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Identification of p130Cas/ErbB2 dependent invasive signatures in transformed mammary epithelial cells.

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Abstract
Understanding transcriptional changes during cancer progression is of crucial importance to develop new and more efficacious diagnostic and therapeutic approaches. It is well known that ErbB2 is over-expressed in about 25% of human invasive breast cancers. We have previously demonstrated that p130Cas over-expression synergizes with ErbB2 in mammary cell transformation and promotes ErbB2-dependent invasion in three-dimensional (3D) cultures of human mammary epithelial cells. Here, by comparing coding and non-coding gene expression profiles, we define the invasive signatures associated with concomitant p130Cas over-expression and ErbB2 activation in 3D cultures of mammary epithelial cells. Specifically, we have found that genes involved in aminoacids synthesis (CBS, PHGDH), cell motility, migration (ITPKA, PRDM1), and angiogenesis (HEY1) are up-regulated while genes involved in inflammatory response (SAA1, S100A7) are down-regulated. In parallel, we have shown that the expression of specific miRNAs is altered. Among these, miR-200b, miR-222, miR-221, miR-R210 and miR-424 are up-regulated while miR-27a, miR-27b and miR-23b are down-regulated. Overall, this study presents, for the first time, the gene expression changes underlying the invasive behavior following p130Cas over-expression in an ErbB2 transformed mammary cell model.
**Introduction**

Breast cancer is the leading cause of cancer-related death in women world-wide \(^1\). Despite significant advances in breast cancer diagnosis and treatment, several major unresolved clinical and scientific problems remain, such as the understanding of the causes of tumor progression and recurrence and how to predict them \(^2\).

ErbB2 is a well-known oncoprotein that belongs to the epidermal growth factor receptor (EGFR) family. It is over-expressed in approximately 25% of invasive breast cancers \(^3\). In particular, over-expression of ErbB2 has been demonstrated to promote breast cancer invasion and metastasis and to correlate with poor patient survival \(^4\)-\(^7\). However, ErbB2 is also over-expressed in non-invasive mammary ductal carcinomas in situ (DCIS) \(^8\). Indeed, ErbB2 amplification or over-expression seems to be crucial but not sufficient for the transition from in situ to invasive cancer and additional hits are required for the progression of ErbB2-positive tumors. Although the molecular and genetic events underlying ErbB2-positive tumor invasion and metastasis are still not well understood, intense investigation has led to the notion that molecules involved in cell adhesion and migration are critical in this process \(^9\). Moreover, it has recently been reported that altered lysosomal biogenesis and distribution play a key role in ErbB2-dependent cancer cell migration, invasion and metastasis \(^10\).

The adaptor protein p130Cas (Crk associated substrate) has been extensively reported to act as a scaffold molecule for signaling platforms that drive cell migration and invasion by regulating intracellular cytoskeletal and effector proteins in response to extracellular matrix and growth factor signals \(^11\). We have previously demonstrated that p130Cas is a crucial regulator of ErbB2-induced mammary tumorigenesis. In particular, p130Cas is required for ErbB2 signal transduction, in vivo tumor growth and lung colonization \(^12\). By using 3D cultures of MCF10A cells modified by Muthuswamy et al.\(^{15}\) to express a chimeric ErbB2 receptor, which can be activated by the synthetic ligand AP1510 (MCF10A.B2 cells), we demonstrated that the concomitant over-expression of p130Cas and ErbB2 activation confers invasive properties to 3D mammary acini. Indeed, in 3D
culture conditions, MCF10A.B2 cells form acini-like spheroids, that recapitulate the terminal ductal lobular unit of human adult breast. Activation of ErbB2 disrupts the morphogenetic process and results in the formation of multiacinar, non-invasive structures, modelling the early stages of breast cancer onset. In contrast, MCF10A.B2 cells that over-express p130Cas give rise to multiacinar structures, that upon stimulation with AP1510, acquire invasive protrusions. This invasive phenotype is characterized by activation of Erk1/2 MAPKs and PI3K/Akt pathways that in turn lead to activation of distinct downstream effectors like mTOR/p70S6K and Rac1, respectively. The clinical relevance of these findings is supported by the in silico analysis indicating that patients with breast cancers with elevated expression of both p130Cas and ErbB2 have a higher risk of developing distant site metastasis compared to patients with low levels of p130Cas. Overall, these data highlight the importance of p130Cas and ErbB2 cooperation in promoting breast cancer progression.

