Cell Cycle 11:18, 3373-3379; September 15, 2012; © 2012 Landes Bioscience

Convergent signaling in the regulation of connective tissue growth factor in malignant mesothelioma

TGFβ signaling and defects in the Hippo signaling cascade

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Malignant mesothelioma (MM) is a neoplasm that arises from serosal surfaces of the pleural, peritoneal and pericardial cavities with worldwide incidence, much of which is caused by asbestos exposure. Patients suffer from pain and dyspnea due to direct invasion of the chest wall, lungs and vertebral or intercostal nerves by masses of thick fibrotic tumors. Although there has been recent progress in the clinical treatment, current therapeutic approaches do not provide satisfactory results. Therefore, development of a molecularly targeted therapy for MM is urgently required. Our recent studies suggest that normal mesothelial and MM cell growth is promoted by TGFβ, and that TGFβ signaling together with intrinsic disturbances in neurofibromatosis type 2 (NF2) and Hippo signaling cascades in MM cells converges upon further expression of connective tissue growth factor (CTGF). The formation of a YAP-TEAD4–Smad3-p300 complex on the specific CTGF promoter site with an adjacent TEAD and Smad binding motif is a critical and synergistic event caused by the dysregulation of these two distinct cascades. Furthermore, we demonstrated the functional importance of CTGF through the mouse studies and human histological analyses, which may elucidate the clinical features of MM with severe fibrosis in the thoracic cavity.

Keywords: CTGF, Hippo pathway, malignant mesothelioma, mesothelial cells, p300, Smad, targeted therapy, TEAD, TGFβ, YAP

Abbreviations: COL1A1, Prepro-alpha1(I) collagen; CTGF, connective tissue growth factor; ECM, extracellular matrix; NF2, neurofibromatosis type 2; MM, malignant mesothelioma; MMP, matrix metallopeptidase; shRNA, short hairpin RNA; TAZ, transcriptional co-activator with PDZ binding domain; TβRI, TGFβ type I receptor; TEAD, TEA domain family member; TGFβ, transforming growth factor beta; YAP, Yes-associated protein

Submitted: 06/29/12

Accepted: 07/06/12

http://dx.doi.org/10.4161/cc.21397

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Introduction

Characterization of both normal and MM cells is important, because no conventional drug is currently effective for treating MM patients. MM is an aggressive disease without an effective treatment, including chemotherapy or/and surgery. One of the reasons for the lack of efficacy of conventional drugs in MM is that it does not originate from epithelial cells and has characteristics distinct from those of carcinomas, for which most anticancer drugs have been developed. Both basic and clinical MM studies are lagging behind compared with research on other malignancies. Since most MM patients are diagnosed at advanced stages, development of a molecularly targeted therapy is urgently required.

TGFβ Signaling in Normal Mesothelial Cells and MM Cells

The mesothelium is composed of a single layer of flattened cells originating from the mesoderm, called mesothelial cells, which line the visceral and parietal surfaces of the pleural, pericardial and peritoneal cavities to provide a slippery surface that facilitates visceral movements. Mesothelial cells have the ability to secrete growth factors, extracellular matrix (ECM) molecules[1] and to form a basement membrane on which they reside. After injury or other stimulus to the serosal surface, mesothelial cells become metabolically active[1] and morphologically change to a cuboidal shape.[1-3]

During serosal tissue repair, mesothelial cells proliferate at distant sites from the wound, most likely due to diffuse activation of the mesothelium in response to mediators probably released by inflammatory cells into serosal fluid or by cell-cell contact.[1,4,5] Clinically, normal reactive mesothelial cells that detach from the serosal membrane into pleural fluid often make differentiation between noncancerous pleural effusion and MM by cytological examination difficult.

