Receptor-Independent Ectopic Activity of *Prolactin* Predicts Aggressive Lung Tumors and Indicates HDACi-Based Therapeutic Strategies

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Abstract

Aims: Ectopic activation of tissue-specific genes accompanies malignant transformation in many cancers. *Prolactin* (*PRL*) aberrant activation in lung cancer was investigated here to highlight its value as a biomarker. **Results:** *PRL* is ectopically activated in a subset of very aggressive lung tumors, associated with a rapid fatal outcome, in our cohort of 293 lung tumor patients and in an external independent series of patients. Surprisingly *PRL* receptor expression was not detected in the vast majority of *PRL*-expressing lung tumors. Additionally, the analysis of the *PRL* transcripts in lung tumors and cell lines revealed systematic truncations of their 5' regions, including the signal peptide-encoding portions. *PRL* expression was found to sustain cancer-specific gene expression circuits encompassing genes that are normally responsive to hypoxia. Interestingly, this analysis also indicated that histone deacetylase (HDAC) inhibitors could counteract the *PRL*-associated transcriptional activity. **Innovation and Conclusion:** Altogether, this work not only unravels a yet unknown oncogenic mechanism but also indicates that the specific category of *PRL*-expressing aggressive lung cancers could be particularly responsive to an HDAC inhibitor-based treatment. *Antioxid. Redox Signal.* 23, 1–14.

Introduction

THE DEMONSTRATION OF large-scale ectopic activations of tissue-restricted genes in many unrelated cancers (1, 4, 9, 19, 23, 30) indicates that cancer cell hallmarks could be sustained by largely unknown mechanisms involving not only genetic alterations but also tissue-specific genes that are wrongly activated (9, 24, 25, 31, 37). The out-of-context activity of these tissue-specific genes, aberrantly expressed due to genetic or epigenetic upheavals, could be highly oncogenic. A clear example is that of NUT, a testis-specific factor expressed in a very aggressive midline carcinoma known as NUT midline carcinoma (NMC) in fusion with BRD4, a double bromodomain-containing factor (12). The dissection of the molecular actions of NUT in this context demonstrated the ability of NUT to sequester CBP/p300 in nuclear foci, subsequently leading to the inability of cancer

Innovation

Diverse and tumor-specific genetic and epigenetic upheavals underlie the occurrence of many yet unknown oncogenic mechanisms. Part of these oncogenic events comprises ectopic activation of normally tissue-specific genes (30). One of these genes is *prolactin* (*PRL*) reported here to be illegitimately activated in very aggressive lung tumor and associated with particularly poor prognosis. In breast and prostate cancer, oncogenic *PRL* acts through its receptor (*PRL-R*) following an autocrine or paracrine pathway (16). In this study, we show that a combination of genetic alterations and ectopic activation of *PRL* is at the heart of a completely unexpected receptor-independent oncogenic mechanism that can be counteracted by histone deacetylase (HDAC) inhibitors.

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cells to respond to various stimuli (29, 32). These molecular studies also indicated therapeutic strategies based on the histone deacetylase inhibitor (HDACi) (29, 32) or bromodomain inhibitors (11). In the case of lung cancer, a systematic and detailed investigation of ectopic gene activations showed that illegitimate expressions of a subset of germline and placenta-specific genes is specifically associated with highly aggressive tumors. This observation strongly supports the hypothesis that, among the hundreds of ectopically activated tissue-specific genes, some decisively contribute to the very aggressive nature of the cells. This hypothesis receives strong support from data obtained following the use of a brain tumor model induced in a specific *Drosophila* line. In this setting, tumor induction resulted in the ectopic activation of a panoply of germline genes that proved to be indispensable for sustaining tumor cell proliferation (21). Other examples show that in human cancers, individual germline-specific genes strongly contribute to the establishment/maintenance of the malignant states. For instance, various members of the MAGE family of cancer-testis genes have been shown to regulate the different signaling systems involving p53 (6, 27, 36, 38). Additionally, ectopic activation of germline-specific genes in cancers could also confer resistance to a specific anticancer treatment. An example is the illegitimate activation of *CYCLON*, a gene normally expressed in testis, in diffuse large B-cell lymphoma (DLBCL). *CYCLON* ectopic activation specifically interferes with the cell response to rituximab and is associated with poor clinical outcome in DLBCL (8).

Prolactin (PRL) and its receptor have now been convincingly shown to be involved in breast and prostate tumorigenesis (10), and consequently targeting the corresponding signaling systems could be considered as a reasonable therapeutic strategy in different cancers, especially in prostate cancer (16). To date, the tumor-promoting action of PRL has been thought to be dependent on its receptor (PRL-R), which is also expressed by cancer cells and enables them to respond, through paracrine or autocrine loops, to circulating PRL from the pituitary gland or to local PRL produced by the cancer cells themselves (10).

In this study, we report the investigation of the out-of-context expression of *PRL* in lung cancer to explore its re-

levance as a potential biomarker for prognosis and as a guide in orienting a particular therapeutic approach.

