

# Survivin Expression in Tuberous Sclerosis Complex Cells

Stephana Carelli,<sup>1,3</sup> Elena Lesma,<sup>1</sup> Simona Paratore,<sup>1,3</sup> Vera Grande,<sup>1</sup> Giorgia Zadra,<sup>1</sup> Silvano Bosari,<sup>2</sup> Anna Maria Di Giulio,<sup>1</sup> and Alfredo Gorio<sup>1,3</sup>

<sup>1</sup>Laboratory of Pharmacology, Dept. of Medicine, Surgery and Dentistry, University of Milan, Polo H. San Paolo, Milano, Italy;

<sup>2</sup>Pathology Unit, Dept. of Medicine, Surgery and Dentistry, University of Milan, Polo H. San Paolo, Milano, Italy; <sup>3</sup>Clinical Pharmacology, IRCCS Humanitas, Rozzano, Milano, Italy

Tuberous Sclerosis Complex (TSC) is a tumor suppressor gene disorder with mutations of TSC1/TSC2 genes. This leads to the development of hamartomas that most frequently affect central nervous system, kidney, and skin. Angiomyolipomas are abdominal masses made up of muscle vessels and adipose tissues that grow mostly in proximity to kidneys and liver. Bleeding and kidney failure are the major justification for surgery. This study shows that angiomyolipoma-derived human smooth muscle TSC2<sup>-/-</sup> cells express the apoptosis inhibitor protein survivin when exposed to IGF-1. Survivin expression is also triggered whenever culture conditions perturb normal TSC2<sup>-/-</sup> cell function, such as the omission of EGF from the growth medium, the supplementation of anti-EGFR, blockade of PI3K and ERK, or inhibition of mTOR. Interestingly, single or simultaneous inhibition of PI3K by LY294002 and ERK by PD98059 does not prevent IGF-1-mediated survivin expression. Apoptogenic Smac/DIABLO, which is constitutively expressed by TSC2<sup>-/-</sup> A<sup>+</sup> cells, is down-regulated by IGF-1 even in the presence of LY294002 and PD98059. These cells release IGF-1 by means of a negative feedback-regulated mechanism that is overrun when they are exposed to antibodies to IGF-1R, which increases the released amount by more than 400%. The autocrine release of IGF-1 may therefore be a powerful mechanism of survival of the tightly packed cells in the thick-walled vessels of TSC angiomyolipoma and in lymphangioleiomyomatosis (LAM) nodules. Future experimental therapies for TSC and LAM may result from the targeted inhibition of survivin, which may enhance sensitivity to TSC2 therapy.

Online address: <http://www.molmed.org>

doi: 10.2119/2006-00091.Carelli

## INTRODUCTION

Tuberous Sclerosis Complex (TSC) is an autosomal dominant syndrome with 95% penetrance, and a birth incidence of 1 in 11,000 (1). It is characterized by the multi-organ development of benign and occasionally malignant tumors that most frequently affect the central nervous system, abdomen, and skin (2). In particular, the kidney tumors include angiomyolipomas and renal cell carcinomas. Angiomyolipomas consist of smooth muscle cells, adipose tissue, and disorganized thick-walled vascular channels (3). Their abnormal vasculature may lead to spontaneous life-threatening bleeding (4). Angiomyolipomas increase in size and/or number in about 60% of children affected

by TSC, highlighting the need for close surveillance of the kidneys and the entire abdomen (5). TSC can occur in association with pulmonary lymphangioleiomyomatosis (LAM), a progressive and often fatal interstitial lung disease characterized by the diffuse proliferation of abnormal smooth muscle cells and cystic degeneration of lung parenchyma. The TSC2 smooth muscle cells in angiomyolipomas are very similar to those of pulmonary LAM, and genetic data suggest that LAM may be the result of benign cell metastases, a highly unusual disease mechanism (6,7).

TSC is characterized by mutations in the tumor suppressor genes TSC1 on chromosome 9q34 or TSC2 on chromo-

some 16p13, which seem to act together as a complex of the encoded hamartin (TSC1) and tuberlin (TSC2) proteins (8). These mutations lead to similar clinical phenotypes, which are more severe in the case of the TSC2 subtype (9). The TSC1/TSC2 complex negatively regulates cell size and proliferation (10,11) is a direct target of Akt, a PI3K-regulated effector that promotes cell growth and survival by means of a mammalian target of rapamycin (mTOR)-dependent mechanism (12). Most of the extracellular and intracellular signaling pathways involved in the regulation of growth factor- and nutrient-mediated cell growth are integrated by mTOR (13). Rapamycin is a microbial product that counteracts these effects by inhibiting mTOR, and Akt is a pro-survival and pro-oncogenic protein that is phosphorylated following the activation of growth factor receptors (13). Akt phosphorylation decreases the ability of TSC2 to inhibit the phosphorylation of the mTOR substrates S6 kinase and eukaryotic initiation factor 4E binding protein-1 (13-15), and excessive Akt, mTOR,

---

**Address correspondence and reprint requests to Alfredo Gorio, Department of Medicine, Surgery and Dentistry, School of Medicine, University of Milan, Polo San Paolo, Via A. Di Rudini 8, 20142 Milano, Italy. Phone: + 39 0250323032; Fax: + 39 0250323033; E-mail: [alfredo.gorio@unimi.it](mailto:alfredo.gorio@unimi.it)**

Submitted October 25, 2006; Accepted for publication January 4, 2007.

and S6K activation causes various types of tumor, including hamartomas (2).

The inhibitors of apoptosis (IAP) gene family (16) is one of the most pursued targets among the regulators of apoptosis. Survivin is a structurally unique IAP protein (17) that is organized as a stable dimer (18) and contains a single baculovirus IAP repeat (BIR) and a COOH terminus coiled-coil domain (19). It has been suggested recently that survivin has dual function, as it is involved in both mitosis and apoptosis (20,21). Survivin expression is transcriptionally controlled in a cell cycle-dependent manner, triggered in the G2/M phase, and localized near the microtubules of centrosomes and mitotic spindle (22), and its down-regulation leads to multiple cell division defects with supernumerary centrosomes, multipolar mitotic spindles, and multinucleation (23-25).

Survivin also is involved in the suppression of cell death triggered by extrinsic or intrinsic apoptotic stimuli (26,27). When survivin expression is enhanced by genetic manipulation in transgenic mice, phenotypes consistent with its cytoprotective activity and enhanced cell viability are observed (28), whereas interference with its expression/function by means of antisense oligonucleotides, dominant negative mutants, ribozymes, or siRNA oligonucleotides triggers caspase-dependent apoptosis and increases apoptotic stimuli (24,29-32). Its anti-apoptotic mechanism of action is not completely understood, but it has been suggested recently that it may affect the upstream initiation of mitochondrial-mediated apoptosis (33). Furthermore, its phosphorylation at threonine 34 allows the formation of a complex with caspase-9 and thus leads to the inhibition of apoptosis (34). In addition to preventing caspase-9 activation within a functional apoptosome (35), survivin binds the apoptogenic Smac/DIABLO released by mitochondrial membranes (36).

One of the most significant features of survivin is its expression in cancers and embryonic and fetal tissues (37), whereas its expression is very low or undetectable

in normal differentiated tissues (38). Survivin up-regulation occurs in response to growth factor signaling (39,40), STAT activation (41), oncogene activation (42), loss of p53 (43,44), and PI3 kinase/Akt signaling (45).

We have isolated and characterized two cell populations from an angiomyolipoma in a patient affected by TSC2: one consists of  $\beta$  actin-positive TSC2 LOH smooth muscle-like cells ( $A^+$ ) and the other of keratin 8/18 positive TSC2 non-LOH epithelial-like cells ( $R^+$ ). Both cell types are HMB45 positive and require EGF for growth and proliferation (46). We report here that an angiomyolipoma in a TSC2 patient includes a small population of survivin-expressing cells, and that the same expression is also present in purified TSC2<sup>+/-</sup> epithelioid cells ( $R^+$ ). Survivin is not detectable in TSC2<sup>-/-</sup> smooth muscle-like cells ( $A^+$ ), but the addition of IGF-1 or removal of EGF from the growth medium, or the exposure of  $A^+$  cells to specific EGFR antibodies (Merck Biosciences, Darmstadt, Germany), promote survivin expression.  $A^+$  cells release IGF-1, and agents such as anti-EGFR antibodies increase IGF-1 release by up to about 13 ng/1.5  $\times 10^5$  cells/24 h.