TGFβ is a pleiotropic polypeptide growth factor that mediates the transformation of non-neoplastic rat kidney and murine AKR-2B fibroblasts,[6-8] while it induces growth arrest and apoptosis in epithelial cells. Subsequent studies revealed that TGFβ acts as a tumor suppressor in premalignant epithelial cells, whereas it exerts pro-oncogenic effects in metastatic tumors.[9,10] Upon TGFβ stimulation, Smad2 and Smad3 are activated by phosphorylation of a C-teminal phosphorserine motif by the TGFβ type I receptor (TβRI) kinase. After forming complexes with the common mediator Smad4, these activated Smads accumulate in the nucleus, thereby regulating expression of various target genes.[11]

Our recent paper clarified intracellular and molecular mechanisms of cell growth induced by TGFβ stimulation in malignant mesothelial cells.[12] We used MeT-5A cells, a normal human pleural mesothelial cell line, transformed by SV40 early region DNA.[13] MeT-5A cells were previously reported to increase DNA synthesis upon TGFβ stimulation.[14] To examine whether TGFβ signaling affects monolayer cell growth, we compared cell growth and Smad3-dependent intercellular signaling after exposure to a TβRI kinase inhibitor in both MeT-5A and MM cell lines. In both cell types, treatment of TβRI kinase inhibitor suppressed cellular growth. Furthermore, oral gavage with TβRI kinase inhibitor prolonged the survival of mice with thoracically implanted MM cells.

The same responses to TGFβ in both normal mesothelial cells and MM cells were also observed in other aspects, such as each induced expression of CTGF, autocrine induction of TGFβ, proliferated faster and increased ECM production (**Fig. 1**). Immunohistochemical analysis of clinical samples demonstrated that, in most cases, the nuclei of both MM and normal reactive mesothelial cells adjacent to the tumor masses were intensely stained by the p-Smad2 antibody. This indicates that MM and normal reactive mesothelial cells maintain a consistent trait of exhibiting TGFβ signaling activation, possibly because of high levels of TGFβ or its intact receptors and Smad2. Sporadic staining of nuclei by the p-Smad2 antibody in the flattened normal mesothelial cells was observed, suggesting that the TGFβ signal is not sufficient for directly inducing the cuboidal appearance of normal reactive mesothelial cells. Furthermore, the observation of normal reactive mesothelial cells with sporadic nuclear p-Smad2 staining in normal pleural tissue from a lung cancer patient also indicates that additional factors may be required for transition to the cuboidal appearance (data not shown).

Pleural fluid from MM patients frequently contains normal reactive mesothelial cells and fibroblasts detached from the serosal membranes as well as MM cells. A higher TGFβ level in pleural effusions was observed in MM patients compared with breast cancer patients.[15] Inflammatory cells, surrounding fibroblasts, normal reactive mesothelial cells and MM cells may contribute to further TGFβ accumulation in pleural fluid of patients, which may increase MM cell activation. There are reports indicating that the systemic administration of TGFβ antagonists can suppress MM growth by reactivation of antitumor immune responses in a mouse model.[16,17] We demonstrated that these antagonists have important inhibitory effects on the tumor parenchyma as well;[12] hence, the large amount of TGFβ accumulation in pleural fluid and the surrounding mesothelioma environment may be a significant disadvantage in MM treatment.

Hippo Signaling Inactivation in MM Cells and Tumors

Recent studies have shown that the protein kinase Hippo signaling pathway and its downstream target YAP regulate cell growth and organ size and play a critical role in human disease. Hippo signaling components, including Hippo, Salvador, and Warts, were originally identified in Drosophila and are highly conserved in mammals. Mammalian Ste20-like serine/threonine kinase 1/2 (MST1/2) is the homolog of Drosophila Hippo,[18] and large tumor suppressor 1/2 (Lats1/2) is the homolog of Drosophila Warts. Merlin (encoded by the neurofibromatosis type 2 gene, *NF2*) was found to activate Hippo signaling in mammals.[19-21] This pathway regulates the activation of YAP, which was initially identified as an oncogene via phosphorylation and subcellular localization in response to cell growth and density.[22,23] At a low cell density, YAP is predominantly found in the nucleus, whereas at high density, it is translocated to the cytoplasm following phosphorylation by Lats. YAP works as a transcriptional co-activator in the nucleus by associating with TEA-domain family member (TEAD),[24] which has a DNA-binding domain and regulates the expression of target genes. Amplification of the YAP gene locus 11q22 has been reported in many types of human cancers, including malignant mesothelioma,[25] ependymoma,[26] hepatocellular carcinomas[27] and esophageal squamous cell carcinomas.[28] Moreover, YAP overexpression has been reported in tumors of the colon,[29] lung, ovary,[30] squamous cells,[31] liver and prostate.[20,32]