Results

Ectopic PRL activation in a subset of very aggressive lung tumors

Recently, we reported transcriptomic data from a cohort of 293 lung tumors (30). These data were used to monitor the expression of *PRL*, which was found to be expressed in 27 lung tumors out of our series of 293 (9.2%) (Fig. 1A, B). A search of expressed sequence tag (EST) database (Supplementary Fig. S1A; Supplementary Data are available online at www.liebertpub.com/ars), experimentally confirmed by RT-PCR and RT-qPCR approaches on RNA from various human tissues (Supplementary Fig. S1B, C), showed that *PRL* is normally highly expressed in the pituitary gland and also, at reduced levels, in several other tissues, including the placenta. This comprehensive investigation of *PRL* expression showed that, although *PRL* expression is not strictly restrained to the pituitary gland and can be detected in some other extrapituitary sources, no ESTs or transcripts were detected in normal lung (Supplementary Fig. S1A–C). To confirm the absence of the *PRL* transcript in normal lung, an RT-PCR-based detection of *PRL* mRNA was setup using the placenta as a positive control and three *PRL*+ [#1, 3, and 6] and three *PRL*– tumors [#2, 4, and 5]. The result obtained (Fig. 1C) along with the *in silico* studies described above definitively confirmed the absence of *PRL* mRNA in normal lung.

We became particularly interested in the functional consequences of the ectopic activation of *PRL* in lung tumors following the observation that most of the patients with *PRL*+ tumors died rapidly (p -value <0.0001; Hazard ratio = 3.23) (Fig. 1D). This association between the ectopic *PRL* expression and adverse clinical outcome was confirmed in a completely independent study (transcriptomic data publically available under the GEO reference GSE19188), where the patients with *PRL*-expressing tumors (10.9%) also exhibited a very poor prognosis (Fig. 1E). Since neuroendocrine lung tumors are more prone to express neuronal genes (13, 35), we wondered whether the ectopic *PRL* activation could only occur in tumors of the neuroendocrine

FIG. 1. *PRL* expression detected by a transcriptomic analysis in 9.2% of pulmonary tumors is associated with a patient's poor prognosis. (A, B) Expression ratios of *PRL* probe set (205455_at, 11 probes recognizing exons 4 and 5 of *PRL* gene sequence, whose positions are shown on the lower panel) obtained by transcriptomic analysis (Affymetrix Technology) of 293 lung tumors of the cohort. Expression ratios were obtained by dividing the RMA normalized signal values by the mean value of the *PRL* signal obtained from in 109 normal human somatic tissues (not including the pituitary gland). We considered that the *PRL* gene was expressed in a given tumor if this ratio was higher than the threshold (blue line: this threshold corresponds to the mean value of the *PRL* signal obtained from in 109 normal human somatic tissues as above). Twenty-seven tumors (9.2% of the cohort) exhibiting a *PRL* expression ratio higher than the threshold were considered *PRL*-expressing tumors; patients were labeled “*PRL*+ patients.” (A) Patients were randomly assigned. (B) Patients were classified in an ascending order of *PRL* expression ratios. (C) RT-PCR analysis with *PRL*-specific primers of six patients' RNA samples. *U6* amplification was used as a loading control. Negative control (–) was performed with H₂O. The *PRL* status (+: expressing or –: not expressing), as determined by the transcriptomic analysis, is indicated for each patient. The scheme on the left shows the position of the exons in *PRL* mRNA and those of the primers used (numbered). (D–F) Survival probability curves (established by the Kaplan–Meier method) for *PRL*– and *PRL*+ patients. The significance of the difference in prognosis was assessed by the univariate log-rank test. The hazard ratio estimates the difference in death rates between the two groups. (D) Survival probability curves in the lung cancer (CHU Grenoble) cohort ($n=293$ patients with lung cancer) for *PRL*– ($n=266$) and *PRL*+ patients ($n=27$). (E) Validation performed on the GSE19188 external study ($n=82$ patients with lung cancer). (F) Survival probability curves in the neuroendocrine group ectopically expressing (*PRL*+) or not (*PRL*–) *PRL*. LCNE ($n=56$) (left) and SCLC ($n=22$) (right) are shown. LCNE, large cell neuroendocrine; PRL, prolactin; SCLC, small cell lung cancer. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

subtype. The analysis of the lung tumors of our series demonstrated that the majority of the *PRL*-expressing tumors indeed belonged to the neuroendocrine types of LCNE or small cell lung cancers (SCLC). However, we also found that not all LCNE and SCLC tumors expressed *PRL* and that, within each histological subtype, the ectopic activation of *PRL* was associated with a particularly aggressive behavior of the tumors (Fig. 1F). These observations, together with a multivariate analysis quantifying the implication of various parameters (histological classification, tumor node-metastasis [TNM] parameters, *PRL* expression) in the survival (Supplementary Fig. S1D), suggested that *PRL* activation

was not simply a phenomenon associated with neuroendocrine lung tumors, but could itself be a strong predictor of poor prognosis.

Receptor-independent action of ectopically activated PRL

Since *PRL* is believed to act through a specific receptor, we wanted to know if the *PRL*-expressing lung tumor cells also expressed the corresponding receptor (*PRL-R*). The analysis of our series of 293 lung tumor transcriptomes showed that 59 tumors ectopically activated *PRL-R*. However, only 5 of

these tumors also expressed *PRL*, so that 22 out of the 27 *PRL*+ tumors did not show any expression of *PRL-R* (Fig. 2A). We were also able to demonstrate that *PRL-R* expression is not associated with tumor aggressiveness (Fig. 2B). According to the EST databanks (Supplementary Fig. S2A), and consistent with our RT-qPCR data (data not shown), the receptor is normally expressed in the breast, placenta, and uterus, but not in the lung.

