Transforming Growth Factor β-regulated MicroRNA-29a Promotes Angiogenesis through Targeting the Phosphatase and Tensin Homolog in Endothelium

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Background: The TGF-β pathway is critical for angiogenesis. Endothelial miR-29a is up-regulated by TGF-β in a Smad4-dependent way to promote angiogenesis via targeting PTEN.

Results: TGF-β promotes angiogenesis by up-regulating miR-29a.

Conclusion: We demonstrate how TGF-β signaling exerts its angiogenic function by up-regulating proangiogenic miRNA.

Angiogenesis refers to the formation of mature vasculature from a pre-existing primary plexus, occurring during embryonic development and adult life (1). Upon stimulation of angiogenic signals, endothelial cells (ECs) turn to be activated. ECs detach adhesions from their neighbors, sprout toward proangiogenic factors, proliferate to form temporary tubes, recruit pericytes, and finally remodel and prune to form a functional network. Various signaling pathways, including TGF-β and phosphatase and tensin homolog (PTEN)/AKT signaling, sophisticatedly regulate distinct cellular processes involved in angiogenesis, of which endothelial migration is an essential event (2).

The TGF-β superfamily contains more than 30 members such as TGF-βs, bone morphogenetic proteins, and activins. Members of the TGF-β superfamily transduce their signals initially via binding specific transmembrane serine/threonine kinases receptors and then through intracellular Smad proteins. Receptor-activated Smad (R-Smad), Smad2, and Smad3, are activated by the TGF-β receptor II-ALK5 complex, whereas Smad1, Smad5, and Smad8 are activated by the TβRII-ALK1 complex. Activated R-Smads associate with Smad4, the unique central mediator of TGF-β signaling, to translocate into the nucleus, where they participate in the transcriptional regulation of downstream target genes (3).

The crucial roles of TGF-β in angiogenesis have been revealed by genetic studies in humans and mice. Mutations in TGF-β signaling pathway components, including ENDOLGIN, ALK1, and SMAD4, account for most hereditary hemorrhagic telangiectasia clinical cases (4). In mice, deletion of various TGF-β signaling members, including TGF-β1, Tgfbr2, Alk5, Alk1, endoglin, Smad1, Smad4, and Smad5, leads to embryonic lethality because of severe vascular abnormalities, including a vascular remodeling defect and the absence of mural cell formation (5–13). In vitro, TGF-β differentially modulates endothelial migration and proliferation through distinct TβRI path-
ways, emphasizing the complexity of TGF-β signaling in endothelial function. Furthermore, effects of TGF-β during different stages of angiogenesis are usually dose-dependent and largely depend on its cellular context (14, 15).

Recent studies are starting to reveal posttranscriptional mechanisms underlying cellular responses of ECs to known angiogenic pathways. MicroRNAs (miRNAs) are a class of endogenous, 18- to 25-nucleotide, small, noncoding RNAs that function by negatively regulating target mRNAs either through translational inhibition or destabilization of mRNA (16). A number of miRNAs have been demonstrated to have pro- or antiangiogenic effects through regulating endothelial migration, survival, or the cell cycle. miR-126, the miR-23~27~24 cluster, miR-424, miR-130a, miR-296, the miR-30 family, and miR-210 promote angiogenesis (17–25), whereas the miR-17~92 cluster, miR-214, miR-200b, miR-1, miR-206, miR-221, and miR-222 block angiogenesis (26–32). Increasing evidence has shown that specific miRNAs can modulate the endothelial responses to blood flow, hypoxia, serum, or VEGF and exert their pro- or antiangiogenic effects. miR-126 regulates angiogenesis by activating VEGF signaling in response to blood flow (18, 33). MiR-130a promotes angiogenesis in response to fetal bovine serum by down-regulating antiangiogenic homeobox genes (23). miR-424 and miR-210 induced by hypoxia stimulate angiogenesis via regulating hypoxia-induced factor α isoforms as well as VEGF-driven cell migration (20, 21, 34). On the other hand, miR-125b induced by VEGF or ischemia inhibits angiogenesis through translational suppression of VE-cadherin (35).

