

MCP-1 overexpressed in tuberous sclerosis lesions acts as a paracrine factor for tumor development

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Patients with tuberous sclerosis complex (TSC) develop hamartomatous tumors showing loss of function of the tumor suppressor TSC1 (hamartin) or TSC2 (tuberin) and increased angiogenesis, fibrosis, and abundant mononuclear phagocytes. To identify soluble factors with potential roles in TSC tumorigenesis, we screened TSC skin tumor-derived cells for altered gene and protein expression. Fibroblast-like cells from 10 angiofibromas and five periungual fibromas produced higher levels of monocyte chemoattractant protein-1 (MCP-1) mRNA and protein than did fibroblasts from the same patient's normal skin. Conditioned medium from angiofibroma cells stimulated chemotaxis of a human monocytic cell line to a greater extent than conditioned medium from TSC fibroblasts, an effect blocked by neutralizing MCP-1-specific antibody. Overexpression of MCP-1 seems to be caused by loss of tuberin function because Eker rat embryonic fibroblasts null for *Tsc2* (EEF *Tsc2*^{-/-}) produced 28 times as much MCP-1 protein as did EEF *Tsc2*^{+/+} cells; transient expression of WT but not mutant human *TSC2* by EEF *Tsc2*^{-/-} cells inhibited MCP-1 production; and pharmacological inhibition of the Rheb-mTOR pathway, which is hyperactivated after loss of *TSC2*, decreased MCP-1 production by EEF *Tsc2*^{-/-} cells. Together these findings suggest that MCP-1 is an important paracrine factor for TSC tumorigenesis and may be a new therapeutic target.

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Abbreviations used: EEF, Eker rat embryonic fibroblast; IGFBP2, insulin-like growth factor-binding protein 2; LOH, loss of heterozygosity; MCP-1, monocyte chemoattractant protein-1; TSC, tuberous sclerosis complex; VEGF, vascular endothelial growth factor.

Patients with tuberous sclerosis complex (TSC) are predisposed to developing tumors in the brain, eyes, heart, kidneys, lung, and skin. Although typically benign, these tumors cause significant morbidity, including seizures, mental retardation, and disfigurement. The tendency to form multiple tumors is a consequence of inactivating mutations in a tumor-suppressor gene, either *TSC1* or *TSC2* (1). The products of these genes, hamartin and tuberin, seem to function as a complex to regulate many cellular processes, in particular signaling through the PI3K-Akt-TSC1/2-Rheb-mTOR pathway (2). Loss of function of the hamartin-tuberin complex in TSC tumors enhances mTOR signaling leading to increased cell numbers and cell size.

Skin tumors, including multiple facial angiofibromas and periungual fibromas, are observed in ~90% of patients with TSC (3). Histologically, angiofibromas and periungual fibromas show increased vessels and fibrosis. There are also increased numbers of cells in the interstitial dermis, predominantly spindle-shaped, fibroblast-

like cells together with stellate cells that seem to be monocyte-derived DCs, based on immunoreactivity for factor XIIIa (4–6). These features are shared variably by TSC tumors in other organs. Increased angiogenesis is observed in TSC-associated tumors of the kidney, lung, and brain (7), and increased numbers of cells positive for factor XIIIa have been observed in subependymal giant cell astrocytomas and angiomyolipomas (4). More recently, tubers have been reported to contain increased numbers of cells expressing CD68, a marker used to identify cells of the monocyte/macrophage/DC lineage (8). It has not been determined what induces the mixture of cell populations composing these hamartomatous tumors and whether this cellular heterogeneity is related to loss of hamartin-tuberin function.

The mixed cellular composition of TSC tumors points to a role for soluble growth factors in their development. Prompted by the increased vascularity of TSC tumors, others have investigated angiogenic factors. TSC tumors of the

skin, brain, and kidney produce vascular endothelial growth factor (VEGF) (7, 9, 10). Furthermore, overexpression of VEGF is related to loss of tuberlin function and is at least partially mTOR-dependent (11, 12). It has been proposed that overexpression of VEGF is a unifying feature of hamartoma syndromes (13).

To identify soluble growth factors involved in the development of TSC skin tumors, we profiled cytokine mRNA levels and protein production in cultured angiofibroma and periungual fibroma cells. TSC skin tumor cells overexpressed MCP-1, a chemokine that stimulates angiogenesis, fibrosis, and recruitment of monocytes. The relationship between MCP-1 production and loss of tuberlin function was investigated using EEF *Tsc2*^{-/-} cells and pharmacological inhibitors of the PI3K-Akt-mTOR pathway. These experiments indicate that overexpression of MCP-1 in EEF *Tsc2*^{-/-} cells is mTOR-dependent and is related to loss of tuberlin function.

RESULTS

Angiofibromas and periungual fibromas show increased vessels, fibrosis, and mononuclear phagocytes

Angiofibromas and periungual fibromas contained greater numbers of dermal cells positive for CD68 than did patient's normal-appearing skin (Fig. 1). These positive cells were stellate or polygonal in shape. As previously reported for angiofibromas (4–6), more cells positive for factor XIIIa, another marker for monocyte-derived cells, were seen in angiofibromas and periungual fibromas than in normal-appearing skin (unpublished data). As expected, angiofibromas and periungual fibromas had greater numbers of endothelial cells positive for CD34 and dermal cells positive for vimentin than did normal-appearing skin (unpublished data). There were sparse but elevated numbers of cells positive for S100 in the papillary dermis, and there were almost no cells in the dermis positive for HMB-45 (unpublished data). The intensity of staining for smooth muscle actin was greater in perivascular cells of angiofibromas and periungual fibromas than in normal-appearing skin (unpublished data). Thus, increased cellularity in these skin tumors derives from several cell lineages.

