Calpain 2 Regulates Akt-FoxO-p27Kip1 Protein Signaling Pathway in Mammary Carcinoma*

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Background: Calpains are intracellular calcium-dependent proteases implicated in cancer.

Results: Calpain 2 knockdown in breast cancer cells correlated with reduced in vitro proliferation, migration, and invasion rates, soft agar colony formation efficiency, and migration rates, indicating roles for calpain 2 in mitogenesis, survival, and motogenesis. Biochemical analysis showed increased levels of protein phosphatase 2A and reduced levels of activated Akt in calpain 2-deficient cells, and this correlated with increased levels of the FoxO3a target gene product p27Kip1, a key regulator of cell proliferation. Calpain 2 deficiency in the AC2M2 cells correlated with enhanced nuclear localization of FoxO3a, consistent with it being in a derepressed state capable of regulating transcriptional targets. Orthotopically engrafted calpain 2 knockdown AC2M2 cells generated tumors with reduced growth rates and enhanced in vivo expression of p27Kip1. In summary, calpain 2 deficiency correlated with reduced Akt activity, increased protein phosphatase 2A levels, derepression of FoxO3a, and enhanced expression of the p27Kip1 tumor suppressor. These observations argue that calpain 2 promotes tumor cell growth both in vitro and in vivo through the PI3K-Akt-p27Kip1 signaling cascade. Inhibition of calpain 2 might therefore provide therapeutic benefits in the treatment of cancer.

Conclusion: Calpain 2 represents a potential therapeutic target in breast cancer.

Significance: Calpain 2 is a potential therapeutic target in breast cancer.

We investigated the role of the ubiquitously expressed calpain 2 isoform in breast tumor cell growth, migration, signaling, and tumorigenesis. RNAi-mediated knockdown of the capn2 transcript was used to manipulate expression of the catalytic subunit of calpain 2 in the AC2M2 mouse mammary carcinoma cell line. Stable knockdown of capn2 correlated with reduced in vitro proliferation rates, soft agar colony formation efficiency, and migration rates, indicating roles for calpain 2 in mitogenesis, survival, and motogenesis. Biochemical analysis showed increased levels of protein phosphatase 2A and reduced levels of activated Akt in calpain 2-deficient cells, and this correlated with increased levels of the FoxO3a target gene product p27Kip1, a key regulator of cell proliferation. Calpain 2 deficiency in the AC2M2 cells correlated with enhanced nuclear localization of FoxO3a, consistent with it being in a derepressed state capable of regulating transcriptional targets. Orthotopically engrafted calpain 2 knockdown AC2M2 cells generated tumors with reduced growth rates and enhanced in vivo expression of p27Kip1. In summary, calpain 2 deficiency correlated with reduced Akt activity, increased protein phosphatase 2A levels, derepression of FoxO3a, and enhanced expression of the p27Kip1 tumor suppressor. These observations argue that calpain 2 promotes tumor cell growth both in vitro and in vivo through the PI3K-Akt-p27Kip1 signaling cascade. Inhibition of calpain 2 might therefore provide therapeutic benefits in the treatment of cancer.

Calpains are a family of calcium-dependent intracellular thiol proteases that are implicated in a wide range of molecular, cellular, and physiological functions (for review, see Ref. 1). Of the 14 known mammalian isoforms, the ubiquitously expressed calpain 1 (μ-calpain) and calpain 2 (m-calpain) have been studied most extensively. These heterodimers consist of distinct large catalytic subunits of 80 kDa encoded by capns1 and capn2, respectively, and a small common regulatory subunit of 28 kDa encoded by capns1/capn4. Targeted disruption of capns1 in mice abolished both calpain 1 and calpain 2 activities and resulted in embryonic lethality (2–4). Capn1 disruption in mice resulted in more subtle phenotypes (5) whereas capn2 knockout was embryonic lethal (6). Thus, although some functional redundancy between calpain 1 and 2 may exist, knock-out phenotypes in mice, as well as observations from isoform specific knockdown experiments in fibroblasts, indicate that calpain 2 plays prominent and essential roles.

