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## Department Title

# Inflammation, but not hypoxia, mediated HIF-1 $\alpha$ activation depends on COX-2

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**Abbreviations:** COX, cyclooxygenase; HIF, hypoxia inducible factor; PGE, prostaglandin E; VEGF, vascular endothelial growth factor; LDHA, lactate dehydrogenase A; SLC, solute carrier; SCID, severe combined immunodeficiency; LOX, lysyl oxidase

**Key words:** breast cancer, inflammation, COX-2, COX-2-silencing, hypoxia, HIF-1 $\alpha$

The COX pathway has been a target for pharmaceutical intervention in diseases with a high inflammatory component ranging from asthma and Alzheimer's to arthritis and cancer. A major transcriptional promoter of the malignant phenotype, HIF-1 $\alpha$ , has been observed to be regulated by the COX-2 product PGE<sub>2</sub>. Here we show that HIF-1 $\alpha$  protein significantly accumulated in human breast cancer MDA-MB-231 cells in response to the pro-inflammatory cytokine IL-1 $\beta$ , but not in COX-2-silenced MDA-MB-231 cells. In contrast HIF-1 $\alpha$  expression could be detected in COX-2-silenced cells in response to the hypoxia-mimetic agent CoCl<sub>2</sub> and hypoxia. Gene expression profiling in COX-2-containing and COX-2-silenced cells showed that the hypoxia-induced transcriptional response is largely unaffected by COX-2 silencing. These data suggest that the profound effects of COX-2 silencing on inhibiting invasion, tumor growth and metastasis from MDA-MB-231 cells are dependent on the induction of IL-1 $\beta$ -dependent COX-2 and HIF-1 $\alpha$  but are independent of hypoxia.

## Introduction

To grow larger than a few millimeters solid tumors have to adapt to decreasing oxygen availability.<sup>1,2</sup> The hypoxia-induced transcriptional response is mediated by the stabilization of hypoxia-inducible factor (HIF) and involves the induction of genes associated, among others, with altered metabolism, angiogenesis and increased invasion and metastasis.<sup>3-6</sup> Another important physiological environment encountered by growing tumor cells, circulating cancer cells and metastasizing cells involves the inflammatory response. Immune cells orchestrating the response to pathogens as well as transformed cells secrete pro-inflammatory cytokines in the local microenvironment, among them IL-1 $\beta$ .<sup>7,8</sup> IL-1 signaling is responsible for the induction of cyclooxygenase 2 (COX-2) an enzyme that catalyzes

the conversion of arachidonic acid to secondary lipid mediators such as prostanoids.<sup>9</sup> The role of prostanoids in cancer is under intense scrutiny particularly in situations of chronic inflammation or in cancerous lesions overexpressing COX-2. Data from several studies have suggested a co-operation between COX-2 and HIF-1 $\alpha$ .<sup>10-13</sup>

We previously reported that COX-2-silenced cells fail to metastasize to the lung in an experimental model of metastasis and displayed reduced tumor onset when grafted in the mammary fat pad of SCID mice.<sup>14</sup> Since hypoxia is known to mediate tumor angiogenesis through vascular endothelial growth factor (VEGF)-A as well as to increase metastasis,<sup>5</sup> we examined differences in the hypoxia and inflammation-driven functional activation of HIF-1 $\alpha$  in COX-2-containing and COX-2-silenced cells. Previous studies with pharmacologic agents selective for COX-2 inhibition have given conflicting results regarding their effect on HIF-1 $\alpha$  levels.<sup>10-13</sup> The nonselective COX inhibitors ibuprofen, diclofenac and ketorolac have been shown to reduce HIF-1 $\alpha$  and -2 $\alpha$  levels in response to hypoxia; however, the reduction of HIF levels was also seen in prostate cancer DU145 cells that did not express COX-2.<sup>15</sup> Additionally, in the same study, the selective COX-2 inhibitor NS-398 did not significantly inhibit HIF-1 $\alpha$  accumulation in response to hypoxia in neither "COX-2-positive" PC-3 cells, nor "COX-2-negative" DU145 cells. In contrast, another study reported that a similar concentration of NS-398 significantly reduced HIF-1 $\alpha$  levels<sup>11</sup> in the COX-2-containing PC-3 prostate carcinoma cells and the COX-2-negative HCT116 colon carcinoma cells. Interestingly, a ten-fold lower dose of NS-398 inhibited HIF-1 $\alpha$  transcriptional activity, as measured by hypoxia response element activity and VEGF production, in the HT-29 colorectal cancer cell line in a separate study.<sup>13</sup> HIF-1 $\alpha$  was also shown to directly bind to the COX-2 promoter thereby regulating the expression of COX-2 protein in two colorectal carcinoma cell lines, HCT116 and HT29, although HCT116 cells are generally referred to as COX-2 negative<sup>11,13</sup> even in response to hypoxia.<sup>13</sup> Since IL-1 $\beta$  signaling also induces HIF-1 $\alpha$  stabilization in a pathway dependent on functional NF $\kappa$ B, COX-2 and VHL<sup>10</sup> even under normoxic conditions, here, for the first time, we have examined the effect of siRNA-mediated silencing of COX-2 on the expression of HIF-1 $\alpha$  in response to inflammation and hypoxia.