## MATERIALS AND METHODS

### Materials

The SDS-PAGE materials came from Biorad (Hercules, CA, USA); LY294002, PD98059, rapamycin, staurosporine-A, wortmannin, cycloheximide (CHX), and recombinant human EGF (rhuEGF) came from Sigma-Aldrich (St. Louis, MO, USA); the fetal bovine serum and media were purchased from Euroclone (Paignton, UK); recombinant human IGF-1 (rhuIGF-1) was provided by Peprotech EC Ltd.; the polyclonal antibodies against survivin (FL-142), caspase-3 (H-277), RhoA, and  $\beta$ -actin came from Santa-Cruz Biotechnology (Santa-Cruz, CA, USA); the polyclonal antibody against  $\beta$ -actin came from Sigma-Aldrich, that against keratin 8/18 from Neomarkers (Freemont, CA, USA), and those against Smac/DIABLO and phospho-S6 from

Cell Signaling (Beverly, MA, USA); the mouse monoclonal antibody against EGFR came from Merck Biosciences (Darmstadt, Germany), and that against IGF-1R from Calbiochem (Darmstadt, Germany). All of the other reagents were of high analytical grade and were purchased from Sigma-Aldrich.

### Cell Cultures

The primary cultures were isolated from the angiomyolipoma of a 42-year-old female TSC2 patient previously described by Lesma et al. (46). The study was approved by the Institutional Review Board of Milan's San Paolo Hospital. The  $\beta$  actin-positive TSC2 LOH smooth muscle-like cells ( $A^+$ ) and keratin 8/18 positive TSC2 non-LOH epithelial-like cells ( $R^+$ ) were kept in culture medium containing a 50/50 mixture of DMEM/Ham F12, supplemented with hydrocortisone 2  $\times 10^{-7}$  mol/L, rhuEGF 10 ng/mL, sodium selenite 5  $\times 10^{-8}$  mol/L (Sigma), insulin 25  $\mu$ g/mL (Sigma), transferrin 10  $\mu$ g/mL (Sigma), ferrous sulfate 1.6  $\times 10^{-6}$  (Sigma), and 15% heat-inactivated fetal bovine serum (FBS). C2C12 (mouse muscle myoblast) cells, CT/G vascular smooth muscle cells from human aorta (VSMCs), and A549 (human lung adenocarcinoma) cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and maintained in accordance with the manufacturer's instructions: C2C12 in DMEM medium, A549 in RPMI 1640 medium, and VSMCs in F12 medium. FBS was added to all of the media at a concentration of 10%. All of the cell lines were kept at 37°C in a humidified atmosphere (5% CO<sub>2</sub>; 95% air). The cell cultures (except that of C2C12) were routinely sub-cultivated by means of trypsinisation to maintain long-phase growth. For the comparative studies of survivin expression, the cells were sub-confluent grown as previously described.

Cell proliferation was evaluated in the presence of various agents added to the culture media by counting the cells in Neubauer chamber.

### Western Blotting

At the end of the treatments, the cell plates were placed on ice, the medium was removed, and the cells were lysed in SDS-containing buffer (2% SDS, 0.1 M sodium acetate, 0.5 mM EDTA, 1 mM phenylmethylsulphonylfluoride, 1 mM aprotinin, 1 mM leupeptin, 2 mM sodium orthovanadate, 2 mM sodium fluoride). Angiomyolipoma was homogenized under isotonic condition (25 mM TrisHCl, PH 7.4, 250 mM sucrose, 1% Triton X-100, 1% NP-40, 5 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulphonylfluoride, 1 mM aprotinin, 1 mM leupeptin, 2 mM sodium orthovanadate, 2 mM sodium fluoride) using an Ultra-Turrax T25. The homogenates were centrifuged at 1500g for 20 min at 4°C, and the supernatant was collected and used for Western blot analysis.

Protein concentrations were determined using the Lowry Ciocalteau method with BSA as standard. Equal amounts (75 µg) of solubilized protein were dissolved in Laemmli sample buffer (Biorad, Hercules, CA, USA), separated by SDS-PAGE in reducing conditions, and electroblotted onto nitrocellulose membranes. The blots were blocked in 5% non-fat dry milk, 0.05% Tween-20 in TBS, pH 7.5, and probed with specific antibodies overnight at 4°C. The membranes were probed with horseradish peroxidase-conjugated goat anti-rabbit antibody (1:10,000) (Chemicon, Temecula, CA) at room temperature for 1 h. The protein was visualized by means of the enhanced chemiluminescence detection system (ECL; Pierce, Rockford, IL, USA), and the autoradiograms were analyzed using Kodak 1D 3.6 Software after acquisition on a Kodak image station 440 CF (Kodak).

### Immunocytochemistry

Paraffin-embedded tissue sections of angiomyolipoma were stained with the anti-survivin polyclonal antibody raised against the full length of the protein (Santa Cruz). Briefly, the slides were dewaxed and rehydrated, treated with 3% hydrogen peroxide in distilled water to

inhibit endogenous peroxidase activity and, after extensive washing in TBS/Triton X-100, incubated overnight at 4°C with a 1:200 dilution of the anti-survivin antibody. Sections were then rinsed and incubated with biotinylated goat anti-rabbit antibody (Pierce), and visualized using a standard ImmunoPure Ultrasensitive ABC Detection Kit (Pierce) with 3,3'-diaminobenzidine (DAB) as the chromogenic substrate. Positive and negative controls were included.

The cells were seeded and maintained in culture for 24 h before staining for survivin. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 1 min at room temperature. Non-specific background was eliminated by incubating the cells with 1.5% BSA in 0.2% Triton X-110 in PBS for 90 min, after which they were incubated overnight at 4°C in a humidified chamber with polyclonal antibody against survivin in 0.1% BSA. They were then rinsed and incubated with biotinylated goat anti-rabbit antibody (Chemicon) for 90 min at room temperature, and visualized using a standard ImmunoPure Ultrasensitive ABC Detection Kit (Pierce) with 3,3'-diaminobenzidine (DAB) as chromogen.

### Cell Immunofluorescence Microscopy

The cells were cultured on glass slides, permeabilized with 70% methanol for 10 min, and dried in air, and the primary antibodies against  $\beta$ -actin (1:100; Sigma), RhoA (1:100; Santa Cruz) and keratin 8/18 (1:100; Neomarkers) were applied overnight at 4°C. The samples were incubated for 3 h at room temperature with fluorescein isothiocyanate-conjugated rabbit anti-mouse antibody (Chemicon) for  $\beta$ -actin and keratin 8/18, and fluorescein-conjugated donkey anti-goat antibody (Chemicon) for RhoA. After washing, the slides were mounted in 50% glycerol with 1 µg/mL 4,6-diamino-2-phenylindole.

### IGF-1 Release Assay

The IGF-1 released in the culture media was measured by means of an enzyme-linked immunosorbent assay (ELISA) for human IGF-1 (Biosource

International) according to the manufacturer's instructions. Briefly, the cells were seeded in 5 cm Petri dishes at a dilution of  $1.5 \times 10^5$  cells/mL and, before testing, maintained in culture for different times (1, 5, 14 or 21 days) or treated as stated in the Results. Fresh media were collected and immediately stored at -80°C.

### Statistical Analyses

The data are expressed as mean values  $\pm$  SEM, and were statistically analyzed using Student t-test or ANOVA followed by Tukey's test; significance is indicated for *P* values of \* < 0.05. All of the analyses were made using GraphPad Prism 4.0 software.

## RESULTS

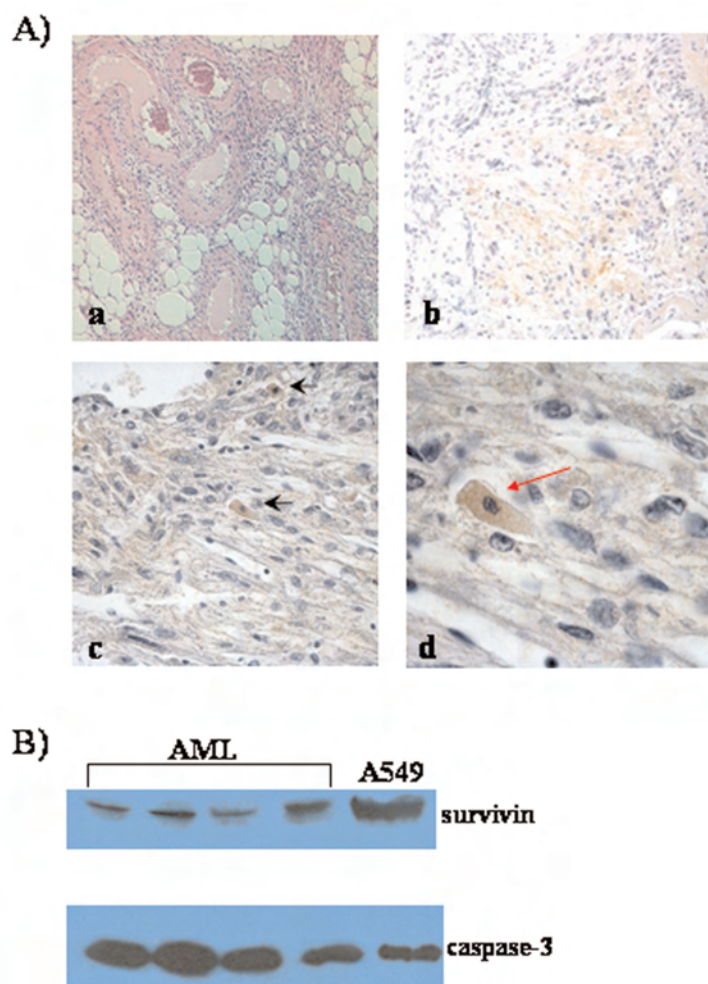
### Survivin Expression in TSC2 Angiomyolipoma

Figure 1A (panel a) shows the three typical cell components of angiomyolipoma (adipocytes, smooth muscle cells, and vascular structures with thick muscular walls) in hematoxylin- and eosin-stained sections of a specimen surgically removed from a TSC2 patient. Survivin-positive cells were most frequently detected in the areas of the angiomyolipoma in which smooth muscle cells were rare (Figure 1A, panels b and c). A detail is shown in Figure 1A, panel d.

