αB-Crystallin is a novel oncoprotein that predicts poor clinical outcome in breast cancer

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Recent gene profiling studies have identified a new breast cancer subtype, the basal-like group, which expresses genes characteristic of basal epithelial cells and is associated with poor clinical outcomes. However, the genes responsible for the aggressive behavior observed in this group are largely unknown. Here we report that the small heat shock protein α-basic-crystallin (αB-crystallin) was commonly expressed in basal-like tumors and predicted poor survival in breast cancer patients independently of other prognostic markers. We also demonstrate that overexpression of αB-crystallin transformed immortalized human mammary epithelial cells (MECs). In 3D basement membrane culture, αB-crystallin overexpression induced luminal filling and other neoplastic-like changes in mammary acini, while silencing αB-crystallin by RNA interference inhibited these abnormalities. αB-Crystallin overexpression also induced EGF- and anchorage-independent growth, increased cell migration and invasion, and constitutively activated the MAPK kinase/ERK (MEK/ERK) pathway. Moreover, the transformed phenotype conferred by αB-crystallin was suppressed by MEK inhibitors. In addition, immortalized human MECs overexpressing αB-crystallin formed invasive mammary carcinomas in nude mice that recapitulated aspects of human basal-like breast tumors. Collectively, our results indicate that αB-crystallin is a novel oncoprotein expressed in basal-like breast carcinomas that independently predicts shorter survival. Our data also implicate the MEK/ERK pathway as a potential therapeutic target for these tumors.

Introduction

Breast cancer is the most frequently diagnosed cancer in women and is responsible for 411,000 deaths per year in women worldwide (1). Although clinical indices such as tumor size and grade and axillary lymph node metastases are useful prognostic factors in breast cancer, there is an urgent need to identify molecular characteristics of breast carcinomas that more accurately predict clinical outcome and guide specific therapies for individual patients (2). Recent gene expression profiling of human breast cancer has led to the identification of several subtypes of breast cancer with different clinical outcomes: 2 estrogen receptor–positive (ER-positive) subtypes, a subtype with high expression of the erythroblastic leukemia viral oncogene homolog 2/HER-2 (ErbB2/HER-2) proto-oncogene, a normal breast-like subtype, and a basal-like subtype that expresses genes characteristic of basal epithelial cells and normal breast myoepithelial cells, such as cytokeratin 5 (CK5) and CK17, and does not express ER or ErbB2/HER-2 (3–6). Of these subtypes, the ErbB2/HER-2 and basal-like groups are associated with the shortest overall and relapse-free survival. Unlike the ErbB2/HER-2 subtype, the genes in the basal-like cluster responsible for these cells’ clinically aggressive behavior are unknown. Identification of these genes could lead to new targeted therapies for basal-like breast tumors, which account for 15–20% of breast cancer cases.

By exploring existing breast cancer cDNA microarray data (3, 4), we observed that α-basic-crystallin (αB-crystallin) was commonly expressed in basal-like breast carcinomas and postulated that αB-crystallin might contribute to their aggressive behavior, for several reasons. αB-Crystallin is a member of the small heat shock protein family (which also includes Hsp27), whose members function as stress-induced molecular chaperones to inhibit the aggregation of denatured proteins, thereby promoting cell survival (7). In addition, ectopic expression of αB-crystallin in diverse cell types confers protection against a broad range of apoptotic stimuli, including TNF-α, TNF-related apoptosis-inducing ligand (TRAIL), etoposide, growth factor deprivation, and oxidative stress, while silencing αB-crystallin expression by RNA interference (RNAi) sensitizes cells to apoptosis (8–11). We have previously demonstrated that αB-crystallin inhibits apoptosis by disrupting the proteolytic activation of caspase-3, whereas a pseudophosphorylation mutant αB-crystallin that mimics stress-induced phosphorylation (S19E, S45E, and S59E, abbreviated 3XSE) fails to suppress caspase-3 activation and apoptosis induced by TRAIL or growth factor deprivation (9–11). Others have reported that αB-crystallin binds to the proapoptotic Bcl-2 family members Bax and Bcl-xS and pre-
vents their translocation to the mitochondria (12). Intriguingly, proteomics analyses revealed that both αB-crystallin and Hsp27 are expressed at greater than 14-fold higher levels in preinvasive ductal carcinoma in situ (DCIS) compared with matched normal breast tissue (13). αB-Crystallin is also expressed in breast cancer and other human cancers, such as gliomas and prostate and renal cell carcinomas (14–16). These findings suggest that the apoptosis resistance conferred by αB-crystallin may contribute to the aggressive behavior of basal-like breast carcinomas.

We report here that αB-crystallin was expressed in approximately half of basal-like breast tumors and predicted shorter patient survival independent of several established prognostic markers. We also show for the first time to our knowledge that overexpression of WT αB-crystallin, but not the pseudophosphorylation mutant, induced neoplastic-like changes in mammary acini grown in 3D culture and transformed immortalized human mammary epithelial cells (MECs). αB-Crystallin overexpression also increased cell migration and invasion in vitro. Strikingly, the transformed
phenotype induced by αB-crystallin was suppressed by inhibitors of the MEK/ERK pathway, which is constitutively activated by αB-crystallin overexpression. Immortalized human MECs overexpressing αB-crystallin formed mammary carcinomas in nude mice that resembled basal-like breast tumors in several respects. Taken together, our results indicate that αB-crystallin is a novel oncoprotein expressed in basal-like breast carcinomas that independently predicts poor clinical outcome. Our data also suggest that inhibition of the MEK/ERK pathway may be an effective therapy for basal-like breast carcinomas expressing αB-crystallin.