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## Results

COX-2-silenced cells accumulate HIF-1 $\alpha$  in response to the hypoxia-mimetic agent CoCl<sub>2</sub> and hypoxia. To examine the role of COX-2 in the hypoxic response we initially probed HIF-1 $\alpha$  accumulation in COX-2-containing and COX-2-silenced cells in a dose-dependent experiment using CoCl<sub>2</sub>. CoCl<sub>2</sub> is a hypoxia-mimetic agent known to inhibit the oxygen-dependent degradation of HIF-1 $\alpha$ . HIF-1 $\alpha$  accumulated in COX-2-silenced cells (Clone 2, Pooled) although in smaller amounts than in COX-2-containing cells (MDA-MB-231) in a dose-dependent manner (Fig. 1A). We then probed the response of COX-2-silenced Clone 2 and Pooled cells, to hypoxia (pO<sub>2</sub>~0.7%—Fig. 1B). A robust and comparable HIF-1 $\alpha$  accumulation was observed in both COX-2-containing and COX-2-silenced cells in response to hypoxia (Fig. 1B).

**COX-2 silencing does not affect the hypoxia-dependent HIF-1 $\alpha$  response.** Quantitative PCR and microarray analysis were performed to examine differences among hypoxia-inducible transcripts between COX-2-containing and COX-2-silenced cells. We probed the induction of three known hypoxia-responsive genes VEGF, LDHA and SLC2A1 in response to hypoxia. HIF-1 $\alpha$ -responsive transcripts VEGF and LDHA were regulated in a similar manner in the presence (MDA-MB-231) or absence of COX-2 (Clone 2 and Pooled) following 24 h of hypoxia (pO<sub>2</sub>~0.7%) as shown by qPCR in two independent experiments. A modest increase in the induction of SLC2A1 was observed in both Clone 2 and Pooled COX-2 silenced cells (Fig. 1C). To further address the role of COX-2 in the hypoxia-induced response we performed microarray analysis on total RNA isolated from cells subjected to hypoxia and normoxia for 24 h and examined the expression of 9 genes known to be HIF targets (Fig. 1D). Expression of HIF-induced transcripts was not affected by the absence of COX-2. To further understand the response of hypoxia-induced transcription in COX-2-containing and COX-2-silenced cells we selected transcripts that have been significantly induced by hypoxia in parental MDA-MB-231 cells (posterior probability >0.5) and compared their expression with that of the same probesets in Clone 2 and Pooled cells. Of the 21 Affymetrix probeset IDs that fulfilled the criteria outlined in the materials and methods section, and have been assigned a gene title, only lysyl oxidase (LOX) was differentially regulated by hypoxia in COX-2-containing and COX-2-silenced cells to a significant extent (higher than 3-fold in both Clone 2 and Pooled cells, Fig. 1E). Higher levels of LOX mRNA were observed in COX-2-silenced cells relative to parental cells following hypoxia, although normoxic COX-2-silenced cells contained lower levels of LOX mRNA than parental cells (data not shown). The remaining 20 genes were similarly regulated by hypoxia regardless of the presence or absence of COX-2.