The expression of survivin in human angiomyolipoma was also assayed by means of the Western blotting of four different samples (Figure 1B), with A549 cells being used as positive control (47). As survivin clearly plays an anti-apoptotic role (48), we also investigated caspase-3 by means of an antibody recognizing both the pro-peptide and the active form, and found that it was abundantly expressed in all of the angiomyolipoma samples (Figure 1B).

### Survivin Expression in Isolated TSC2 Cells

Two pure homogeneous cell populations were isolated from the angiomyolipoma of a TSC2 patient and characterized by sub-cloning at limiting dilution



**Figure 1.** Survivin immunocytochemical localization in a TSC2 patient angiomyolipoma. A: Adjacent sections were stained with hematoxylin and eosin (a), and exposed to a polyclonal antibody against survivin (b-d). Immunocytochemistry revealed survivin-positive cells scattered in the parenchyma and localized in areas that were relatively poor in smooth muscle cells; survivin was mainly localized in the cytoplasm (d, arrow). B: Western blot evaluation of survivin expression in four different samples of angiomyolipoma. A549 cells were used as a positive control of survivin and caspase-3 expression.

(46). One population consisted of elongated, flat cells highly positive for smooth muscle-specific  $\beta$ -actin (Figure 2a); the cells also are labeled by anti-HMB45 antibody (46), a TSC marker (49,50), but are negative for S100 and keratin 8/18. These smooth muscle-like cells ( $A^+$ ) are LOH (a mutation on chromosome 16p13.3 at exon 18 consisting of an A/T substitution) and do not express tuberlin (46). Conversely, the epithelioid cells ( $R^+$ ) were highly positive for keratin 8/18 and RhoA (Figure 2b), and negative for  $\beta$ -actin and

S100; they are not LOH and express tuberlin in both its phosphorylated and non-phosphorylated forms (46).

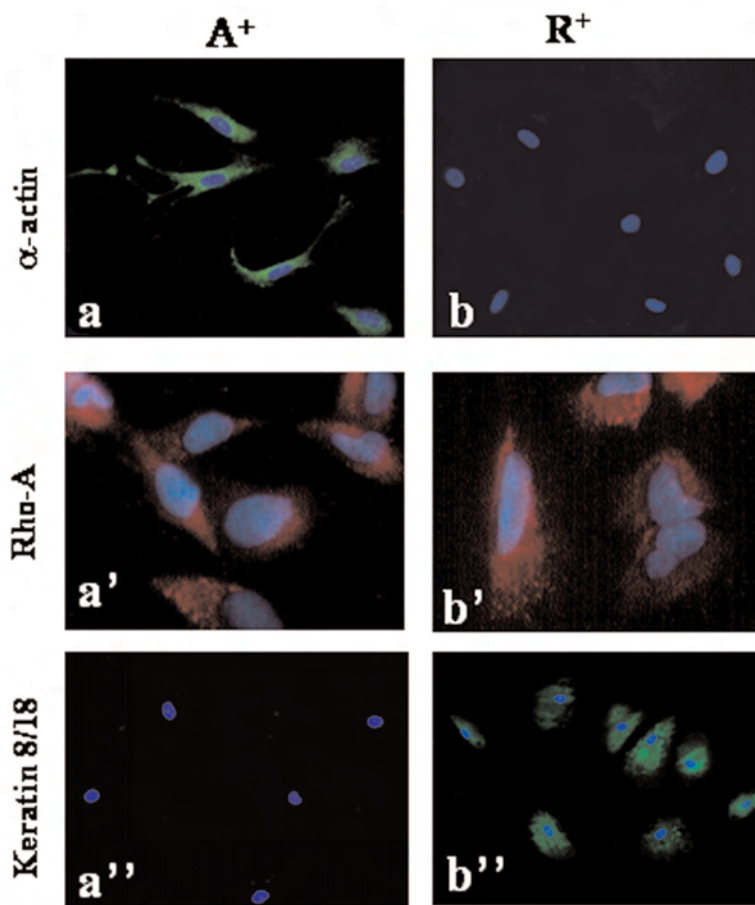
Hamartin is expressed quite abundantly in both  $A^+$  and  $R^+$  cells (46). The immunocytochemical localization of survivin clearly labeled  $R^+$  cells (Figure 3A, panels b and b'), but the  $A^+$  cells were negative (Figure 3A, panels a, a'). In particular, survivin was detected in both the nuclear and extra-nuclear portions of  $R^+$  cells. Survivin also was expressed in the control A549 cells (Figure 3A, panel c

and c'). Western blotting confirmed the absence of survivin expression in the  $A^+$  cells, and its abundance in  $R^+$  cells (Figure 3B).

#### Pharmacological Regulation of Survivin Expression in Isolated TSC2 Cells

TSC2 is a direct substrate of Akt, a PI3K target involved in regulating cell growth and proliferation (13). Forty-eight h exposure of  $A^+$ ,  $R^+$ , and A549 cells to mTOR-inhibiting rapamycin (100 ng/mL) or PI3K-inhibiting LY294002 (LY, 100  $\mu$ M) induced survivin expression in the  $A^+$  cells, and did not change it in the  $R^+$  and A549 cells (Figure 4A). Exposure to staurosporine (1  $\mu$ M) for 48 h also triggered survivin expression in the  $A^+$  cells (data not shown). Caspase-3 levels were not changed under these conditions (Figure 4A). The induction of survivin expression also was established morphologically by means of immunocytochemistry (Figure 4B). Human aorta smooth muscle cells (VSMCs) were used as controls for the human smooth muscle TSC2 cells. Under physiological conditions, as well as in the presence of rapamycin (100 ng/mL), LY (100  $\mu$ M), wortmannin (320 nM), and staurosporine (1  $\mu$ M), survivin was undetectable in VSMCs (Figure 4C); pro-caspase-3 was expressed, but none of the experimental conditions affected its level of expression (Figure 4C).

Apoptosis can be triggered by multiple stimuli, including cytokines, free radicals, toxins, and growth factor withdrawal (51); furthermore,  $A^+$  and  $R^+$  cells are routinely grown in the presence of 10 ng/mL EGF, and exposure to anti-EGFR leads to their death (46). We grew  $A^+$  and  $R^+$  cells for 48 h in standard medium with and without EGF, or with the addition of IGF-1 (50 ng/mL) in the absence of EGF. The removal of EGF from standard medium and exposure to IGF-1 in the absence of EGF triggered the expression of survivin in the  $A^+$  cells, and the same experimental conditions increased the expression of survivin in  $R^+$  cells (Figure 5A, upper panel). Pro-



**Figure 2.** Two types of purified TSC2 cells. Exposure to an  $\beta$ -actin antibody (a,b) specific for smooth muscle cells showed that the antigen was present in flat, elongated TSC2<sup>-/-</sup> cells (A<sup>+</sup>) but undetectable in epithelioid TSC2<sup>+/-</sup> cells (R<sup>+</sup>). Rho-A was very abundant in R<sup>+</sup> cells (a',b'), which were labeled with the anti-keratin 8/18 antibody (b''). No anti-keratin 8/18 labeling was observed in A<sup>+</sup> cells (a'').

caspase-3 levels were apparently unaffected in the A<sup>+</sup> cells, and only slightly increased in the R<sup>+</sup> cells (Figure 5A, lower panel).

The induction of survivin expression in A<sup>+</sup> cells also was triggered when IGF-1 was added to the standard culture growth medium containing EGF (Figure 5B). IGF-1-mediated survivin expression in A<sup>+</sup> cells was accompanied by the down-regulation of Smac/DIABLO. This was not observed when IGF-1 was added to the growth medium without EGF, and the increased survivin level was accompanied by the expression of Smac/DIABLO (Figure 5B). The absence

of both growth factors led to the same survivin and Smac/DIABLO results. It is well known that survivin-mediated apoptosis suppression may occur via a direct interaction with the caspase activator Smac/DIABLO (36). None of the experimental conditions affected the expression of phospho-S6 and caspase-3. The pharmacological induction of survivin in TSC2 cells was quite rapid and did not require serum: it was expressed as little as 2 h after the addition of IGF-1 or LY to serum-deprived standard medium, and the inhibition of PI3K by LY did not antagonize the effect of IGF-1 on survivin expression (Figure 5C).