miRNAs have been found to mediate TGF-β signaling and participate in TGF-β-regulated biological processes. MiR-155 is induced by TGF-β and promotes epithelial-mesenchymal transformation by targeting RhoA (36). TGF-β inhibits myogenic differentiation by down-regulating miR-24 (37). TGF-β also protects cardiomyocytes from hypertrophic growth by reducing miR-27b (38). TGF-β and bone morphogenetic protein signaling promotes the differentiation of human vascular smooth muscle cells by up-regulating miR-21 (39), and miR-21 accelerates re-epithelialization during wound healing in mice (40) and also participates in TGF-β-induced endothelial-to-mesenchymal transformation (41). Several studies have shown that miR-29 reduces fibrotic response and is down-regulated by TGF-β1 in cultured fibroblasts, tubular epithelial cells, and myogenic C2C12 cells. Nevertheless, the role of TGF-β-regulated miRNAs involved in angiogenesis is poorly investigated (42, 43). In this study, we revealed that endothelial miR-29a is up-regulated by TGF-β1 in a Smad4-dependent way to promote angiogenesis via targeting PTEN.

**EXPERIMENTAL PROCEDURES**

**Vector Construction**—For the promoter assay, a 1.7-kb genomic fragment upstream of the transcriptional start site of the miR-29a precursor (EU154353) was amplified by PCR using the primer pairs reported previously (44) and cloned into the PGL3-basic vector to obtain the PGL3–29a promoter vector. To delete potential Smad-binding sites in the promoter region, appropriate primer sets were used to amplify several deletion mutant fragments. WT/mut vector (–433 to –420 bp deleted) was obtained by putting fragments (–1706 to –433 and –420 to +1) together with the KpnI restriction enzyme site. Mut/WT (–1327 to –1309 deleted) was constructed by linking fragments (–1706 to –1327 and –1309 to +1) together with KpnI sites. Both the –1327 and –433 regions were deleted in the mut/mut vector. The amplicon was cloned into pGL3. The correct sequence was confirmed by sequencing. Sequences for Smad4 and PTEN RNA interference (RNAi) were cloned into the pSuperRetropuro vector described previously (6).

**Small RNA Transfection**—ECs were transfected with 20 nmol/liter miR-29a mimic, antagonir, or Scrambled oligonucleotides (GenePharma) using Lipofectamine 2000 (Invitrogen).

**Cell Culture**—The bEnd.3 cell line was purchased from ATCC. Human umbilical vein endothelial cell (HUVEC) and mice primary ECs were isolated and cultured as described previously (6). Mice were anesthetized with an intraperitoneal injection of sodium pentobarbital (25–50 mg/kg body weight) and then killed by cervical dislocation before isolation of tissues (brain). The depth of anesthesia was confirmed by lack of tail pinch response. All experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health after securing the approval of the Committee of Animal Care of the Beijing Institute of Biotechnology.

**Reporter Assay**—bEnd.3 cells in 24-well plates were transfected with wild-type or mutant pGL3–29a promoters cotransfected with or without the HA-Smad4 plasmid. 24 h later, the cultures were treated with 5 ng/ml TGF-β1 for another 8 h. Luciferase activities were detected with dual luciferase reporter assay reagents (Promega). We used the PGL3-basic plasmid for normalization of luciferase values.

**ChIP**—bEnd.3 cells were pretreated with 5 ng/ml TGF-β1 for 4 h. ChIP assays were performed according to the instructions of the manufacturer with a SimpleChIP enzymatic chromatin immunoprecipitation kit (Cell Signaling Technology, Inc.). Antibodies used for ChIP were purchased from Cell Signaling Technology, Inc. DNA fragments of the miR-29a promoter region flanking the potential Smad4 binding site were amplified with the following primers: –551 to –392 bp, 5’-TGA-CTGGAGCATTAAACCTTGCA-3’ and 5’-TGTCCTACATAAGGCTTGTGA-3’; –1385 to –1219 bp, 5’-ACTGAGAAAAGGACCGCTTGGG-3’ and 5’-TCTATGGGTCGTCACTTTGATTG-3’). The distal regions of the miR-29a promoter were amplified as a control with primers 5’-CATGACAGCTCC-TGTGAAAG-3’ and 5’-AGTCAACAGGAGTGGGACT-CGGT-3’.