Disruption of calcium homeostasis is often associated with cell death. Although calpain activation may sometimes be a consequence rather than a cause of cell death, there is compelling evidence for pro-apoptotic calpain functions. Indeed, capns1 knock-out fibroblasts are resistant to many cytotoxic challenges (7–9). Paradoxically, there is also evidence for pro-survival functions of calpain (9–11). Thus, the ultimate contribution of calpain to survival or death may depend upon context in which the cell is challenged.

Calpain cleaves cytoskeletal proteins and may thereby contribute to the regulation cellular processes including migration and invasion. In human colon cancer cells, the calpain inhibitor ALLN was shown to block FAK cleavage, cell adhesion, and migration mediated by integrin α2β1 signaling (12). These observations implicate calpain in the regulation of cell adhesion and migration through interactions with integrins. In fibroblasts, disruption of calpain reduces cell migration (13), at least in part through inhibition of actin remodeling associated with lamellipodial dynamics at the leading edge (14).

Studies have shown that the calpain system is dysregulated in cancer. Calpain 2 is overexpressed in human colorectal adenocarcinomas (15). The endogenous inhibitor of calpain, calpastatin, is down-regulated in nasopharyngeal carcinoma (16). Calpain inhibitors blocked proliferation of cancer cell lines PC-3, HeLa, Jurkat, and Daudi cells (17). These observations suggest that calpain may be a useful therapeutic target in cancer.

The PI3K-Akt signaling cascade is important in regulating cell survival, and up-regulation of this pathway has been observed in various cancers. PTEN mutation has been found in 60–80% of prostate cancers, and this leads to a constitutive activation of the PI3K-Akt-mTor pathway. Increased Akt activity also correlates with reduced activity of the growth suppres-
Calpain 2 Knockdown Attenuates Mammary Tumorigenesis

EXPERIMENTAL PROCEDURES

Cell Culture, Plasmids, and Reagents—The highly metastatic AC2M2 mouse mammary carcinoma cell line (22) was routinely cultured in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS; Sigma), 2 mM l-glutamine, and antibiotics/antimycotics (Invitrogen) and maintained at 37 °C with 5% CO2 in a humidified incubator. The expression of calpain 2 was depleted using RNAi sequences designed using the pSUPER Design Tool from Oligoengine to target the mouse capn2 transcript, as described previously (23). The target sequence in capn2 was 5′-GGATGGCCGATTTCTGCACTC-3′, and a nonsilencing control sequence that lacked homology to known Mus musculus mRNAs was 5′-TTCTCCGAACGTTGTCACGT-3′. These shRNA-encoding sequences were excised using the XhoI and EcoR I sites from the pSuper-retro vectors provided by Anna Huttenlocher (23) and inserted into the pWPXLd vector, provided by Didier Trono, at the SnoB1 site. AC2M2 cells were then infected with the control (nonsilencing) shRNA or capn2 shRNA lentiviruses, and selection was performed to obtain clones that showed a significant level in calpain 2 knockdown. FoxO expression plasmids, pECE-HA-FoxO3a WT (wild type) and pECE-HA-FoxO3aTM (triplet mutant T32A/S253A/S315A) were purchased from Addgene. FoxO3aWT and FoxO3aTM DsRed fusion protein expression plasmids were generated by subcloning the FoxO3a coding sequences into the pDsRed1N1 vector (Clontech).

Proliferation and Colony Forming Assays—Equal numbers of AC2M2 control and calpain 2 knockdown cells were cultured in 12-well plates. Cells were harvested and counted using a hemacytometer after 48 h. In the colony forming assay, the soft agar was composed of a base agar and a top agar. The bottom layer was made of 0.5% agarose with culture medium, whereas the top layer contained 20,000 cells in 0.25% agarose with culture medium. The assay was carried out using 6-well plates, and the plates were incubated at 37 °C for 2 weeks. Colonies grew in the soft agar were stained with 0.05% crystal violet solution and quantified using a light microscope.