Nothing is known about gene expression changes underlying the transition from non invasive to invasive phenotype in the presence of p130Cas over-expression and ErbB2 activation. Emerging technologies such as coding and non coding genes microarrays have greatly improved the capability to identify key molecular targets involved in a specific cellular process. In particular, the past decade has shown remarkable advances in our knowledge of microRNAs and their functional importance in cancer predisposition, initiation and progression, with several small non coding RNAs demonstrating oncogenic and/or tumor suppressor properties.

To identify transcriptional changes occurring during invasion, we have performed a comparative microarray analysis of coding and non coding genes between MCF10A.B2 acini over-expressing p130Cas with or without activation of ErbB2. We have found that genes involved in biological processes like amino acid synthesis (CBS, PHGDH), cell motility and migration (ITPKA, PRDM1), angiogenesis (HEY1) are up-regulated in invasive p130Cas over-expressing and ErbB2 activated mammary acini while genes implicated in inflammatory diseases (S100A7 and SAA1) are down-regulated. In parallel, we have also identified a microRNA invasive signature in which miR-200b,
miR-222, miR-221, miR-210 and miR-424 result to be up-regulated and miR-27a, miR-27b and miR-23b are down-regulated. Interestingly, we have also found that the target genes of miR-27a, miR-27b and miR-23b are differentially expressed in the invasive signature.

Our results provide new insights on the genetic program underlying p130Cas/ErbB2 dependent invasion that may be useful for prognostic and therapeutic purposes. Indeed, this invasive signature may serve as a prognostic tool to discriminate between non invasive and invasive ErbB2-positive tumors.
Results

Whole genome expression analysis reveals two invasive p130Cas/ErbB2 dependent signatures

It has previously been shown that activation of ErbB2 in MCF10A.B2 cells treated with AP1510 (B2) leads to the formation of multiaclinar structures that resemble the DCIS condition of human breast cancer \(^\text{15}\). Similarly, the over-expression of p130Cas in MCF10A.B2 cells (Cas) induces perse multiaclini formation \(^\text{13}\). Remarkably, the activation of ErbB2 in Cas cells (Cas/B2) induces invasion into the surrounding extracellular matrix in about 50-60 % of the acinar structures \(^\text{12}\) (Figure 1A).

In order to define the gene expression pattern specifically associated with invasiveness of ErbB2-activated acini in presence of p130Cas over-expression, we performed protein-coding microarray analyses (Illumina, HumanHT-12/V3) on high quality RNA from invasive acini (Cas/B2) and non-invasive (Cas or B2) multiaclinar structures as illustrated in Figure 1A. According to our data, two invasive signatures can be identified. The gene profile obtained from the comparison of Cas/B2 versus B2 acini revealed a signature of 91 differently expressed genes among which 67 up-regulated and 24 down-regulated (Figure 1 and Supplemental Table S1). On the other hand, the comparison of Cas/B2 versus Cas acinar structures revealed a signature of 232 genes, among which 160 up-regulated and 72 down-regulated (Supplementary Figure S1 and Supplemental Table S2). These findings support that deregulation of specific gene subsets may contribute to the invasive phenotype observed in Cas/B2 transformed cells.