**In Vitro Tube Formation Assay**—ECs were plated onto 48-well plates precoated with a thin layer of Matrigel (BD Biosciences) in culture medium containing 5% fetal calf serum and allowed to form tube-like structures for 12 h. Measurements were performed as described previously (11).

**Wound Healing Assay**—The confluent cell monolayer in a 12-well plate was wounded by manually scraping the cells with a white pipette tip. The cells were treated with 5 ng/ml TGF-β1 in serum-free medium. Cell migration into the wound surface was monitored at various times. Quantitation was done by measuring the distance of the wound edge of the migrating cells...
from the start point to the migrated point in three independent experiments.

Chick Chorioallantoic Membrane (CAM) Assay—Fertilized eggs were incubated at 37 °C and 60% humidity for 10 days. A square window was made on the air sac to expose the CAM. Sterile 0.25 cm-diameter filter papers were applied onto the window. The eggs were fixed with 2% paraformaldehyde in PBS for 30 min, permeabilized with 0.2% bovine serum albumin in PBS for 30 min, and incubated with propidium iodide and RNase A at 37 °C for 30 min. Cell cycle analysis was evaluated by flow cytometry and the data were analyzed.

MTS Assay—bEnd.3 were seeded at a concentration of 5000 cells per well in 96-well plates. Relative cell numbers were quantified every day via (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2H-tetrazolium)(MTS) assay. For each well, medium was removed, and 1 l of 5 mg/ml MTS was added. After 4 h of incubation at 37 °C, 150 µl of dimethyl sulfoxide was added to each well, and the absorbance was measured at 492 nm on a multifunction microplate reader.

Cell Cycle Analysis—bEnd.3 were transfected with miR-29a mimic or scramble oligo for 24h. Then cells were harvested and fixed in 70% alcohol for 30 min on ice. Cells were then stained with propidium iodide and RNase A at 37 °C for 30 min. Cell cycle was assessed by flow cytometry and the data were analyzed.

Cytoskeleton Labeling—bEnd.3 were transfected with miR-29a antisense oligos as described above. 24 h later, cells were fixed with 2% paraformaldehyde in PBS for 30 min, permeabilized with 0.2% bovine serum albumin in PBS for 30 min, and incubated with 5 µg/ml of phalloidin-TRITC and 2 µg/ml of DAPI for 30 min. Pictures were obtained using a fluorescence microscope and a digital camera.

Real-time RT-PCR—RNA was extracted from pretreated cells with TRIzol reagent (Invitrogen) and reverse-transcribed to cDNA. A Taqman miRNA RT kit with multiplex RT primer (Applied Biosystem) was used to quantitate miR-29a. Real-time PCR was performed with a Roche LightCycler 2.0 system. Primers were purchased from Invitrogen Corp. Primers for PTEN were as follows: 5'-TGATGAGCTTCAGTTGGACCT-3' and 5'-GGGTGCTCATAAGATGCTAG-3'. Primers for VEGF and Smad4 were as described previously (6).

Northern Blot Analysis—Total RNAs were isolated using TRIzol reagent (Invitrogen) on the basis of the suggested protocol. Northern blot analysis was performed as described using 20 µg of total RNA from each sample. Probes were purchased from Invitrogen Corp. as follows: miR-29a, 5'-TACCGATTTCAGATGCTAAGC-3'; miR-106a, 5'-CTACTGTGACTCTGGCTTAA-3'; miR-21, 5'-AAACAGAAGCAGTCTAAGCTA-3'; and miR-29a, 5'-TCAACATGCTCTGATAAGCTA-3'.

Western Blot Analysis—20 µg of protein were electrophoresed on 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes. Immunoblotting was performed according to the instructions of the manufacturer using the following antibodies: PTEN, Smad4, AKT, phosphorylated AKT, and GAPDH (Abcam).

Statistical Methods—Data were evaluated using Student’s two-tailed t test. p < 0.05 and p < 0.01 were taken to be statistically significant. The error bars on the graphs represent the mean ± S.D.