Migration Assays: Wound Healing and Transwell Migration Assays—In wound healing assays, mouse mammary carcinoma AC2M2 cells were seeded on 6-well plates to form a confluent monolayer. Then a scrape wound was made on the cell monolayer using a pipette tip, and cell migration was allowed for the designated time. Pictures were taken at time 0 and 6 h. The cell migration was assessed by subtracting the width of the wound at each time point from that at time 0 to determine the percentage wound closure. In the Transwell migration assay, membrane inserts (8-μm pore size) coated with 10 μg/ml fibronectin were used. Fifty thousand cells were loaded into the top chamber and allowed to migrate through the membranes toward the bottom chamber containing medium with 10% FBS for 8 h at 37 °C. Cells were then fixed with 3% paraformaldehyde for 30 min at room temperature and stained with crystal violet for 1 h. The membranes were removed, mounted on glass slides, and the number of migrated cells was counted using a light microscope with a 20× objective and represented as the average of five random fields.

Engraftment Mouse Tumor Model and Metastasis Studies—AC2M2 cells (7,500 cells in 10 μl) were injected into the mammary fat pad of nu/nu mice (Ncr-Foxn1nu/nu, Taconic). Tumor growth was monitored every 2 or 3 days, starting on day 13 and up to day 27 using ultrasound imaging (VisualSonics™ Vevo 770®). In metastasis studies, primary tumors were removed from the mice 22 days after tumor cell injection to promote the growth of lung metastatic lesions. On day 35, mice were euthanized, lungs were dissected, and GFP-expressing metastatic nodules were imaged using a Pan-A-See-Ya Panorama imaging system. Images were captured using a Hamamatsu B/W ORCA-ER digital camera with excitation filter at 470 nm/20 nm and an emission filter at 525 nm/20 nm. Mice were housed in the Animal Care Facility, and procedures were carried out according to the guidelines of the Canadian Council on Animal Care, with the approval of the institutional animal care committee.

Western Blotting and Casein Zymography—Cell or tumor lysates were prepared in buffer containing 150 mM NaCl, 10 mM Tris, pH 8, 10% glycerol, 1% Nonidet P-40, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 100 μM PMSF, and 100 μM sodium orthovanadate. SDS-PAGE was carried out, and proteins were then transferred onto PVDF membranes. Membranes were blocked and incubated with primary antibodies against phospho-ERK, ERK, phospho-Akt, Akt, calpain 2, PP2A, cyclin D1, p27Kip1, and Bim. Quantification of band density was performed using ImageJ software. Casein zymography was carried out to determine calpain activity in cell lysates. Equal amounts of protein (20 μg) were resolved on 8% nondenaturing poly-

2The abbreviations used are: PP2A, protein phosphatase 2A; TM, triple mutant.
Calpain 2 Knockdown Attenuates Mammary Tumorigenesis

Knockdown of Calpain 2 Expression in Mouse Mammary Carcinoma AC2M2 Cells—Mouse mammary carcinoma AC2M2 cells were transduced with control lentiviruses or lentiviruses encoding shRNA directed against capn2, and clones were selected. The capn2-encoded 80-kDa calpain 2 catalytic subunit was down-regulated by ~80% in cells transduced with the capn2 shRNA compared with the cells transduced with the control shRNA. The capns1-encoded 1/2 small subunit heterodimer partner was also reduced by ~70% (Figs. 1A and 3). Zymogram analysis indicated a reduction in calpain 2 activity which correlated with the reduction in calpain 2 protein (Fig. 1B). Calpain 1 activity and expression of the capn1-encoded 80-kDa catalytic subunit were unchanged in capn2 knockdown cells (Fig. 1). Clone 5 was selected for subsequent analysis.

Depletion of Calpain 2 Correlated with Reduced Proliferation, Colony Formation, and Migration—We first examined the effect of calpain 2 depletion on proliferation, anchorage-independent growth, and migratory properties of AC2M2 cells. Calpain 2-deficient cells displayed significantly reduced proliferation relative to control cells (Fig. 2A, p = 0.017) and attenuated colony forming potential in soft agar (Fig. 2B, p = 0.011). In scrape wound assays, calpain 2-deficient cells showed a significant reduction in migration relative to the control cells (Fig. 2C, p < 0.05). A similar difference was observed in the Transwell migration assay when FBS was used as a chemoattractant (Fig. 2D, p < 0.05). We also attempted to investigate the signaling cascades involved in AC2M2 cell migration. Using PI3K inhibitor LY294002, we observed reduction in cell migration, suggesting that the PI3K-Akt pathway may play a key role in AC2M2 cell migration (data not shown). However, we have not yet ruled out the possibility that this is due to a reduction in cell survival. Thus, calpain 2 deficiency in the AC2M2 breast carcinoma cell line correlated with reduced proliferation, colony formation, and migration.

