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長鏈非編碼 RNA *Smyca*藉由控制 TGF-β 訊號通路的回饋機制以促進腫瘤進程

Long non-coding RNA *Smyca* promotes tumor progression by controlling a feedback mechanism of TGF-β signaling

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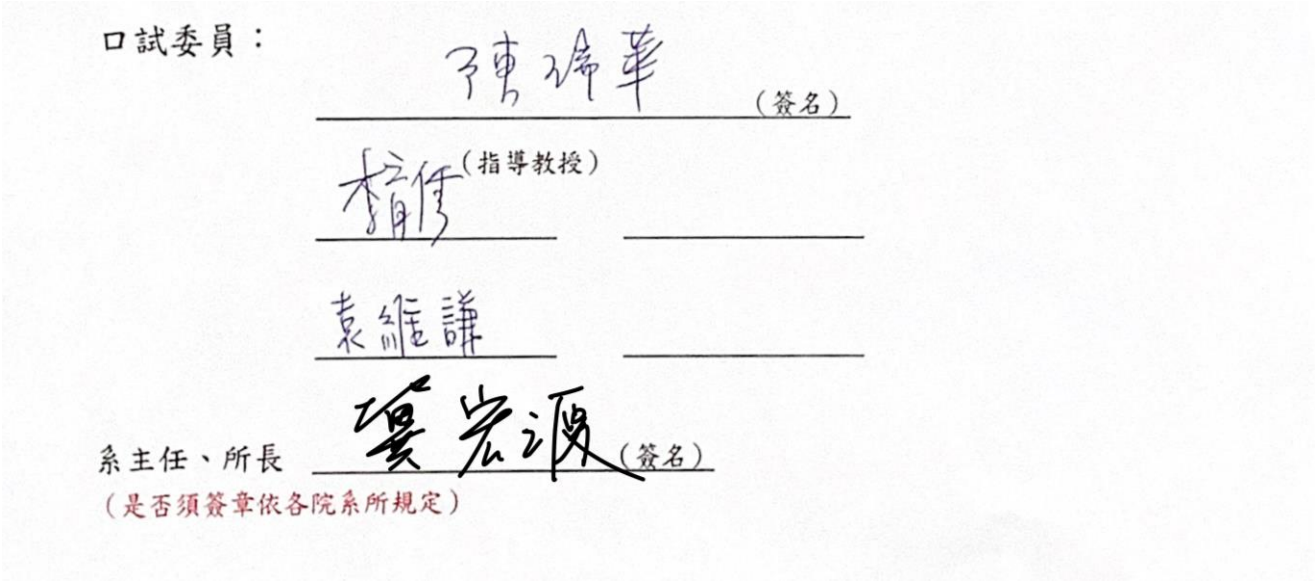
本論文係簡茹因君（R07B46002）在國立臺灣大學生化科學研究所完成之碩士學位論文，於民國 110 年 07 月 29 日承下列考試委員審查通過及口試及格，特此證明



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謝辭

感謝我的指導教授陳瑞華老師透過自身的經驗教導，讓我對於研究領域又有更深一層的體悟。初來實驗室時，我對於自己的實驗技巧感到非常自卑，但是經由老師及忻怡學姊嚴謹扎實的訓練下，我的實驗技巧開始進步，對於研究的自信心也跟著一點一滴的增長。看著陳老師對於研究的熱忱與毅力，我心懷敬佩，也以此作為榜樣，勉勵自己要勤奮向學。老師總是不辭辛苦地栽培實驗室的每位學生，並在每次實驗室會議時耐心地傳授知識給每位學生。老師對於研究領域始終抱持著堅持不懈、嚴謹、熱愛的態度，也因此激勵我在科研領域需抱持著奮發向上、學而不怠的精神。