These observations suggested a possible and previously undetected *PRL*-dependent, but receptor-independent oncogenic mechanism and prompted us to investigate the issue. Accordingly, to identify *PRL*+ cell lines, we screened the available transcriptomic data from lung cancer cell lines (data not shown) and from various cancer cell lines available to us (Fig. 3A). Following these approaches, we chose four cell lines, all from SCLC, three (H146, H524, and H69) expressing *PRL* at variable, but low amounts (not exceeding the placental expression level), and one (H526) showing no *PRL* expression (Fig. 3B, C). The analysis of the expression of *PRL-R* showed that none of our three *PRL*+ cell lines expressed the receptor (Fig. 3B). In this experiment, to make sure that we confidently defined the state of *PRL-R* expression, a PCR primer set was chosen in a region present in all *PRL-R* encoding mRNAs (2, 20) (Supplementary Fig. S2B) and confirmed the absence of *PRL-R* expression in the nonpathological lung and in our three *PRL*+ cell lines (Supplementary Fig. S2C). These cell lines appeared therefore as good model systems to investigate the molecular basis of *PRL* activity in lung cancer.

Ectopically expressed PRL mRNAs in lung cancers bear 5' truncations and systematically lack the signal peptide-encoding region

To better understand the possible receptor-independent cellular functions of the ectopically activated *PRL* in lung cancers, we first addressed the question whether an important functional determinant of the *PRL* protein, its signal peptide, was present in the ectopically expressed *PRL* mRNA. The absence or the presence of a nonfunctional signal peptide would lead to the production of a nonsecreted *PRL* and

the accumulation of the protein in the cells, which would have the potential to affect various cellular functions as opposed to a secreted protein that, in the absence of *PRL-R*, would not have any effects.

We designed a series of primers to better characterize *PRL* transcripts expressed in our *PRL*+ lung cell lines. We first focused on the two known transcript variants of *PRL*, differing only in the 5'UTR and encoding the same protein, which are expressed either from a proximal promoter in the pituitary gland (variant 1) or from a distal promoter used in the extrapituitary tissues (variant 2) (15). Although we could detect the expected transcripts in the pituitary gland and placenta, the transcripts detected in our lung cancer cell lines lacked the 5' regions, which are normally present in transcripts produced from either of the promoters (Fig. 4A).

A more detailed analysis of these transcripts with sets of primers spanning the *PRL* transcript from a fix 3' position toward the 5' end showed that in all the three *PRL*+ cell lines (H146, H524, and H69), *PRL* transcripts lacked its 5' exons (exon 1 and even a portion of exon 2) (Fig. 4B). Since the beginning of the signal peptide is encoded by exon 1, all the *PRL* transcripts produced in the three studied cell lines lack at least a portion of the signal peptide-encoding region.

Following this observation, we also tested 50 lung tumors, including 31 *PRL*+ and 19 *PRL*- samples. The 31 *PRL*+ group included 23 *PRL*+ tumors detected by the affymetrix transcriptomic analysis as well as 9 additional *PRL*+ tumors, which were identified by RT-PCR among the tumors originally classified as *PRL*- following affymetrix analysis, thanks to the sensitivity of PCR approaches.

Scanning *PRL* transcripts with some of the above-mentioned primers showed that 29 out of the 31 *PRL*+ tumors were missing regions encoding the signal peptide, with various extents of 5' deletions (Fig. 4C and Supplementary Fig. S3). These data therefore indicate that in lung tumors and cell lines, not only *PRL* is ectopically activated but also genetic alterations almost systematically affect the genomic region encoding the 5' part of the transcript that covers the signal peptide.

We also wondered whether the ectopic expression of *PRL* could be associated with amplification of the gene or its

FIG. 2. Expression pattern of *PRL-R* gene in Grenoble lung tumors. (A) Expression of *PRL* and *PRL-R* genes in the 293 patients from the Grenoble lung tumors cohort. Expression values corresponding to the *PRL-R* probe set (227629_at) were extracted from the transcriptomic data (Affymetrix Technology) of 293 patients. The *PRL-R* gene was considered expressed if the *PRL-R* expression ratio was higher than a threshold corresponding to the mean signal for the *PRL* signal in 112 human somatic tissues + 2 standard deviations. *PRL-R* expressing tumors are labeled “*PRL-R*+.” (B) Survival probability curves (established by the Kaplan–Meier method) for *PRL-R*- ($n=234$) and *PRL-R*+ patients ($n=59$). The univariate log-rank test showed no significant differences between the two tumor groups. *PRL-R*, prolactin receptor. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

FIG. 3. Expression patterns of the *PRL* and *PRL-R* genes in human cancer cell lines: identification of SCLC cell lines expressing *PRL* and not expressing *PRL-R*. (A–C) RNA extracted from various human cancer cell lines were submitted to RT-PCR and RT-qPCR analyses with specific *PRL* primers. (A) RT-qPCR analysis on the indicated human cancer cell cDNAs. (B) RT-PCR analyses with primers specific for *PRL* and *PRL-R* on five human lung cancer cell cDNAs as indicated. (C) Expression ratios of the *PRL* gene in five human lung cancer cell lines (and comparison with tissues *PRL* expression ratios) obtained from six independent RT-qPCR experiments, with normalization using two different control genes (*Actin* and *U6*). One hundred percent indicates the mean value of *PRL* expression ratios from the six *PRL*-expressing tissues. Error bars represent the standard deviation.

genomic region. Data from comparative genomic hybridization (CGH) arrays, obtained for 247 of our 293 lung cancer patients, demonstrated that, although the genomic region corresponding to the *PRL* gene was amplified in a proportion of patients, there was no correlation between amplification and expression (Fig. 4D). Moreover, survival comparisons between groups of patients according to the presence or absence of amplification within this region demonstrated that there was no association between the amplification of this region and prognostic (data not shown).

