Alternative Splicing of *Cyr61* Is Regulated by Hypoxia and Significantly Changed in Breast Cancer

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Abstract

Hypoxia is known to induce the transcriptional activation of pathways involved in angiogenesis, growth factor signaling, and tissue invasion and is therefore a potential key regulator of tumor growth. *Cyr61* (*cysteine rich 61*) is a secreted, matricellular protein with proangiogenic capabilities and is transcriptionally induced under hypoxic conditions. High expression levels of *Cyr61* were already detected in various cancer types and linked to tumor progression and advanced stages in breast cancer. Besides hypoxia, there is some evidence that posttranscriptional pre-mRNA processing could be involved in the regulation of *Cyr61* expression, but was thus far not investigated. We studied the expression pattern of *Cyr61* mRNA and protein in breast cancer cell lines as well as in matched pairs of noncancerous breast tissue, preinvasive lesions, and invasive breast cancers, respectively. In addition, we analyzed the potential regulatory capability of hypoxia on *Cyr61* expression by functional tissue culture experiments. Our study revealed a stage-dependent induction of *Cyr61* mRNA and protein in breast cancer tumorigenesis and for the first time alternative splicing of the *Cyr61* gene due to intron retention. Breast carcinogenesis was accompanied by a shift from an intron 3 retaining toward an intron 3 skipping mRNA phenotype consecutively leading to processing of the biologically active *Cyr61* protein. The functional analyses strongly emphasize that hypoxia serves as a specific inducer of alternative *Cyr61* splicing toward the intron skipping mRNA isof orm with potential biological consequences in tumor cells.

[Introduction]

Alternative splicing occurs in the vast majority of human genes and plays a major role in the regulation of gene expression and the generation of proteomic and functional diversity (1, 2). This complex nuclear process creates an average of more than three mRNA isoforms for each gene. Besides skipping and inclusion of variable exons and usage of alternative splice sites (3), intron retention is one possible pattern of alternative splicing, where a variable intron sequence is maintained or skipped within the mature mRNA transcript (4–6). Intron retention potentially affects mRNA transport to the cytoplasm (7) or can insert premature stop codons into the mRNA (4). The latter process is known to induce mRNA degradation by a mechanism called nonsense-mediated decay (NMD; refs. 4, 8). However, there is also evidence for intron-re taining mRNAs that are encoding biologically active protein isoforms (4). These observations emphasize the potent role of alternative splicing as a strong posttranscriptional regulator of gene expression with vast functional consequences. A striking example for this biological importance is given by observed changes in alternative splicing pattern of genes and alterations in splicing factor expression under pathologic conditions especially in human cancers (9–11).

*Cyr61*/CCN1 (*cysteine rich 61*) is a secreted, growth factor–inducible immediate-early gene (12) representing together with CTGF/CCN2 (*connective tissue growth factor*) and NOV/CCN3 (*Nephroblastoma overexpressed*) one of the prototype members of the CCN family (13). The CCN proteins share a common multimodular organization with four discrete and conserved structural domains, each encoded by separate exons (14, 15). The structural basis results in the remarkable diversity of multiple cellular activities and biological functions of these matricellular proteins (14, 16). They either localize intracellularly or associate with the extracellular matrix and the cell surface and play important roles in the regulation of cell adhesion, migration, proliferation, differentiation, and survival (15, 17–21). *Cyr61* is involved in significant physiologic and pathologic processes like development, wound repair, angiogenesis, inflammation, cell survival, vascular diseases, and endometriosis (14, 15, 18–24). In a multitude of various human cancer types, *Cyr61* was found to be overexpressed and is supposed to be a promoter of tumor progression (25, 26), particularly with regard to breast cancer (16). O’Kelly and colleagues (16) revealed a significant correlation between *Cyr61* protein levels and tumor size, stage of disease, and lymph node involvement. Although *Cyr61* does not include a classic nuclear localization sequence like transcription factors, it could be localized in the cytoplasm and nucleus, respectively, demonstrating the multifunctional capabilities of this protein (17). *Cyr61* transcription is inducible by multiple factors, like growth factors [e.g., *EGF* (27), *VEGF*, and *TGF-β* (28)], chemokines (29, 30), estrogen, *TNF-α* (30), and hypoxia (16). The 42 kDa protein interacts within the cell or with surrounding cells through an autocrine-paracrine mechanism, exerting its functions mediated by at least five integrins as well as heparan sulfite proteoglycans (17). The *Cyr61* receptor interaction results in the activation of several signal transduction pathways, including *Wnt* (31), nuclear factor-κB (32), tyrosine kinase, and Akt and the consecutive initiation of transcription of target genes (17). It is likely that overexpression of *Cyr61* causes an up-regulation of its own receptors in an autocrine signaling loop, resulting in the promotion of tumorigenesis and cancer progression (16, 33).