Calpain 2-deficient AC2M2 Cells Display Dysregulated Akt Signaling—We next compared the steady-state signaling properties of calpain 2-deficient and control cells (Fig. 3). Calpain 2 knockdown cells displayed a similar expression level and activation status of ERK compared with the control cells. However, the activation status of Akt was reduced by 80% in calpain 2 knockdown cells. This is in agreement with previous studies by us and others showing a reduction of Akt phosphorylation in calpain small subunit (capns1/capn4)-null embryonic fibroblasts (9, 19). PP2A has also been shown to contribute to the regulation of Akt (20, 21). Interestingly, we observed an 80% increase in PP2A in calpain 2-deficient cells, suggesting that it might contribute to suppression of Akt activity in AC2M2 cells.

FoxO transcriptional regulators are important targets of Akt. FoxO3a transcriptional activity is suppressed by Akt phosphorylation, 14-3-3 binding, and cytoplasmic sequestration (24). Activation of FoxO3a by dephosphorylation, 14-3-3 dissociation and nuclear translocation can lead to increased expression of target genes including p27Kip1 and Bim (25), which inhibit proliferation and promote apoptosis, respectively. Transcriptional regulation of these and other FoxO target genes provides a mechanistic link for Akt-based regulation of cell survival, proliferation, and migration. When we compared the expression of the FoxO target genes p27Kip1, Bim, and cyclin D1 in control and calpain 2-deficient AC2M2 cells, calpain 2 knockdown correlated with 2-fold higher levels of p27Kip1, but only slightly elevated levels of Bim and cyclin D1 (Fig. 3). Collectively, these observations argue that calpain 2 deficiency could lead to dysregulation of the Akt-FoxO-p27Kip1 signaling axis and thereby contribute to increased p27Kip1 and reduced proliferation.

To explore further the role of calpain 2 in regulation of FoxO3a, AC2M2 cells were transected with plasmids encoding DsRed-FoxO3aWT or DsRed-FoxO3aTM, and the subcellular distribution of the DsRed tagged proteins was evaluated. In the control AC2M2 cells, 68% of cells expressing DsRed-FoxO3aWT displayed exclusive cytoplasmic localization, whereas mixed distribution in both cytoplasm and nucleus was observed in 23% of the cells, and exclusive nuclear expression was found in 9% of the cells (Fig. 4A). As expected, the distribution of DsRed-FoxO3aTM, which lacks multiple negative regulatory Akt phosphorylation sites, shifted significantly to the nucleus. Only 10% of control cells showed exclusive cytoplasmic localization, whereas the remaining 90% of DsRed-FoxO3aTm-expressing control cells displayed localization...
either exclusively in the nucleus (46%) or in both the cytoplasm and the nucleus (44%). These data are consistent with the expectation that blocking Akt-mediated phosphorylation enhances FoxO3a nuclear translocation. In the calpain 2 knockdown AC2M2 cells, only 20% of DsRed-FoxO3aWT expressing cells showed cytoplasmic localization (Fig. 4B), whereas the remaining 80% displayed either exclusively nuclear localization (36%) or distribution in both cytoplasm and the nucleus (44%). Upon transfection with the DsRed-FoxO3aTM construct, only 1% of the calpain 2 knockdown cells displayed an exclusive cytoplasmic localization, whereas 64% showed exclusive nuclear localization and 35% displayed mixed nuclear/cytoplasmic localization. These data indicate that depletion of calpain 2 in AC2M2 cells correlates with a dramatic shift toward nuclear localization of FoxO3a. This shift phenocopies the effect of mutating Akt phosphorylation sites in FoxO3a, which further argues for an important role of calpain in regulating Akt-mediated suppression of FoxO3a functions in breast cancer cells.