**COX-2-silencing inhibits IL-1 $\beta$ -dependent accumulation of functional HIF-1 $\alpha$ .** We next examined the role of COX-2 and HIF-1 $\alpha$  in response to the pro-inflammatory cytokine IL-1 $\beta$ . COX-2-containing, MDA-MB-231 but not COX-2-silenced (Clone

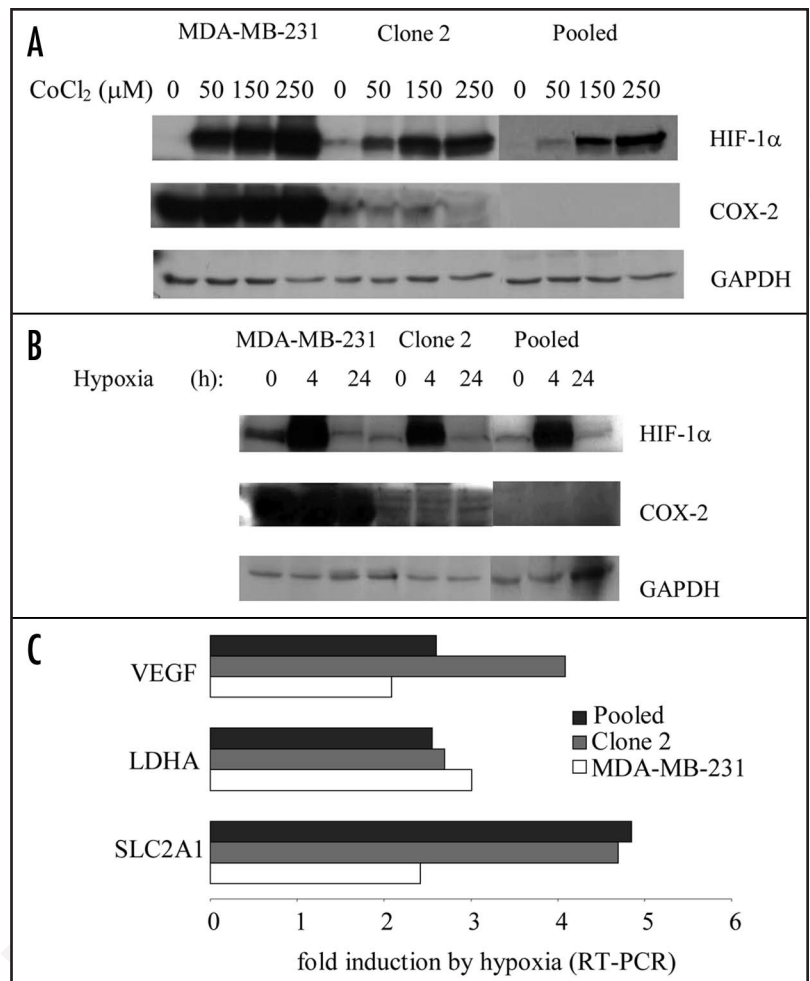


Figure 1. COX-2-silenced cells accumulate functional HIF-1 $\alpha$  and maintain an intact hypoxic response. (A) Immunoblotting for HIF-1 $\alpha$  in COX-2-containing parental MDA-MB-231 (231) cells, and COX-2-silenced Clone 2 and Pooled cells subjected to the indicated amounts of CoCl<sub>2</sub> for 24 h. Total cell lysate from each cell line was subjected to immunoblotting for HIF-1 $\alpha$ , COX-2 and GAPDH. (B) Immunoblotting for HIF-1 $\alpha$  in COX-2-containing parental MDA-MB-231 (231) cells, and COX-2-silenced Clone 2 and Pooled cells subjected to 0.7% hypoxia for the indicated times. Total cell lysate from each cell line was subjected to immunoblotting for HIF-1 $\alpha$ , COX-2 and GAPDH. (C) Fold induction of HIF-1 $\alpha$ -induced transcripts SLC2A1, VEGF and LDHA in COX-2-containing (MDA-MB-231) and COX-2-silenced cells (Clone 2 and Pooled) in response to 24 h of hypoxia. Fold induction was calculated using the 2 <sup>$\Delta\Delta$ Ct</sup> method using 18S Ct values as control.

2 and Pooled) cells accumulated HIF-1 $\alpha$  in response to IL-1 $\beta$  (Fig. 2A). The accumulating HIF-1 $\alpha$  protein was functional as it induced the expression of the known HIF-1 $\alpha$  target genes CXCR4 and VEGFA in COX-2 containing cells (Fig. 2B). The fold-induction of VEGF in COX-2-silenced cells suggests that IL-1 $\beta$  may induce VEGF independently of COX-2 and HIF-1 $\alpha$ .