Results

αB-Crystallin is expressed in basal-like breast carcinomas and predicts shorter survival. Recent cDNA microarray studies have identified a new breast cancer subtype, termed the basal-like group, which expresses genes characteristic of basal epithelial cells and normal breast myoepithelial cells, such as CK5, and is associated with poor clinical outcomes (3–6). An expanded view of the gene expression data from 115 tumors presented by Sorlie et al. (4) showed that αB-crystallin was most consistently expressed in the basal-like tumors and the normal breast samples (Figure 1A). Consistent with these gene expression results, immunohistochemistry (IHC) revealed that αB-crystallin protein was coexpressed with CK5/6 in 2 known basal-like breast tumors (Figure 1, B and C) and was predominantly expressed in myoepithelial cells in normal breast tissue (Figure 1D). We next examined the expression of αB-crystallin by IHC in a tissue microarray (TMA) of invasive breast carcinomas with linked clinical and pathological data (median follow-up, 10.8 yr; range, 0.3–20.0 yr) (17). Tumors were scored as strongly positive (Figure 1E, left), weakly positive (Figure 1E, middle) or negative (Figure 1E, right) for αB-crystallin expression (IHC results for all tumors can be viewed at https://www.gpecimage.ubc.ca/tma/web/viewer.php). αB-Crystallin was expressed in 39 of 361 (11%) breast carcinomas (25 tumors were weakly positive and 14 were strongly positive). Using an IHC surrogate to identify basal-like tumors (negative for ER and ErbB2/HER-2 and positive for EGFR/HER-1 and/or CK5/6) that was validated in an independent breast cancer series (18), we observed that αB-crystallin was expressed in 18 of 40 basal-like breast carcinomas (45%) in the TMA, but only 17 of 288 (6%) nonbasal tumors expressed αB-crystallin (P = 8 × 10−18 by Fisher’s exact test). Kaplan-Meier analyses revealed that expression of αB-crystallin in breast carcinomas was associated with shorter disease-specific survival (Figure 1F, left; 10-year disease-specific survival, 59% for αB-crystallin–positive tumors versus 74% for αB-crystallin–negative tumors; P = 0.0054). In addition, the levels of expression of αB-crystallin correlated inversely with disease-specific survival: strongly positive tumors were associated with shorter survival than were weakly positive tumors (Figure 1F, right; 10-year disease-specific survival, 46% for strongly positive tumors versus 66% for weakly positive tumors; P = 0.0125). In contrast, expression of the related small heat shock protein Hsp27 was not associated with survival in this cohort (ref. 17 and data not shown), thereby underscores the specificity of our observation. Multivariate Cox regression analysis revealed that αB-crystallin expression predicted shorter survival (hazard ratio, 2.23; P = 0.001) independent of tumor grade, lymph node status, and ER and ErbB2/HER-2 expression status (tumor size was not included because accurate measures were unavailable for many cases). These results indicate that αB-crystallin is commonly expressed in basal-like breast carcinomas and is an independent predictor of poor clinical outcome.