RESULTS

TGF-β1 Up-regulates miR-29a Expression in ECs—First we checked the expression of some miRNAs that are mentioned to be regulated by TGF-β and highly expressed in ECs in previous studies (45–47). Northern blot analysis showed that treatment of TGF-β1 at 5 ng/ml induced up-regulation of mature miR-29a in HUVECs dramatically. MiR-21 was increased as reported (39), whereas the expression of miR-106a and miR-222 was hardly changed (Fig. 1A). Through bioinformatic analysis we found that the sequence and seed region of miR-29a were conserved among species, including mouse, human, and chicken (Fig. 1B). We compared the relative expression abundance of mature miR-29a in several kinds of primary cells and normal cell lines, including mouse and human primary ECs (HUVEC), cardiomyocytes, smooth muscle cells,
chondrocytes, fibroblasts (3T3), and keratinocytes (HaCaT), showing that miR-29a was highly expressed in ECs and fibroblasts (Fig. 1C). The stimulating effect of TGF-β1 could also be observed in bEnd.3 cells, which is an immortalized mouse brain microvascular EC line, and TGF-β1 increased miR-29a expression in a dose-dependent manner (Fig. 1D). Next we examined the expression of primary miR-29a and mature miR-29a, respectively, in response to TGF-β1 at different time points. After 5 ng/ml TGF-β1 treatment, primary miR-29a increased first, starting at about 15 min. The up-regulation of mature miR-29a was not obvious until 1 h. Both forms of miR-29a expression were at maximums about 2 h after TGF-β1 treatment (Fig. 1E). This result implied that TGF-β1 might regulate miR-29a at the transcriptional level.

**Smad4 Is Required for the Transcriptional Activation of miR-29a by TGF-β1**—As Smad4 is the unique central mediator of canonical TGF-β signaling, we determined the endogenous need of TGF-β signaling for the up-regulation of miR-29a in ECs by using Smad4 knockdown bEnd.3 cells (Fig. 2A). Smad4 RNAi led to down-regulation of miR-29a in bEnd.3 cells and largely blocked the stimulating role of TGF-β1 on miR-29a expression (Fig. 2B). Furthermore, we checked miR-29a expression in primary brain ECs derived from cerebrovascular endothelial-specific Smad4 knockout mice (6), showing that miR-29a was decreased by 50% in the Smad4-deficient cells (Fig. 2C). Next we performed a luciferase assay in the Smad4 knockdown bEnd.3 cells. Knockdown of Smad4 apparently reduced basic miR-29a promoter activity and absolutely abolished the activating role of TGF-β1 on the miR-29a promoter, strongly suggesting that TGF-β-regulated miR-29a expression is largely dependent on Smad4 (Fig. 2D). Smads regulate gene transcription through their physical association with the Smad binding elements (SBE) in the promoters of target genes. A bioinformatics analysis was carried out to find potential SBEs in the miR-29a promoter with the rVista tool. Sequence analysis of the miR-29a promoter showed that there are several conserved SBEs in the promoter region of miR-29a. As shown in Fig. 2E, we constructed various miR-29a promoter luciferase constructs with SBE isolate or double deletions. TGF-β1 treatment and/or HA-Smad4-cotransfected increased the promoter activity. Moreover, the increases were largely blocked in the absence of either or both SBEs (Fig. 2E), indicating that SBE regions are essential for TGF-β regulation of miR-29a transcription. Next we examined whether Smads physically interact with the miR-29a promoter at these sites. We performed a ChIP assay in bEnd.3 cells that were treated with TGF-β1 for 4 h. DNA that coprecipitated with Smads was analyzed by PCR using primers specific for the two regions containing the -1327- or -433-bp Smad binding regions. Smad1/5/8, Smad2/3, and Smad4 antibodies all pulled down the miR-29a promoter region. TGF-β1 treatment increased amplification pull-down by Smad4, Smad1/5/8, and Smad2/3 antibodies (Fig. 2F). These data indicate that TGF-β1 up-regulates miR-29a in a Smad4-dependent way.