Cells cultured from angiofibromas and periungual fibromas express fibroblast markers

We hypothesized that the abnormal cellular composition of TSC skin tumors was related to altered expression of soluble growth factors. To generate an in vitro system for study, cells were grown from angiofibromas, periungual fibromas, and the patient's normal-appearing skin using standard methods for culturing fibroblasts. Cells grown from angiofibromas and periungual fibromas were bipolar or multipolar, as previously reported (14, 15). Immunocytochemistry was used to distinguish cells from different lineages, using, for example, an antibody to the collagen chaperone HSP47 to detect fibroblasts (16). Fibroblasts from normal-appearing skin of a TSC patient (hereafter referred to as TSC fibroblasts) and angiofibroma cells from the same patient expressed HSP47 but not

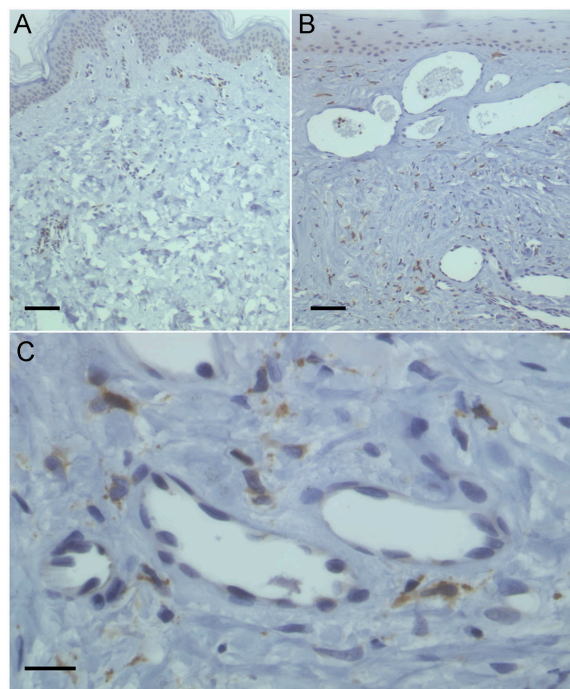


Figure 1. CD68-positive cells are more abundant in TSC skin tumors than in normal-appearing skin from a patient with TSC. (A) In sections of normal-appearing skin, cells staining for CD68 (brown) are sparse in the dermis. Bar, 65 μ m. (B) In sections of a periungual fibroma, there are dilated vessels and fibrosis and a more cellular stroma. Many stromal cells stain positive for CD68. Bar, 65 μ m. (C) CD68-positive cells are located near vessels among CD68-negative, fibroblast-like cells. Bar, 15 μ m. Similar results were observed in four angiofibromas and four periungual fibromas from six patients.

CD68 (Fig. 2, A–F). They also expressed vimentin but not CD14, S100, or smooth muscle actin (unpublished data).

Angiofibroma and periungual fibroma cells show altered expression of soluble growth factors

A cytokine cDNA array was used to compare gene expression, using total RNA from paired cultures of TSC fibroblasts and angiofibroma cells from three patients. In the studies reported below, cells were incubated in DMEM plus 1% FBS for 1 d to reduce extrinsic stimulation of cytokine production, unless noted otherwise. Four of 268 genes were overexpressed by a mean of threefold or more, including MCP-1 (9.8-fold), insulin-like growth factor-binding protein 2 (IGFBP2) (8.1-fold), secreted frizzled-related protein 2 (3.2-fold), and IGFBP5 (3.0-fold). For VEGF, the mean ratio of tumor to TSC fibroblasts was 1.4. It is interesting that IGFBP3 expression was reported in an earlier study of angiofibroma cells (17), and that IGFBP1, IGFBP2, IGFBP4, IGFBP5, and IGFBP6 are expressed in pulmonary lesions of lymphangiomyomatosis, that also exhibit mutations in TSC genes (18).

Because changes in mRNA expression are not always reflected at the protein level, the levels of MCP-1 and 10

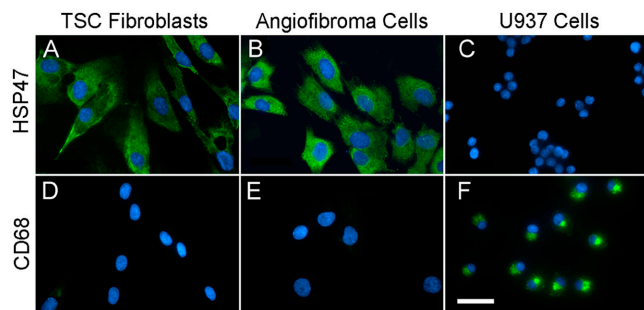


Figure 2. Cells cultured from TSC skin tumors express the fibroblast marker HSP47 but not CD68. The cytoplasm of TSC fibroblasts (A) and angiofibroma cells (B) stain positive for HSP47 (green), whereas U937 cells (C), a human monocytic cell line, are negative. In contrast, the cytoplasm of TSC fibroblasts (D) and angiofibroma cells (E) are negative for CD68, and the cytoplasm of U937 cells (F) stains positive (green). Nuclei fluoresce blue with DAPI. Bar, 15 μ m. Similar results were observed in cells from two other patients.

other cytokines (selected based on cDNA array data or potential roles in TSC tumorigenesis) were measured in culture supernatants using an ELISA array. After 24 h incubation, only MCP-1 was higher in supernatants from angiofibroma cells than TSC fibroblasts in all paired samples from seven patients ($P = 0.018$; Fig. 3). Because of the altered expression observed using both cDNA and ELISA arrays, MCP-1 was selected for further studies.

Angiofibroma and periungual fibroma cells overexpress MCP-1 capable of stimulating migration of monocytes

MCP-1 mRNA levels were quantified using real-time PCR in cells derived from angiofibromas and periungual fibromas.

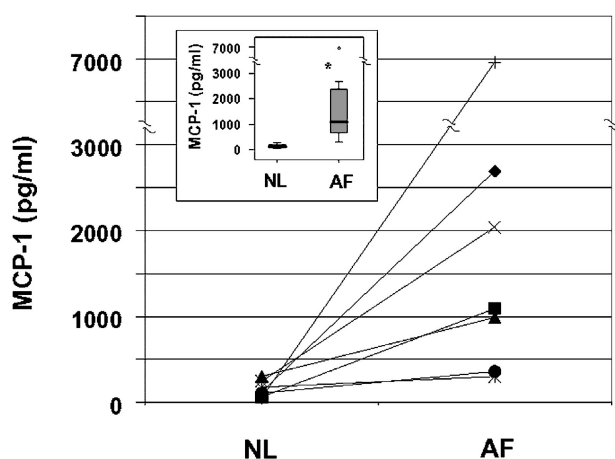


Figure 3. Measurement of cytokines in culture supernatants reveals increased production of MCP-1 by angiofibroma cells. Fibroblasts from normal-appearing skin (NL) or angiofibroma cells (AF) from seven patients were incubated in 1% FBS/DMEM for 24 h, and supernatants were collected for cytokine measurement using ELISA. Lines connect paired samples for each patient. The inset shows the data as a box plot. *, $P = 0.018$.