A genetic characteristic of MM tumors is the deletion or mutation of tumor suppressor genes, such as $p16^{INK4a}$/$p14^{ARF}$ and NF2.[33,34] Although several other cancer cell types have also been reported to have homologous somatic mutations of the NF2 gene,[35] MM has an extremely high mutational frequency in this pathway. The fact is that at least 75% of MM cases have a mutation in the NF2 pathway and components of the Hippo signaling pathway, Salvador1 and LATS2, indicating that this type of tumor greatly relies on the inactivation of this pathway for oncogenesis.[36]

Mesothelial cells are formed as a single monolayer, firmly bound together by well-developed tight junctions, which provide physical support and a protective barrier against invading organisms. On the other hand, the parietal surface of mesothelium functions by releasing pleural fluid for cushioning the gap between the visceral

and parietal mesothelia to facilitate visceral movements. When compared with epithelial cells, mesothelial cells have a structure to maintain physical strength and enable the release of pleural fluid. The control of mesothelial cell density may be important in maintaining mesothelial functions; therefore, we speculated that the NF2-Hippo signaling cascade plays a critical role, particularly in mesothelial cells, in monitoring cell density and cell-cell contact to avoid overgrowth of cells. A disturbance in the NF2-Hippo signaling cascade may lead to dysregulation of the monitoring system, thereby leading to occurrence of MM. These observations could be used to elucidate the origin of MM tumors that employ the Hippo signaling pathway as the main route of tumor suppression.

Association of the TGFβ Pathway with Oncogenic Mutations of MM Cells

It has been established that TGFβ induces connective tissue growth factor (CTGF), which is also a known target of the Hippo signaling pathway.[37,38] Among the numerous other target genes induced by TGFβ or Hippo-YAP signaling, we found that CTGF expression was induced by TGFβ treatment but attenuated by YAP knockdown in MM cells.[12] Most other genes activated by TGFβ, such as *Smad7*, *MMP-2*, *COL1A1* and *fibronectin* were not affected by YAP depletion in MM cells. This is in agreement with the finding that CTGF has a specific promoter site that is regulated by Smad3 and YAP.

Several studies have reported the interactions between Smad and YAP/TAZ.[39-41] Varelas et al.[39] showed that YAP/TAZ controls the nuclear-cytoplasmic shuttling of Smad2/3–4 complexes and regulates the nuclear accumulation of Smad complexes in mouse embryonic stem cells. Alarcón et al.[41] showed that Smad1 (a component of the BMP signaling pathway) and YAP interact to regulate the transcription of target genes during neural differentiation in mouse embryonic stem cells, and Smad3/YAP binding was extremely weak compared with Smad1/YAP binding. This finding is consistent with our results that Smad3/YAP binding was weak despite an obvious crosstalk between these proteins as indicated by the results of a reporter assay and a CTGF protein expression assay.[12] Although an immunoprecipitation assay using the HEK293 cell line demonstrated that exogenous Smad3 overexpression showed weak binding with endogenous YAP, endogenous Smad3 and YAP binding could not be detected in MM cells. Therefore, we hypothesized that other components strengthened YAP and Smad3 binding. We selected several candidates that were likely to promote gene transactivation and found that p300 was probably a component in the Smad complex[42] as well as TEAD, which was found to be a strong binding partner with YAP.[43,44] We also found that Smad3 binds more preferably to TEAD than to YAP. This hypothesis enabled us to demonstrate the functional implications of Smad3/TEAD4 and Smad3/YAP binding in MM cell growth induced by TGFβ (**Fig. 2**).

Although CTGF is a known target of both TGFβ and the Hippo signaling pathway, the transcriptional mechanism that converges on CTGF expression involved in the crosstalk between these pathways in MM cells has been described for the first time in our study.[12] The crosstalk between the TGFβ and Hippo signaling pathways found in MM cells may be specific to this type of cell, but this finding may be applicable to other tumor types with defective NF2 function or mesenchymal origin. Besides the direct effect of TGFβ on cancer cells, TGFβ may promote further malignancies by cooperating with the genetic disturbances in cancer.