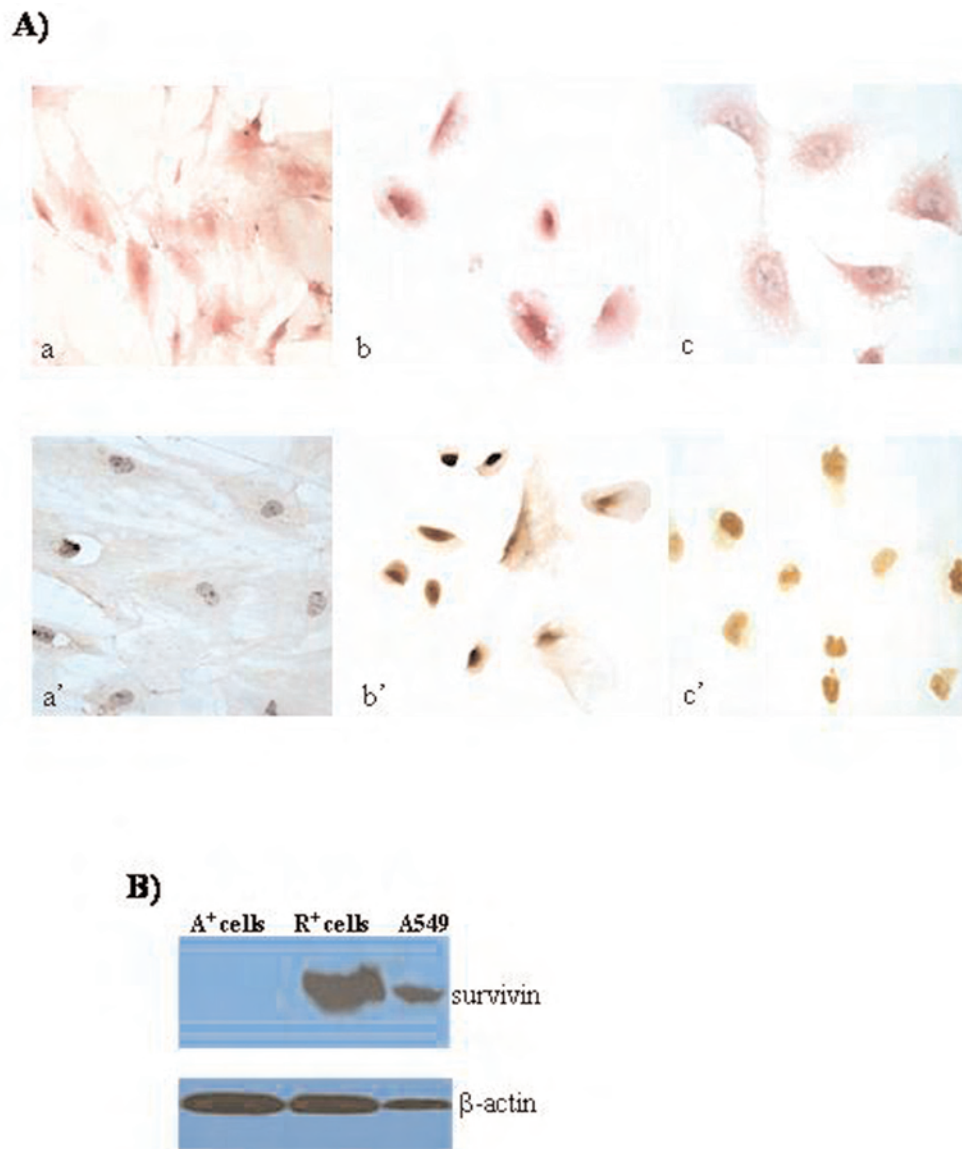
### Cell Proliferation and Survivin Expression

Adding EGF to the culture medium allows the proliferation of A<sup>+</sup> cells (46) and, as described above, keeps the expression of survivin at undetectable levels. However, when these cells were exposed for 48 h to EGFR antibodies (5  $\mu$ g/mL) raised against the receptor domain that interacts with the ligand (clone 225), survivin expression was greatly enhanced (Figure 6A). The induction mediated by the blockade of EGFR was boosted by co-incubation with IGF-1. The inhibition of MAPK by PD98059 (PD, 30  $\mu$ M) and LY (100  $\mu$ M) also increased the expression of survivin in A<sup>+</sup> cells (Figure 6A), which was enhanced further by the co-incubation of IGF-1 with LY and PD, and even the simultaneous co-addition of PD (30  $\mu$ M) and LY (100  $\mu$ M) did not prevent the IGF-1 promotion of survivin expression (data not shown). This evidence suggests that IGF-1 may also act through a pathway other than those regulated by PI3K/Akt and MAPK. Smac/DIABLO expression was down-regulated by IGF-1, without counteracting the effects of PI3K and MAPK blockade even when the inhibitors were added simultaneously; conversely, the effects were enhanced by anti-EGFR (Figure 6A). When exposure to anti-EGFR was extended from 48 h to five days, the period in which its killing action begins to take place (46), survivin expression was significantly reduced in the TSC2<sup>-/-</sup> A<sup>+</sup> cells (Figure 6B), and the same occurred when rapamycin was administered for 5 days. However, prolonged anti-EGFR or rapamycin treatment did not affect Smac/DIABLO expression (Figure 6B).

Only rapamycin and anti-EGFR affected the proliferation rate during 48 h exposure, which suggests that the increased expression of survivin may be unrelated to proliferation in this instance (Figure 6C).

### IGF-1 Release

We have reported previously that A<sup>+</sup> cells are capable of releasing IGF-1 into

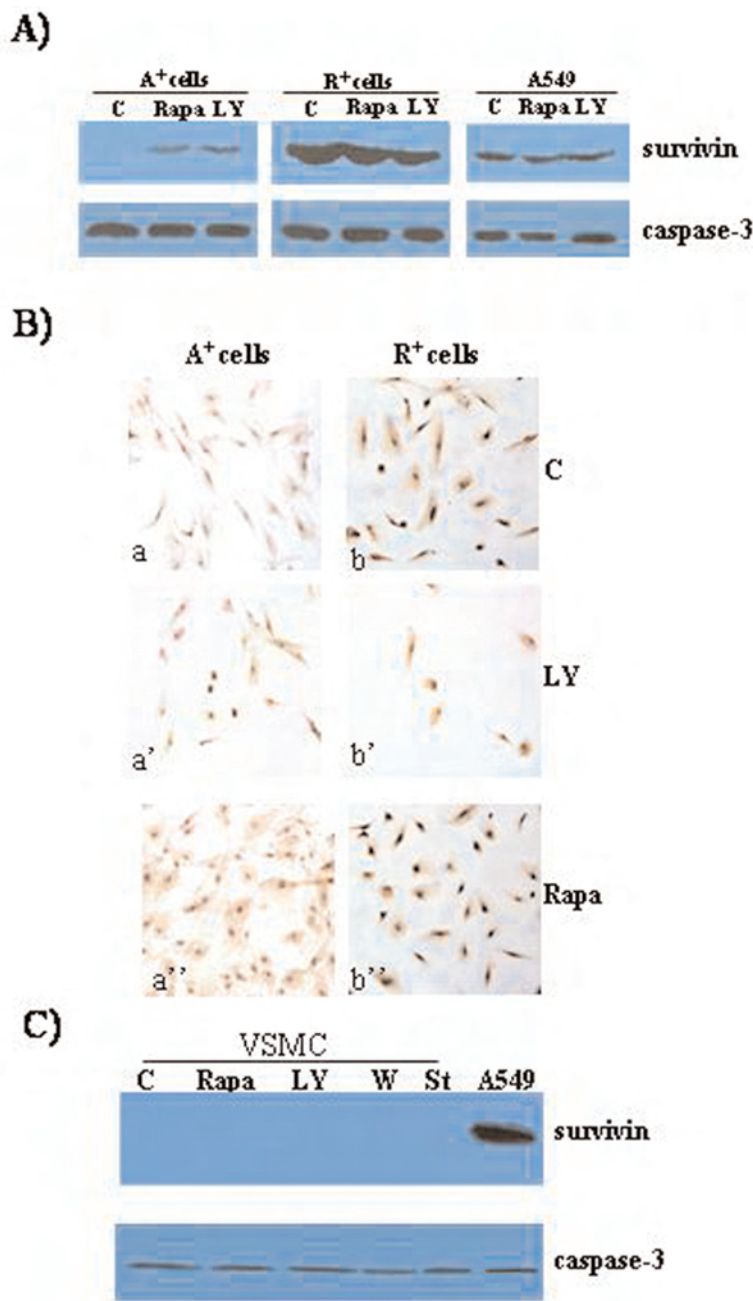


**Figure 3.** Survivin expression in TSC2 cells. A: Hematoxylin and eosin staining of TSC2<sup>-/-</sup> A<sup>+</sup> cells (a), TSC2<sup>+/-</sup> R<sup>+</sup> cells (b), and A549 cells (c); immunocytochemical staining revealed that the R<sup>+</sup> (b') and A549 cells (c') were rich in survivin, whereas the A<sup>+</sup> cells were negative (a'). B: Western blot analysis of survivin expression in A<sup>+</sup>, R<sup>+</sup>, and A549 cells (upper panel); as an internal control of gel loading, the membrane also was decorated with β-actin antibody (lower panel). Western blotting did not detect survivin in the TSC2<sup>-/-</sup> A<sup>+</sup> cells, but showed its abundance in TSC2<sup>+/-</sup> R<sup>+</sup> and A549 cells.