Overexpression of αB-crystallin disrupts mammary acini and induces neoplastic abnormalities. In 3D basement membrane culture, human immortalized MECs, such as MCF-10A cells, form acinar-like structures consisting of a single cell layer of polarized, growth-arrested MECs surrounding a hollow lumen (19). Activation of oncoproteins such as ErbB2/HER-2 induces neoplastic-like changes in mammary acini, including loss of polarity, increased proliferation, diminished apoptosis, and luminal filling (19, 20). To examine whether overexpression of αB-crystallin induces similar neoplastic abnormalities, we generated by retroviral infection MCF-10A pools that stably expressed either WT αB-crystallin (αB-WT) or a mutant αB-crystallin that mimics stress-induced phosphorylation (αB-3XSE) and is impaired in its antiapoptotic function (10, 11, 21). pLXSN (vector) and parental MCF-10A acini expressed modest levels of αB-crystallin, while the levels in αB-WT and αB-3XSE acini were 11.7-fold and 13.1-fold greater, respectively, than those in parental MCF-10A acini (Figure 2A). Importantly, these levels of αB-WT overexpression are comparable to those observed in DCIS compared with matched normal breast tissue (14.1-fold increase) (13). Although MCF-10A–pLXSN cells formed normal acini similar to those formed by parental cells, MCF-10A–αB-WT cells formed strikingly abnormal acini at a high frequency that were grossly enlarged, disorganized, and hypercellular and contained filled lumens (Figure 2, B and C). Each abnormal αB-WT acinus was formed from a single cell (data not shown). MCF-10A cells stably overexpressing oncoenic H-RasV12 also formed enlarged, disorganized acini with filled lumens (Figure 2B). In contrast, MCF-10A–αB-3XSE cells infrequently formed abnormal acini (Figure 2, B and C). Importantly, αB-WT overexpression also induced similar neoplastic changes in MCF-12A cells (Figure 2B), an immortalized, nontransformed human MEC line unrelated to MCF-10A cells (22). The morphological abnormalities in MCF-10A–αB-WT acini were dependent on αB-crystallin expression: silencing αB-crystallin by retroviral RNAi suppressed the abnormal phenotype of αB-WT acini (Figure 2D). αB-WT (but not αB-3XSE) mammary acini were also characterized by disruption of apicobasal polarity, as determined using GM130 as an apical marker and the integrin β4 subunit and laminin-5 as basalolar markers (Figure 2, E and F). In addition, αB-WT acini had higher expression levels of integrin β4 and laminin-5 than did pLXSN or αB-3XSE acini (Figure 2E and data not shown). αB-WT acini also had diminished luminal apoptosis on day 8, as detected by reduced active caspase-3 immunostaining compared with pLXSN or αB-3XSE acini (Figure 2G, upper panels, and Figure 2H). Furthermore, while pLXSN and αB-3XSE acini underwent proliferative arrest by day 12 as determined by minimal Ki-67 staining, αB-WT acini contained Ki-67–positive cells at day 12 (Figure 2G, lower panels, and Figure 2H). Of note, some αB-3XSE acini exhibited a mild phenotype, characterized by persistence of a few luminal cells at day 12 (Figure 2E, upper panel, and Figure 2G, lower panel). These results indicate that αB-crystallin overexpression in immortalized MECs induces profound abnormalities in acinar architecture that resemble features of preinvasive breast tumors (e.g., DCIS), including enlarged acini with filled lumens, loss of polarity, diminished luminal apoptosis, and increased proliferation (19, 20, 23).
Figure 2
αB-Crystallin overexpression disrupts mammary acinar morphology. (A) Immunoblot of day-12 acini formed by parental MCF-10A cells and MCF-10A pools (pLXSN, αB-WT, and αB-3XSE). αB, αB-crystallin. (B) Phase contrast images of day-12 acini formed by parental MCF-10A cells, MCF-10A pools, or an MCF-12A pool stably expressing αB-WT. (C) Day-12 acini formed by parental MCF-10A cells or pools were scored for abnormal morphology (enlarged structures with filled lumens), diameter, and cell number per cross section (mid-acini). (D) Silencing αB-crystallin in MCF-10A–αB-WT cells by RNAi suppressed the abnormal morphology of αB-WT acini. (E) Day-12 pLXSN, αB-WT, or αB-3XSE acini were immunostained with antibodies to αB-crystallin (green) and GM130, an apical marker of polarized epithelium (red, upper panels), or the integrin β4 subunit, a basolateral marker (red, lower panels). Confocal images of mid-acini cross sections are shown. Inset: Higher magnification of the same acinus (magnified ×2.5 of original). (F) Percentage of day-12 acini with polarized epithelium. (G) pLXSN, αB-WT, and αB-3XSE acini were immunostained with antibodies to αB-crystallin and active caspase-3 (upper panels, day 8) or Ki-67 (lower panels, day 12). (H) Percentage of acini that contained ≥2 cells positive for active caspase-3 (day 8) or Ki-67 (day 12). In A–H, data are mean ± SD (n = 3, except RNAi in D and laminin-5 polarity in F, which were n = 2, each in duplicate). *P < 0.05 versus control; ***P < 0.001 versus pLXSN. Scale bars: 100 µm (B and D); 50 µm (E and G).
cells expressed higher levels of total and phosphorylated ERK1/2, Akt, and p38 than did parental MCF-10A, MCF-10A–αB-3XSE, and MCF-10A–pLXSN cells (Figure 3A). MCF-10A pools were then plated on Matrigel in the presence of vehicle, 10 μM SB 203580 (SB), 10 μM PD 98059 (PD), 1 μM U 0126 (U), 10 μM LY 294002 (LY), or 0.1 μM wortmannin ( Wort). Representative phase contrast images of day-12 acini. (C) Representative confocal images of day-12 αB-WT acini cultured from day 0 in the presence of vehicle, SB 203580, or PD 98059 (mid-acini cross sections). Acini were immunostained with antibodies to GM130 (red; αB-crystallin, green), laminin-5 (red; αB-crystallin, green), integrin β4 (red; αB-crystallin, green) or Ki-67 (green; αB-crystallin, red). Nuclei were stained with DAPI (blue). (D) Percentage of day-12 acini with abnormal morphology (enlarged structures with filled lumens). MCF-10A pools were treated as in B. Data are mean ± SD (n = 3; at least 100 acini scored per experiment). ***P < 0.001 versus control. Scale bars: 100 μm (B); 50 μm (C).

Figure 3
The neoplastic abnormalities in mammary acini overexpressing αB-crystallin are MEK dependent. (A) Immunoblot analysis of MCF-10A cells or MCF-10A pools grown as monolayers in low serum without added EGF. p-, phosphorylated. (B) MCF-10A pools were plated on Matrigel in the presence of vehicle, 10 μM SB 203580 (SB), 10 μM PD 98059 (PD), 1 μM U 0126 (U), 10 μM LY 294002 (LY), or 0.1 μM wortmannin ( Wort). Representative phase contrast images of day-12 acini. (C) Representative confocal images of day-12 αB-WT acini cultured from day 0 in the presence of vehicle, SB 203580, or PD 98059 (mid-acini cross sections). Acini were immunostained with antibodies to GM130 (red; αB-crystallin, green), laminin-5 (red; αB-crystallin, green), integrin β4 (red; αB-crystallin, green) or Ki-67 (green; αB-crystallin, red). Nuclei were stained with DAPI (blue). (D) Percentage of day-12 acini with abnormal morphology (enlarged structures with filled lumens). MCF-10A pools were treated as in B. Data are mean ± SD (n = 3; at least 100 acini scored per experiment). ***P < 0.001 versus control. Scale bars: 100 μm (B); 50 μm (C).

Overexpression of αB-crystallin confers EGF- and anchorage-independent growth and MEK-dependent enhanced migration and invasiveness. Because growth factor– and anchorage-independent growth are defining properties of transformed cells (24), we examined whether overexpression of αB-crystallin was sufficient to confer these
Overexpression of αB-crystallin in MECs confers EGF- and anchorage-independent growth and MEK-dependent enhanced migration and invasiveness. (A) Growth of parental MCF-10A cells and MCF-10A pools in low serum (without added EGF) as determined using an MTS viability assay. The results are expressed as fold change in viable cells relative to t = 0. (B) Representative soft agar colony formation of parental MCF-10A cells and MCF-10A pools; entire well (upper panels) and higher magnification (lower panels). (C) Representative micrographs of the wound closure assay of MCF-10A pools treated with vehicle, 10 μM SB 203580 or 10 μM PD 98059 for 18 hours. (D) Percentage wound closure. (E) MCF-10A pools were placed in a transwell invasion chamber in the presence of vehicle, SB 203580, or PD 98059, and the number of cells invading through Matrigel-occluded pores was determined at 24 hours. The number of invading cells is expressed as fold change from that observed in untreated MCF-10A–pLXSN cells. In A–E, data are mean ± SD (n = 3). ***P < 0.001. Scale bars: 500 μm (B); 100 μm (C).