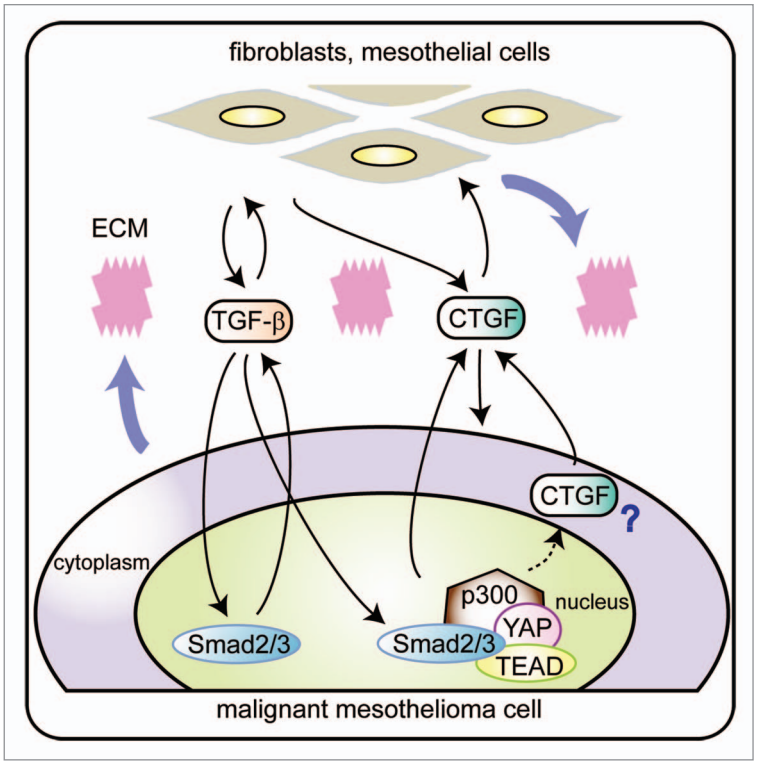

Figure 1. A model environment of MM cells and surrounding fibroblasts and mesothelial cells mediated by TGFβ and CTGF. Autocrine stimulation by TGFβ in MM cells causes further production of TGFβ and other ECM-related proteins independently from disturbances in the Hippo signaling cascade. CTGF may exert its effect on MM cells, fibroblasts and mesothelial cells to promote growth.

The Role of CTGF in MM

CTGF is a 36–38-kD cysteine-rich expressed in the early developmental stages of the embryo in cartilage, bone and at other sites where connective tissue is deposited.[45-48] TGFβ induces enhanced expression of CTGF, extracellular matrix-related proteins and autocrine induction of TGFβ both in normal fibroblasts and mesothelial cells (**Fig. 1**). In the context of oncogenic