culture medium (46), and the findings of this study show that this release continues even when the period of culture is extended to 21 days. During the early stages, the A<sup>+</sup> cells released smaller amounts of IGF-1 (4.58 ng/1.5 × 10<sup>5</sup> cells/24 h after one, and 4.71 ng/1.5 × 10<sup>5</sup> cells/24 h after 5 days), but this markedly and highly significantly in-

creased to 9.14±0.21 ng/1.5 × 10<sup>5</sup> cells/24h and 14.21±0.65 ng/1.5 × 10<sup>5</sup> cells/24h after respectively 14 and 21 days (Figure 7A). This release was dependent on protein synthesis (it was significantly reduced by the addition of cycloheximide 10 μg/mL to the culture medium) and was unrelated to cell proliferation or mTOR activity as rapamycin

(5 ng/mL) had no effect (Figure 7B). Interestingly, blocking EGFR or IGF-1R by means of their specific antibodies (clone 225 and αIR3) greatly increased IGF-1 release (Figure 7C). Evaluation of the time-course of IGF-1 release over 24 h showed that it was quite constant during the 1st 10 h, and then sharply decreased (0-5 h: 0.912 ng/h/1.5 × 10<sup>5</sup> cells, 0-10 h:



**Figure 4.** mTOR and PI3K inhibition promotes survivin expression in TSC2<sup>-/-</sup> cells. Twenty-four h after plating, the cells were incubated with rapamycin (Rapa, 100 ng/mL) or LY294002 (LY, 10  $\mu$ M) for 48 h. A: Western blot detection of survivin and procaspase-3 expression. Under control conditions, the TSC2<sup>-/-</sup> A<sup>+</sup> cells did not express detectable levels of survivin, which only became apparent after mTOR and PI3K blockade; procaspase-3 was expressed under all experimental conditions. The R<sup>+</sup> and A549 cells show survivin and procaspase-3 expression under all experimental conditions. B: Immunocytochemical detection of survivin. The A<sup>+</sup> cells became positive to antibody labeling after exposure to rapamycin and LY. C: Twenty-four h after plating in specific standard medium, VSMC cells were treated with rapamycin (100 ng/mL), LY (100  $\mu$ M), wortmannin (W, 320 nM) and staurosporine (St, 1  $\mu$ M) for 48 h, and survivin and procaspase-3 expression was assessed by means of Western blotting. The VSMCs showed the expression of procaspase-3, but not survivin.

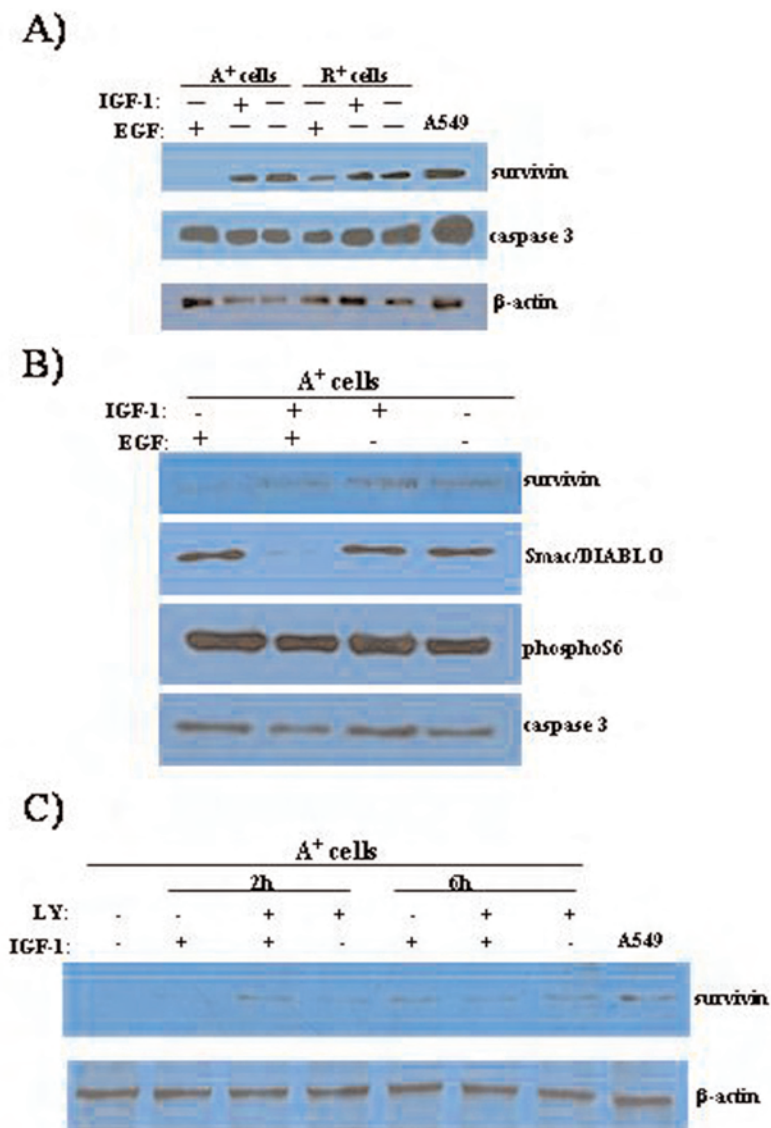
1.006 ng/h/1.5  $\times$  10<sup>5</sup> cells, 0-24 h: 0.279 ng/h/1.5  $\times$  10<sup>5</sup> cells). This may have been due to the accumulation of IGF-1 in the collection medium, which may exert a negative feedback control on the release mechanism. The more effective action of anti-IGF-1R may be due to a blockade of this negative feedback regulation.

## DISCUSSION

The results of this study show that angiomyolipoma cells from a TSC2 patient express the apoptosis inhibitor survivin. Two pure homogenous cell populations were isolated from the angiomyolipoma and characterized: TSC2<sup>-/-</sup> A<sup>+</sup> smooth muscle cells and TSC2<sup>+/-</sup> R<sup>+</sup> epithelioid cells. The latter show detectable levels of survivin, whereas the former express the protein when the growth medium is supplemented with IGF-1. Furthermore, survivin is expressed when the cells are exposed to conditions that perturb normal TSC2<sup>-/-</sup> cell function, such as the omission of EGF from the growth medium, the addition of anti-EGFR, the blockade of PI3K and ERK, or the inhibition of mTOR.

Interestingly, single or dual inhibition of PI3K by LY and ERK by PD does not prevent the triggering of survivin expression in TSC2<sup>-/-</sup> A<sup>+</sup> cell exposed to IGF-1, therefore IGF-1 may play a role in the survival of these cells via a pathway that does not require PI3K and ERK function. Such a role is also supported by the IGF-1-mediated down-regulation of apoptogenic Smac/DIABLO, which is constitutively expressed by TSC2<sup>-/-</sup> A<sup>+</sup> cells. Once again, the inhibition of PI3K by LY and ERK by PD does not prevent the marked reduction of Smac/DIABLO expression caused by IGF-1 exposure but, unlike that of survivin, its expression is not affected by LY or PD applied alone.

We found that the amounts of IGF-1 released by TSC2<sup>-/-</sup> A<sup>+</sup> cells into the culture medium become progressively larger over time, being much higher on day 21 than on day one or five. This is probably an auto-regulated process with a feedback mechanism activated by the



**Figure 5.** Modulation of survivin expression in  $TSC2^{-/-}$  A<sup>+</sup> cells by IGF-1 and EGF. A,B: The cells were cultured for 48 h in standard medium, or in the same medium devoid of EGF in the presence or absence of IGF-1 (50 ng/mL). C: After 24 h in serum-deprived medium, IGF-1 (50 ng/mL), LY294002 (LY, 100  $\mu$ M), or both were added to the serum-deprived medium for 2 or 6 h, and survivin, Smac/DIABLO, caspase-3, and the phosphorylated form of S6 were assessed by means of Western blotting. Removing EGF from the growth medium (A), or adding IGF-1 in the presence or absence of EGF (B), triggered the expression of survivin. The expression of proapoptotic Smac/DIABLO in A<sup>+</sup> cells was down-regulated by IGF-1, but only in the presence of EGF (B). The omission of EGF counteracted the suppressive action of IGF-1 on Smac/DIABLO expression (B). Serum-deprivation and LY, a PI3K inhibitor, did not inhibit the rapid IGF-1 induction of survivin expression, which was already evident 2 h after drug exposure (C).

accumulation of IGF-1 in the growth medium because, during the first 10 h of 24 h collection, the amounts of IGF-1 released remained quite constant and were