Characteristics on MCF-10A MECs. While parental MCF-10A cells and MCF-10A–pLXSN cells failed to grow in 1% horse serum without added EGF, MCF-10A–αB-WT cells grew rapidly under these conditions, as determined by a 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) viability assay (Figure 4A). MCF-10A–αB-3XSE cells exhibited little growth under these conditions. Similar results were obtained through counting cells by trypan blue exclusion (data not shown). Furthermore, MCF-10A–αB-WT cells, but not parental MCF-10A or MCF-10A–pLXSN cells, formed abundant large colonies in soft agar; only rare colonies were observed with MCF-10A–αB-3XSE cells (Figure 4B). These experiments reveal that αB-crystallin overexpression confers EGF- and anchorage-independent growth.

Careful inspection of αB-WT acini revealed that some cells formed protrusions that penetrated the Matrigel (data not shown), suggesting that αB-crystallin overexpression may promote migration and invasion, 2 essential features of malignant tumors (24). To investigate this possibility, we performed cell migration (wound closure) and MCF-10A cells overexpressing αB-crystallin form invasive mammary carcinomas in nude mice. Human MCF-10A MECs are not tumorigenic in nude mice and are a highly stringent model to examine tumorigenicity of oncogenes in vivo (25, 26). To determine whether overexpression of αB-crystallin was sufficient to induce mammary tumors in vivo, we inoculated both mammary fat pads of female athymic nude mice with parental MCF-10A cells or MCF-10A pools (5 × 10⁶ cells in Matrigel) or Matrigel alone. MCF-10A cells were inoculated in a Matrigel suspension to simulate the tumor microenvironment (27). Strikingly, 18 of 20 mammary fat pad inoculated with MCF-10A–αB-WT cells developed tumors (mean volume at 10 weeks, 591 ± 120 mm³), while only 3 of 20 mammary fat pad inoculated with MCF-10A–αB-3XSE cells developed small tumors (mean volume at 10 weeks, 85.8 ± 27.3 mm³; P < 0.01; Figure 5A). In contrast, none of the mice inoculated with Matrigel alone, parental MCF-10A cells, or MCF-10A–pLXSN cells developed tumors 13 weeks after inoculation. Small MCF-10A–αB-WT mammary tumors were first palpable 1 week after inoculation and grew pro-
gressively larger throughout the 10-week study, at the end of which some mice were euthanized due to extensive tumor burden. The 3 small MCF-10A-αB-3XSE mammary tumors were first palpable 6 weeks after inoculation and did not grow appreciably (1 tumor completely regressed by week 15; data not shown). Histologic examination of MCF-10A-αB-WT mammary tumors at 5 weeks revealed sheets of elongated mesenchymal-like spindle cells associated with atypical glandular epithelial elements and frank invasion of adjacent muscle and fat, confirming their malignant nature (Figure 5B, upper panels). At week 10, mammary tumors were composed of epithelialoid cells with high grade pleomorphic nuclei and abundant mitoses (Figure 5B, lower left panel, thin arrow) that were arranged in glandular-like structures (Figure 5B, lower left panel, thick arrow) and less prominent high nuclear grade spindle cells (Figure 5B, lower right panel). Both 5- and 10-week MCF-10A-αB-WT mammary tumors expressed mesenchymal (vimentin) and epithelial markers (cytokeratins) as well as αB-crystallin (Figure 5C). These findings indicate that overexpression of WT αB-crystallin in MCF-10A cells is sufficient to induce invasive mammary carcinomas in nude mice.

Discussion

We have demonstrated that the small heat shock protein αB-crystallin is a novel oncoprotein: overexpression of WT αB-crystallin was sufficient to transform immortalized human MCF-10A MECs and to induce mammary carcinomas in vivo. Specifically, overexpression of WT αB-crystallin in MCF-10A cells induced profound abnormalities in mammary acini that resembled features of preinvasive tumors: enlarged masses with filled lumens, loss of polarity, increased proliferation, and diminished luminal apoptosis (19, 20, 23). Notably, the abnormal morphology of αB-WT acini was suppressed by silencing αB-crystallin by RNAi. αB-Crystallin overexpression also induced neoplastic changes in immortalized MCF-12A MECs grown in 3D culture, indicating that the observed oncogenic effects are not cell type–specific. Similar neoplastic abnormalities were induced in mammary acini by activating the ErbB2/HER-2 oncogene, which, like αB-crystallin, inhibits apoptosis and promotes proliferation (19, 20). Moreover, overexpression of αB-crystallin in MCF-10A cells induced EGF- and anchorage-independent growth and promoted cell migration and invasion in vitro, which are defining characteristics of malignant neoplasms.

Figure 5

MCF-10A cells overexpressing αB-crystallin form invasive mammary carcinomas in nude mice. (A) Left: Mean tumor volume ± SEM at weekly intervals in female athymic nude mice inoculated with MCF-10A pools. Right: Volume of tumors in each group at week 10; horizontal line indicates the median tumor volume in each group. Ratios at top indicate the number of tumors per total inoculation sites. **P < 0.01 versus αB-3XSE. (B) H&E staining of representative mammary tumors at 5 (upper panels) and 10 weeks (lower panels) from nude mice inoculated with MCF-10A–αB-WT cells. Upper left panel: Prominent elongated spindle cells and atypical epithelial components with tumor invasion of muscle and fat. Upper middle panel: Mesenchymal spindle cells. Upper right panel: Atypical epithelial components. At 10 weeks, we observed epithelioid cells with pleomorphic high grade nuclei and mitoses (thin arrow) that form glandular like structures (thick arrow, lower left panel) and high nuclear grade spindle cells (lower right panel). (C) Representative IHC of the mammary carcinomas shown in B. Original magnification, ×100 (B, upper left panel), ×200 (B, remaining panels, and C). Furthermore, MCF-10A pools stably expressing WT αB-crystallin formed invasive mammary carcinomas in nude mice, thereby confirming the malignant nature in vivo of MCF-10A cells transformed by αB-crystallin. Importantly, the levels of ectopically expressed αB-crystallin observed in mammary acini are comparable to those previously observed in human DCIS (13). In contrast, overexpression of similar levels of a αB-crystallin phosphorylation mutant that mimics stress-induced phosphorylation and is impaired in its cytoprotective function was largely incapable of transforming MECs or initiating mammary tumors, which suggests that the transforming activity of αB-crystallin may be negatively regulated by phosphorylation. These data indicate that αB-crystallin is a bona fide oncoprotein, which to our knowledge was previously unrecognized.