## Discussion

We have previously shown that silencing of COX-2 expression in MDA-MB-231 cells reduced orthotopic tumor growth in SCID mice and abolished extrapulmonary metastasis in an experimental model of metastasis.<sup>14</sup> Given the importance of hypoxia in tumor growth and metastasis we sought to examine whether COX-2 silencing compromised the hypoxia-dependent response thus

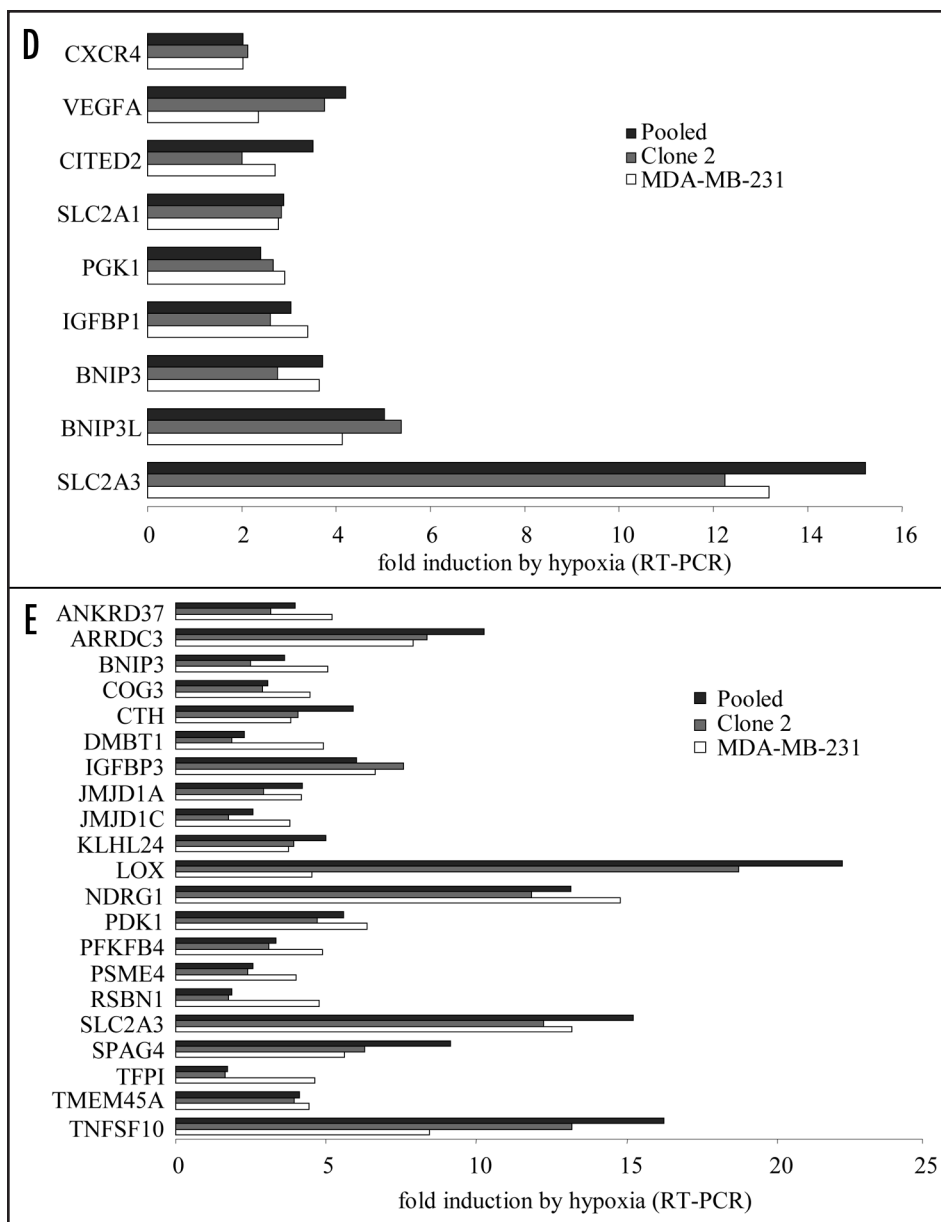


Figure 1. COX-2-silenced cells accumulate functional HIF-1 $\alpha$  and maintain an intact hypoxic response. (D) Microarray analysis of total mRNA in COX-2-containing (MDA-MB-231) and COX-2-silenced cells (Clone 2 and Pooled) in response to hypoxia for 24 h. Fold-induction of known HIF-1 $\alpha$  target genes is shown. (E) Fold induction of transcripts significantly upregulated by hypoxia in MDA-MB-231 cells and comparison with the fold induction of transcripts that were similarly regulated between Clone 2 and Pooled cells.

