD
9	62	+	+	NA	IIB	Ductal	Epi 5.2-fold	Epi 4.4-fold	S/VE 3.4-fold	S/VE 6.2-fold	S/VE 13.9-fold	Epi 4.0-fold	S/VE 1.8-fold	UD
10	82	–	–	NA	IIB	Ductal	Epi 2.0-fold	Epi 3.4-fold	S/VE 24.9-fold	S/VE 21.1-fold	S/VE 34.2-fold	S/VE 1.8-fold	S/VE 4.2-fold	UD
11	70	+	+	NA	IIIC	Lobular	Epi 1.3-fold	Epi 2.4-fold	S/VE 4.0-fold	S/VE 5.9-fold	S/VE 7.7-fold	Epi 1.7-fold	Epi 1.1-fold	Epi 3.4-fold

ER, Estrogen receptor; PR, progesterone receptor; Her2, human epidermal growth factor receptor 2; NA, not available; Epi, epithelial fraction; S /VE, stromal/vascular endothelial fraction; UD, undetermined. *For each transcript, the fraction with higher expression (Epi *versus* S/VE) is indicated, followed by the fold enrichment in that fraction compared to the other fraction.

(44.6-fold) as an outlier, *JAK2* expression in the epithelial fractions of the remaining seven tumors was only 3.1 (1.5-4.7)-fold lower compared to the stromal/ endothelial fraction. Thus, similar to *EPOR*, the extent of depletion of *JAK2* from epithelial fractions was to a lesser extent than the aforementioned extent of depletion of the endothelial markers *CDH5* and *PECAM1*. *JAK2* depletion from epithelial fractions was to the same extent as both *CDH5* and *PECAM1* for only one of these eight tumors (tumor 7, compare Figure 2C, D and G). In three tumors, *JAK2* expression was 1.1 (1.0-1.1)-fold higher in the epithelial fraction compared to the stromal/endothelial fraction (tumors 2, 8, and 11, Figure 2G).

Several studies have reported that EPO secreted by tumor cells supports cell growth (16, 17). Therefore, the types of cells within tumors that express *EPO* mRNA were surveyed. Owing to low expression levels of this gene together with the limiting amounts of RNA, *EPO* was detectable in only 6 out of 11 epithelial fractions and 3 out of 11 stromal/endothelial fractions. In two tumors for which *EPO* levels were determined in both fractions, *EPO* was 9.8- and 3.4-fold higher in the epithelial fraction compared to the stromal/endothelial fraction (tumors 3 and 11, Figure 2H).

Inter-tumoral versus intra-tumoral variation in EPOR and JAK2 levels. Among all eleven tumors, the intra-tumor variation in *EPOR* mRNA levels between epithelial *versus* stromal/ endothelial compartments was maximally 5.7-fold-with a mean of 2.6-fold (Figure 2F), whereas the inter-tumor range of overall *EPOR* expression among all 11 unfractionated tumors was as much as 12.7-fold, with a mean of 6.3-fold (not shown). For *JAK2*, the intra-tumor variation was maximally 44.6-fold, but all of the remaining tumors exhibited intra-tumor variation of less than 4.7-fold, with a mean of 2.5-fold (Figure 2G). The inter-tumor variation in *JAK2* levels was 15.6-fold, with a mean of 8.6 fold (data not shown). Thus, the inter-tumor variation in *EPOR* and *JAK2* levels between different patient tumor samples exceeded the intra-tumor variation between epithelial *versus* stromal /endothelial fractions.

Discussion

While the current study was limited to 11 tumor samples due to the difficulties in obtaining sufficient RNA and the unsuitable morphology of most tumor specimens for lineage fractionation, this was sufficient to reveal that in 10 out of the 11 tumors the endothelial cells were not able to account for all of the *EPOR* and *JAK2* expression. The basis for

elevated *EPOR* and *JAK2* expression in epithelial fractions compared to stromal/endothelial fractions in certain tumors, and the basis for the inter-tumor variation in *EPOR* and *JAK2* levels across patients remain unknown. While tissue processing can affect mRNA levels (18) this study minimized differences in tissue processing, and our results likely reflect biological variation between different patient tumor specimens.

Recently, Liang and others demonstrated that EPO stimulated SRC activation and PTEN inactivation in human breast cancer cell lines, and markedly inhibited the response of $HER2^+$ breast cancer cells to trastuzamab in a murine tumor xenograft model (19). Furthermore, in trastuzamab-treated patients with $HER2^+$ metastatic breast cancer, EPO use correlated with reduced progression-free and overall survival. These results suggest that EPO can act directly on breast tumor epithelial cells. This is consistent with the present demonstration that *EPOR* and *JAK2* are not limited to the endothelial fraction, but are also co-expressed in the epithelial fraction of primary breast tumors.

The observed expression of *EPOR* and *JAK2* in the stromal/endothelial cell fraction is consistent with reports documenting the expression of *EPOR* mRNA and protein in endothelial cells and with reports of the effects of EPO on tumor angiogenesis (20, 21). In this study it was not possible to determine whether stromal cells express *EPOR* and *JAK2* mRNA, or whether the source of *EPOR* and *JAK2* mRNA in the stromal/endothelial fraction was solely due to endothelial cell expression. Future studies aimed at fractionating endothelial *versus* stromal cells using fluorescent-labeled antibodies directed against endothelial and stromal markers will help resolve this issue.

It was previously found that *EPOR* mRNA levels varied 30-fold range across panels of both breast tumors and head and neck tumors (10). In the present study, it was found that the inter-tumor variation in *EPOR* and *JAK2* levels between different patient tumor samples exceeded the intra-tumor variation between epithelial *versus* stromal/endothelial fractions. Thus, archival tissues from completed and ongoing randomized trials of EPO can be used to determine whether tumors with the highest levels of *EPOR* and *JAK2* mRNA are more susceptible to EPO-induced tumor progression. However, the presence of endothelial cells in tumor sections together with the exquisite sensitivity of quantitative PCR precludes the ability to simply characterize tumors as *EPOR* or *JAK2* positive *versus* negative (22). Moreover, the heterogeneity of vasculature or other factors such as hypoxia between different tumors, or even between different regions of the same tumor, may influence measurements of *EPOR* or *JAK2* mRNA obtained using single tumor sections. Nonetheless, it was previously shown, using 23 breast tumors, that *EPOR* and *JAK2* measurements in different pieces of the same tumor sample were significantly correlated, while the endothelial markers *CDH5* and *PECAM1* were not (10). This is consistent with the present demonstration that endothelial cells are not the sole source of *EPOR* and *JAK2* mRNA in tumors. Thus, despite the known heterogeneity in endothelial representation, tumors can be characterized for their overall levels of *EPOR* and *JAK2* mRNA. In addition, tumor epithelial-specific levels of *EPOR* and *JAK2* in single tumor sections may be normalized to the levels of endothelial markers, to adjust for the heterogeneity in tumor vasculature representation.

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