How then does αB-crystallin transform MECs? Although overexpression of αB-WT, but not the 3XSE mutant, induced EGF-independent activation of multiple signaling pathways, the transformed phenotype of MCF-10A–αB-WT cells was selectively suppressed by MEK inhibitors. Indeed, treatment of αB-WT mammary acini with MEK inhibitors restored apicobasal polarity, suppressed proliferation, and promoted apoptosis of centrally located MECs, resulting in morphologically normal acini despite their transformed genotype. These findings are consistent with previously published reports indicating that MEK inhibition suppresses, at least in part, the neoplastic phenotype of breast cancer cells grown in 3D basement membrane culture (28, 29). Constitutively active MEK transforms NIH 3T3 cells, while a dominant-negative MEK
Inhibitor suppresses transformation by v-Src or H-Ras, indicating a critical role for the Raf/MEK/ERK pathway in transformation (30, 31). Overexpression of αB-crystallin increased levels of total and phosphorylated ERK1/2 protein, a pattern commonly observed in human breast cancer and in MCF-10A cells transformed by H-Ras (32, 33). Although ERK is activated by EGFR/HER family members and integrins in breast cancer (19, 28, 29), our results suggest that αB-crystallin contributes to ERK activation in basal-like tumors. Consistent with this notion, activation of the MEK/ERK pathway produces many of the same consequences we observed by overexpressing αB-crystallin, namely, increased proliferation, reduced apoptosis, and increased cell migration and invasion (34). Clearly, the mechanisms by which αB-crystallin activates ERK have yet to be elucidated. As a molecular chaperone, αB-crystallin may regulate ERK1/2 protein stability and/or phosphorylation/dephosphorylation as Hsp90 regulates Akt and Raf kinase activity (35, 36). Nevertheless, our data indicate unequivocally that the MEK/ERK pathway plays a key role in MEC transformation by αB-crystallin.

We have also demonstrated that MCF-10A–αB-WT cells induced mammary carcinomas in nude mice. In contrast, MCF-10A cells constitutively overexpressing oncogenic H-RasV12, cyclin D1, or ErbB2 do not induce tumors in nude mice, despite promoting anchorage-independent growth in vitro (26, 37, 38), thereby underscoring the tumorigenic potency of αB-crystallin. Early-stage MCF-10A–αB-WT carcinomas had prominent mesenchymal-like spindle cells that expressed αB-crystallin and both mesenchymal (vimentin) and epithelial markers (cytokeratins). These characteristics are strongly suggestive of epithelial-mesenchymal transition (EMT), a process implicated in carcinoma progression, whereby epithelial cells lose polarity and cell-cell adhesion and acquire mesenchymal characteristics such as motility (39). Intriguingly, late-stage carcinomas continued to express αB-crystallin, vimentin, and cytokeratins and retained a less prominent spindle cell component. Although it remains to be determined whether the observed differences in early and late mammary carcinomas represent a temporal progression, it is striking that overexpression of αB-crystallin promoted some aspects of EMT in vitro (disruption of epithelial polarity and enhanced cell migration and invasion) and corresponding histological features in vivo (spindle cells and vimentin expression). Consistent with its potential role in neoplastic EMT, αB-crystallin expression is induced at an early stage during cardiac and skeletal muscle differentiation (mesodermal tissue) and protects myoblasts from apoptosis (10, 40). Both early and late tumors were ER-negative, progesterone receptor–negative (PR-negative), and ErbB2/HER-2–negative (data not shown) and resembled human metastatic breast carcinomas, a histopathologic subtype notable for mesenchymal spindle cells mixed with epithelial glandular elements (41, 42). Metastatic carcinomas are also predominantly ER-, PR-, and ErbB2/HER-2–negative and frequently express vimentin and CK5 (43, 44), an expression pattern shared by basal-like breast carcinomas. Hence, the mammary carcinomas induced by αB-crystallin overexpression recapitulated aspects of human basal-like breast tumors, and this xenograft model may be useful for testing new breast cancer therapies. In addition to its role in transformation and tumorigenesis, we recently demonstrated that overexpression of αB-crystallin in breast carcinoma cells promotes their growth as xenograft mammary tumors (11), suggesting that αB-crystallin may also play a role in breast cancer progression.

Although p53 is commonly mutated in basal-like breast tumors, and BRCA1 carriers tend to develop these tumors (4, 5), additional genes likely contribute to their aggressive nature. The potent transforming and tumorigenic activity of αB-crystallin, coupled with our observation that αB-crystallin was expressed in approximately half of basal-like breast tumors, suggests that αB-crystallin may contribute to the aggressive behavior of these basal-like tumors. Indeed, we observed that αB-crystallin predicted shorter disease-specific survival independent of tumor grade, lymph node status, and ER and ErbB2/HER-2 status, suggesting that αB-crystallin may provide additional prognostic information for breast cancer patients independent of these established factors. Our findings agree in part with those of a recent study indicating that αB-crystallin expression in breast cancer was associated with lymph node involvement and shorter survival in univariate analyses (14). In contrast to our findings, αB-crystallin expression was not predictive of survival independent of lymph node status in the study by Chelouche-Lev et al. (14). These investigators observed that 88% of breast carcinomas expressed αB-crystallin by IHC, in contrast to the 11% we noted. It is unclear whether these disparities reflect methodological or patient cohort differences. However, our IHC results are consistent with gene expression data from an independent breast cancer cohort indicating that only a minority of breast carcinomas highly express the αB-crystallin gene. We are currently examining the expression of αB-crystallin in additional breast cancer cohorts to determine whether αB-crystallin may be a clinically useful predictor of prognosis or drug response. Indeed, the suppression of the transformed phenotype of MCF-10A–αB-crystallin cells by MEK inhibitors in vitro suggest that MEK inhibitors may be an effective therapy for basal-like breast tumors expressing αB-crystallin. Orally active MEK inhibitors have been shown to potently suppress colon cancer growth and melanoma metastasis in xenograft models (45, 46), underscoring the potential feasibility of this therapeutic strategy.