Levels of MCP-1 mRNA in angiofibroma cells and periungual fibroma cells were 1.3- to 33-fold ($n = 10$, $P = 0.001$) and 1.8- to 4.9-fold ($n = 5$, $P = 0.007$), respectively, greater than those in TSC fibroblasts (Table I). MCP-1 protein was measured using ELISA, and results were normalized to total cellular ATP levels after demonstrating the same linear relationship of cell number and cellular ATP in TSC fibroblasts and angiofibroma cells (unpublished data). Almost all of the MCP-1 produced was released into the medium, with only 5% recovered in cell lysates (unpublished data). TSC angiofibroma cells and periungual fibroma cells released 1.4- to 104-fold ($P = 0.001$) and 1.4- to 4.1-fold ($P = 0.015$), respectively, as much MCP-1 into the medium as TSC fibroblasts (Table I). The fold changes in MCP-1 mRNA correlated with those of MCP-1 protein ($n = 15$; Pearson correlation = 0.868; $P < 0.001$). Serum stimulated MCP-1 production by both TSC fibroblasts and angiofibroma cells ($P < 0.001$), but the effect was greater in angiofibroma cells ($P < 0.001$; Fig. 4).

To test biological activities of MCP-1 produced by TSC tumor cells, we performed proliferation and chemotaxis assays. Proliferation of normal human fibroblasts, TSC fibro-

Table I. Cells derived from angiofibromas and periungual fibromas express higher levels of MCP-1 mRNA and protein than do fibroblasts from the same patient

Patient	Tumor	MCP-1	
		mRNA ^a	Protein ^b
		<i>ratio: tumor/normal^c</i>	
1	AF	33	15
	PF	2.1	1.7
2	AF	12	19
	PF		
3	AF	6.4	6.0
	PF		
4	AF	1.3	1.4
	PF	2.0	1.4
5	AF	4.5	1.9
	PF	2.9	2.0
6	PF	4.9	4.1
	AF		
7	AF1	1.6	3.9
	AF2	31	104
8	AF	20	32
	PF		
9	AF	2.9	3.5
	PF	1.8	3.2
10	AF	5.2	15

^aReal-time PCR was used to measure MCP-1 mRNA levels in triplicate. Results were normalized to 18S rRNA, and results were expressed as a ratio of angiofibroma or periungual fibroma to TSC fibroblasts.

^bAn ELISA was used to measure MCP-1 in culture supernatants in triplicate. Results were normalized by total cellular protein and expressed as a ratio of tumor to normal.

^cEqual numbers of cells were incubated for 24 h in DMEM plus 1% FBS.

AF, angiofibroma; PF, periungual fibroma.

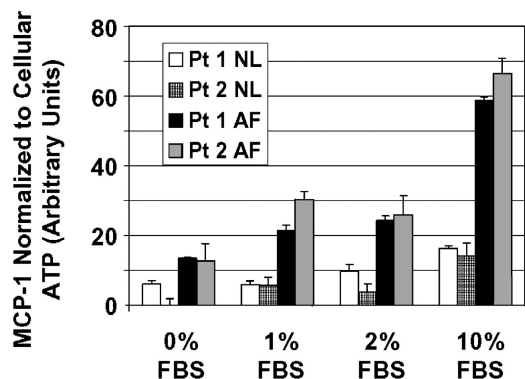


Figure 4. MCP-1 production is stimulated by FBS. Paired cultures of angiofibroma cells (AF) and TSC fibroblasts (NL) from two patients were seeded at 10,000 cells/well in 96-well plates, in DMEM containing 0%, 1%, 2%, or 10% FBS (1% BSA was added to the 0% FBS condition). After a 12-h incubation, MCP-1 concentration in the medium was measured by ELISA (pg/ml), and expressed per total cellular ATP (luminescence units) as an indication of cell number. Results are means \pm SD of values from triplicate wells. Fresh medium with 10% FBS does not contain detectable MCP-1.

blasts, and angiofibroma cells was similar whether growing in conditioned medium from angiofibroma cells (containing 740 pg/ml MCP-1) or from TSC fibroblasts (containing 44 pg/ml MCP-1; unpublished data). Likewise, recombinant MCP-1 (1,000 pg/ml) had no significant effect on proliferation of these cells (unpublished data). Thus, MCP-1 did not seem to be a major mitogen for angiofibroma cells, but MCP-1 produced by angiofibroma cells did stimulate chemotaxis of THP-1 cells, a human monocytic cell line. THP-1 cell migration in response to conditioned medium from angiofibroma cells was greater than migration in response to medium from TSC fibroblast alone ($P = 0.019$) or plus control IgG ($P = 0.007$), whereas the addition of neutralizing antibody to MCP-1 abrogated the effect on migration (Fig. 5). Findings were similar using recombinant MCP-1 (5 ng/ml) as a positive control: IgG had no effect, and anti-MCP-1 antibody blocked chemotactic activity (unpublished data). Thus, MCP-1 produced by angiofibroma cells may play a significant role as a chemotactic factor, recruiting cells into the tumor.

Overexpression of MCP-1 by tuberin-null rat fibroblasts is inhibited by *TSC2* transfection or inhibition of mTOR signaling

To investigate the relationship between MCP-1 production and tuberin function in a homogeneous, genetically defined cell population, we compared EEF *Tsc2*^{-/-} cells with those expressing tuberin (EEF *Tsc2*^{+/+}). EEF *Tsc2*^{-/-} cells produced significantly more MCP-1 than did EEF *Tsc2*^{+/+} cells ($P < 0.01$ at all times tested; Fig. 6). To evaluate further whether loss of tuberin leads to increased MCP-1 production, we transiently transfected EEF *Tsc2*^{-/-} cells with full-length human *TSC2* cDNA constructs including one WT, two human polymorphisms, and two mutants identified in TSC pa-

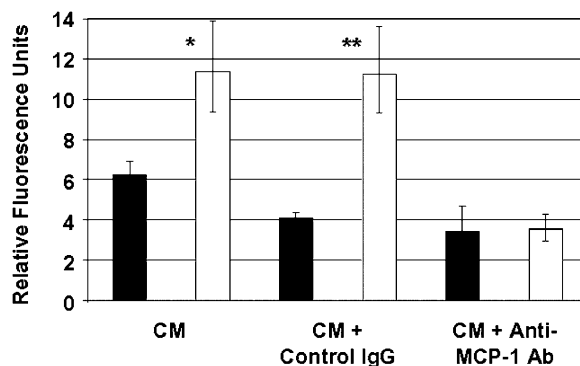


Figure 5. MCP-1 produced by TSC tumor cells is chemotactic for monocytes. Conditioned medium (CM), from TSC fibroblasts (closed bars) or angiofibroma cells (open bars), CM plus control antibody, or CM plus anti-MCP-1 antibody was added to the bottom chamber of cell migration plates. THP-1 cells (a human monocytic cell line) were added to the top chamber. Cells that migrated through 8- μ m pores to the feeder tray after a 2-h incubation were lysed and detected by CyQuant GR dye that exhibits enhanced fluorescence upon binding cellular nucleic acids. Results are geometric means \pm SD of values from three separate migration chambers. *, $P = 0.019$ and **, $P = 0.007$ as compared with CM from TSC fibroblasts. Conditioned medium from angiofibroma cells or TSC fibroblasts contained 1640 and 60 pg/ml MCP-1, respectively. Similar results were observed in three separate experiments.