Calpain 2 Knockdown Attenuates Mammary Tumorigenesis

FIGURE 2. Calpain 2 regulates proliferation, colony formation, and migration in AC2M2 cells. A, AC2M2 cells (calpain 2 knockdown or control) were plated at 5,000 cells/ml and cultured for 48 h. Cells were then harvested and counted using a hemocytometer. Cell proliferation is represented as the average of replicates of four (p = 0.017). B, colony formation potential was examined by mixing 20,000 cells in 0.25% agar and plating on a 0.5% agar layer with culture medium. Cultures were incubated for 2 weeks at 37 °C. Colonies were then stained with 0.05% crystal violet and quantified using a light microscope. The results shown are the mean of three individual experiments (p = 0.011) and are representative of a total of 15 experiments. C, wound healing assays were performed on confluent monolayers of AC2M2 cells. A scrape wound was induced using a pipette tip, and cells were allowed to migrate into the wound. Pictures were taken at t = 0 and t = 6 h. The differences in distance were calculated and expressed as percent of wound healing (p < 0.05). D, Transwell migration assays were carried out on fibronectin-coated membranes (8 μm pore size). 50,000 cells in serum-free medium were loaded on the top chambers, and 10% FBS-containing medium was placed in the bottom wells as a chemoattractant. Cells were allowed to migrate for 8 h. Cells that migrated to the bottom side of the membrane were fixed and stained with crystal violet. Migrated cells were counted using a light microscope. Cell migration is expressed as the average of cells in five 20× fields from each membrane, with replicates of four in each condition (p = 0.05). Error bars, S.E.

FIGURE 3. Calpain 2 knockdown affects signaling properties in AC2M2 cells. AC2M2 control or calpain 2 knockdown cell lysates containing equal amounts of protein (20 μg) were analyzed by immunoblotting using antibodies directed against phospho-ERK, total ERK, phospho-Akt, total Akt, PP2A, cyclin D1, p27Kip1, Bim, and calpain 2 (detects both capn2-encoded 80kDa and capns1-encoded 28-kDa subunits).
in calpain 2 knockdown AC2M2 cells, we observed no significant effect on metastasis to the lung in this in vivo tumor model (Table 1).

**DISCUSSION**

In this study, we show that knockdown of calpain 2 correlates with reduced in vivo tumor growth in an orthotopic engraftment model of mammary tumorigenesis. Biochemical analysis revealed that Akt activation was compromised in calpain 2 knockdown tumor cells, and this correlated with increased expression of the cyclin-dependent kinase inhibitor p27Kip1, which is a key target of the FoxO3a transcription factor. We further showed that FoxO3a subcellular location was skewed toward nuclear localization in calpain 2-deficient cells. This is consistent with a model where Akt-mediated FoxO3a phosphorylation correlates with nuclear exit of FoxO3a (for review, see Ref. 26). Increased in vivo p27Kip1 expression was also observed in engrafted tumors generated with calpain 2 knockdown AC2M2 cells. These observations argue that calpain 2 promotes proliferation of mammary carcinoma cells through the Akt-FoxO3a-p27Kip1 signaling pathway.
Previous studies have demonstrated roles for calpain in cell migration and invasion. *Capn*1 knock-out mouse embryonic fibroblasts, which are deficient in both calpain 1 and 2, have abnormal organization of the actin cytoskeleton and displayed reduced *in vitro* migration and invasion (23). Others have suggested complex roles for calpain in regulating cell migration and invasion by influencing membrane-actin cytoskeletal remodeling and focal adhesion dynamics through cleavage of substrates including cortactin (14), Fak (27), talin (28), and paxillin (29). In the DU-145 prostate cancer cell model, an antisense knockdown approach implicated calpain 2 in promoting cell migration and invasion *in vitro*, and xenograft experiments suggested that calpain inhibition might also reduce the *in vivo* invasive potential of these prostate cancer cells (30). In our study, calpain 2-deficient AC2M2 cells displayed reduced migration *in vitro*, suggesting that calpain 2 might indeed promote *in vivo* metastatic potential. However, when metastasis from the orthotopic tumor site to the lung was evaluated *in vivo*, we saw no effect of calpain 2 knockdown (Table 1). These observations argue that calpain 2 is not required for metastasis *in vivo*, at least not in this particular model system. This was a surprising result because calpain 2 deficiency has been correlated extensively with reduced *in vitro* migration and invasion. However, Huttenlocher and colleagues have implicated calpain 2 in both inhibitory and stimulatory roles in cell migration and invasion (29, 31). We must also consider the possible involvement of calpain 1 or other calpain isoforms *in vivo*. It was recently shown that tissue-specific knock-out of both calpain 1 and 2 in T cells, through disruption of *capn*1, did not compromise integrin-mediated adhesion and migration *in vitro* (32). It will be interesting to see whether these *capns*1 knock-out T cells display *in vivo* migration defects. These observations underscore the importance of evaluating calpain isoform functions *in vivo* in appropriate animal model systems when exploring potential therapeutic applications of calpain inhibition.