**Methods**

**TMA and IHC analyses.** This cohort of 438 patients with invasive breast carcinoma and the corresponding TMA from archival tumor blocks were described previously (17). αB-Crystallin expression was detected by standard IHC methods using a mAb (SPA-222, Stressgen Biotechnologies). Briefly, antigen retrieval was performed by steaming TMA slides in citrate buffer (pH 6.0) for 30 minutes. Endogenous peroxidases were blocked by incubation with 3% hydrogen peroxide for 10 minutes at room temperature (RT). Next, slides were incubated for 30 minutes in Dako protein block at RT and incubated overnight at 4°C with the αB-crystallin mAb (1:200 dilution in Dako antibody dilution buffer). After rinsing in PBS/Tween 20 (0.2%), the slides were incubated with anti-mouse, Dako-labeled polymer for 30 minutes at RT and again rinsed in PBS/Tween 20 (0.2%). Slides were then stained with Nova Red (Vector Laboratories) for 10 minutes at RT, rinsed in distilled water, and counterstained with hematoxylin. αB-Crystallin immunostaining was detected by stan-
MCF-10A and MCF-12A cells were cultured in growth medium (DMEM/ F12 medium [Invitrogen Corp.] supplemented with 5% horse serum [Invitrogen Corp.], 20 ng/ml EGF [Sigma-Aldrich], 0.5 mg/ml hydrocortisone [Sigma-Aldrich], 100 ng/ml cholera toxin [Sigma-Aldrich], 10 μg/ml insulin [Sigma-Aldrich], and Pen/Strep [Invitrogen Corp.]) as previously described (48). SB 203580, PD 98059, U 0126, 10 μM LY 294002, or 0.1 mM NaF. In other experiments, subconfluent monolayers of MCF-10A cells were grown in assay media (described above) supplemented with 1% horse serum (without added EGF) for 18 hours; cell lysates were prepared as described above. Total protein concentration was determined by BCA Protein Assay Kit (Pierce Biotechnology Inc.). Protein (10–20 μg total) was loaded on SDS-PAGE gels and transferred to PVDF membranes (Millipore) using a semidy transfer apparatus (BioRad Laboratories). Membranes were blocked with blocking buffer (5% nonfat milk in TBS with 0.1% Tween 20 [TBS/T]) for 1–2 hours at RT and then incubated with the primary antibody (in TBS/T with 5% BSA) overnight at 4°C. The following primary antibodies were used: α-crystallin and Hsp27 mAbs (Stressgen Biotechnologies); Akt and phosphorylated Akt mAbs (Ser473), ERK1/2 polyclonal Ab and phosphorylated ERK1/2 (Thr202/Tyr204) rabbit mAb (Cell Signaling Technology); and p38 mAb and phosphorylated p38 (Thr180/Tyr182) polyclonal Ab (BioSource International). After washing with TBS/T, membranes were incubated for 1 hour at RT with the appropriate secondary antibody conjugated to HRP (diluted in TBS/T). Proteins were visualized using the ECL Western Lightning Chemiluminescence kit (PerkinElmer).

Cell migration and invasion assays. For cell migration (wound closure) assays, confluent monolayers of MCF-10A cells (6 × 10^5 cells/well) in 6-well plates were wounded with a 200-μl pipette tip, washed with PBS, and cultured at 37°C in assay medium (described above) supplemented with 1% horse serum (without added EGF). After 18 hours, the wounds were photographed, and the percentage of the wound closed was quantified. For the invasion assays, 2.5 × 10^5 cells were seeded on top of a Matrigel invasion chamber (8-μm pore diameter; BD Biosciences) in assay medium (described above) supplemented with 1% horse serum (without added EGF); for the lower chamber, assay medium supplemented with 5% horse serum (without added EGF) was used as a chemoattractant. Plates were placed in an incubator at 37°C for 24 hours. Cells invading the lower chamber were stained with 0.5% crystal violet and counted in an inverted microscope.

Mammmary tumorigenesis in nude mice. The mammary fat pads of 4- to 5-week-old female athymic nude (nu/nu) mice (Harlan) were inoculated bilaterally with parental MCF-10A cells, MCF-10A pools (5 × 10^5 cells in 200 μl Matrigel), or Matrigel alone. Tumor volume was measured with Vernier calipers as described previously (11). Tumors were harvested from euthanized mice; formalin-fixed and paraffin-embedded tumor tissue sections were stained with H&E by standard methods. IHC of tumors was performed using mAbs to α-crystallin (1:200 dilution; Stressgen Biotechnologies), vimentin (clone V9, 1:200 dilution; Sigma-Aldrich) or pan-cytokeratins (AE1/AE3, 1:200 dilution; Dako). All animal procedures were approved by the Animal Care and Use Committee of Northwestern University.
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Is the small heat shock protein αB-crystallin an oncogene?

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In the last 5 years, global gene expression profiling has allowed for the subclassification of the heterogeneous disease of breast cancer into new subgroups with prognostic significance. However, for most subgroups, the nature of the contributions of individual genes to the clinical phenotypes remains largely unknown. In this issue of the JCI, Moyano and colleagues further examine the oncogenic potential of the small heat shock protein α-basic-crystallin, commonly expressed in tumors of the basal-like breast cancer subtype associated with poor prognosis, and show that it is an oncogenic protein in the breast (see the related article beginning on page 261).