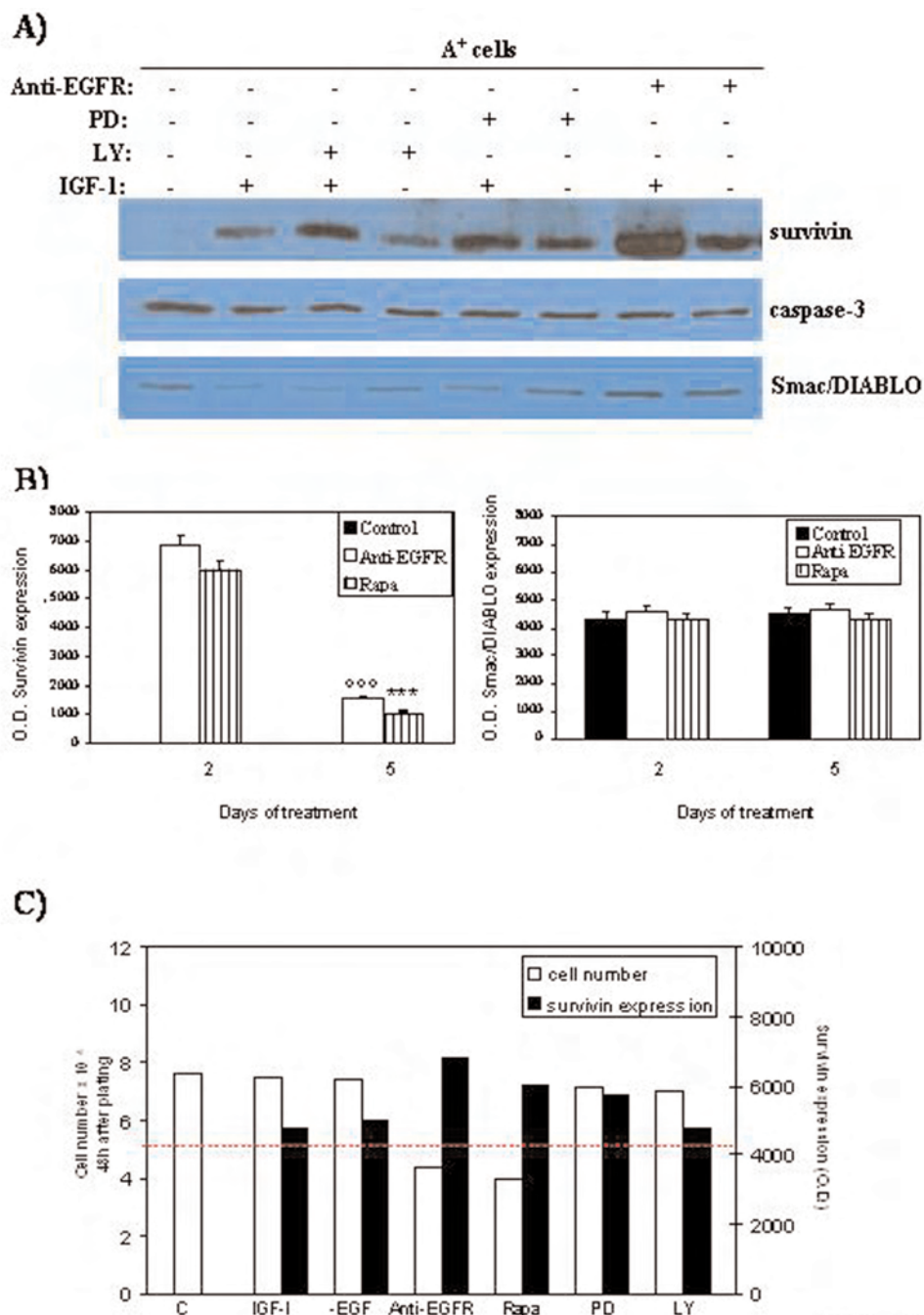
larger than later. However, this feedback regulation is overcome if  $TSC2^{-/-}$  A<sup>+</sup> cells are exposed to antibodies to IGF-1R, which increases the released amount by

more than 400%. We have reported previously that  $TSC2^{-/-}$  A<sup>+</sup> cells exposed to anti-IGF-1R gradually die in 12-14 days (46), and so the autocrine release of IGF-1 seems to be a powerful survival mechanism activated by  $TSC2^{-/-}$  smooth muscle cells which are tightly packed in the thick-walled vessels of TSC angiomyolipoma and lymphangioliomyomatosis (LAM) nodules.

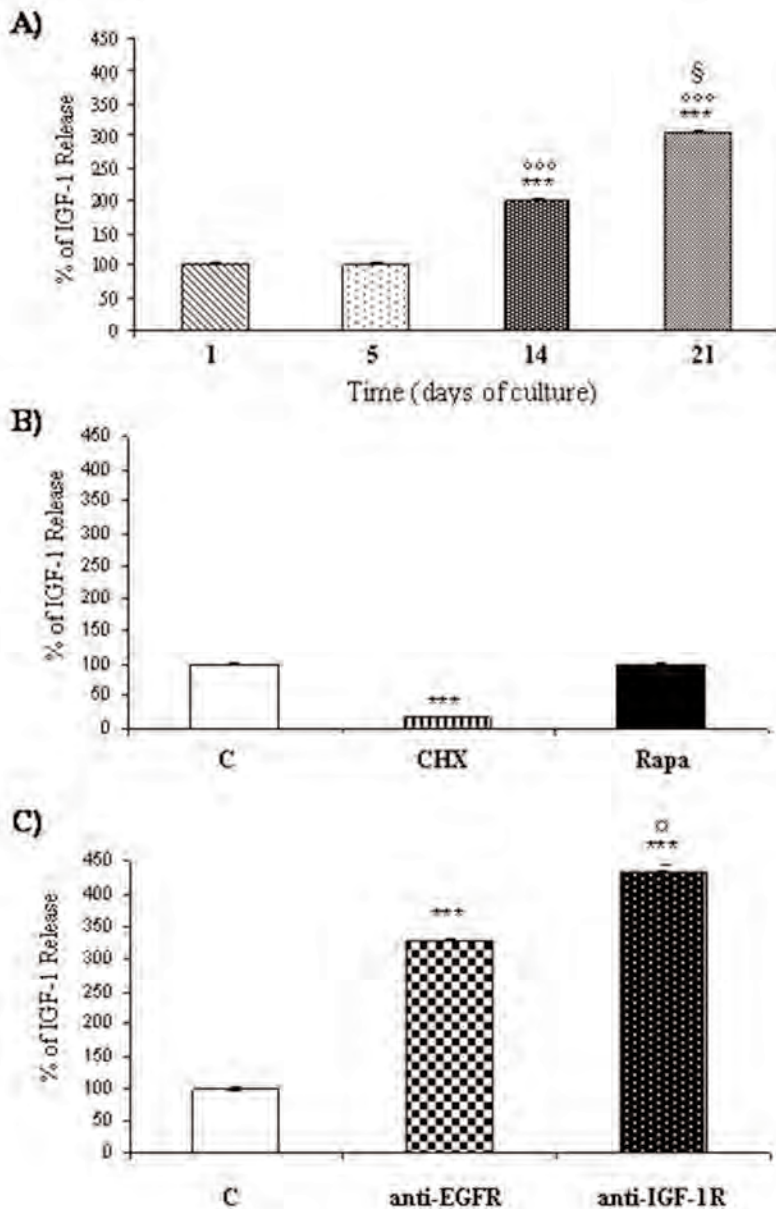
Survivin originally was isolated as a member of IAP family, and it was suggested that it plays a fundamental role in inhibiting apoptosis and regulating the mitotic process and cell proliferation (19,20). Its expression profile is indicative of this role as it is detectable in embryonic and fetal tissues, but poorly or not expressed in normal adult tissues (37). However, it is expressed in cancer cells, and higher levels of expression have been correlated with a poor prognosis (38); its expression also has been correlated with resistance to chemotherapeutic agents, thus suggesting that lower expression may indicate greater tumor sensitivity to chemotherapy (52,53). Survivin affects the initiation of mitochondrial-mediated apoptosis by binding the Smac/DIABLO released from mitochondria membranes, and then inhibiting caspase-9 activation (33-36).