Validation of microarray data

Among the genes differentially expressed in Cas/B2 versus Cas or B2 acini, we selected some genes for validation by qRT-PCR analysis with a fold change of at least ± 1.5 (Supplementary Table S1, S2). As shown in Figure 2A, among the most up-regulated genes in the Cas/B2 versus B2 we validated \(\text{DUSP5, CBS, PLAU, PHGDH, TEK, SERPIN2, HEY1, SPRY2, PRDM1} \) and \(\text{ITPKA}\). Instead, among the down-regulated genes, we validated \(\text{SAA1}\). In the Cas/B2 versus Cas
comparison, PHGDH, SERPIN2, DUSP5, ITGA5 and PLAU resulted validated among the up-regulated genes while SAA1, S100A7 and FGFR3 among the down-regulated components (Fig. 2B). Some of these genes were also validated at protein levels, consistently with mRNA modulations, as shown in Figure 2C. Some of the validated genes were more strongly modulated (up or down) in Cas/B2 invasive acini than in Cas non invasive acini, suggesting that p130Cas and ErbB2 cooperate in a synergistic manner. Differential expression of some of the genes associated with cell invasiveness was also evaluated in 2D growth conditions and, as shown in Supplementary Fig. S2, in most cases the expression of these genes is modified accordingly. However, CBS, one of the most up-regulated gene in Cas/B2 versus B2 signature was not differentially expressed in 2D culture condition, suggesting that 3D cultures can unravel gene expression changes relevant for invasion that are lost in 2D cultures.

These results support the reliability of our microarray analyses for the determination of gene deregulation during invasion.

**Functions of the genes belonging to the invasive signatures**

To determine whether the differentially expressed genes in invasive versus non invasive 3D cultured cells shared a particular biological function, we conducted a gene ontology (GO) analysis. Notably, our analyses revealed that a significant portion of the over-represented GO annotations points to biological processes linked to tumor progression. As shown in Figures 3A and 3B, the main GO categories for over-represented genes in both Cas/B2 compared to B2 or to Cas were cancer, cell death and survival, cell growth and proliferation, cellular development and movement, with a p value <0.001. Overall this analysis highlighted that the most represented categories are those linked to transformation and invasion.

To further evaluate the functional pathways in which the identified genes are involved in p130Cas/ErbB2 dependent invasion we used the Ingenuity Pathway Analysis (IPA) system. One main molecular network was identified for Cas/B2 versus B2 gene signature (Figure 4A). This
network includes 35 genes associated with *cellular growth and proliferation and cellular movement*, such as tissue inhibitor of metalloproteinases (Timp1), the serine/threonine kinase Mitogen-activated protein kinase kinase kinase kinase 4 (MAP4K4), the fibroblast growth factor receptor 3 (FGFR3), PR domain zinc finger protein 1 (PRDM1), Erk1/2 and Hairy/enhancer of split related with YRPW motif 1 (HEY1). In parallel, as shown in Figure 4B, we were able to identify a *Cell Movement and Cancer-related* network for the Cas/B2 versus Cas gene signature. Although these two networks share some similarities, most of the genes are not overlapping, indicating that the two invasive signatures might result from the integration of differentially activated signaling pathways. This is in line with the pathway analysis shown in Figure 5A and 5B in which it is evident that only some common signaling exist between the Cas/B2 versus B2 or versus Cas signatures. Specifically, the most representative signaling in the Cas/B2 versus B2 signature were those related to ammoniac synthesis and degradation, invasiveness and glucose metabolism. In parallel, the Cas/B2 versus Cas signature was characterized by a higher number of predicted signaling pathways involved in ammoniac transport and metabolism, proliferation, survival, adhesion and invasion. Interestingly, when we evaluated the correlation of our 91 (Cas/B2 versus B2) and 232 (Cas/B2 versus Cas) differentially expressed genes with patient survival in different human breast cancer datasets we found that in Miller dataset both up- and down-regulated genes of the 91 signature correlated with worse survival (*Supplementary Figure S3A e S3B*). The same correlation with survival was also observed for the up-regulated genes of the 91 gene signature with the Wang dataset (*Supplementary Figure S3C*). In addition, the entire 232 gene signature (up and down-regulated genes together) showed a significant correlation with survival in the Sotiriou data set (*Supplementary Figure S3D*), suggesting the relevance of our data in various large patient cohorts.