tients (19). Under conditions that yielded a transfection efficiency of 50% for GFP (unpublished data), transfection with WT tuberin or two nonpathogenic missense polymorphisms (M286V and R367Q) decreased MCP-1 production by \sim 50% ($P = 0.005$; Fig. 7). A dose-response for transfection with WT tuberin showed that tuberin significantly inhibited MCP-1 production in a dose-dependent manner (Fig. 7). In contrast, two nontruncating mutants (G294E and I365del) known to disrupt binding of tuberin to hamartin (19) had no effect on MCP-1 production (Fig. 7). Because tuberin negatively regulates signaling through the PI3K-Akt-TSC1/2-

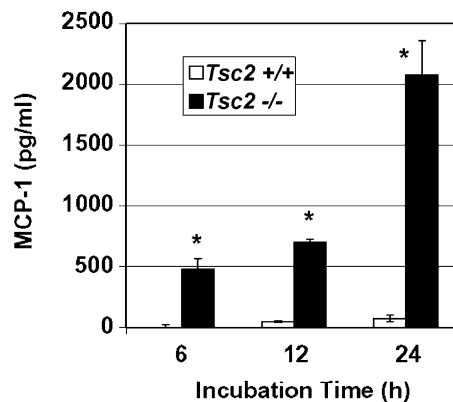


Figure 6. EEF *Tsc2*^{-/-} fibroblasts produced more MCP-1 than normal (EEF *Tsc2*^{+/+}) fibroblasts. Culture supernatants were collected after incubating EEF cells for the indicated time, and MCP-1 in the medium was measured by ELISA. Results are means \pm SD of triplicate supernatants.

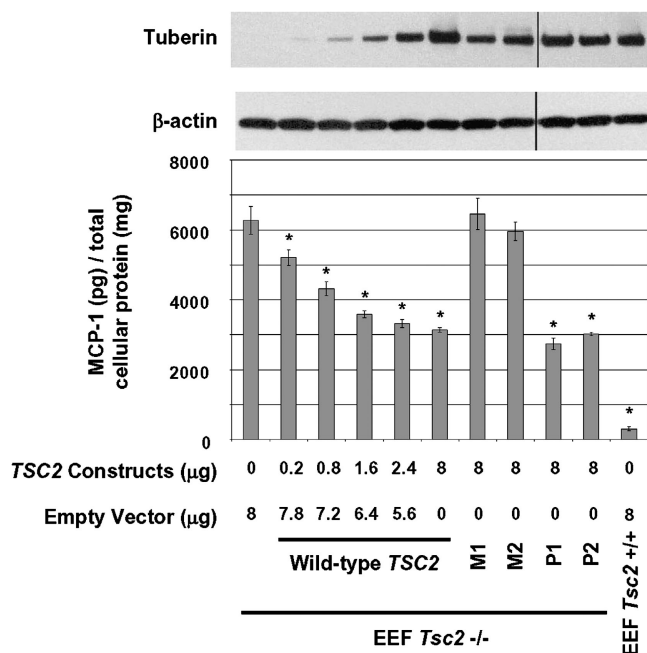


Figure 7. Transfection of WT but not mutant *TSC2* into EEF *Tsc2*^{-/-} cells inhibits MCP-1 production. EEF *Tsc2*^{-/-} cells were transfected with the indicated amounts of human *TSC2* constructs and/or empty vector. In the last lane, EEF *Tsc2*^{+/+} cells were transfected with empty vector. After transfection, cells were maintained in DMEM with 10% FBS overnight before the culture medium was replaced with DMEM containing 2% FBS; then incubation continued for an additional 24 h. The cells were harvested, and tuberin expression was detected by Western blot. Culture supernatants were collected, and MCP-1 release into the medium was measured by ELISA. Results are the mean ± SD of three separate wells. M1, mutation G294E; M2, mutation I365del; P1, polymorphism M286V; P2, polymorphism R367Q. *P, < 0.01 compared with empty vector control. Similar results were obtained in three separate experiments.

Rheb-mTOR pathway, we tested the effects of pharmacological inhibition of this pathway on MCP-1 production by EEF *Tsc2*^{-/-} cells. The inhibitors used were rapamycin, a specific inhibitor of mTOR, FTI-277, a farnesyl transferase inhibitor that blocks production of active Rheb, and LY294002, a PI3K and mTOR inhibitor. Each of these compounds significantly suppressed MCP-1 production in a concentration-dependent manner (*, P < 0.01; Fig. 8). None of the compounds was cytotoxic at the concentrations tested (unpublished data). Thus, overexpression of MCP-1 by EEF *Tsc2*^{-/-} cells was mTOR-dependent.

DISCUSSION

The cellular composition of TSC tumors is mixed, implicating paracrine factors in their development. Previous studies have focused on the increased vessels in TSC tumors and have shown increased expression of VEGF (7, 9–12). We found that TSC skin tumors, in addition to having more blood vessels and fibrosis than normal skin, show greater numbers of CD68-positive cells, and that cells derived from TSC skin tumors overexpress MCP-1, a chemokine with

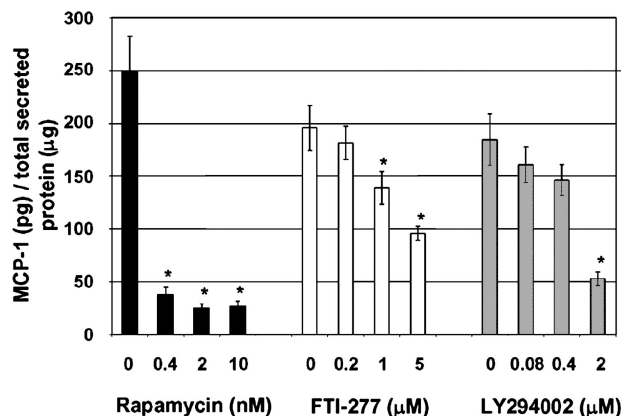


Figure 8. MCP-1 production by EEF *Tsc2*^{-/-} cells is inhibited by rapamycin, FTI-277, and LY294002. Rat MCP-1 was measured in culture supernatants by ELISA after incubating equal numbers of cells 24 h in serum-free medium without or with inhibitor at the indicated concentration. MCP-1 concentration was expressed per total secreted protein at the end of the incubation and is reported as the mean ± SD of triplicate wells. *, P < 0.01 compared with control. Similar results were obtained in three separate experiments.

roles in angiogenesis, fibrosis, and recruitment of monocytes. We also showed that conditioned medium from cultured TSC skin tumor cells was chemotactic for human monocytic cells, and neutralizing antibody against MCP-1 inhibited the chemotactic activity. These data suggest that MCP-1 is a paracrine factor that contributes to TSC tumorigenesis.