Small heat shock proteins

α-Basic-crystallin (αB-crystallin) is a member of the mammalian small heat shock protein (sHsp) superfamily, whose 10-protein membership is defined by the presence of a conserved, approximately 90-aa region (termed the α-crystallin core) with divergent amino- and carboxyterminal domains (1–3). The sHsps function as cytoprotective molecular chaperones, preventing stress-induced aggregation of denaturing proteins as well as keeping aggregation-prone proteins in reservoirs of nonnative refoldable intermediates by holding proteins within large, soluble, multimeric structures. sHsps also appear to have a structural role; for example, αB-crystallin together with α-Acidic-crystallin comprise as much as 40% of the cytoplasmic proteins in lens cells of the eye and are thought to play an essential role in maintaining its transparency. However, in other tissues in the human body the 2 α-crystallins are expressed constitutively at much lower levels. αB-Crystallin shares a close homology with another sHsp, Hsp27, and both of these proteins are activated in response to stresses such as heat shock, radiation, oxidative stress, and exposure to anticancer drugs. Moreover, both proteins have been shown to have antiapoptotic functions by interfering with the activity of various apoptotic proteins (2). Indeed, the literature suggests that αB-crystallin is important in the pathology of cancer, as overexpression of αB-crystallin has been observed in gliomas (4), renal carcinomas (5), breast carcinomas (6, 7), and ductal carcinoma in situ compared with matched normal breast tissue (8). Until now, the importance of αB-crystallin in cancer has been vaguely attributed only to its antiapoptotic functions.

αB-Crystallin as an oncoprotein

In this issue of the JCI, Moyano and colleagues demonstrate properties of αB-crystallin not previously described to our knowledge that define it as a potential oncogenic protein (9). Using various cell-culture systems, including reconstituted 3D basement membrane cultures, they report that constitutive overexpression of αB-crystallin in 2 human mammary epithelial cell lines, MCF-10A and MCF-12A, induced neoplastic-like changes such as EGF- and anchorage-independent growth, loss of polarity, disorganized acinar structure, increased proliferation, diminished apoptosis, and increased migration and invasion (Figure 1). These morphological changes were reliant on αB-crystallin overexpression, since retroviral RNA interference–mediated silencing of αB-crystallin expression suppressed the abnormal phenotype. Moreover, although this transformation induced the expression and phosphorylation of ERK1/2, AKT, and p38, the authors show that the neoplastic changes were dependent on the ERK/MAPK pathway, as inhibition of only this pathway by highly specific MAPK kinase (MEK) inhibitors completely negated transformation. Transformation also appears to be dependent on the phosphorylation state of αB-crystallin, as a pseudophosphorylation mutant of αB-crystallin, which mimics stress-induced phosphorylation, did not confer neoplastic changes. Furthermore, the authors demonstrate that αB-crystallin meets one of the “gold standards” for classification as an oncogenic protein: human mammary epithelial cells overexpressing WT αB-crystallin were shown to form invasive carcinomas in nude mice. In contrast, constitutive overexpression of the recognized oncogenes H-RasV12, cyclin D1, and erythroid leukemia viral oncogene homolog 2 (Erbb2) in these cells does not induce tumors in nude mice (10–12), suggesting that αB-crystallin is a more potent oncoprotein in this model. Finally, the authors demonstrate the clin-
αB-Crystallin overexpression causes neoplastic-like transformation of human mammary epithelial cells. MCF-10A, immortalized human mammary epithelial cells, form acinar-like structures consisting of a single layer of polarized cells surrounding a hollow lumen, resembling normal breast ducts, when grown in 3D basement membrane culture. Activation of oncogenes such as ErbB2 induces neoplastic-like changes in these mammary acini. In this issue of the JCI, Moyano et al. (9) demonstrate that MCF-10A cells stably overexpressing WT αB-crystallin (αB-WT) form large, abnormal acini with filled lumen in contrast to the normal acini formed by empty vector–infected control cells (pLXSN) and cells overexpressing a pseudophosphorylation mutant of αB-crystallin (αB-3XSE). Immunostained cross sections of the acini displayed higher levels of αB-crystallin (green) in αB-WT acini and marked disruption of normal acinar polarity, as evidenced by the localization pattern of the apical marker GM130 (red; upper panels) and the basolateral marker integrin j4 (red; lower panels), compared with pLXSN and αB-3XSE cells. In addition, the dramatic loss of polarity and disruption of acinar morphology in αB-crystallin–overexpressing MCF-10A cells was also accompanied by increased EGF- and anchorage-independent growth, increased proliferation, diminished apoptosis, and increased migration and invasion, and these cells formed tumors in nude mice. These findings clearly indicate that αB-crystallin is an oncogenic protein in human mammary epithelial cells.

Regulation of αB-crystallin and its targets

Moyano et al. (9) bring the importance of αB-crystallin in breast cancer to the forefront, but it is still unknown whether αB-crystallin overexpression in breast tumors is driven by promoter transactivation, loss of transcriptional inhibition, increased DNA copy number, or other means, such as mutation of promoter elements. Moreover, it is not yet known whether overexpression of αB-crystallin is a major initiating oncogenic change in vivo. And what is the mechanism of the αB-crystallin–dependent activation of the ERK/MAPK pathway? One may speculate that αB-crystallin stabilizes an activator of the pathway, such as Ras GTPases or one of the Rafs, or enhances ubiquitination and degradation of a MEK inhibitor, such as Raf kinase inhibitor protein, or have a completely novel function. The relationship between αB-crystallin and the ERK/MAPK pathway may also be cell type specific or context dependent, as a recent report (13) shows that in rabbit lent cells, in contrast to Moyano et al.’s observations in human mammary epithelial cells (9), αB-crystallin inhibits the ERK/MAPK pathway, thereby disrupting calcium-activated, p53-dependent apoptosis. In this respect, it is interesting to note that mutation of p53 occurs in over 80% of basal-like breast tumors, a rate much higher than most other subtypes (6). Furthermore, since ERK/MAPK has over 70 known substrates and modulates many fundamental cellular processes (e.g., cell proliferation, survival, differentiation, apoptosis, motility, and metabolism) (14), it remains to be seen which of these effectors is important in αB-crystallin–mediated transformation of mammary epithelial cells.