accounting for reduced tumor onset and loss of metastatic potential observed in these cells. COX-2-silenced cells maintained the capacity to induce HIF-1 $\alpha$  to a significant, although slightly reduced, extent in response to the hypoxia-mimetic agent CoCl<sub>2</sub> or hypoxia. Real-time RT-PCR and microarray analysis of total RNA demonstrated that COX-2 silencing did not significantly affect hypoxia-driven gene expression. One notable exception was the higher levels of LOX mRNA in COX-2-silenced cells relative to parental cells following hypoxia. LOX was recently shown to be necessary for metastasis of MDA-MB-231 cells in lungs and livers of nude mice<sup>17</sup> an observation attributed to the reduction of hypoxia-stimulated cell invasion. More recently the accumulation of LOX was found to inversely correlate with LOX activity in response to hypoxia in poorly

invasive MCF-7 and T47D breast cancer cells<sup>18</sup> due to the decreased oxygen availability necessary for enzyme function. It was proposed that poorly invasive breast cancer cells subjected to hypoxia overexpress LOX but that its enzymatic function is reduced. This observation is consistent with our finding that poorly invasive COX-2-silenced MDA-MB-231 cell<sup>14</sup> express more LOX in response to hypoxia. Our data suggest that COX-2 modulates the expression of LOX, but the interplay between inflammation, hypoxia and the effect of these environments on LOX-dependent metastasis remains to be more precisely defined.

In contrast to the intact response to hypoxia in COX-2 silenced cells, we found that IL-1 $\beta$ -dependent induction of functional HIF-1 $\alpha$  depended on COX-2 expression in poorly differentiated breast cancer MDA-MB-231 cells consistent with an earlier study.<sup>10</sup> Our results indicate that COX-2 is important for IL-1 $\beta$ , but not hypoxia, driven HIF-1 $\alpha$  activation. Our data also suggest that the previously observed inability of COX-2-silenced cells to metastasize and their significantly reduced ability to establish tumor growth in the mammary fat pad cannot be attributed to differential responses to hypoxia. Instead it is likely, that the decreased tumor onset we previously reported<sup>14</sup> is mediated, at least in part, as a result of silencing COX-2-dependent HIF-1 $\alpha$  activation. Some of the research on the influence of inflammation on hypoxia has been conducted using pharmacologic inhibitors that are now known to have a wide array of functions independent of the COX-2 pathway.<sup>19</sup> Our results support the conclusion that COX-2-dependent HIF-1 $\alpha$  activation is an important factor in the cellular response to IL-1 $\beta$  but not hypoxia.<sup>12</sup> Research results presented here and elsewhere<sup>14</sup> indicate that the signaling mediated

through the IL-1 $\beta$ -COX-2-HIF-1 $\alpha$  axis plays an important role in the tumorigenicity and metastatic potential of poorly differentiated breast tumors.

The role of IL-1 $\beta$  and pro-inflammatory signaling is currently being investigated for the induction promotion, or exacerbation of cancer,<sup>8</sup> diabetes<sup>20</sup> premature labor,<sup>21</sup> auto-immune,<sup>22</sup> neurodegenerative<sup>23</sup> and cardiovascular diseases.<sup>24</sup> Our results show that treatment of illnesses with exacerbated inflammatory components should also account for the implications of functional HIF-1 $\alpha$  activation even under normoxic conditions; targeting COX-2 could minimize these effects. Trauma from cancer treatments such as surgery and ionizing radiation are also likely to activate the inflammatory response. Our data support the administration of anti-inflammatory agents



immediately following surgery and ionizing radiation treatment of patients to minimize activation of the IL-1 $\beta$ -COX-2-HIF-1 $\alpha$  axis of oncogenic signaling.

## Materials and Methods

**Cell culture and hypoxia studies.** All cells were cultured with RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO) supplemented with 8.25% FBS (Sigma-Aldrich, St. Louis, MO) without penicillin or streptomycin. Cells used in the microarray experiment were seeded ( $1 \times 10^6$ ) in 100 mm dishes and allowed to attach to the plate overnight. The following day cells were placed in a hypoxic chamber at 0.7% oxygen or a normoxic incubator for 24 h at 37°C. Oxygen tension in the hypoxia chamber was monitored in real-time using the ProOXc system (Model 110, Biospherix, Ltd.).