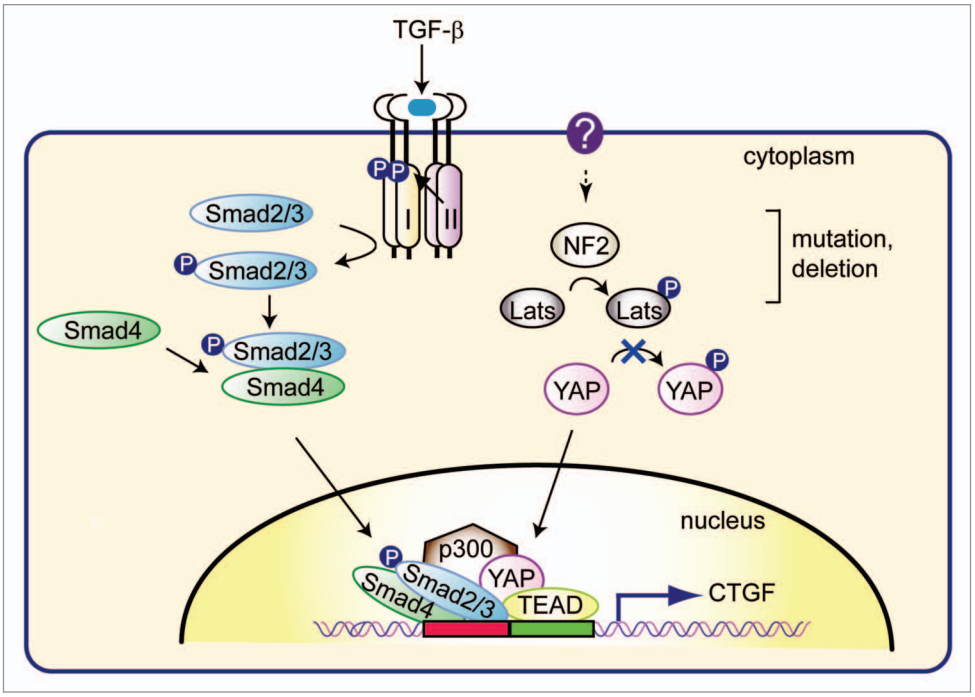

Figure 2. Schematic model of *CTGF* promoter activation through TGFβ/Smad signaling and disturbance of the NF2/Hippo pathways in MM cells. Because of the genetic disturbance in NF2 and/or Lats2, Yap was dephosphorylated and constitutively translocated to the nucleus. On the other hand, upon TGFβ stimulation, Smad2/3 and Smad4 associate, move to the nucleus, make a complex with YAP/TEAD, and recruit p300 to the promoter to activate CTGF expression.

properties, CTGF expression was observed in the stroma of tumors and affects vascularization, migration, and epithelial-mesenchymal transition (EMT).[49,50]

Recent studies indicated that tumor cell-derived CTGF plays an important role in the proliferation of breast cancer cells[22] and growth of pancreatic tumors.[37] We demonstrated a crosstalk mechanism between the TGFβ and Hippo signaling pathways, in which both were strong regulators of MM cell growth, suggesting that CTGF is an important regulator of MM tumor growth.

In our experiment, CTGF expression was predominantly overexpressed in sarcomatoid and biphasic tumor cells in tissues samples, although little was observed in epithelioid tissues.[12] Using a short hairpin (sh) CTGF lentivirus-based vector, we showed that the survival of nude mice thoracically implanted with CTGF-knockdown malignant mesothelioma cells (NCI-H290) survived longer than mice with control NCI-H290 cells.[12] Although most of the mice with control NCI-H290 cells showed severe emaciation with pale and dry skin, CTGF-knockdown mice were healthy and moving actively 21 days after the thoracic implantation (**Fig. 3**). The ratios of mice with emaciation at the time of death were 7/8 with the control, 3/8 with shCTGF #1 cells and 1/8 with shCTGF #2 cells ($p < 0.05$ vs. control). The lungs from mice with control NCI-H290 cells showed that MM cells cover the lung surface and eventually adhere to the pleura, which may induce respiratory failure in mice. Interestingly, the lung surfaces of shCTGF NCI-H290-implanted mice were relatively free from tumor cells, which preferably remained in the mediastinal space. CTGF may influence the speed of migration and spread in the thoracic cavity of mice due to cellular changes as well as mutual interactions with normal mesothelial cells of the host. The cause of death of shCTGF tumor-bearing mice may be different from that of control tumor-bearing mice, because a feature of their last stage was not emaciation, but an unexpected hydroperitoneum and occasional systemic edema. Several causes of this symptom may be suspected, including hypoalbuminemia from malnutrition, chronic inflammation accompanied by tumor growth or lymph edema due to impaired lymphatic vessels. Since visible tumor cell dissemination was not observed in the peritoneal cavity, we speculated that this may occur from impaired circulation in the thoracic cavity due to a slow but sustained progression of CTGF-knockdown tumors compared with the control tumors. Although small amounts of ascites were observed in some of the mice with control tumors, respiratory failure caused by the aggressive spread over the lung surface may lead to early death.

We verified the histological changes in these tumors but could not discern any effect by CTGF knockdown, suggesting that CTGF affects MM cell growth and migration; therefore, mice implanted with CTGF-knockdown MM cells survived longer.