MCP-1 and its receptor, CCR2, are expressed by a variety of cells including skin fibroblasts, and are up-regulated in a variety of pathological processes (20, 21). MCP-1 expression has been associated with tumor vascularity (22) and is critical for hemangioendothelioma proliferation (23). MCP-1 can stimulate angiogenesis directly (24) and indirectly through recruitment of monocytes (25). MCP-1 is overexpressed in fibrotic processes and has a variety of fibrogenic effects (21, 26, 27). Thus, it is likely that MCP-1 overexpression in TSC skin tumors stimulates angiogenesis and fibrosis.

MCP-1 is chemotactic for monocytes and for peripheral blood myeloid DCs (28). Expression of MCP-1 is associated with the appearance of tumor-associated macrophages (29) and with recruitment of DCs into skin (30). It has been proposed that mononuclear phagocytes both stimulate tumor formation through effects on angiogenesis and release of mitogens and, in other systems, inhibit tumor growth through immunologic effects (29). In TSC skin tumors, the balance of growth-promoting and growth-inhibitory effects of tumor-associated mononuclear phagocytes may lead to the long-term stable size of cutaneous tumors. Disruption of this balance may represent a new therapeutic approach for these tumors.

TSC skin tumors are heterogeneous in cell lineages and genetically. Although it has been possible to show loss of heterozygosity (LOH) at the *TSC2* locus in other TSC tumors, angiofibromas and periungual fibromas often do not show LOH (31), probably because of the mixture of

TSC2^{+/-} cells and *TSC2*^{-/-} cells in such lesions. Immunohistochemical studies of hamartin and tuberin expression are consistent with this interpretation. When loss of hamartin and tuberin expression is observed in angiofibromas, the cells showing decreased staining are the interstitial cells, not endothelial cells (32). We have not detected LOH in our cultured angiofibroma cells or periungual fibroma cells, in agreement with the results of others (33). However, most of these cultures show allelic deletion of the *TSC2* gene in a minority of the cells (34). In addition, angiofibroma cells and periungual fibroma cells exhibit hyperphosphorylation of ribosomal protein S6 under conditions of serum starvation (35), which is also observed in other TSC-related tumors as a consequence of loss of tuberin function and activation of mTOR signaling. Together, these observations suggest that increased MCP-1 production by these angiofibroma cells and periungual cells is related to defective tuberin function, although the possibility of additional genetic or epigenetic alterations cannot be excluded.

To explore the relationship of defective tuberin function and MCP-1 production, we used EEF *Tsc2*^{-/-} cells. Much more MCP-1 was produced by EEF *Tsc2*^{-/-} cells than by EEF *Tsc2*^{+/+} cells. MCP-1 production by EEF *Tsc2*^{-/-} cells was reduced by transient transfection with human tuberin and was suppressed by rapamycin, a specific inhibitor of mTOR. These results indicate that MCP-1 overexpression by EEF *Tsc2*^{-/-} cells is caused by defective tuberin function and dysregulated mTOR-dependent signaling. Recently, it was shown that transgenic mice overexpressing a dominant-negative allele of tuberin develop fibrovascular collagenomas in the skin, and that these skin lesions show increased expression of MCP-1 as compared with WT skin (36). Others have found that rapamycin inhibits MCP-1 production in animal models of atherosclerosis (37) and transplant rejection (38, 39). It is interesting that loss of tuberin function and increased activation of the Rheb/mTOR/S6K pathway induce insulin receptor substrate-1 depletion and insulin resistance (40, 41), and that baseline levels of MCP-1 are increased in insulin-resistant 3T3-L1 adipocytes and in insulin-resistant obese mice (42).

In conclusion, MCP-1 is overexpressed by TSC skin tumor cells and probably exerts paracrine effects leading to angiogenesis, fibrosis, and recruitment of monocytes. Blocking MCP-1 has been effective for treatment of a variety of conditions in experimental animals, including atherosclerosis (43), pulmonary fibrosis (27), and renal fibrosis (26), and antibodies to MCP-1 completely blocked the development of hemangiopericytomas in mice (44). Therefore, MCP-1 may present a new therapeutic target for TSC skin tumors in humans.

MATERIALS AND METHODS

Tumor samples and cell culture. Adult patients, diagnosed with TSC according to clinical criteria (45) were enrolled after institutional review board approval (National Heart, Lung, and Blood Institute Institutional Review Board-approved protocol 00-H-0051). Cells cultured from explants of angiofibromas, periungual fibromas, and patients' normal-appearing skin as previously described (14), from normal adult human fibroblasts (Cambrex Bio Science), and from EEF *Tsc2*^{-/-} and EEF *Tsc2*^{+/+} cells (46) were

grown in DMEM supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100 µg/ml). Human monocyte cell lines U937 and THP-1 (ATCC), were grown in RPMI 1640 medium with 10% FBS.

Immunohistochemical and immunocytochemical staining. 6-µm sections of four angiofibromas, four periungual fibromas, and four samples of normal-appearing skin from six patients were immunostained using the autostainer (DakoCytomation) according to the manufacturer's recommendations with antibodies against S100 (1:600), HMB-45 (1:50), smooth muscle actin (1:200), CD34 (1:75), CD68 (1:200), vimentin (1:200) (DakoCytomation) and factor XIIIa (Bio-Genex). An avidin-biotin peroxidase detector kit (DakoCytomation) was used with diaminobenzidine as the chromogen and Gill's hematoxylin as the counterstain.

TSC fibroblasts, angiofibroma cells, or human monocytic cells (5 × 10⁴ cells per chamber) on four-chamber slides (Nalge Nunc International) were incubated overnight in DMEM plus 10% FBS, followed by DMEM plus 1% FBS for 24 h. After fixation, permeabilization, and blocking, cells were incubated with mouse anti-Hsp47 monoclonal antibody (3 µg/ml; Stress-Gen Biotechnologies), anti-CD68 monoclonal antibody (1:100 dilution; DakoCytomation), Texas red α-smooth muscle actin (1:100; Sigma-Aldrich), or HMB45 (1:25; DakoCytomation) overnight at 4°C, and then with Alexa Fluor 488 goat anti-mouse IgG (1:1,000; Molecular Probes) at room temp for 1 h before nuclei were stained with DAPI.