Epithelioid  $TSC2^{-/-}$  R<sup>+</sup> cells constitutively express survivin, but it is undetectable in smooth muscle  $TSC2^{-/-}$  A<sup>+</sup> cells both in vivo and in vitro. However, its expression increases dramatically when the cells are exposed to agents that affect their function, such as LY, PD, and the mTOR inhibitor rapamycin. At a dose of 5 ng/mL, rapamycin affects the proliferation of smooth muscle  $TSC2^{-/-}$  A<sup>+</sup> cells (46), but LY and PD do not; the addition of anti-EGFR to the growth medium affects the rate of proliferation and promotes the expression of survivin. Both rapamycin and anti-EGFR increase survivin levels without affecting the constitutively expressed Smac/DIABLO, but their effects are time related: after five days' treatment, the survivin-promoting effects of anti-EGFR or rapamycin have almost vanished, whereas Smac/DIABLO is still ex-





**Figure 6.** Pharmacological regulation of survivin expression in TSC2<sup>-/-</sup> A<sup>+</sup> cells. The cells were cultured for 48 h in standard medium, or in the same medium in the presence of IGF-1 (50 ng/mL), LY294002 (LY, 100  $\mu$ M), PD98059 (PD, 30  $\mu$ M) or anti-EGF receptor antibody (C225, 5  $\mu$ g/mL). A: Survivin expression was triggered by IGF-1 and all of the other agents. The action of IGF-1 was enhanced by the co-administration of any of the other agents. The IGF-1-mediated attenuation of Smac/DIABLO expression was counteracted only by anti-EGFR. B: Survivin and Smac/DIABLO expression evaluated by means of Western blotting after two and five days' exposure to anti-EGFR (C225, 5  $\mu$ g/mL) or rapamycin (Rapa, 5 ng/mL). OD quantification showed that the expression of survivin decreased over time, whereas that of Smac/DIABLO did not. <sup>ooo</sup>  $P < 0.001$  vs. two days of anti-EGFR treatment; <sup>\*\*\*</sup>  $P < 0.001$  vs. two days of rapamycin treatment. C: A total of  $50 \times 10^4$  cells were plated and counted 48 h later in a modified Neubauer chamber. Rapamycin and anti-EGFR inhibited cell proliferation (the horizontal line represents cell number at plating); the other agents had no effect, and the rate of cell growth was the same as under control condition. Despite their different effects on cell proliferation (white bars), all of the treatments enhanced survivin expression (black bar).



**Figure 7.** TSC<sup>-/-</sup>A<sup>+</sup> cells release IGF-1 in culture medium. The medium was removed and the cells washed once, and then fresh medium was added and collected 24 h later for IGF-1 assay. The collected media were immediately stored at -80°C until IGF-1 determination by ELISA. IGF-1 release is shown as a percentage of controls (fixed at 100%). A: The cells were cultured for the indicated times (days) in standard medium. IGF-1 release was quite constant during the first week in culture, and then progressively increased to peak levels. B: The cells were grown for five days, and incubated for 24 h with fresh medium (control, C) with and without the presence of cycloheximide (CHX, 10 μg/mL) or rapamycin (Rapa, 5 ng/mL). CHX greatly reduced IGF-1 release, whereas rapamycin did not change it. C: The cells were grown for five days in standard medium (control, C) with and without the presence of anti-EGFR (clone 225, 5 μg/mL) or anti-IGF-1R antibody (αIR3, 5 μg/mL). Both antibodies greatly enhanced IGF-1 release, but anti-IGF-1R was significantly more effective than anti-EGFR. Mean values ± SEM; between-group differences evaluated by ANOVA test: \*\*\* *P* < 0.001 vs control; °°° *P* < 0.001 vs 5 days; § *P* < 0.05 vs 14 days; € *P* < 0.05 vs anti-EGFR treatment.

pressed at normal levels. We have reported previously that anti-EGFR-induced TSC<sup>-/-</sup>A<sup>+</sup> cell death begins after five and seven days' exposure, and so it is possible that the progressive down-regulation of survivin may be one of the mechanisms triggered by anti-EGFR in killing these cells. In addition, anti-EGFR is probably a highly unfavorable signal for cells that require EGF to grow and proliferate (46); the 24-hour application of anti-EGFR enhances the autocrine release of IGF-1, which is comparable to that observed when the IGF-1 receptor is blocked by means of the specific antibody.

Epithelioid TSC<sup>+/+</sup>R<sup>+</sup> cells are very sensitive to EGF and its omission from the culture medium leads to a quick death, whereas smooth muscle TSC<sup>-/-</sup>A<sup>+</sup> cells stop proliferating but do not die when EGF is not added (46). Furthermore, R<sup>+</sup> cells secrete significantly smaller amounts of IGF-1, about 10% of that released by A<sup>+</sup> cells (data not shown). Both TSC<sup>-/-</sup> cell types are therefore sensitive to EGF, but the extent of their dependence is rather different and R<sup>+</sup> cells have a less developed ability to release IGF-1. Smooth muscle TSC<sup>-/-</sup>A<sup>+</sup> cells have developed a survival strategy that links the self-secretion of IGF-1 to the expression of anti-apoptotic survivin and the down-regulation of Smac/DIABLO. This survival mechanism seems to be quite specific as TSC<sup>-/-</sup>A<sup>+</sup> cells also show the constitutive expression of p53, which is genetically normal and without any mutation (unpublished data), and remains unaffected by IGF-1 or anti-EGFR exposure, and more generally by all of the experimental procedures used in this study (unpublished data).

Furthermore, IGF-1 supplementation promotes the proliferation of VSMCs and inhibits that of A<sup>+</sup> cells. The role of IGF-1 has thus changed from inducing proliferation in normal smooth muscle to auto-secreted survival activity with no proliferative ability in smooth muscle TSC<sup>-/-</sup>A<sup>+</sup> cells (46).

The mechanisms developed by TSC<sup>-/-</sup>A<sup>+</sup> cells in terms of IGF-1 action and sur-

Survivin expression are thus rather different from those described in cancer, in which the inhibition of PI3K leads to the down-regulation of survivin expression and increased sensitivity to chemotherapy (54-57), and CD34<sup>+</sup> cells, in which the selective inhibition of PI3K and MAPK blocks the up-regulation of survivin by growth factors (58).

In conclusion, Tuberous Sclerosis Complex (TSC) is a tumor suppressor gene disorder characterized by mutations in the TSC1 or TSC2 genes that lead to the development of benign tumors involving smooth muscle cells, and also cause life-threatening LAMs. TSC2 is the form most often correlated with a poor clinical outcome. We have isolated and grown in vitro a pure colony of TSC2<sup>-/-</sup> smooth muscle cells from angiomyolipoma (christened A<sup>+</sup>) that require EGF to grow, and die in 12-14 days after exposure to EGFR and IGF-1R antibodies (46). These cells secrete much IGF-1 that is greatly increased when they are subjected to killing insults, such as exposure to EGFR and IGF-1R antibodies. Pharmacological treatments capable of interfering with the function of key enzymes such as PI3K, ERK, and mTOR, or with vital EGFR functions, such as the omission of EGF from growth medium or exposure to anti-EGFR, trigger the expression of survivin by TSC2<sup>-/-</sup> smooth muscle cells. The autocrine release of IGF-1 seems to be a powerful survival mechanism activated by TSC2<sup>-/-</sup> smooth muscle cells, which are tightly packed in the thick-walled vessels of TSC angiomyolipomas and LAM nodules, and in which IGF-1 concentrations may increase to reach effective anti-apoptotic concentrations.

Future experimental therapies for TSC and LAM will need to overcome this obstacle to achieve their goal, and a novel approach may be the targeted inhibition of survivin, which may enhance sensitivity to TSC2 therapy.

#### ACKNOWLEDGMENTS

The authors acknowledge financial support to Alfredo Gorio from the Italian

Ministry of Education and Research program, FIRB 2003.