The ectopically expressed truncated PRL is a potent regulator of gene expression

Our data show that all the *PRL*+ lung tumor samples and cell lines express a truncated form of a *PRL* transcript lacking

the 5' part of the mRNA to various extents. Since this almost systematic 5' deletion includes the translation initiation ATG codon, it is unlikely that *PRL* is produced. Nevertheless, we decided to investigate the consequences of the ectopic expression of these transcripts. This was achieved through a siRNA knockdown in two *PRL*+ cell lines (H146 and H524) followed by a whole genome transcriptomic analysis. With a threshold of 1.2 for mRNA variations between control siRNA and the anti-*PRL* siRNA, the number of genes affected was found to be proportional to the level of *PRL* expression. In the H146 cell line, expressing the highest level of *PRL* mRNA (Figs. 3C and 6A), *PRL* silencing resulted in the upregulation and downregulation of 2559 and 2402 genes, respectively, whereas in the H524 cells, which express about half the level of *PRL* mRNA (Figs. 3C and 6A), 800 and 763 genes were, respectively, upregulated and downregulated (Fig. 5A).

Interestingly, the majority of the genes affected by *PRL* silencing in the H524 cell line were also *PRL*-dependent genes in the H146 cells. Indeed, 74% and 68% of genes, respectively, upregulated and downregulated in H524 cells were included in the corresponding gene lists from the H146 cell line after *PRL* knockdown (Fig. 5A). These data strongly suggested that *PRL* affects the same regulatory pathways in these two different cell lines and that the extent of these effects depends on the amount of *PRL* expressed. Within the genes affected in both cell lines, we chose 12 for validation by RT-qPCR using H146 RNA from cells treated with control or anti-*PRL* siRNAs, and the data confirmed the robustness of the analysis (Fig. 5B). Hence, these results reveal an important gene regulatory function for these truncated *PRL* RNAs in both cell lines.

In an attempt to identify the cellular regulatory circuits controlled by *PRL*, a Gene Set Enrichment Analysis (GSEA) was performed on the list of genes differentially regulated after *PRL* knockdown in the H146 cell line. This analysis showed that the group of genes requiring *PRL* to be expressed (downregulated after *PRL* knockdown) significantly overlaps with previously established lists of genes upregulated in non-small cell lung tumors as well as in a variety of unrelated cancers (Fig. 5C).

Interestingly, the GSEA also revealed that in these cells, which are here maintained under normoxic culture conditions, the ectopic *PRL* expression activates a group of genes that were found to be also expressed in response to both hypoxia and HIF1 α (7) (Fig. 5C).

Remarkably, this analysis also revealed a highly significant overlap with genes that are upregulated either after treatment with the HDACi trichostatin A (TSA) or after the knockdown of HDAC1, HDAC2, or HDAC3 (18, 33) in independent studies and cell systems (Fig. 5C). These observations suggested that in *PRL*+ lung cancers, *PRL* somehow controls cellular regulatory circuits involving histone acetylation-dependent transcriptional activities, since both *PRL* knockdown and HDAC1–3 inhibition or knockdown affect a significant number of common genes. To better understand the relationship between *PRL* transcripts/peptides and HDACs, we treated our three *PRL*+ and one *PRL*– cell lines with TSA, as well as with the FDA approved HDAC

inhibitor, SAHA, and measured the level of *PRL* mRNA by RT-qPCR. Interestingly, and consistent with the GSEA results, we observed that treatment with both TSA and SAHA leads to a remarkable decrease in the steady-state amounts of *PRL* mRNA (Fig. 5D), indicating that by downregulating *PRL*, a treatment by HDAC inhibitors could also affect a significant number of common genes.

PRL downregulation sensitizes lung cancer cells to genotoxic treatments

Since in many cases, cancer cell treatment with HDAC inhibitors restores cell cycle check points and apoptotic cell responses (22, 26), we wondered if *PRL* downregulation could also render cells more sensitive to a cytotoxic treatment. Our three *PRL*+ and one *PRL*– lung cancer cell lines were treated with either a control or the two anti-*PRL* siRNAs (Supplementary Table S1A), leading to a significant knockdown of *PRL* expression in all *PRL*+ cell lines (Fig. 6A).

We then assessed the effect of *PRL* silencing on cell apoptosis. We first observed that the loss of *PRL* expression does not affect the basal mortality. We then subjected SCLC cells to genotoxic stress, through a treatment with actinomycin D (Acti D). In the three *PRL*+ cell lines (H146, H524, and H69), *PRL* knockdown increased the proportion of apoptotic cells following the Acti D treatment, while the *PRL* siRNA transfection in the *PRL*– cell line did not significantly modify the proportion of apoptotic cells. The latter cells appeared to be more responsive to a genotoxic treatment, which is consistent with the absence of *PRL* expression (Fig. 6B). This figure also shows the original FACS data for one experiment with the H524 cell line treated with control or anti-*PRL* siRNA (siRNA *PRL* #1) (Fig. 6C). These data show that *PRL* downregulation in this cell line under genotoxic treatment almost doubled the number of dying cells, which approached the totality of the population. It is of note that the dosage of Acti D used to induce genotoxic effects is below the amount required to block the *PRL* gene activity (Supplementary Fig. S4).