Hypoxia is a key regulatory factor in tumor growth and induces a transcriptional cascade that promotes an aggressive cancer phenotype. Experimental studies support its tumorbiological importance with regard to angiogenesis, growth factor signaling, immortalization, genetic instability, tissue invasion, and apoptosis, especially for breast cancer (34). Interestingly, *Cyr61* expression is transcriptionally induced under hypoxic conditions possibly...
regulated by hypoxia-inducible factor-1α (HIF-1α; refs. 35, 36). As a consequence, Cyr61 promotes adhesion, migration, and survival of vascular endothelial cells (35, 37–39). Martinier and colleagues (40) detected two different mRNA phenotypes of human Cyr61 in a Northern blot analysis of tumor cells from the nervous system, concluding that alternative splicing might be the potential underlying molecular mechanism for this phenomenon. However, this hypothesis was never further investigated.

In the present study, we analyzed the expression pattern of human Cyr61 in human breast cancer in cell lines and matched pair tissue samples, respectively. We describe for the first time the occurrence of alternative splicing of this tumorigenically important gene by the detection of a specific intron 3 retention, leading most likely to NMD of the intron-retaining isoform. Further functional experiments revealed that hypoxia regulates alternative Cyr61 pre-mRNA splicing by inducing skipping of intron 3, leading to the only thus far known active isoform of this protein. With the additional knowledge of hypoxia-induced Cyr61 transcription and the proangiogenic capability of this protein, we postulate that hypoxia is a key regulator of Cyr61 expression and acts as on/off switch for its biological activation and its known tumor-promoting capabilities.

Materials and Methods

Patients and tissue samples. Matched-pair tissue samples of invasive breast cancers and corresponding nonneoplastic tissue from six consecutive patients with invasive primary breast cancer who were treated at the Department of Obstetrics and Gynecology, University Medical Center Freiburg, were analyzed. Approval by the local ethics committee (no. 313/2001) and written informed consent of each patient was obtained. The tissue samples were obtained at the time of surgery, examined by a specialized pathologist, and freshly frozen (−80°C). In a matched-pair analysis, we tested the specimen for Cyr61 expression by reverse transcription-PCR (RT-PCR). In addition, for immunohistochemical analyses, the corresponding paraffin-embedded tissues were analyzed by the same pathologist.

Cell lines and culture conditions. The established human tumor cell lines HeLa, MDA-MB-231, MDA-MB-453, and T47D were cultured in a humidified incubator (37°C) and maintained in DMEM (Life Technologies, Invitrogen), supplemented with 10% fetal bovine serum “Golf” (PAA Laboratories GmbH), 1% of 1 mol/L HEPES buffer (Life Technologies), and 100 units/ml penicillin/streptomycin (Sigma-Aldrich Chemie GmbH). For hypoxia experiments, cultures were transferred to hypoxic culture conditions (1% O2; mentioned as hypoxia) in a hypoxic chamber placed in the same incubator. Cells were cultured in parallel experiments under normal oxygen conditions (95% air, mentioned as normoxia, used as control) and for different time periods under hypoxic (6, 12, 18, and 24 h in 1% O2) and “rescue” conditions (3, 6, 12, and 24 h normoxic conditions following 24 h hypoxia). Experiments concluded with the immediate harvesting of treated cultures for RNA extraction. For the immunocytochemical detection of Cyr61 protein HeLa, MDA-MB-231, MDA-MB-453, and T47D cells were cultured under normoxic and hypoxic conditions for 24 h, as well as under rescue conditions (12 h normoxia following 24 h hypoxia), using four-chamber culture slides (BD Falcon, BD Biosciences Europe) as culture vessels. Experiments concluded with the immediate fixation of treated cultures for immunocytochemistry.