**miR-29a Mediates TGF-β1-induced Angiogenesis**—As TGF-β1 is an important angiogenic factor and induced miR-29a expression in ECs, we then attempted to study the role of miR-29a in TGF-β1-induced angiogenesis. We performed a CAM assay for in vivo angiogenesis evaluation. We first studied the effect of miR-29a in angiogenesis. Real-time PCR analysis of CAM tissues confirmed efficient overexpression of miR-29a packaged with Lipofectamine (Fig. 3A). Compared with scramble controls, incubation with the miR-29a mimic apparently induced more radial formation of new blood vessels (Fig. 3B). Then TGF-β1 together with miR-29a antagonir were applied onto the CAM surfaces to test whether miR-29a participates in...
miR-29a Targets PTEN in Endothelial Cells—To identify the targets of miR-29a in ECs, we used three algorithms (Pictar, miRanda, and Targetscan) to predict potential direct targets. PTEN is one of the candidates predicted by all three algorithms that has two potential binding regions in the 3’ UTR completely complementary to the seed region of miR-29a. We confirmed that miR-29a down-regulated PTEN in ECs at both mRNA and protein levels (Fig. 5A). Supportively, miR-29a targets PTEN via binding to its 3’ UTR in other cell types (49). PTEN is a multifunctional phosphatase, and its major substrate is phosphatidylinositol-3,4,5-trisphosphate, a lipid second messenger molecule. Phosphatidylinositol-3,4,5-trisphosphate activates numerous downstream molecules, including the serine-threonine kinase PKB/AKT (50). Because TGF-β/Smad4 signaling up-regulated miR-29a and miR-29a directly targeted PTEN, we further examined PTEN/AKT signaling in the absence of Smad4. We confirmed that without endogenous TGF-β/Smad4 activity, PTEN was increased, and phosphorylated AKT was reduced concomitantly (Fig. 5B). Consistently, TGF-β1 activated AKT signaling in ECs (Fig. 5C). Importantly, miR-29a antagonir blocked the effect of TGF-β1 on AKT phosphorylation, implying that TGF-β1-stimulated AKT activity was largely mediated by miR-29a (Fig. 5C). We examined the effect of PTEN on the migrating ability of ECs. As shown in Fig. 5, D and E, knockdown of PTEN caused an obvious increase in AKT phosphorylation and apparently accelerated endothelial migration, recapitulating the role of miR-29a in promoting migration. Most importantly, inhibition of miR-29a had no effect on EC migration in the absence of PTEN (Fig. 5E) in contrast to the negative role of miR-29a antagonist in EC migration, as shown in Fig. 4D. These results suggest that miR-29a promotes endothelial migration by reducing PTEN expression and that PTEN is a specific target of miR-29a in ECs to exert its proangiogenic function.

MiR-29a Promotes Angiogenesis by Activating AKT Signaling—We supposed that miR-29a promoted angiogenesis by activating AKT signaling through targeting PTEN. We used PI3K inhibitor LY294002 to evaluate the role of miR-29a-stimulated AKT activation on endothelial migration and tube formation. Western blotting assured that LY294002 inhibited miR-29a-induced AKT phosphorylation (Fig. 6A). A number of studies have shown that PI3K/AKT signaling induces angiogenesis and can increase VEGF expression (50). We checked the expression of the VEGFA164 isoform in b.End3 cells and showed an increase by miR-29a over-expression, which was completely attenuated by LY294002 treatment (Fig. 6B). Functionally, treatment with LY294002 completely blocked the effect of miR-29a in promoting EC migration in the wound healing assay and three-dimensional tube formation in Matrigel (Fig. 6, C and D). In the CAM angiogenesis assay, addition of LY294002 neutralized the stimulating role of miR-29a (Fig. 6E). Taken together, these in vitro and in vivo functional assays supported the notion that miR-29a promoted angiogenesis largely through activating AKT signaling.

DISCUSSION

Here we showed that TGF-β-regulated miR-29a promoted angiogenesis, demonstrating a novel epigenetic mechanism of
TGF-β signaling in controlling endothelial function. Canonical TGF-β selectively induces the transcription of downstream molecules through distinct type I receptors. Previous studies suggest that the balance between TGF-β/ALK1 versus TGF-β/ALK5 determines the effects of TGF-β on angiogenesis. Activation of ALK5 by TGF-β induces PAI-1 and inhibits migration and proliferation, whereas TGF-β-induced activation of ALK1 up-regulates Id1 expression and stimulates migration and proliferation (14). We provided the first evidence to prove that TGF-β signaling could up-regulate proangiogenic miRNA to exert its angiogenic function.