Gene expression arrays. Early-passage human cells (P = 3–5) were grown until 70–80% confluent. The medium was changed to DMEM plus 1% FBS 24 h before harvesting. Total RNA was extracted from primary cultured cells using a phenol-based kit (TRIzol, GIBCO BRL), followed by further purification using an RNeasy Midi-Column (QIAGEN) and concentration using Microcon YM-30 (Millipore). Radiolabeled cDNA probes, made using a gene-specific primer mix and [³³P]dATP, were hybridized to the Clontech Atlas Human Cytokine/Receptor Array (BD Biosciences), according to manufacturer's instructions. To quantify signal intensity, a phosphorimager (Storm 860, Molecular Dynamics, Inc.) was used at a pixel resolution of 100 µm. These data were analyzed using P-SCAN (peak quantification using statistical comparative analysis) software, version 1.2 and MATLAB 6.1 (The MathWorks, Inc.).

Culture supernatant cytokine array. Human cells (5 × 10⁵ cells/well) were incubated overnight on six-well plates in 10% FBS/DMEM. The medium was switched to 1% FBS/DMEM, and the cells were incubated for 24 h. The levels of 11 cytokines (MCP-1, VEGF, platelet-derived growth factor-bb, TNFα, fibroblast growth factor, IL-7, IL-8, GM-CSF, lymphotaxin, IFN-γ-inducible T cell α chemoattractant, and leukemia inhibitory factor) were measured in supernatants using a multiplexed ELISA (SearchLight Array).

Human MCP-1 mRNA and protein. Human cells (1 × 10⁶ cells/plate) were grown overnight on 10-cm plates in 10% FBS/DMEM, then were switched to 1% FBS/DMEM, and the incubation was continued for 24 h. Supernatants were collected, and total RNA was extracted from the cells using the RNeasy Mini Kit (QIAGEN). MCP-1 mRNA and 18S rRNA were measured using RT-PCR with MCP-1 or 18S rRNA primers and FAM dye-labeled probe (MCP-1, 18S rRNA; Assays-on-Demand, Applied Biosystems) on an ABI PRISM 5700 Sequence Detection System Instrument (Applied Biosystems). MCP-1 was measured in supernatants using an ELISA specific for human MCP-1 (R&D Systems). Cellular lysates were prepared in 10 mM Tris buffer, pH 7.4, 100 mM NaCl, 1% Triton X-100, 10% glycerol, 0.1% SDS, 1 mM PMSF and a mixture of protease inhibitors (Sigma-Aldrich). Total cellular protein was measured using BCA reagent (Pierce Chemical Co.).

Proliferation and chemotaxis assays. Cells (1,500 cells/well in 96-well plates) were incubated overnight in 10% FBS/DMEM, before the medium was changed to 1% BSA/DMEM (no serum) with or without MCP-1 (1 ng/ml) (Pepro Tech) or 2% FBS/DMEM. Cells were incubated for 72 h,

with medium changed every 24 h. Cell number was assessed by measuring cellular ATP content with a luminescence assay (ATPlite, PerkinElmer) and BMG FLUOstar plate reader (BMG Lab Technologies). For preparation of conditioned medium, TSC fibroblasts or angiofibroma cells in 10-cm dishes (1×10^6 cells/dish) were incubated overnight in 10% FBS/DMEM before the medium was replaced with 1% FBS/DMEM and incubation continued for an additional 24 h. Culture supernatants were then collected and stored at -70°C .

Monocyte chemotaxis was assayed using Chemicon QCM 96-well cell migration assay (8- μm pore size) (CHEMICON International, Inc). THP-1 cells were incubated in 1% FBS/DMEM for 24 h before transferring 1.5×10^5 cells/100 μl of the same medium to migration chambers. Conditioned medium (150 μl /well) was added to the feeder tray. Recombinant human MCP-1 was used as the positive control. In neutralization studies, the conditioned medium was incubated with 10 $\mu\text{g}/\text{ml}$ of either anti-human MCP-1 monoclonal antibody (R&D Systems) or control mouse IgG (R&D Systems) for 2 h at 4°C before use in chemotaxis assays.

Rat MCP-1. Rat embryonic fibroblasts (5×10^5 cells/well) were grown overnight on six-well plates in 10% FBS/DMEM before switching to 2% FBS/DMEM, and the incubation continued for the indicated time. Rat MCP-1 was measured in culture medium by ELISA (Pierce Chemical Co.).

Gene transfection. Rat EEF *Tsc2*^{-/-} cells were transfected with WT *TSC2*, mutant *TSC2*, or *TSC2* polymorphism in pCMVTag2 (J. Sampson, Cardiff, UK) or with the empty vector by nucleofection technology using MEF Nucleofector kits (Amaxa Biosystems) according to the manufacturer's instructions. After transfection, cells were maintained in DMEM with 10% FBS overnight before the culture medium was replaced with DMEM containing 2% FBS, and incubation continued for an additional 24 h. Culture supernatants were collected for assay of MCP-1 by ELISA. Cells were lysed, and cell lysates were subjected to SDS-PAGE and immunoblotting for tuberin using rabbit anti-tuberin C-20 (Santa Cruz Biotechnology, Inc.) and monoclonal anti- β -actin antibody AC-15 (Sigma-Aldrich).

Cell treatment. Rat embryonic fibroblasts plated in 12-well plates (2×10^5 cells/well) were incubated in 10% FBS/DMEM overnight before incubation for 4 h with serum-free DMEM containing indicated concentrations of rapamycin (EMD Biosciences, Inc.), FTI-277 (EMD Biosciences, Inc.), or LY294002 (BIOMOL Research Laboratories, Inc.), followed by an additional 24 h in fresh medium with the same inhibitor. Cell culture supernatants were collected, and MCP-1 was quantified by ELISA. The concentrations of total secreted proteins in cell culture supernatants were quantitated by Bio-Rad Protein Assay (Bio-Rad Laboratories). Cell death was measured by lactate dehydrogenase release with the CytoTox96 Non-Radioactive Cytotoxicity Assay (Promega) according to the manufacturer's instructions.

Statistics. Cytokine ELISA data were analyzed using Wilcoxon signed ranks test. MCP-1 mRNA and protein data were analyzed using a one-sample *t* test to compare the average log ratio of fold-changes to the hypothesized value of 0 (no difference). The effects of serum on MCP-1 production by fibroblasts and angiofibroma cells from individuals with TSC were assessed using univariate analysis of variance. Overall significance of the effects of transfections or pharmacological agents on MCP-1 production was evaluated using analysis of variance, followed by Dunnett *t* test to compare each treatment group to control. For all other comparisons, significance was determined using *t* test with equal variances not assumed. Significance was defined as $P < 0.05$.