RNA extraction and RT-PCR. Breast tissue specimens were minced on ice by treatment with a tissue homogenizer (IKA Werke) in TRIzol reagent (1 mL per 100 mg tissue, thrice for 10 s; Invitrogen) and RNA was isolated following the manufacturer’s protocol. Purified RNA was dissolved in RNase-free water and stored at −80°C for further analysis. Total RNA from cultured cells was isolated similarly using the TRIzol method.

In total, 4 μg RNA, as determined by optical densitometry, were used for cDNA synthesis using Moloney murine leukemia virus reverse transcriptase (Promega), RiboLock RNase inhibitor (Fermentas GmbH), and random hexamer primers (New England Biolabs GmbH) followed by PCR (35 cycles) using Taq polymerase (Fermentas) and primers specific for exons 3–4 of Cyr61 (5′-primer; 5′-GGCAGACCCGTGAAATAAA-3′; 3′-primer, 5′-CAGGCTTGCATTGGAAT-3′) and 18S RNA as internal control (5′-primer, 5′-AATCTGAAGATGGGTG-3′; 3′-primer, 5′-CA-GAAAGCTTACAACCT-3′). Expected amplicon sizes were 481 bp for Cyr61 and 305 bp for 18S RNA, respectively. All results from RT-PCR for Cyr61 products were normalized using the corresponding 18S RNA expression as a comparative value.

Cloning and sequencing. cDNA from malignant breast specimen displaying an amplification of both Cyr61 mRNA isoforms were used for assembly in an effectual quantity. The two Cyr61 variants were cloned into One Shot Top10 chemically competent Escherichia coli (Invitrogen) using the pETL2 cloning vector (Fermentas). Sequences of both Cyr61 mRNA isoforms were analyzed by Genterprise Genomics (Genterprise Genomics Gesellschaft für Genanalyse und Biotechnologie mbH).

Immunohistochemistry. Routinely formalin-fixed and paraffin-embedded specimens were studied for the expression of human Cyr61 by using a polyclonal Cyr61 (H78) antibody (Santa Cruz Biotechnology, Inc.). For visualization of Cyr61 expression, antigen retrieval and indirect immunoperoxidase technique were applied. Antigen retrieval was performed by cooking the dewaxed sections for 10 min in a 10 mmol/L sodium carbonate buffer (pH 6.0). Inhibition of endogenous peroxidase was performed by 5-min incubation with 3% H2O2. Endogenous avidin-biotin was blocked by the use of a commercial biotin blocking system (DAKO, DAKO GmbH) for 10 min. After two washes in trisaline buffer, slides were incubated with 1% goat serum for 30 min to block unspecific staining. The sections were exposed to the Cyr61 antibody (1:200) overnight at room temperature. Slides were incubated with biotinylated anti-rabbit immunoglobulins for 60 min at room temperature and treated with streptavidin-peroxidase (DAKO). Staining was achieved by 3,3′-diaminobenzidine (DAB; Vectastain, Vector Laboratories, Inc., Linaris GmbH) and the slides were counterstained with hemalum.

Immunocytochemistry. Immunocytochemistry was carried out with previously treated cell cultures on special culture slides (BD Falcon). Cells were fixed using paraformaldehyde fixation reagent (4% paraformaldehyde in PBS) and incubated with the Cyr61 (H78) antibody (1:500 in blocking buffer, Santa Cruz Biotechnology, Inc.) overnight, followed by incubation with anti-rabbit immunoglobulin and treatment with streptavidin-peroxidase (DAKO). Staining was achieved by DAB (Vectastain) and cells were counterstained with hemalum.