#### REFERENCES

- O'Callaghan FJ, Shiell AW, Osborne JP, Martyn CN (1998) Prevalence of tuberous sclerosis estimated by capture-recapture analysis. *Lancet* 351:1490.
- Young J, Povey S (1998) The genetic basis of tuberous sclerosis. *Mol. Med. Today*. 4:313-9.
- Karbowiczek M, Yu J, Henske EP (2003) Renal angiomyolipomas from patients with sporadic lymphangiomyomatosis contain both neoplastic and non-neoplastic vascular structures. *Am. J. Pathol.* 162:491-500.
- Neumann HP, Schwarzkopf G, Henske EP (1998) Renal angiomyolipomas, cysts, and cancer in tuberous sclerosis complex. *Semin. Pediatr. Neurol.* 5:269-75.
- Henske EP (2005) Tuberous sclerosis and the kidney: from mesenchyme to epithelium, and beyond. *Pediatr. Nephrol.* 20:854-7.
- Henske EP (2003) Metastasis of benign tumor cells in tuberous sclerosis complex. *Genes Chromosom. Cancer.* 38:376-81.
- Carsillo T, Astrinidis A, Henske EP (2000) Mutations in the tuberous sclerosis complex gene TSC2 are a cause of sporadic pulmonary lymphangiomyomatosis. *Proc. Natl. Acad. Sci. USA.* 97:6085-90.
- van Sleightenhorst M et al. (1998) Interaction between hamartin and tuberlin, the TSC1 and TSC2 gene products. *Hum. Mol. Genet.* 7:1053-7.
- Dabora SL et al. (2001) Mutational analysis in a cohort of 224 tuberous sclerosis patients indicates increased severity of TSC2, compared with TSC1, disease in multiple organs. *Am. J. Hum. Genet.* 68:64-80.
- Tapon N, Ito N, Dickson BJ, Treisman JE, Hariharan IK (2001) The drosophila tuberous sclerosis complex gene homologs restrict cell growth and cell proliferation. *Cell* 105:345-55.
- Hengstschlager M et al. (2001) Tuberous sclerosis gene products in proliferation control. *Mutat. Res.* 488:233-9.
- Edinger AL, Thompson CB (2002) Akt maintains cell size and survival by increasing mTOR-dependent nutrient uptake. *Mol. Biol. Cell.* 13:2276-88.
- Inoki K, Li Y, Zhu T, Wu J, Guan KL (2002) TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signaling. *Nat. Cell. Biol.* 4:648-57.
- Dan HC et al. (2002) PI3K/AKT pathway regulates TSC tumor suppressor complex by phosphorylation of tuberlin. *J. Biol. Chem.* 277:35364-70.
- Manning BD, Tee AR, Logsdon MN, Blenis J, Cantley LC (2002) Identification of the tuberous sclerosis complex-2 tumor suppressor gene product tuberlin as a target of the phosphoinositide 3-kinase/akt pathway. *Mol. Cell.* 10:151-62.
- Deveraux QL, Reed JC (1999) IAP family proteins—suppressors of apoptosis. *Genes Dev.* 13:239-52.
- Salvesen GS, Duckett CS (2002) IAP proteins: blocking the road to death's door. *Nat. Rev. Mol. Cell. Biol.* 3:401-10.
- Verdecia MA et al. (2000) Structure of the human anti-apoptotic protein survivin reveals a dimeric arrangement. *Nat. Struct. Biol.* 7:602-8.
- Ambrosini G, Adida C, Altieri DC (1997) A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma. *Nat. Med.* 3:917-21.
- Altieri DC (2004) Molecular circuits of apoptosis regulation and cell division control: the survivin paradigm. *J. Cell. Biochem.* 92:656-63.
- Li F, Ling X (2006) Survivin study: an update of "what is next wave"? *J. Cell. Physiol.* 208:476-86.
- Li F et al. (1998) Control of apoptosis and mitotic spindle checkpoint by survivin. *Nature.* 396:580-4.
- Chen J et al. (2000) Down-regulation of survivin by antisense oligonucleotides increases apoptosis, inhibits cytokinesis and anchorage-independent growth. *Neoplasia.* 2:235-41.
- Li F et al. (1999) Pleiotropic cell-division defects and apoptosis induced by interference with survivin function. *Nat. Cell. Biol.* 1:461-6.
- Olie RA et al. (2000) A novel antisense oligonucleotide targeting survivin expression induces apoptosis and sensitizes lung cancer cells to chemotherapy. *Cancer Res.* 60:2805-9.
- Reed JC, Bischoff JR (2000) BIRing chromosomes through cell division—and survivin' the experience. *Cell.* 102:545-8.
- Grossman D et al. (2001). Transgenic expression of survivin in keratinocytes counteracts UVB-induced apoptosis and cooperates with loss of p53. *J. Clin. Invest.* 108:991-9.
- Grossman D, Kim PJ, Schechner JS, Altieri DC (2001). Inhibition of melanoma tumor growth in vivo by survivin targeting. *Proc. Natl. Acad. Sci. USA.* 98:635-40.
- Kanwar JR, Shen WP, Kanwar RK, Berg RW, Krissansen GW (2001) Effects of survivin antagonists on growth of established tumors and B7-1 immunogene therapy. *J. Natl. Cancer Inst.* 93:1541-52.
- Yamamoto T, Manome Y, Nakamura M, Tanigawa N (2002) Downregulation of survivin expression by induction of the effector cell protease receptor-1 reduces tumor growth potential and results in an increased sensitivity to anticancer agents in human colon cancer. *Eur. J. Cancer* 38:2316-24.
- Zhou M et al. (2002) DNA damage induces a novel p53-survivin signaling pathway regulating cell cycle and apoptosis in acute lymphoblastic leukemia cells. *J. Pharmacol. Exp. Ther.* 303:124-31.
- Beltrami E, Plescia J, Wilkinson JC, Duckett CS, Altieri DC (2004) Acute ablation of survivin uncovers p53-dependent mitotic checkpoint functions and control of mitochondrial apoptosis. *J. Biol. Chem.* 279:2077-84.
- McNeish IA et al. (2005) Survivin interacts with Smac/DIABLO in ovarian carcinoma cells but is redundant in Smac-mediated apoptosis. *Exp. Cell Res.* 302:69-82.

34. O'Connor DS et al. (2000) Regulation of apoptosis at cell division by p34cdc2 phosphorylation of survivin. *Proc. Natl. Acad. Sci. USA.* 97:13103-7.
35. Marusawa H et al. (2003) HBXIP functions as a cofactor of survivin in apoptosis suppression. *EMBO J.* 2:2729-40.
36. Song Z, Yao X, Wu M (2003) Direct interaction between survivin and Smac/DIABLO is essential for the anti-apoptotic activity of survivin during taxol-induced apoptosis. *J. Biol. Chem.* 278:23130-40.
37. Adida C, et al. (1998) Anti-apoptosis gene, survivin, and prognosis of neuroblastoma. *Lancet* 351:882-3.
38. Altieri DC (2001) The molecular basis and potential role of survivin in cancer diagnosis and therapy. *Trends Mol. Med.* 7:542-7.
39. Tran J et al. (1999) Marked induction of the IAP family antiapoptotic proteins survivin and XIAP by VEGF in vascular endothelial cells. *Biochem. Biophys. Res. Commun.* 64:781-8.
40. O'Connor DS et al. (2000) Control of apoptosis during angiogenesis by survivin expression in endothelial cells. *Am. J. Pathol.* 156:393-8.
41. Aoki Y, Feldman GM, Tosato G (2003) Inhibition of STAT3 signaling induces apoptosis and decreases survivin expression in primary effusion lymphoma. *Blood.* 101:1535-42.
42. Sommer KW et al. (2003) Inhibitor of apoptosis protein (IAP) survivin is upregulated by oncogenic c-H-Ras. *Oncogene.* 22:4266-80.
43. Mirza A et al. (2002) Human survivin is negatively regulated by wild-type p53 and participates in p53-dependent apoptotic pathway. *Oncogene.* 21:2613-22.
44. Hoffman WH et al. (2002) Transcriptional repression of the anti-apoptotic survivin gene by wild type p53. *J. Biol. Chem.* 277:3247-57.
45. Dan HC et al. (2004) Phosphatidylinositol-3-OH kinase/AKT and survivin pathways as critical targets for geranylgeranyltransferase I inhibitor-induced apoptosis. *Oncogene.* 23:706-15.
46. Lesma E et al. (2005) Isolation and growth of smooth muscle-like cells derived from tuberous sclerosis complex-2 human renal angiomyolipoma: EGF is the required growth factor. *Am. J. Pathol.* 167:1093-103.
47. Xu ZX et al. (2004) Promyelocytic leukemia protein 4 induces apoptosis by inhibition of survivin expression. *J. Biol. Chem.* 279:1838-44.
48. Altieri DC (2003) Validating survivin as a cancer therapeutic target. *Nat. Rev. Cancer.* 3:46-54.
49. Bonetti F et al. (1994) Clear cell ("sugar") tumor of the lung is a lesion strictly related to angiomyolipoma—the concept of a family of lesions characterized by the presence of the perivascular epithelioid cells (PEC). *Pathology.* 26:230-6.
50. Bjornsson J et al. (1996) Tuberous sclerosis-associated renal cell carcinoma. Clinical, pathological, and genetic features. *Am. J. Pathol.* 149:1201-8
51. Hengartner MO. (2000) The biochemistry of apoptosis. *Nature.* 407:770-6.
52. Ambrosini G, Adida C, Sirugo G, Altieri DC (1998) Induction of apoptosis and inhibition of cell proliferation by survivin gene targeting. *J. Biol. Chem.* 273:11177-82.
53. Kim PJ, Plescia J, Clevers H, Fearon ER, Altieri DC (2003) Survivin and molecular pathogenesis of colorectal cancer. *Lancet.* 362:205-9.
54. Asanuma H et al. (2005) Survivin expression is regulated by co-expression of human epidermal growth factor receptor 2 and epidermal growth factor via phosphatidylinositol 3-kinase/AKT signaling pathway in breast cancer cells. *Cancer Res.* 65:11018-25.
55. Ling X, Bernacki RJ, Brattain MG, Li F (2004) Induction of survivin expression by taxol (Paclitaxel) is an early event, which is independent of taxol-mediated G2/M arrest. *J. Biol. Chem.* 279:15196-203.
56. Zhang M, Latham DE, Delaney MA, Chakravarti A (2005) Survivin mediates resistance to antiandrogen therapy in prostate cancer. *Oncogene.* 24:2474-82.
57. Testa JR, Tsichilis PN (2005) AKT signaling in normal and malignant cells. *Oncogene.* 24:7391-3.
58. Fukuda S, Foster RG, Porter SB, Pelus LM (2002) The antiapoptosis protein survivin is associated with cell cycle entry of normal cord blood CD34<sup>+</sup> cells and modulates cell cycle and proliferation of mouse hematopoietic progenitor cells. *Blood* 100:2463-71.