**Immunoblotting.** Cells were lysed using M-PER (Pierce, Rockford, IL) and a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) was added at a 1:750 dilution. Crude cell extracts were quantified using Dc protein assay (Bio-Rad, Hercules, CA). Fifty micrograms of protein were subjected to SDS-PAGE and immunoblotting following standard protocols. Membranes were probed overnight with antibodies against COX-2 (CaymanChem, Ann Arbor, MI), HIF-1 $\alpha$  (BD Biosciences, Franklin Lakes, NJ) and GAPDH (Santa Cruz, Santa Cruz, CA) and washed and probed with appropriate HRP-labeled secondary antibodies (Jackson ImmunoResearch, West Grove, PA) following standard protocols. Substrate (SuperSignal West Pico-Pierce, Rockford, IL) was added to the probed membranes and films were developed using automatic film processors (Kodak, Rochester, NY—Agfa, Ridgefield Park, NJ).

**Real-time RT-PCR.** One-step real-time RT-PCR was performed as previously described<sup>14</sup> using 10 ng of total RNA isolated following the Qiagen RNeasy purification protocol as described by the manufacturer (Qiagen, Valencia, CA). Primer sequences for 18S transcript used as a control were: 5'-GGTTGATCCTGCCAGTAGC-3' and 5'-GCGACCAAAGGAACCATAAC-3'. QuantiTect real-time primers for CXCR4, VEGF, SLC2A1 and LDHA were also purchased from Qiagen's geneglobe collection and they are catalogue numbers: QT00223188, QT01036861, QT00068957 and QT00001687 respectively. Fold differences were calculated using the  $2^{-\Delta\Delta C_t}$  method.

**Microarray analysis.** RNA from COX-2-containing (MDA-MB-231) and COX-2-silenced (Clone 2 and Pooled) cells was isolated as described above. Samples were subjected to agarose formaldehyde electrophoresis to determine the quality of the RNA by comparing the ratios of 18S and 28S RNA species. All samples were run in commercial arrays from Affymetrix (Santa Clara, CA), using Human Genome U133Plus 2.0 GeneChip arrays as described in the Affymetrix web site (<http://www.affymetrix.com>). Probe hybridization and analysis was run as suggested by the manufacturer in the aforementioned website. These descriptions include all information currently considered under the MIAME supportive guidelines, with which the JHMI Microarray Core Facility abides in all its procedures. Expression signals were obtained by Robust Multiarray Analysis.<sup>16</sup> The posterior probabilities of the differential expression of genes between the treatments were estimated with an empirical Bayes method using Gamma-Gamma modeling by the bioconductor R package, EBarrays. The methodology for selecting transcripts shown in Figure 2C is as follows: the criterion of the posterior probability

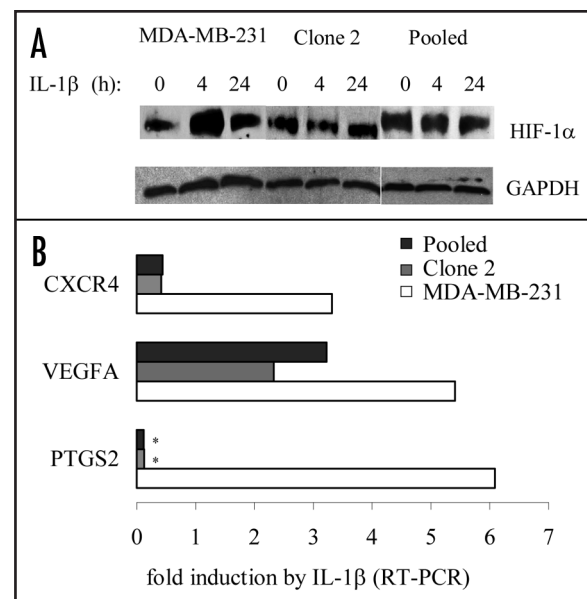


Figure 2. COX-2 silencing inhibits functional HIF-1 $\alpha$  activation by IL-1 $\beta$ . (A) Immunoblotting for HIF-1 $\alpha$  in COX-2-containing parental MDA-MB-231 (231) cells, and COX-2-silenced Clone 2 and Pooled cells in response to the pro-inflammatory cytokine IL-1 $\beta$  (10 ng/ml). (B) Fold induction of HIF-1 $\alpha$ -related transcripts by RT-PCR. \* Below detection limits.

>0.5 was used to produce a list of genes upregulated in hypoxic over normoxic conditions in parental MDA-MB-231 cells. Affymetrix probeset IDs corresponding to transcripts upregulated in hypoxic over normoxic conditions from MDA-MB-231 cells were matched and compared with those from Clone 2 and Pooled cells. Differences in the hypoxic response of MDA-MB-231 cells compared to Clone 2 and Pooled cells higher than 3-fold was considered significant.

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