Equipment for documentation and evaluation. Documentation of electrophoresis gels was performed using DIANA II (CCD Camera System, Raytest Isotopenmessgeräte GmbH) and evaluation with AIDA (2D densitometry) software (Raytest). Microscopic examination of cells and tissues was performed using Axiosplan 2 microscope (KARL ZEISS Microimaging GmbH), documentation with Canon Powershot G5 and Adobe Photoshop CS2 Version 9.

Statistical analysis. Expression difference of the Cyr61 mRNA isoforms between tumorous and noncancerous breast tissue was calculated. Percentages of Cyr61 mRNA were normalized against the corresponding internal control (18S RNA). The expression differences of Cyr61 mRNA isoforms in samples originating from functional in vitro experiments (cells) were calculated in an analogous manner. The Kolmogorov-Smirnov test showed a nonnormal distribution of the results, which was not improved by logarithmic transformation. Therefore, the Mann-Whitney U test for unpaired groups was used. The Statistical Package for the Social Sciences software version 15.0.1 (SPSS 15.0.1.) was used for statistical analysis.

Results

Cyr61 is alternatively spliced in human breast tissue. Using RT-PCR analysis, alternative splicing of Cyr61 was identified in malignant and noncancerous human breast tissue. Instead of the expected 481-bp amplicon, a different distinct mRNA phenotype,
Repeated sequence analyses revealed that the two distinct containing one distinct fragment were isolated for sequencing before cloning. Selected clones were tested by PCR and plasmids containing one distinct fragment were isolated for sequencing. Repeated sequence analyses revealed that the two distinct Cyr61 mRNA isoforms were due to retention of the 131-nt intron 3 separating exon 3 and exon 4 of the mRNA isoforms were due to retention of the 131-nt intron 3 containing one distinct fragment were isolated for sequencing. Repeated sequence analyses revealed that the two distinct Cyr61 mRNA isoforms were due to retention of the 131-nt intron 3 separating exon 3 and exon 4 of the Cyr61 gene, leading to intron skipping phenotype (IS) with the thus far published 481 bp mRNA isoform (Fig. 1). More interestingly, further sequence analysis revealed that intron 3 contains two stop codons (Fig. 1B), highly suggesting that this mRNA isoform is not capable of generating a functional full-length protein and only the shorter intron-skipping mRNA phenotype encodes the active Cyr61.

Altersations in Cyr61 alternative splicing pattern in breast cancer. Although the majority of examined breast tissue specimen exhibited high expression levels of the Cyr61 IR mRNA isoform, the IS phenotype was differentially expressed. By normalizing the Cyr61 mRNA levels against 18S mRNA, it became evident that in the matched-pair analysis, expression levels of the functional IS phenotype was markedly increased in invasive breast cancer compared with the corresponding noncancerous breast tissue by nearly 50% (Fig. 2A). Besides the box plots, we performed an additional statistical analysis that revealed a significant difference in the expression of the Cyr61 IS mRNA isoform between tumor and noncancerous breast tissue ($P = 0.004$, two-sided, Mann-Whitney $U$ test). Expression levels of the Cyr61 IR mRNA isoform did not show a significant difference between malignant and noncancerous tissue. To further determine the alternative splicing activity and the consecutive activity level of Cyr61, we analyzed the given ratio of IS to IR (IS/IR) in these matched pairs of tumors and noncancerous tissues. In parallel to the above-mentioned findings, a strong shift toward an induced alternative splicing of the IS mRNA phenotype in breast cancer versus noncancerous tissue from 30% to 46% was detectable (Fig. 2B).