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REFERENCES

1. Sampson, J.R. 2003. TSC1 and TSC2: genes that are mutated in the human genetic disorder tuberous sclerosis. *Biochem. Soc. Trans.* 31:592–596.
2. Li, Y., M.N. Corradetti, K. Inoki, and K.L. Guan. 2004. TSC2: filling the GAP in the mTOR signaling pathway. *Trends Biochem. Sci.* 29:32–38.
3. Webb, D.W., A. Clarke, A. Fryer, and J.P. Osborne. 1996. The cutaneous features of tuberous sclerosis: a population study. *Br. J. Dermatol.* 135:1–5.
4. Penneys, N.S., K.J. Smith, and A.J. Nemeth. 1991. Factor XIIIa in the hamartomas of tuberous sclerosis. *J. Dermatol. Sci.* 2:50–54.
5. Nemeth, A.J., and N.S. Penneys. 1989. Factor XIIIa is expressed by fibroblasts in fibrovascular tumors. *J. Cutan. Pathol.* 16:266–271.
6. Benjamin, D.R. 1996. Cellular composition of the angiofibromas in tuberous sclerosis. *Pediatr. Pathol. Lab. Med.* 16:893–899.
7. Arbisser, J.L., D. Brat, S. Hunter, J. D'Armiento, E.P. Henske, Z.K. Arbisser, X. Bai, G. Goldberg, C. Cohen, and S.W. Weiss. 2002. Tuberous sclerosis-associated lesions of the kidney, brain, and skin are angiogenic neoplasms. *J. Am. Acad. Dermatol.* 46:376–380.
8. Maldonado, M., M. Baybis, D. Newman, D.L. Kolson, W. Chen, G. McKhann II, D.H. Gutmann, and P.B. Crino. 2003. Expression of ICAM-1, TNF-alpha, NFkappaB, and MAP kinase in tubers of the tuberous sclerosis complex. *Neurobiol. Dis.* 14:279–290.
9. Liu, M.Y., L. Poellinger, and C.L. Walker. 2003. Up-regulation of hypoxia-inducible factor 2alpha in renal cell carcinoma associated with loss of Tsc-2 tumor suppressor gene. *Cancer Res.* 63:2675–2680.
10. Nguyen-Vu, P.A., I. Fackler, A. Rust, J.E. DeClue, C.A. Sander, M. Volkenandt, M. Flaig, R.S. Yeung, and R. Wienecke. 2001. Loss of tuberin, the tuberous-sclerosis-complex-2 gene product is associated with angiogenesis. *J. Cutan. Pathol.* 28:470–475.
11. Brugarolas, J.B., F. Vazquez, A. Reddy, W.R. Sellers, and W.G. Kaelin Jr. 2003. TSC2 regulates VEGF through mTOR-dependent and -independent pathways. *Cancer Cell.* 4:147–158.
12. El-Hashemite, N., V. Walker, H. Zhang, and D.J. Kwiatkowski. 2003. Loss of Tsc1 or Tsc2 induces vascular endothelial growth factor production through mammalian target of rapamycin. *Cancer Res.* 63:5173–5177.
13. Brugarolas, J., and W.G. Kaelin Jr. 2004. Dysregulation of HIF and VEGF is a unifying feature of the familial hamartoma syndromes. *Cancer Cell.* 6:7–10.
14. Kato, M., T. Katsumoto, K. Ohno, S. Kato, F. Herz, and K. Takeshita. 1992. Expression of glial fibrillary acidic protein (GFAP) by cultured angiofibroma stroma cells from patients with tuberous sclerosis. *Neuropathol. Appl. Neurobiol.* 18:559–565.
15. Onodera, K., Y. Ishibashi, M. Sasaki, and G. Kimura. 1989. Abnormal division and gene expression in cultured cells from a patient with tuberous sclerosis. *J. Dermatol.* 16:263–269.
16. Kuroda, K., and S. Tajima. 2004. HSP47 is a useful marker for skin fibroblasts in formalin-fixed, paraffin-embedded tissue specimens. *J. Cutan. Pathol.* 31:241–246.
17. Ishibashi, Y., R. Watanabe, and K. Onodera. 1993. Tuberous Sclerosis: Advances in Clinical and Genetic Research. In *Dermatology: Progress & Perspectives*. W.H.C. Burgdorf, and S.I. Katz, editors. The Parthenon Publishing Group, New York. 764–766.
18. Valencia, J.C., K. Matsui, C. Bondy, J. Zhou, A. Rasmussen, K. Cullen, Z.X. Yu, J. Moss, and V.J. Ferrans. 2001. Distribution and mRNA expression of insulin-like growth factor system in pulmonary lymphangiomyomatosis. *J. Invest. Med.* 49:421–433.
19. Hodges, A.K., S. Li, J. Maynard, L. Parry, R. Braverman, J.P. Cheadle, J.E. DeClue, and J.R. Sampson. 2001. Pathological mutations in TSC1 and TSC2 disrupt the interaction between hamartin and tuberin. *Hum. Mol. Genet.* 10:2899–2905.
20. Yamamoto, T., and K. Nishioka. 2003. Role of monocyte chemoattractant protein-1 and its receptor, CCR-2, in the pathogenesis of bleomycin-induced scleroderma. *J. Invest. Dermatol.* 121:510–516.