What Causes the Histological Difference in MM?

A specific feature of MM is the mixed pathological appearance with epithelioid and sarcomatoid cell types, also called biphasic or mixed types. In a tissue specimen from an MM patient, both histological subtypes were observed with clear borders. Our previous report showed that p-Smad2

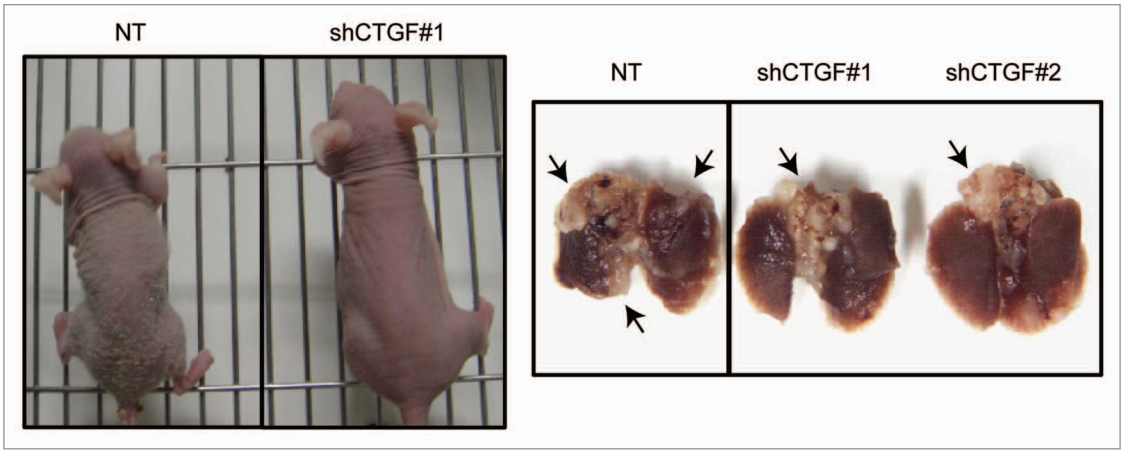

Figure 3. A picture of mice 21 d after thoracic implantation of NCI-H290 cells infected by non-target (NT) and shCTGF lentivirus-based vectors. NT tumor-bearing mice showed severe emaciation at this point (left). Lungs excised at the time of death are shown on the right (NT day 23, shCTGF #1 day 36, and shCTGF #2 day 40). Arrowheads indicate the tumor masses.

staining was observed in all 24 tissue specimens from MM patients, although there was a clear difference in CTGF staining between epithelioid and sarcomatoid tissues, suggesting that CTGF may play a role in histological changes in MMs.[12] Thus far, CTGF overexpression in NCI-H290 cells has not induced any histological change in tissues implanted in the thoracic cavities of mice (data not shown). This may be attributed to yet insufficient CTGF expression or certain unique characteristics of the cell line used in our study. In this regard, there is a possibility that other components are expressed predominantly in sarcomatoid cells but not in epithelioid subtypes involved in this difference. It remains uncertain whether CTGF expression brings about histological changes directly or indirectly, or whether there are some conditions in the sarcomatoid tissues that CTGF expression induces and maintains at a high level. Furthermore, we also found a strong link between CTGF expression in MM cells and the deposition of ECM proteins in both MM cell lines implanted into mice and patient tissue specimens. Again, whether CTGF expression induces ECM deposition or whether deposition of ECM causes CTGF expression should be further investigated.

Targeting CTGF as a Molecular Target Therapy

CTGF plays an important role in regulating cell survival, and we demonstrated its importance through in vivo studies and human histological analyses in the clinical features of substantial spreading of tumor mass accompanied by fibrosis in the thoracic cavity, which suggested the functional importance of CTGF in MM growth.