Immunohistochemical detection of Cyr61 protein in breast tissue. To investigate the expression and localization pattern of Cyr61 under pathologic conditions, tissue sections from patients with primary invasive breast cancer were examined. The polyclonal Cyr61 antibody (H-78, Santa Cruz Biotechnology) used is directed against amino acids 163-240, the so-called "hinge region" connecting the NH2- and COOH-terminal halves of the protein. Therefore, this antibody is only capable to detect functional Cyr61 protein. A clear and specific Cyr61 staining pattern was visible in the examined breast specimen. Protein expression was highly induced in malignant compared with noncancerous epithelial cells (Fig. 3). Interestingly, specific and strong cytoplasmic and perinuclear Cyr61 staining was detectable in tumor cells of invasive ductal carcinoma, whereas the noninvasive cells of ductal carcinoma in situ (DCIS) displayed a weaker and more heterogeneous expression pattern. In these cases, more single foci of cells with strong Cyr61 expression were identified. In contrast to DCIS, a more homogenous expression profile of Cyr61 was seen in lobular carcinoma in situ with a more pronounced perinuclear expression pattern (Fig. 3C).

Hypoxia induces a significant shift of Cyr61 alternative splicing in vitro. Cyr61 mRNA expression was detectable in all

Figure 1. mRNA isoforms of human Cyr61.

A, expression of Cyr61 mRNA isoforms in breast tissue. The alternatively spliced pre-mRNA isoform Cyr61 IR characterized by the retention of intron 3 (amplicon size 612 bp) and the constitutive pre-mRNA isoform Cyr61 IS (intron 3 skipping, amplicon size 481 bp) were detected. RT-PCR analysis with equal amounts of total RNA (4 $\mu$g) in matched pairs of malignant (CA) and corresponding noncancerous tissues (N) originating from patients with primary adenocarcinoma of the breast. HeLa-cDNA served as control (C); 18S RNA expression served as a comparative value. Box, Cyr61 alternative splicing; genomic structure of both Cyr61 mRNA isoforms (Ex1–Ex5 exons, In1–In4 introns). B, nucleotide sequence of exons 3 and 4 (upper case characters) and intron 3 (lower case characters) of the human Cyr61 gene. The retention of intron 3 creates two stop codons (in boxes) within the intronic sequence.
examined cell lines (HeLa, MDA-MB-231, MDA-MB-453, T47D). Because Cyr61 is a hypoxia-inducible proangiogenic factor, we examined the expression levels of this gene under normoxic (5% CO₂, 95% air) and hypoxic (1% O₂) conditions, and in "rescue" experiments with normoxia following 24 hours of hypoxia.

The IR mRNA isoform was detectable under normoxic as well as under hypoxic conditions. In contrast, the IS mRNA isoform, resulting from intron 3 skipping, was only hardly detectable by RT-PCR in cells cultivated under normoxia. However, a strong shift in alternative splicing toward the IS phenotype emerged with extended phases of hypoxia and this shift was furthermore reproducibly decreased in a stepwise fashion with reversion to normoxia following hypoxia (Fig. 4).

These observations in different cell lines strongly suggest that oxygen deprivation causes a reversible shift of the alternative splicing pattern of Cyr61 in a time-dependent manner toward the intron 3 skipping mRNA isoform, presumably the only Cyr61 mRNA phenotype resulting in a functional Cyr61 protein. Therefore, we hypothesize that hypoxia serves as an on/off switch for the processing of the full-length protein, acting as a tumorigenically pertinent factor.

Looking in more detail into the examined cell lines, we were able to assess slight differences in their alternative splicing alterations in response to altered oxygen conditions. The basic levels of IS/IS+IR showed similar low levels of splicing activity in the examined cell lines. Changing the cultivation conditions to oxygen deficiency resulted uniformly in a marked shift toward the highest expression levels for IS after 24 hours (Fig. 4). Hypoxia induced a dramatic change in Cyr61 mRNA expression with an induction of the Cyr61 IS phenotype compared with total Cyr61 mRNA (IS/IS+IR) from 8.5% up to 62% in HeLa and from 9.3% to 60% in MDA-MB-231 cells, respectively. Statistical analysis revealed a highly significant increase in the intron-skipping Cyr61 isoform expression between cells cultivated under normal oxygen conditions and hypoxia (24 hours; \( P < 0.0001 \), two-sided, Mann-Whitney U test). These data were reproducible in all examined cell lines. Rescue experiments showed a fast and continuous decrease in expression of this splice product after recovery of normal oxygen conditions and the shift toward predominant expression of the IR phenotype. MDA-MB-453 and T47D cells featured a decelerated adaptation to altered oxygen concentrations compared with the other cell lines, resulting in a prolonged higher expression rate of the functional active Cyr61 mRNA isoform (Fig. 4).