MiR-29a has been reported as a miRNA that can be regulated by TGF-β. TGF-β down-regulates miR-29a in skeletal muscle cells to influence TGF-β-mediated control of myogenic differ-
entiation (43). Furthermore, TGF-β reduces the level of miR-29a in fibroblast and HK-2 cells to stimulate collagen expression (51, 52). In contrast to most previous studies, we found that TGF-β1 up-regulated miR-29a at transcriptional level in ECs in a Smad4-dependent manner. Importantly, miR-29a expression was reduced 2-fold in primary isolated Smad4-deficient ECs, confirming the positive regulation of miR-29a by TGF-β signaling under physiological conditions. We demonstrated that suppression of miR-29a significantly inhibited TGF-β1-induced CAM angiogenesis. In Smad4-deficient ECs, down-regulation of miR-29a (Fig. 2C) correlated with defective angiogenesis, evidenced by compromised tube-forming capacity (11). These results indicate that TGF-β1 promotes angiogenesis at least partially via up-regulating miR-29a. MiR-29a may not be the only miRNA mediating TGF-β induced angiogenesis. We found that miR-21 was also significantly up-regulated upon treatment of TGF-β1 on HUVECs (Fig. 1A). Previous studies have revealed that miR-21 induces angiogenesis through AKT and ERK activation and hypoxia-induced factor α expression (53). We also showed that similar to miR-29a, miR-21 overexpression promoted EC migration and tube formation. Notably, endothelial PTEN was not regulated by miR-21 overexpression4. Thus, miR-21 must execute its function via other targets in ECs. It would be interesting to explore the synergistic role of miR-29a and miR-21 in TGF-β-regulated angiogenesis in future studies. It is highly possible that TGF-β signaling regulates groups of proangiogenic miRNAs as well as anti-angiogenic miRNAs to elicit their pleiotropic and complex effects on angiogenesis. How ECs choose to activate or inhibit specific miRNAs in response to TGF-β signaling under certain physiological or pathologic conditions needs to be investigated further.

For the first time, we deciphered the role of miR-29a in endothelial cells. We identified miR-29a as a proangiogenic miRNA by positively regulating EC migration and tube formation. It has been reported that the TGF-β/Smad and PTEN/AKT pathways could regulate each other reciprocally in many other systems (54–56). In this study, we showed that TGF-β signaling could up-regulate the miRNA targeting PTEN to activate AKT signaling in ECs. The inhibitor of AKT could significantly diminish miR-29a-promoted angiogenesis, demonstrating that the function of TGF-β-regulated proangiogenic miR-29a is largely dependent on activated AKT signaling. PTEN knockdown or LY294002 treatment had no effect on mature miR-29a expression (data now shown). Thus, it is unlikely that PTEN/AKT play a role upstream of miR-29a to regulate angiogenesis. Previous studies have revealed that PTEN inhibits vascular sprouting and endothelial tube formation induced by VEGF (57). Sustained endothelial activation of AKT1 has been shown to induce the formation of structurally and functionally abnormal blood vessels (58). Notably, embryos deficient for the endothelial p110α catalytic subunit of PI3K develop severe vascular sprouting remodeling defects, leading to embryonic lethality at mid-gestation that highly resembles the phenotype observed in endothelial-specific Smad4 deleted mice (59). Furthermore, p110α promotes endothelial migration and tube formation, similar to the role of TGF-β/Smad4 signaling in ECs (11). MiR-29a has been shown to have an antifibrotic effect by directly targeting a set of extracellular matrix genes in the heart, kidney, and other organs (51, 60, 61). We also found that miR-29a could down-regulate collagen genes in ECs (data not shown). Considering that extracellular matrix degradation is another key step in angiogenesis, we could not exclude the possibility that the stimulating effect of miR-29a in CAM angiogenesis might be partially caused by reduced expression of extracellular matrix-related genes.

In summary, our results suggest a novel mechanism by which TGF-β/Smad4 signaling promote angiogenesis, thwarting PTEN by up-regulating miR-29a and thus activating AKT to promote EC migration and tube formation. Dysregulation of miR-29a has been shown to occur in some types of cancers (62). Whether the dysregulation is mediated by TGF-β signaling and
whether miR-29a plays a role in tumor angiogenesis are worth further investigation.

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