Our results showed that the TβRI inhibitor is effective for in vivo treatment of human MM cells in a mouse model. TGFβ antagonists are currently under clinical trial for the treatment of melanomas and high-grade gliomas. The TβRI inhibitor can suppress MM growth through the reactivation of antitumor immune responses.[16,17] We propose that TβRI inhibitor or antibody be used to suppress activation of the TGFβ pathway and CTGF expression in MM. It is preferable that the Hippo signaling pathway be activated for MM treatment. Regarding the Hippo signaling pathway, since YAP is a practical effector, which itself translocates to the nucleus to transactivate target genes, removing YAP from the nucleus is theoretically the most efficient way to suppress its activation.[51]

We used a shRNA system for in vivo studies to elucidate the role of CTGF in MM, but whether this system is applicable in actual clinical treatment remains uncertain. Therefore, when targeting CTGF directly, the first priority may be treatment with CTGF antibody or antisense therapy. A recent report showed that CTGF activated Smad1/5/8 and MAPK signaling during osteoblast differentiation,[52] although studies of CTGF-induced intracellular signaling mechanisms should be further studied. Our data indicated that the knockdown of CTGF suppresses MM cell growth and proliferation both in vitro and in vivo in the mouse model.

Histological staining of patient tissues revealed that CTGF was strongly stained in the cytoplasm of MM cells rather than in the stroma or on the surface of the cells, suggesting a potential to exert an effect while remaining in the cells. There are many critical questions remaining: is CTGF secreted extracellularly from MM cells? How does it transduce signals to MM cells and surrounding stromal cells and lead to the deposition of ECM protein? Is there any other mechanism to retain a high level of CTGF expression in these cells? It has been reported that TGFβ stimulates CTGF production in both normal and systemic sclerosis fibroblasts, with the latter found to constitutively express higher CTGF levels.[53] Similar mechanisms resulting in induction of an enhanced response of CTGF promoter or prolongation of CTGF expression, which were observed in systemic sclerosis fibroblasts, may be responsible also in the pathogenesis of MM. Considering the manner in which CTGF should be practically targeted as a novel molecular therapy, more information on its functional mechanism and regulation is required.

More choices will be available to regulate CTGF expression if additional targets are included from the TGFβ and Hippo signaling pathways. Our previous

analysis using human MM tissues showed that these two pathways are not perfectly linked to direct CTGF expression, suggesting that besides TGFβ signaling and defects in Hippo signaling, there are additional unknown mechanisms that participate in CTGF regulation. Further research regarding CTGF expression may lead to other methods for suppressing CTGF expression.

Materials and Methods

Animal experiments. Seven–eight-week-old female athymic nude mice of KSN strain (Shizuoka Laboratory Animal Center) were weighed and randomly assigned to different treatment groups. Lentivirally infected or uninfected NCI-H290 cells were then orthotopically injected into the right thoracic cavity of each mouse. The experimental design was approved by the Animal Care Committee of the Aichi Cancer Center Research Institute, and the animals were cared for in accordance with institutional guidelines as well as the Guidelines for Proper Conduct of Animal Experiments (Science Council of Japan, June 1, 2006).

Conclusion

We demonstrated that the TGFβ pathway and disturbances in the Hippo signaling pathway synergistically promote MM cell growth.[12] To study the crosstalk between distinct pathways is important while searching for molecular therapy targets, because the blockade of one pathway may be insufficient to obtain a maximum effect. Certain target genes converge in these significant and distinct pathways, both of which have a strong association with oncogenic properties in MM cells, and may play important roles in their growth. Our findings showed that CTGF is a strong candidate for molecularly targeted therapy for MM patients, which may be effective in both MM cell growth and tumor microenvironment.

Acknowledgments

We thank L.M. Wakefield for comments on the manuscript and Y. Matsudaira, H. Ito, N. Saito and M. Tsuji for technical assistance. This work was supported by funds from the 24th General Assembly of the Japanese Association of Medical Sciences (to M.F.), funds from the Aichi Cancer Research Foundation (to M.F.), and Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (20590420 and 23592788 to M.F.) and partly by Grant-in-Aid for Third-Term Comprehensive Control Research for Cancer from the Ministry of Health, Labor and Welfare of Japan (to Y.S.), Grant-in-Aid for Project for Development of Innovative Research on Cancer Therapeutics from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to Y.S.), and Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (22300338 to Y.S.).

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