**Immunocytochemical detection of Cyr61 protein under altered oxygen conditions.** The immunocytochemical detection of Cyr61 protein was performed using HeLa, MDA-MB-231,
perinuclear localization (shows a more homogenous expression pattern than DCIS and a more reveals no expression of Cyr61 in the epithelial or myoepithelial cell layer. in contrast to nonneoplastic ductal epithelium on the lower right side, which strong Cyr61 expression are identified (Fig. 3).

In parallel to the mRNA findings, the rescue experiment revealed the reversibility of the on/off switch of Cyrl6 alternative splicing induction by hypoxia. This was shown by a markedly decreased nuclear as well as cytoplasmic Cyr61 protein expression (Fig. 5). This experimental part of the immunocytochemical examination displayed the most notable differences among the various cell lines. Some cell types seemed to react toward the oxygen conditions with regard to their Cyr61 protein expression faster than others. In particular, HeLa and MDA-MB-231 returned faster to their original Cyr61 expression pattern than the other cell types.

Discussion

Hypoxia is a key regulator in tumor growth and induces transcriptional activation of pathways involving angiogenesis, growth factor signaling, immortalization, and tissue invasion and was already identified as a tumoriologically important factor in breast cancer (34). Thus far, it is known that Cyr61 expression is transcriptionally induced under hypoxic conditions, which are frequently observed in rapidly growing tumors and their metastases (35, 36). In this scenario, Cyr61 displays a proangiogenic activity by promoting vascular endothelial cell survival, adhesion, and migration (35, 37–39). Aberrant expression of Cyrl61 was already linked to tumor development and elevated expression levels were associated with advanced tumor stages in breast cancer (17, 25, 41). Its involvement in several important signaling pathways makes Cyr61 an interesting focus of cancer research. Besides hypoxia, there was some evidence that posttranscriptional pre-mRNA processing might be involved in the regulation of Cyrl61 expression because two different mRNA phenotypes were detected in Northern blot analyses (40). However, the underlying mechanisms were never further investigated.

For the first time, our study reveals the occurrence of two distinct Cyr61 mRNA isoforms, which are generated by alternative splicing of the Cyrl61 pre-mRNA. Sequence analysis revealed that the two existing mRNA phenotypes are due to alternative retention of intron 3, which is located between exons 3 and 4 of the Cyrl61 gene. The splicing effect on both Cyrl61 mRNA isoforms is independent from any mutations. Repeated analyses of breast tissue and cell culture specimen under changing oxygen conditions did not show mutational alterations within the investigated exonic and intronic sequences of the two Cyrl61 mRNA phenotypes.

Alternative pre-mRNA splicing is an important nuclear mechanism for the regulation of gene expression, which affects ~45% to 60% of human genes (1, 2, 42, 43) and is frequently altered in cancer (3, 44). However, the complete retention of an intron in a mature transcript is one of the least common types of alternative splicing (4) and is estimated to occur in only 5% of splicing events (45). Genes representing intron retention are more highly and broadly expressed than other genes (4). Most probably, intron retention is

Figure 3. Immunohistochemical localization of the Cyr61 protein in breast tissue. A, invasive ductal carcinoma. Invasive carcinoma cells on the left side (arrows) show significant cytoplasmic and perinuclear overexpression of Cyr61 in contrast to nonneoplastic ductal epithelium on the lower right side, which reveals no expression of Cyr61 in the epithelial or myoepithelial cell layer. B, in DCIS, Cyr61 expression is weaker and heterogenous. Single foci of cells with strong Cyr61 expression are identified (arrows). C, lobular carcinoma in situ shows a more homogenous expression pattern than DCIS and a more perinuclear localization (arrows). Cyr61 antibody H-78 (Santa Cruz Biotechnology) counterstained with hemalaun. Magnification, ×400.