We concluded that the *PRL* ectopic expression in SCLC cells is involved in their resistance to cell death following a genotoxic treatment.

FIG. 4. The *PRL* mRNAs ectopically expressed in lung cancer cell lines or in tumor samples are lacking the 5' region corresponding to the signal peptide, but are not correlated with genomic amplification of the *PRL* gene region. (A) RT-qPCR analysis of *PRL* mRNAs from lung tumor cell lines. The top panel shows the position of the primers (numbered) used in (A–C). Primers 11, 16, and 17 are located within the *PRL* coding sequence (CD). Primers 1 and 2 are located within the 5'UTR sequence specific of the pituitary *PRL* mRNA (variant 1). Primers 4 and 5 are located in the exon 1a sequence, which is the 5'UTR sequence specific of the extrapituitary *PRL* mRNA (variant 2). RNAs extracted from human tissues and cancer lung cell lines were submitted to RT-PCR, with the indicated primer pairs. *U6* amplification was used as a control. Negative control (–) was performed with H₂O. (B) Cartography of *PRL* mRNAs shows that exon 1 and exon 1a sequences are absent from the transcripts from the SCLC cell lines. Cartography of the *PRL* mRNA expressed in the SCLC *PRL*+ cell lines was performed by using a reverse primer in 3'UTR (#19) and the indicated forward primers along the *PRL* transcript sequences (numbered). Negative control (–) was performed with H₂O. (C) Cartography of *PRL* mRNAs in tumor samples shows the extended absence of 5' sequences. Cartography of the *PRL* mRNAs generated in lung tumors was performed by using a reverse primer in 3'UTR (#19) and five different forward primers corresponding to every exon junction sequence (numbered). *U6* amplification was used as a control. Negative control (–) was performed with H₂O. (D) Analysis of variations in copy number (deletion and amplification) at the *PRL* gene locus by CGH array in 247 lung tumors of the cohort. For each tumor, the copy number at the *PRL* gene locus is indicated on the x-axis and the corresponding *PRL* expression value is indicated on the y-axis. The correlation between *PRL* expression and *PRL* copy number is evaluated by the nonparametric Spearman's coefficient. A control analysis on nontumor lung samples from 35 patients (right panel) is also shown. CGH, comparative genomic hybridization; UTR, untranslated region. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

Discussion

Genome instability and subsequent gross and subtle genome alterations associated with a profoundly modified epigenetic signaling, systematically accompany malignant

cell transformation (34). Growing and invading cancer cells harbor new states of gene expression involving not only activation and repression of normal genes but also *de novo* creation of genes that never existed in normal cells. Such genes result from chromosomal translocations, mutations,

loss and gain of specific genomic regions. The combined action of these created new functional factors, with gene silencing and ectopic activations of hundreds of tissue-restricted genes, gives birth to cells that acquire new properties (9, 24, 31) allowing them to sustain growth and dissemination, while avoiding destruction (17). In this study, we show that *PRL*, a gene never expressed in normal lung cells, becomes ectopically activated in lung cancer tumors, essentially in a fraction of the neuroendocrine subtype. This activation of *PRL* is strongly associated with adverse clinical outcomes, not only in our population of lung cancer patients from the Grenoble university hospital but also in a completely unrelated group of patients. Unexpectedly, we also found that, in both groups of patients, the majority of *PRL*+ lung tumors did not express detectable amounts of *PRL-R* transcripts. However, our present knowledge of *PRL* biology suggested that, in the absence of a receptor, the produced *PRL* should have no effects on the *PRL*-expressing tumor cells, since most of the synthesized proteins would be secreted out of the cells and no signaling could be initiated in the absence of the receptor. This was inconsistent with our clinical data, showing a systematic association between *PRL* expression and rapid fatal outcome, which suggested that the produced *PRL* should have an effect on the cells and somehow promote their aggressive behavior. Therefore, we made the hypothesis that, in the absence of its receptor, the only way *PRL* expression could have an impact on cells would be that the protein somehow would lose its signal peptide, and hence becomes trapped inside the cells. Our investigations in the selected *PRL*+ cell line models and tumors revealed genome-altering events, which almost systematically affected the first exon of *PRL*. In fact, in most of the tested cases, the transcript did not contain the translation initiation codon ATG. Even in the five cases of tumors expressing *PRL-R* and *PRL* mRNA, the *PRL* transcripts were truncated.

The important question following this observation is whether these mRNAs could give rise to a polypeptide. The use of an antibody able to detect large amounts of

PRL in the pituitary gland, did not allow us to visualize the protein neither in lung tumors nor in our lung cancer cell lines (data not shown). Therefore, at this point, the absence of the translation initiation codon ATG in the transcripts, suggests that actually no easily detectable peptides are expressed. Nevertheless, the detailed investigation of our *PRL*+ cells revealed clear and characteristic molecular responses to the knockdown of *PRL* mRNA. Most remarkably, *PRL* mRNA silencing was associated with very consistent alterations in gene expression in two different cell lines. This gene expression response was proportional to the level of *PRL* expression: a higher number of genes were affected in the cell line expressing the highest amount of *PRL* mRNA. Additionally, the majority of the genes affected in the cell line expressing a lower amount of *PRL* mRNA were included in the list of differentially expressed genes in the first cell line.

This observation suggests that the truncated *PRL* mRNAs could titrate out other regulatory molecules, and hence, the higher the concentration of *PRL* mRNAs, the stronger the effect. One very attractive hypothesis could be that *PRL* mRNAs act as endogenous competing RNAs (ceRNA) and titrate out specific microRNAs [for review see de Giorgio et al. (5)]. However, important investigations are required to unravel the exact molecular mechanism underlying the oncogenic activity of truncated *PRL* mRNAs.