21. Yamamoto, T. 2003. Potential roles of CCL2/monocyte chemoattractant protein-1 in the pathogenesis of cutaneous sclerosis. *Clin. Exp. Rheumatol.* 21:369–375.
22. Ohta, M., Y. Kitadai, S. Tanaka, M. Yoshihara, W. Yasui, N. Mukaida, K. Haruma, and K. Chayama. 2003. Monocyte chemoattractant protein-1 expression correlates with macrophage infiltration and tumor vascularity in human gastric carcinomas. *Int. J. Oncol.* 22:773–778.
23. Gordillo, G.M., D. Onat, M. Stockinger, S. Roy, M. Atalay, M. Beck, and C.K. Sen. 2004. A key angiogenic role of monocyte chemoattractant protein-1 in hemangioendothelioma proliferation. *Am. J. Physiol. Cell Physiol.* 287:C866–C873.
24. Salcedo, R., M.L. Ponce, H.A. Young, K. Wasserman, J.M. Ward, H.K. Kleinman, J.J. Oppenheim, and W.J. Murphy. 2000. Human endothelial cells express CCR2 and respond to MCP-1: direct role of MCP-1 in angiogenesis and tumor progression. *Blood.* 96:34–40.
25. Yoshida, S., A. Yoshida, H. Matsui, Y. Takada, and T. Ishibashi. 2003. Involvement of macrophage chemotactic protein-1 and interleukin-1 β during inflammatory but not basic fibroblast growth factor-dependent neovascularization in the mouse cornea. *Lab. Invest.* 83:927–938.
26. Wada, T., K. Furuichi, N. Sakai, Y. Iwata, K. Kitagawa, Y. Ishida, T. Kondo, H. Hashimoto, Y. Ishiwata, N. Mukaida, et al. 2004. Gene therapy via blockade of monocyte chemoattractant protein-1 for renal fibrosis. *J. Am. Soc. Nephrol.* 15:940–948.
27. Inoshima, I., K. Kuwano, N. Hamada, N. Hagimoto, M. Yoshimi, T. Maeyama, A. Takeshita, S. Kitamoto, K. Egashira, and N. Hara. 2004. Anti-monocyte chemoattractant protein-1 gene therapy attenuates pulmonary fibrosis in mice. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 286: L1038–L1044.
28. de la Rosa, G., N. Longo, J.L. Rodriguez-Fernandez, A. Puig-Kroger, A. Pineda, A.L. Corbi, and P. Sanchez-Mateos. 2003. Migration of human blood dendritic cells across endothelial cell monolayers: adhesion molecules and chemokines involved in subset-specific transmigration. *J. Leukoc. Biol.* 73:639–649.
29. Mantovani, A., T. Schioppa, S.K. Biswas, F. Marchesi, P. Allavena, and A. Sica. 2003. Tumor-associated macrophages and dendritic cells as prototypic type II polarized myeloid populations. *Tumori.* 89:459–468.
30. Nakamura, K., I.R. Williams, and T.S. Kupper. 1995. Keratinocyte-derived monocyte chemoattractant protein 1 (MCP-1): analysis in a transgenic model demonstrates MCP-1 can recruit dendritic and Langerhans cells to skin. *J. Invest. Dermatol.* 105:635–643.
31. Niida, Y., A.O. Stemmer-Rachamimov, M. Logrip, D. Tapon, R. Perez, D.J. Kwiatkowski, K. Sims, M. MacCollin, D.N. Louis, and V. Ramesh. 2001. Survey of somatic mutations in tuberous sclerosis complex (TSC) hamartomas suggests different genetic mechanisms for pathogenesis of TSC lesions. *Am. J. Hum. Genet.* 69:493–503.
32. Fackler, I., J.E. DeClue, H. Rust, P.A. Vu, H. Kutzner, A. Rutten, S. Kaddu, C.A. Sander, M. Volkenandt, M.W. Johnson, et al. 2003. Loss of expression of tuberin and hamartin in tuberous sclerosis complex-associated but not in sporadic angiofibromas. *J. Cutan. Pathol.* 30:174–177.
33. Wataya-Kaneda, M., Y. Kaneda, O. Hino, H. Adachi, Y. Hirayama, K. Seyama, T. Satou, and K. Yoshikawa. 2001. Cells derived from tuberous sclerosis show a prolonged S phase of the cell cycle and increased apoptosis. *Arch. Dermatol. Res.* 293:460–469.
34. Darling, T.N., J. Wang, F. Takeuchi, T.C. Lei, S. Pack, Z. Zhuang, and J. Moss. 2002. Allelic deletion of the TSC2 gene in tuberous sclerosis skin tumors and cultured stromal cells. *J. Invest. Dermatol.* 119:215.
35. Takeuchi, F., J. Wang, J. Moss, and T. Darling. 2003. Hyperphosphorylation of p70S6k and ribosomal protein S6 in tuberous sclerosis skin tumors is inhibited by rapamycin. *J. Invest. Dermatol.* 121:A165.
36. Govindarajan, B., D.J. Brat, M. Csete, W.D. Martin, E. Murad, K. Litani, C. Cohen, F. Cerimele, M. Nunnelley, B. Lefkove, et al. 2005. Transgenic expression of dominant negative tuberin through a strong constitutive promoter results in a tissue-specific tuberous sclerosis phenotype in the skin and brain. *J. Biol. Chem.* 280:5870–5874.
37. Castro, C., J.M. Campistol, D. Sancho, F. Sanchez-Madrid, E. Casals, and V. Andres. 2004. Rapamycin attenuates atherosclerosis induced by dietary cholesterol in apolipoprotein-deficient mice through a p27 Kip1-independent pathway. *Atherosclerosis.* 172:31–38.
38. Oliveira, J.G., P. Xavier, S.M. Sampaio, C. Henriques, I. Tavares, A.A. Mendes, and M. Pestana. 2002. Compared to mycophenolate mofetil, rapamycin induces significant changes on growth factors and growth factor receptors in the early days post-kidney transplantation. *Transplantation.* 73:915–920.
39. Wasowska, B.A., X.X. Zheng, T.B. Strom, and J.W. Kupiec-Weglinski. 2001. Adjunctive rapamycin and CsA treatment inhibits monocyte/macrophage associated cytokines/chemokines in sensitized cardiac graft recipients. *Transplantation.* 71:1179–1183.
40. Harrington, L.S., G.M. Findlay, A. Gray, T. Tolkacheva, S. Wigfield, H. Rebholz, J. Barnett, N.R. Leslie, S. Cheng, P.R. Shepherd, et al. 2004. The TSC1-2 tumor suppressor controls insulin-PI3K signaling via regulation of IRS proteins. *J. Cell Biol.* 166:213–223.
41. Shah, O.J., Z. Wang, and T. Hunter. 2004. Inappropriate activation of the TSC/Rheb/mTOR/S6K cassette induces IRS1/2 depletion, insulin resistance, and cell survival deficiencies. *Curr. Biol.* 14:1650–1656.
42. Sartipy, P., and D.J. Loskutoff. 2003. Monocyte chemoattractant protein 1 in obesity and insulin resistance. *Proc. Natl. Acad. Sci. USA.* 100: 7265–7270.
43. Kitamoto, S., and K. Egashira. 2003. Anti-monocyte chemoattractant protein-1 gene therapy for cardiovascular diseases. *Expert. Rev. Cardiovasc. Ther.* 1:393–400.
44. Gordillo, G.M., D. Onat, M. Stockinger, S. Roy, M. Atalay, F.M. Beck, and C.K. Sen. 2004. A key angiogenic role of monocyte chemoattractant protein-1 in hemangioendothelioma proliferation. *Am. J. Physiol. Cell Physiol.* 287:C866–C873.
45. Roach, E.S., M.R. Gomez, and H. Northrup. 1998. Tuberous sclerosis complex consensus conference: revised clinical diagnostic criteria. *J. Child Neurol.* 13:624–628.
46. Soucek, T., R.S. Yeung, and M. Hengstschlager. 1998. Inactivation of the cyclin-dependent kinase inhibitor p27 upon loss of the tuberous sclerosis complex gene-2. *Proc. Natl. Acad. Sci. USA.* 95:15653–15658.