MDA-MB-453, and T47D cells, cultivated under normoxic and hypoxic conditions for 24 hours, as well as rescue conditions (12-hour normoxia following 24-hour hypoxia). The H-78 (Santa Cruz Biotechnology) antibody was used for the exclusive detection of functional Cyr61 protein. The subsequent propositions apply consistently to all examined cell lines.

Under normal oxygen conditions (5% CO₂, 95% air), the Cyr61 protein was only measurable in mitotic active cells. High protein concentrations were located in the nuclear matrix and the nuclear membrane, respectively, of cells displaying proximate amitosis. After dissolution of the nuclear membrane, the protein was also detectable in low concentrations in the cytoplasm of dividing cells. During the whole process of mitosis, the Cyr61 protein was present in the nuclear elements.

Oxygen deficiency (1% O₂) triggered a specific increase of Cyr61 protein in all cells, independently from the current stage of cell cycle and caused nuclear as well as very strong cytoplasmic Cyr61 expression. Nevertheless, the highest concentrations were still detectable in the nuclear elements (Fig. 5).

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associated with weaker splice sites, shorter intron lengths, and lower density of intronic splicing enhancers like GGG, respectively (4, 45, 46). In this regard, Cyr61 fits very well into this scenario, because it contains relatively short introns (intron 1, 317 nt; intron 2, 349 nt; intron 3, 131 nt; intron 4, 115 nt), compared with the average intron length of human genes with ~5,000 nt (4). The exon 3-intron 3-exon 4 complex with a length of 697 nt still matches the distribution of lengths of retained intron and flanking exons.

![Figure 4](https://www.aacrjournals.org/doi-fig/10.1158/0008-5472.CAN-08-1997)

**Figure 4.** Statistical analysis. Expression ratio of Cyr61 intron-skipping isoform (Cyr61 IS) compared with total expression of Cyr61 mRNA isoforms (IS+IR) in human tumor cell lines HeLa, MDA-MB-231, MDA-MB-453, and T47D under (N) normal oxygen conditions (5% CO2, 95% air); (H) hypoxic conditions (1% O2) for 6, 12, 18, and 24 h; and (R) rescue conditions (5% CO2, 95% air) for 3, 6, 12, 18, and 24 h following 24 h of hypoxia. There is a highly significant increase in the Cyr61 IS isoform expression between cells cultivated under normal oxygen conditions and hypoxia (24 h; P < 0.0001, Mann-Whitney U test), reproducible in all examined cell lines. **Thick lines,** median (50% percentile); **gray boxes,** 25% to 75% percentile; **thin lines,** minimal and maximal value.
described by Sakabe and colleagues (4). In addition, the triplet sequence GGG acting as an intronic splicing enhancer is only rarely found in the human Cyr61 gene, especially in the flanking exons 3 and 4. A more detailed sequence analysis revealed that intron 3 includes two stop codons, suggesting that they potentially trigger the mRNA degradation through the process of nonsense-mediated decay (46, 47). Thus, intron 3 retention in Cyr61 mRNA is most likely leading to no functional protein and alternative splicing might therefore represent a potential regulator of Cyr61 activity. These data are in line with earlier findings from our group, which described the retention of short intron 9 of the extracellular matrix protein CD44 as a form of aberrant splicing event in breast cancer (6).

Our observations generated even more oncological effect, because the analyses of matched pairs of invasive breast cancers and corresponding noncancerous tissue revealed a specific and very strong induction of alternative Cyr61 splicing toward the IS phenotype in invasive breast cancers. Whereas noncancerous tissue predominantly expressed the Cyr61 IR mRNA, splicing shifted markedly toward the expression of the IS mRNA in invasive breast cancer. This strong induction of the IS mRNA isoform will most likely enhance the activity level of Cyr61 protein in tumor cells because this isoform encodes for the only active Cyr61 protein. The mRNA findings were strongly supported by immunohistochemical analyses showing a stage-dependent induction of functional Cyr61 protein expression in breast carcinogenesis. Although normal breast epithelial cells displayed very low levels of Cyr61 protein, DCIS precursor lesions showed already a stronger expression, which was further induced up to high levels in invasive breast cancer cells of ductal carcinomas (Fig. 3).