GSEA also indicated a tight link between the genes repressed following *PRL* knockdown in lung cancer cell lines cultured under normoxic conditions and genes that become expressed following hypoxia or a pharmacological stabilization of HIF1 α by a 2-oxoglutarate-dependent dioxygenase inhibitor (7). It has also been shown that HIF1 α contributes to important gene expression programs affecting processes, such as DNA repair, and that a hypoxic microenvironment could enhance genetic alterations in apoptosis-deficient cells (39). By regulating such a specific hypoxia-dependent gene expression program, *PRL* ectopic activation, could confer to cells under normoxic conditions, specific oncogenic properties that normally appear only under hypoxia.

FIG. 5. Identification of *PRL*-responsive genes. (A) In two different *PRL*+ SCLC cell lines (H146 and H524), the transcriptomes of cells treated with control and anti-*PRL* siRNAs were compared to identify upregulated or downregulated genes following *PRL* silencing. The value distribution and mean fold change were calculated and the probe sets corresponding to mRNAs consistently differentially expressed in at least four samples out of the five biological replicates, with a fold change greater than 1.2 retained. After collapsing the probe sets to the genes, a number of differentially expressed genes in both cell lines were represented as Venn diagrams. (B) Validation by RT-qPCR of the 12 differentially expressed genes (common to both cell lines) chosen among those exhibiting a marked fold change after *PRL* knockdown. One hundred percent corresponds to the expression ratio of the values si*PRL*/siControl obtained after RT-qPCR and normalization with *Actin* and *U6*. Fold changes observed in transcriptomic data are indicated below each histogram. (C) A GSEA was performed on differentially expressed genes according to our transcriptomic approach between H146 SCLC cells expressing *PRL* and the same cells silenced for *PRL* expression (si*PRL*). In addition to the results shown here, the analysis performed on the C5 group of gene sets, corresponding to genes grouped by gene ontology terms, shows that i) genes downregulated by si*PRL* (upregulated by *PRL* expression) are significantly enriched in the terms related to actin-based processes, cytoskeleton organization and biogenesis, vesicle and Golgi apparatus-mediated transport, amine and carbohydrate metabolic processes, and oxidoreductase activity; ii) genes upregulated by si*PRL* (downregulated by *PRL* expression) are enriched in terms related to RNA processing and ribosomal constituents, nuclear factors, mitochondrion, DNA repair, DNA replication, and cell cycle. (D) The inhibition of HDACs by TSA or by SAHA induces a downregulation of *PRL* mRNA. The SCLC cell lines were treated with 100 ng/ml TSA (left panel) or SAHA (right panel) for 6 h. The expression values of the *PRL* gene were obtained from six independent RT-qPCR experiments. The expression values were normalized to the *Actin* control gene and 100% corresponds to the *PRL* expression value in nontreated H146 cells. The significance of the differences in *PRL* expression between treated and untreated conditions was assessed by a Student's *t*-test of mean comparison (***p*-value < 0.05). GSEA, Gene Set Enrichment Analysis; HDAC, histone deacetylase; TSA, trichostatin A. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

GSEA also revealed a clear and significant overlap between genes upregulated after the knockdown of *PRL* and genes upregulated after a HDAC inhibitor (TSA) treatment or after a targeted knockdown of *HDAC3*, *HDAC2*, and *HDAC1* (18, 33). This observation is reminiscent of the results reported by Schwartz *et al.*, where the knockdown of *BRD4-NUT* in NMC led to a gene activation that was comparable to the effect observed after treatment of cells with HDAC inhibitors (32). In the case of NMC, the molecular dissection of the underlying mechanism showed that the fusion protein *BRD4-NUT* traps CBP/p300 in nuclear foci that can be released after *BRD4-NUT* knockdown (29). Although we have not here been able to identify the molecular basis of the control of gene expression by *PRL*, it is possible to speculate on a similar direct or indirect effect of *PRL* on the activity of major cellular HATs, so that *PRL* knockdown could restore normal acetylation and the underlying gene expression. Additionally, we showed that a TSA treatment can directly downregulate *PRL*. This could also account for the overlap between HDACi gene responses and our cell line response after *PRL* knockdown. The fact that *PRL* is a strong and reliable predictor of poor clinical outcome in the case of lung cancer and that HDAC inhibitors could downregulate *PRL* or activate a shift of the *PRL*-associated aggressive gene signature toward that observed in tumors with better prognosis, suggests that the treatment of *PRL*+ lung tumors with HDACi alone or in combination with a more conventional chemotherapy, should be a reasonable therapeutic strategy for this category of *PRL*+ lung tumor patients.

Finally, this work highlights once more that cancer cells use many yet unknown and unexpected molecular mechanisms to ensure their malignant states and that a comprehensive understanding of cancer cannot be approached without a global consideration of all the oncogenic events.

Materials and Methods

Material

H146, H524, H69, H526, and H1299 cell lines were obtained from the American Type Culture Collection (ATCC). H146, H524, H69, and H526 are derived from small cell lung carcinomas from Caucasian males, respectively, 59, 63, 55, and 55 years old. H1299 is a non-SCLC from a Caucasian 43-year-old male.

Human tissue RNAs were purchased from the Clontech society (Human Total RNA, Master Panel II), except for

placenta RNAs, which were a kind gift from Dr Nadia Al-faidy-Benharouga (CEA).