αB-Crystallin as a marker for basal-like tumors

In the 1980s, it was first reported that breast tumors could be classified based on their expression of various epithelial cell cytokeratins (CKs), which are highly conserved cytoskeletal structural poly-peptides found in all epithelial tissues. In these early studies, tumors that expressed the stratified CKs (such as CK5, CK14, and CK17) of the basal/myoepithelial cell lineage were associated with poor survival (15–17). With the advent of global gene expression profiling, this tumor subgroup, termed basal-like, has garnered renewed interest in recent years due to the fact that these tumors display a distinct transcriptional profile (18) that correlates with a poor prognosis (6, 19, 20). In addition, a link between this tumor subgroup and genotype has shown that hereditary breast cancer tumors with mutations in breast cancer 1, early onset (BRCA1) almost invariably display the basal-like phenotype (19). Although the basal-like subgroup appeared distinct when defined using gene expression data in a number of studies (6, 18–20), it should be noted that the formal definition and molecular markers that specify a basal-like tumor are still evolving (21–23).

Moyano and colleagues (9) use an immunohistochemistry-based (IHC-based) panel of protein expression (22) to identify the basal-like subgroup (estrogen receptor–negative [ER-negative], ErbB2/HER-2–negative, and CK5/6-positive and/or EGFR-positive). In this context, the association between αB-crystallin and the basal-like subtype of breast cancer, while attractive, is not as conclusive as the rest of their study. About half of the basal-like tumors characterized in a study by Sorlie...
et al. (6) and in the Moyano et al. study (9) did not overexpress αB-crystallin (mRNA and protein, respectively). Furthermore, about half of the αB-crystallin IHC-positive tumors identified by Moyano et al. did not belong to their identified basal-like group. It is unknown whether the non-basal-like, αB-crystallin–positive tumors are misclassified basal-like cases, or whether they are found randomly among the other breast cancer subtypes or enriched in certain subtypes. It should be noted that approximately half of the tumors belonging to the so-called “normal-like” subtype (which does not have such a poor prognosis as the basal-like subtype) have also been shown to express αB-crystallin message (6). That being said, the imperfect correlation between basal-like breast tumor marker expression and αB-crystallin expression may actually provide support for the idea that αB-crystallin is an oncogene, as it would be unlikely for an oncogene to be activated in all tumors of the imperfectly homogeneous basal-like subgroup. Nevertheless, Moyano et al. state that the αB-crystallin–transformed tumors that grew in the nude mice displayed basal-like characteristics; however, just how well these MCF-10A cell–derived tumors recapitulate the basal-like phenotype is not clear, as specific staining for basal CKs was not performed, nor were they analyzed for basal-like gene expression patterns. In this respect, any result should be interpreted within the context that MCF-10A cells themselves already appear to display a basal-like gene expression profile (24, 25). Additionally, while the authors show that αB-crystallin protein expression was associated with a shorter disease-specific survival independent of tumor grade, lymph node status, and ER and ErbB2 expression, it is not clear whether the prognostic value of αB-crystallin is independent of basal-like status. It also remains to be validated whether αB-crystallin is a general prognostic marker (in absence of therapy), a predictive marker for response to a particular therapy, both, or neither, as the patients studied by Moyano et al. (9) were diagnosed over several decades and had substantially varied (and unspecified) adjuvant therapies (26). This is particularly important given that increased expression of αB-crystallin in cell line models has been associated with acquired resistance to the DNA-damaging agents cisplatin, etoposide, and fotemustine (27) and in light of recent evidence that the basal-like, but not the normal-like, subtype is particularly sensitive to preoperative paclitaxel followed by S-Fluorouracil, doxorubicin, and cyclophosphamide chemotherapy (28). As with any groundbreaking work, the study by Moyano and colleagues (9) opens up as many questions as it convincingly answers. Their impressive study marks a significant step forward in our understanding of how αB-crystallin contributes to cancer; clearly, αB-crystallin is involved in many more ways than simply suppressing apoptosis. Furthermore, if validation studies demonstrate that human breast tumors with high αB-crystallin levels have an activated ERK/MEK pathway, then a tailored therapy for this phenotype may soon be clinically testable, as at least 2 drugs active against this pathway are currently being evaluated: CI-1040 (also known as PD 183432), an orally available and well-tolerated MEK1/2 inhibitor, has shown encouraging results in phase I and II clinical trials as an anticancer agent and well-tolerated MEK1/2 inhibitor, has shown encouraging results in phase I and II clinical trials as an anticancer agent and has been shown to be generally efficacious in the investigation of a number of human tumors. Therefore, MEK1/2 inhibitors may be effective in patients whose tumors express high levels of αB-crystallin, suggesting that these patients may benefit from MEK1/2 inhibitor therapy. It is also worth noting that in vitro studies have shown αB-crystallin upregulates the expression of RTKs, which are known to play a role in the development and progression of breast cancer. Therefore, it is possible that αB-crystallin may play a role in the proliferation and survival of breast cancer cells. Furthermore, αB-crystallin has been shown to be a predictor of poor outcome in breast cancer patients. In a study of 100 patients with breast cancer, it was found that patients with high levels of αB-crystallin had a significantly worse survival rate compared to patients with low levels of αB-crystallin. Therefore, it is possible that αB-crystallin may be a predictor of poor outcome in breast cancer patients.

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