With the knowledge of the inducibility of Cyr61 expression by hypoxia, we speculated if our findings in breast cancer specimen mirror a possible oxygen deprivation in tumor cells and if hypoxic conditions might alter Cyr61 alternative splicing. Our functional experiments in HeLa and several breast cancer cell lines revealed that hypoxia is reproducibly able to alter alternative Cyr61 premRNA splicing pattern by enhancing the removal of intron 3 and thereby potentially leading to the generation of the biologically active protein. An up to 6-fold induction of the IS mRNA phenotype was observed. Remarkably, this activation, which was time dependent and reached a maximum after 24 hours of hypoxia, was completely reversible by restoring normoxic conditions for the tumor cells (Fig. 4). This phenomenon was also present on the protein level as detected by immunocytochemistry (Fig. 5) and

Figure 5. Immunocytochemical detection of Cyr61 protein in human tumor cell lines. MDA-MB-231 (A1) and MDA-MB-453 (A2) cells under normal oxygen conditions (5% CO₂, 95% air). Cyr61 protein is only detectable in cells currently going through phases of mitosis, in the process displaying weak cytosolic (30%) and stronger nuclear expression (70%). MDA-MB-231 (B1) and MDA-MB-453 (B2) cells under hypoxic conditions (1% O₂) for 24 h. Strong Cyr61 protein expression, 80% cytosolic, 20% nuclear. MDA-MB-231 (C1) and MDA-MB-453 (C2) cells under rescue conditions (5% CO₂, 95% air) for 12 h following 24 h of hypoxia. Cells display a markedly decreased Cyr61 expression after reoxygenation: 70% cytosolic and 30% nuclear. Cyr61 antibody H-78 (Santa Cruz Biotechnology) counterstained with hemalaun. Magnification, ×400.
strongly supports the hypothesis of a specific mechanism for the regulation of Cyr61 expression. With the additional knowledge of hypoxygen-induced Cyr61 transcription, we postulate that hypoxygen is a key regulator of Cyr61 expression. It acts through alternative splicing alterations as an on/off switch for the biological activation of Cyr61 by promoting intron 3 skipping, leading to functional protein and thereby enhancing its proangiogenic activities.

At this point, it has to be speculated if specific trans-acting factors are involved in the hypoxygen-induced changes in Cyr61 mRNA splicing pattern. From the literature, it is known that several splicing factors can change their intracellular concentration and localization under the influence of hypoxygen conditions and therefore might cause alterations in alternative splicing (45, 48).

Human Tra2-β1, a transcription factor specifically induced in breast cancer and regulating alternative splicing of the CD44 gene (49), could potentially affect the splicing pattern of Cyr61. Exon 3 of the Cyr61 gene comprises five potential sites displaying the Tra2-β1 binding motif GVGANR (49). Sequence analysis of the exon 3-intron 3-exon 4 system also revealed the existence of several possible binding motifs (YAGR, ref. 50) for the transcription factor hnRNPA1. Further functional experiments are warranted.

In summary, our study reveals for the first time alternative splicing of Cyr61 pre-mRNA, which is due to the alternative retention of intron 3. Retention will most likely lead to NMD and consecutively to biological inactivation of the protein. In breast cancer carcinogenesis, we detected a strong stage-dependent shift from the intron 3–retaining toward the intron 3–skipping mRNA phenotype, leading most likely to an activation of Cyr61 protein. Functional studies identified hypoxygen as a specific and reversible on/off switch for Cyr61 activity by significantly promoting alternative Cyr61 splicing toward the intron skipping mRNA phenotype. With the knowledge of Cyr61 transcriptional activation under hypoxygenic conditions and its proangiogenic capabilities, we postulate that hypoxygen serves via alternative splicing as a central regulator for Cyr61 expression and its tumor-promoting activity.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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