The Grenoble lung cancer cohort, established by Pr. Elisabeth Brambilla and Christian Brambilla (CHU), includes 293 patients who underwent surgery at the University Hospital of Grenoble. For over 10 years, clinical data (patient age and gender, smoking status, date of diagnosis, histological and TNM classifications of the tumor, treatment received, and date of death) and biological samples (biopsies, tumor sections, and RNAs) were collected. For each patient, a transcriptomic study was performed on a tumor sample collected before implementation of treatment, and in some cases, on a sample of adjacent noncancerous lung tissue. Detailed clinical and biological information relative to these patients is available in Rousseaux *et al.* (30).

PRL+ versus PRL− cell line transcriptomes

All transcriptomic data in lung cancer cell lines were obtained using the Illumina HumanHT-12V4.0 expression beadchip and were processed exactly as described in Refs. (14, 28). The raw and processed data from the array are deposited in the GEO databank under the accession GSE49544.

Transcriptomic and gene copy number variants from Grenoble lung cancer tumor cohort

Transcriptomic data from 293 pulmonary tumors of the lung cancer cohort were obtained on the Affymetrix Human Genome U133 Plus 2.0 Array (HG-U133_Plus_2) as described earlier (30). These data are available on the GEO website (reference number GSE30219).

For 247 of the same tumor samples, gene copy numbers were also calculated thanks to parallel hybridization of genomic DNAs on Illumina SNP HumanCNV370 chips (Illumina). The GISTIC version 2.0 algorithm (www.broadinstitute.org/cancer/pub/GISTIC2) was used to identify significant regions of amplification or deletion. The frequencies of aberrations contributing to significant peak regions were compared using chi-square tests.

Analysis of PRL and PRL-R expression in tumors

The expression data of *PRL* and *PRL-R* genes in lung tumors were extracted from the Affymetrix RMA normalized signal values corresponding to probe sets *205455_at* (*PRL*) and *205455_at* (*PRL-R*). For each gene, a threshold value was defined corresponding to a positive expression, by using data

FIG. 6. Effect of *PRL* silencing on cell response to genotoxic treatment in *PRL*+ and *PRL*− SCLC cell lines. SCLC cell lines were transfected with siRNA scrambled (si-) or targeting *PRL* (siPRL, two different siRNA duplex) for 48 h, and treated for 12 h by 250 mM Acti D or control treatment. (A) Silencing of the *PRL* gene assessed by RT-qPCR. Expression values of the *PRL* gene were obtained from four independent RT-qPCR experiments. Expressions were normalized to the *Actin* gene. One hundred percent indicates *PRL* expression value in H146 treated with si-Control (scrambled). Silencing efficiencies are indicated. The significance of the differences in *PRL* expression between si- and siPRL conditions was assessed by a Student's *t*-test of mean comparison (***p*-value < 0.05; **p*-value < 0.10). (B) (Top panel) The percentage of apoptotic cells was measured by flow cytometry after incubation with an antiactivated caspase-3 antibody in six independent experiments. (Bottom panel). The ratio of cell mortality between Acti D-treated and nontreated cells was calculated for each of the six experiments. The significance of the differences in the apoptotic cell proportions between si- and siPRL conditions was assessed by a Student's *t*-test of mean comparison (***p*-value < 0.05; **p*-value < 0.10). (C) Example of flow cytometry data. Each dot represents one H524 cell. The PE fluorescence of the antiactivated caspase-3 staining is represented on the y-axis. The FSC size parameter is represented on the x-axis. The P3 delimited region comprises apoptotic cells. Acti D, actinomycin D; FSC, forward-scattered light; PE, phycoerythrin. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

A *PRL* expression ratio

B Cell mortality (% activated caspase 3 positive cells)

C

from a series of normal human somatic tissue samples, as described previously (30). For the *PRL* gene, the threshold above which it was considered expressed was the mean value of the 205455_at probe set signal in 109 samples of normal human somatic tissues (excluding the pituitary samples). For the *PRL-R* gene, the threshold above which it was considered expressed was the mean + 2 standard deviations of the 205455_at of the signal values in 112 normal human somatic tissue samples.

RNA extraction and reverse transcription

SCLC cells growing in suspension were harvested by pipetting 2 days after seeding (and eventual transfection of siRNA or drug treatment). The extraction of total RNA was performed with the NucleoSpin RNA II (Macherey-Nagel) kit according to the supplier's recommendations. The RNA content and purity were assessed using NanoDrop 1000 (Thermo Scientific). The reverse transcription reaction was performed on 1 µg of total RNA using the kit SuperScript III RT First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen) according to the supplier's recommendations.

PCR reactions

Primers were designed with the help of the three following online software: Universal Probe Library Assay Design Center (Roche Applied Science), Primer 3 (<http://frodo.wi.mit.edu/>), or Primer Bank (<http://pga.mgh.harvard.edu/primerbank/>), and their specificity was checked using BLAST (Basic Local Alignment Search Tool) (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The used primers and their sequences are listed in Supplementary Table S1B.