In the AC2M2 cells used in this study, we achieved ~80% knockdown of the *capn*2-encoded 80-kDa catalytic subunit. Zymography analysis showed a corresponding reduction in calpain 2 activity, but we could not precisely quantitate this. Unfortunately, calpain-specific *in vivo* activity assays are still lacking. We also failed to observe any compensatory up-regulation of calpain 1 (protein or activity) in the *capn*2 knockdown cells. In fact, the levels of the *capns*1-encoded 28-kDa regulatory subunit for calpain 1 and 2 were also reduced in *capn*2 knockdown cells, which is consistent with the regulatory subunit being unstable in the absence of stoichiometric levels of its partner catalytic subunits.

PI3K promotes the activation of Akt through generation of phosphatidylinositol 3,4,5-triphosphate, which recruits pleckstrin homology domain-containing PDK1 and Akt to the membrane. PDK1 and mTORC2 then phosphorylate Akt at Thr-308 and Ser-473, respectively, resulting in its activation. Using *capn*1 knock-out embryonic fibroblasts, we had previously shown that calpain is important for Akt activation in response to a number of cytotoxic challenges (9). We had also observed that the protective effect of calpain against staurosporin-induced cytotoxicity was negated by PI3K inhibition (9). However, this did not tell us whether calpain was acting upstream or downstream of PI3K. Our attempts to determine whether there was less *in vivo* PI3K activity in calpain 2 knockdown cells using localization of an Akt-pleckstrin homology domain-GFP fusion protein as a surrogate indicator were inconclusive (data not shown). The activation status of PDK1, as assessed by PDK1 phosphorylation at Ser-241, did not appear to be affected by calpain 2 knockdown (data not shown). Thus, although it is clear that calpain contributes to Akt activation, we have not yet determined its specific molecular functions.

PP2A has been shown to inhibit Akt through dephosphorylation (33). Interestingly, PP2A expression was enhanced in calpain 2 knockdown cells compared with the control AC2M2 cells, suggesting that elevated levels of PP2A might play a role in reduced Akt activation. A previous study has demonstrated PP2A associates more tightly with Akt in *capns*1 knock-out mouse embryonic fibroblasts compared with wild-type cells, and this correlated with increased FoxO3a nuclear localization and expression of p27Kip1 and Bim (19). Those authors also reported similar effects upon siRNA-based knockdown of *capns*1 in the estrogen-dependent MCF-7 breast cancer cells.

Inhibition of PI3K–Akt signaling correlates with increased expression of p27Kip1. Hereceptin-induced p27Kip1 expression has been observed in Her2* +* breast cancer cell lines SKBR3 and BT474 (34) whereas p27Kip1 down-regulation has been correlated with hereceptin resistance in breast SKBR3 cells (35). Interestingly, a recent report showed that calpain inhibition could resestiment herceptin-resistant variants of these HER2* +* cells (36). Calpain knock-out in the chondrocyte lineage in mice has also been associated with decreased proliferation correlating with increased p27Kip1 expression (37). Our observations in this breast tumor model system are consistent with a role for calpain in regulating PI3K–Akt–FoxO–p27Kip1 signaling.