**Modulation of CBS activity and of S100A7 and PHGDH expression levels is sufficient to block p130Cas-dependent invasion of ErbB2 transformed acini**
In order to investigate the relevance of the identified invasive signatures, we performed gain and loss of function studies. As shown in Figure 6A, when mammary acini grown in 3D were treated with the chemical inhibitor Hydroxylamine (Hydr.) for CBS, one of the most up-regulated gene in the Cas/B2 versus B2 signature, (Supplementary Table S1), the invasive phenotype was abrogated in Cas/B2 acini while no effects were observed in B2 cells (Figure 6A, panel e and f). Conversely, CBS activation, induced by treatment with S-adenosyl methionine (AdoMet), promoted invasion of B2 acini but was not able to induce invasion of Cas acini in absence of ErbB2 activation (Fig. 6A, panels g and h). These results indicate that CBS hyper-activation contributes to the invasive phenotype in an ErbB2 dependent manner. Since we have previously demonstrated that Erk1/2 MAPKs and PI3K/Akt activation are both involved in p130Cas dependent invasion of ErbB2 transformed acini and CBS was reported to induce Akt and Erk1/2 MAPKs activation in colon cells, we tested whether the phosphorylation levels of MAPKs and Akt were affected by treatment with CBS inhibitor and activator. Both Akt and Erk1/2 MAPKs activation were decreased upon treatment with Hydr. and increased in presence of AdoMet (Figure 6B), indicating that Akt and Erk1/2 MAPKs are implicated in CBS-dependent invasion.

In parallel, Transwell invasion assays performed through a layer of Matrigel/collagen-coated membranes indicated that the over-expression of S100A7, one of most down-regulated genes in the Cas/ErbB versus Cas signature, was sufficient to inhibit cell invasion in p130Cas over-expressing ErbB2 transformed acini (Figure 6C and D). In addition, lowering the expression levels of PHGDH, that is up-regulated in Cas/B2 versus B2 or Cas signatures, impairs ErbB2-dependent cell invasion of p130Cas over-expressing acini (Figure 6C and D).

These data indicate that alteration of S100A7 and PHGDH expression induced by p130Cas are instrumental to induce invasiveness of ErbB2-transformed acini.

miRNA expression analysis revealed a p130Cas/ErbB2-dependent signature
Increasing evidence points out miRNAs are master regulators of gene expression in many biological and pathological processes, including mammary gland development and breast cancer. Therefore, our aim was to evaluate the contribution of specific miRNAs in the acquisition of invasive properties of p130Cas over-expressing cells upon ErbB2 activation. Ultra pure RNA from B2 and Cas/B2 was prepared for miRNA expression analysis by using the Agilent “Human miRNA Microarray Kit (V3)” for 866 human and 89 human viral miRNAs. Using SAM two class-paired we found that 40 miRNAs were differentially expressed with a False Discovery Rate (FDR) = 8.9%, 20 up-regulated and 20 down-regulated (Figure 7A, Supplemental Table S3). In order to validate these results, we performed qRT-PCR expression analysis for the differentially expressed microRNAs miR-210, miR-222, miR-424, miR-221, miR-23b, miR-27b and miR-455-3p. As shown in Figure 7B miRNA expression patterns were largely consistent between the microarray and the qRT-PCR data.

Since miR-210 has been recently described to be involved in tumor proliferation, invasion and poor clinical outcome in breast cancer, to gain functional insights on the role of this miRNA in mediating invasion, Mock, Cas, B2 or Cas/B2 cells were transfected with pre-control or pre-miR-210 and used in a matrigel/collagen Tranwell invasion assay (Fig. 7C). Transfected cells were treated with AP1510 (ErbB2 activator) immediately before seeding the cells. Up-regulation of miR-210 is shown in Figure 7D. As shown in Figure 7C, as expected, invasion was promoted only in Cas/B2 cells in control conditions. Interestingly, the transfection of pre-miR-210 was sufficient to promote invasion in B2 cells and to further increase invasion in Cas/B2 cells. These results indicate that miR-210 is indeed playing a critical role in ErbB2 transformed cell invasion and that the concomitant over-expression of p130Cas and miR-210 in ErbB2 transformed cells boosts their invasive behavior.

The identification of miRNA targets is crucial to understand the biological role of miRNAs. Since mRNA destabilization is one of miRNA-mediated gene repression mechanism, we used the
TargetScan (v. 5.1) algorithm to identify putative targets of the differentially expressed miRNAs in