The PCR reactions were carried out in a final volume of 25 µl containing 0.5 µl dNTPs (1 mM, 5'), 0.5 µl of each primer, forward and reverse (10 µM), 2.5 µl of 10× buffer, 0.5 µl of TaqPol enzyme (5 U/µl, 5'), and cDNAs (RT product diluted 1/10). The PCR program was as follows: denaturation at 95°C for 1 min, then 35 cycles (denaturation 95°C 30 s, annealing 30 s, elongation 68°C), and finally 5 min of terminal extension at 68°C. The elongation time varied depending on the length of the PCR product expected (1 min/kb). The annealing temperature was chosen according to the T_m of primers (often 65°C, sometimes down to 55°C). A negative control was achieved by replacing the cDNA by DEPC H₂O. The PCR products were analyzed by electrophoresis in a gel containing 1% agarose (Invitrogen) and 5 µl of BET (10 mg/ml; Sigma-Aldrich) in 100 ml of 1× TAE buffer. DNA (25 µl of PCR product) was mixed with 3 µl of loading buffer (bromophenol blue, 40% sucrose) and loaded onto the gel, together with a size marker (DNA Ladder, Fermentas or 1 kb-Plus; Invitrogen). Migration was performed in 1× TAE at 100 V for about 30 min. The reading was done under a UV lamp.

qPCR reactions

qPCR analysis was performed using Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (3). Primers were designed so that the length of the amplicon is between 70 and 200 bp. qPCR was performed in 96-well plates in a final volume of 20 µl. Each well contained 0.03 µl of each primer, forward and

reverse (100 µM), 10 µl of Brilliant SYBR Green 2× III (Agilent Technology), and 4 µl of cDNA (RT product diluted 1/10). For each condition, a technical duplicate and a control point where the cDNA is replaced by the extracted RNA (no RT) were added. The reaction was carried out in the Mx3005P qPCR cyclers (Agilent Technology) with the following program: 3 min of initial denaturation at 95°C, then 40 cycles with 20 s of denaturation at 95°C, and 20 s of hybridization and elongation at 60°C.

The qPCR analysis was performed using the MxPro software (Agilent Technology). After verifying that a given pair of primers gives a single peak of dissociation, the value of the threshold cycle (C_t) was given by the number of cycles necessary for the measured fluorescence value to exceed the threshold value (threshold = 235). When the C_t exceeded 35, we considered that there was no expression. When the C_t value obtained for the no RT control point was too close (NoRT-RT < 2), we did not take the value into account. The analysis was performed on the average of the two C_t values obtained for the two technical replicates (difference < 1). Expression values were normalized with the selected control genes (*Actin*, *U6*, *RELA*, and/or *AUPI*). The expression values were given as a percentage of the value corresponding to a reference condition (reference tissue such as pituitary or placenta or breast, or nontransfected control condition for cell lines) by the method of Delta C_t (18): $2^{\Delta C_t}$ gene of interest (reference condition – condition of interest) / $2^{\Delta C_t}$ control gene (reference condition – condition of interest).

Cell culture and transfection

H524 and H526 cells were cultured in the Roswell Park Memorial Institute medium (RPMI 1640; Gibco) supplemented with 10% fetal bovine serum (PAA), 2 mM L-glutamine (Gibco), and 1% penicillin–streptomycin (Gibco). For H69 cells, the complete RPMI medium was supplemented with 10 mM HEPES (Gibco), 1 mM sodium pyruvate (Gibco), and 2.5 g/L glucose (Gibco). For H146 cells, the complete RPMI medium was supplemented with 10 mM HEPES (Gibco), 1 mM sodium pyruvate (Gibco), 4.5 g/L glucose (Gibco), and 1.5 g/L sodium bicarbonate (Gibco). Cells were maintained in 75-cm² flasks at 37°C in a humidified 5% CO₂ incubator.

siRNAs targeting human *PRL* and nonspecific control siRNAs were designed with the DSIR software (<http://biodev.extra.cea.fr/DSIR/>). *PRL* siRNA sequences are shown in Supplementary Table S1A. Transfections were carried out in six-well plates with 1.5 µl siRNA duplex (20 µM), 5 µl Lipofectamine RNAiMAX Reagent (Invitrogen), 500 µl of Opti-MEM medium (Gibco), and 250,000 cells in an antibiotic-free culture medium.

Two days after transfection, the culture medium was complemented with 250 nM Acti D (Sigma-Aldrich) for 12 h or 100 ng/ml TSA (Sigma-Aldrich) for 6 h.

Quantification of apoptotic cells

Cells were harvested and washed in PBS. Active caspase-3 was detected using the Active caspase-3 antibody kit (BD Pharmingen) following the manufacturer's instructions. Briefly, cells were fixed and permeabilized for 30 min, and then incubated with the phycoerythrin-conjugated antiactive caspase-3 antibody. Analysis of active caspase-3-positive

cells was performed by counting 10,000 events on a Becton Dickinson FACScan flow cytometer and data were fit using DIVA6 software (Becton Dickinson).

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Author Disclosure Statement

The authors declare that there is no competing financial interest.

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Abbreviations Used

Acti D	= actinomycin D
CGH	= comparative genomic hybridization
DLBCL	= diffuse large B-cell lymphoma
EST	= expressed sequence tags
FSC	= forward-scattered light
GSEA	= Gene Set Enrichment Analysis
HDAC	= histone deacetylase
HDACi	= histone deacetylase inhibitor
LCNE	= large cell neuroendocrine
NMC	= NUT midline carcinoma
PE	= phycoerythrin
PRL	= prolactin
PRL-R	= prolactin receptor
(q)PCR	= (quantitative) polymerase chain reaction
RT	= reverse transcription
SCLC	= small cell lung cancer
TNM	= tumor node metastasis
TSA	= trichostatin A
UTR	= untranslated region