FoxO expression is dysregulated in several types of tumors including breast cancer (38) and prostate cancer (18). Nuclear exclusion of FoxO3 correlates with active Akt or IKK in primary tumors, and this seems to link with poor prognosis in patients with breast tumors (38). Overexpression of FoxO1 and FoxO3a in a prostate cancer cell line-induced apoptosis and enhanced TRAIL expression (18). Genetic studies from knock-out mice have also shown that disruption of all three FoxO genes (FoxO1, FoxO3a, and FoxO4) correlated with increased tumorigenesis, further indicating a tumor suppressor role of FoxO (39).

### Table 1: Lung metastasis was not affected by calpain 2 disruption

| Cells injected | No. of mice engrafted | No. of mice with lung metastasis | Percent with metastasis |
|---------------|-----------------------|---------------------------------|------------------------|
| **Experiment 1** |                       |                                 |                        |
| Control       | 5                     | 2                               | 40%                    |
| capn2 knockdown | 6                  | 3                               | 50%                    |
| **Experiment 2** |                     |                                 |                        |
| Control       | 6                     | 2                               | 33%                    |
| capn2 knockdown | 8                  | 4                               | 50%                    |
A recent study has suggested that PP2A interacts with FoxO3a (40). The 14–3–3 proteins bind to FoxO3a at phosphorylated Thr-32 and Ser-253, facilitating nuclear export of FoxO3a. However, inactivation of PI3K-Akt signaling leads to dephosphorylation of FoxO3a at these sites by PP2A, resulting in 14–3–3 dissociation, nuclear translocation, and transcriptional regulation by FoxO3a. Interestingly, PP2A is a proposed substrate of calpain, further suggesting a link between calpain and FoxO signaling (19). We also observed reduced Akt phosphorylation and elevated PP2A levels in calpain 2-deficient AC2M2 cells, and this correlated with increased nuclear localization of FoxO3a and up-regulation of p27kip1.

Another recent study using conditional deletion of capns1 showed a role for calpain in embryonic chondrocyte proliferation (37). Disruption of calpain 1 and 2 in chondrocytes reduced cell cycle progression at G1/S transition, reduced cyclin D1 transcription, but enhanced the accumulation of cell cycle proteins cyclin D, cyclin E, and p27kip1. Our data indicate that knockdown of calpain 2 in breast cancer cells leads to reduction of Akt activation, and this correlates with enhanced p27kip1 expression. The phosphorylation status and nuclear/cytoplasmic translocation of p27kip1 regulate its function. Nuclear p27kip1 interacts with various cyclin-Cdk complexes to inhibit cell cycle progression whereas cytosolic p27kip1 facilitates cell motility through inhibition of RhoA activation (41). There appears to be a role of p27kip1 in human tumors. Low expression of p27kip1 is associated with enhanced cell proliferation and is linked to several types of human tumors including breast cancer (42). Targeted disruption of p27kip1 gene results in an increase of body size in mice, enlargement of organs, and development of pituitary tumors (43, 44). Reduced level or mislocalization of p27kip1 correlates with poor prognosis in tumor (45). Several types of cancers, including breast, colon, esophagus, and thyroid, show reduction of nuclear p27kip1 level (46). The p27kip1-null fibroblasts demonstrate a significant decrease in cell migration compared with wild-type fibroblasts (41). This regulation of migration appears to be independent of the cell cycle regulatory function of p27kip1. The p27kip1-null fibroblasts are found to have increased actin stress fibers, focal adhesions, and activation of RhoA.

Interestingly, calpain has been shown to degrade p27kip1 (47, 48). Calpain degrades p27kip1 during the mitotic clonal expansion of 3T3-L1 preadipocyte differentiation, and calpain activity is required during the early stage of adipocyte differentiation (48). In a choroidal melanoma tumor-derived cell line OCM-1, p27kip1 expression level was found to be degraded by calpains through a MAPK-dependent process (47).

In conclusion, we have shown that calpain 2 expression correlates with cell proliferation and migration in AC2M2 carcinoma cells. It appears that calpain 2 regulates phosphorylation of Akt, which then leads to transcription regulation of FoxO3a and its downstream target, p27kip1. Data from our mouse engraftment experiments also demonstrate a role of calpain 2 in tumor growth in vivo. Thus, our results provide further evidence that calpain may be a potential target in the development of novel cancer therapeutics.

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