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中文摘要

近幾年內，諸多研究指出長鏈非編碼 RNA 在癌症進程中扮演著致癌的角色。然而，長鏈非編碼 RNA 對於影響腫瘤生長及轉移的詳細機制仍待釐清。在本篇研究，我們發現了一個與癌症相關的長鏈非編碼 RNA 並取名為 *Smyca* (Smad/Myc coactivator)，它的高度表現與多種癌症病人的不良預後呈正相關。從結構上觀察，*Smyca* 的初級轉錄產物中，*Smyca* 序列的 5’端連接著一群微小 RNA (miRNA)，而經過 Drosha 蛋白酶的切割後，則能使 *Smyca* 與這群微小 RNA 分開。為了研究 *Smyca* 在癌症進程中具有哪些功能，我們首先利用能與 *Smyca* 序列互補的短髮夾 RNA (shRNA)以有效降低 *Smyca* 的表現量，且不影響其上游端的微小 RNA 群表現。我們發現降低乳癌細胞中 *Smyca* 的表現量，會誘導其細胞型態會從間質型態轉換為上皮型態。隨後，我們實驗室使用 RNA 定序及生物資訊的分析，發現 *Smyca* 可能參與 TGF-β訊號通路。而實驗也發現，過度表現 *Smyca* 的正常乳腺細胞會促進 TGF-β訊號通路的中下游目標基因的表現量及啟動子的活性。然而，*Smyca* 並不會影響 TGF-β訊號通路過程中 Smad 蛋白的表現量及其磷酸化程度。在實驗室先前的研究成果中，發現 *Smyca* 本身的表現能被 TGF-β所誘導。而後續的實驗證實，在過度表現 *Smyca* 的組別中會增長 TGF-β訊號通路的強度與時間長度，因此表明 *Smyca* 會利用與 TGF-β訊號通路形成的正回饋機制，加以調控 TGF-β訊號通路。此外，我們實驗室也發現 *Smyca* 會連結到 MYC 蛋白並促進 MYC 下游基因表現。通常 MYC 需要與 MAX 結合形成一個穩定的異二聚體以誘導下游基因表現。因此，我們決定研究 *Smyca* 是否會透過促進 MYC-MAX 聚合物的形成，以影響 MYC 下游基因的表現。儘管如此，我們發現過度表現或降低表現 *Smyca* 都不會影響 MYC 與 MAX 的結合力，這暗示著 *Smyca* 可能透過其他機制以促進 MYC 下游基因的表現。總結，我們的實驗結果揭示了一個嶄新的長鏈非編碼 RNA 調節 TGF-β訊號通路的回饋機制，也發現此種長鏈非編碼 RNA 還能參與提升 MYC 訊號通路。此外，我們的研究也闡釋了 *Smyca* 在癌症進程中的功能，及其詳細的分子調控機制。

關鍵字: 長鏈非編碼 RNA，*Smyca*，癌症進程，間質上皮細胞轉化，TGF-β，MYC

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Abstract

In recent years, emerging evidences have indicated that long noncoding RNAs (lncRNAs) play an oncogenic role in cancer progression. However, the detailed mechanisms of lncRNAs in promoting tumor growth and metastasis have not been completely understood. Here, we uncover an lncRNA termed *Smyca* (Smad/Myc coactivator), whose high expression correlates with poor prognosis of several cancer types. The primary transcript of *Smyca* contains a miRNA cluster at the 5' region of *Smyca*, which is separated after Drosha cleavage. To investigate the functional mechanisms of *Smyca* in cancer progression, we first used *Smyca* shRNAs to efficiently knockdown *Smyca* but not miRNAs in the cluster. We found that *Smyca* knockdown induces mesenchymal–epithelial transition (MET). Next, our lab used RNAseq followed by bioinformatics analysis and revealed *Smyca*-regulated gene signature correlates TGF-β-regulated gene signature. Indeed, we showed that *Smyca* overexpression promotes TGF-β-induced downstream gene expression and promoter activity in normal breast epithelial cells. However, *Smyca* does not affect Smad expression and phosphorylation. Previous study in our lab found that *Smyca* can be induced via TGF-β treatment. Consistently, *Smyca* overexpression increases the amplitude and duration of TGF-β signaling, thus demonstrating a role of *Smyca* in controlling a positive feedback mechanism of TGF-β signaling. In addition, our lab also found *Smyca* binds MYC to promote its ability to induce gene expression. To activate gene transcription, MYC requires the formation of a stable heterodimer with MAX. Thus, we decided to study whether *Smyca* regulates the transcription of MYC target genes by promoting MYC-MAX complex formation. Nevertheless, we found that MYC-MAX binding could not be affected by *Smyca* upregulation or downregulation, which implies that *Smyca* uses other mechanism to promote MYC-induced gene expression. Hence, our results uncover the novel lncRNA-mediated mechanisms for feedback control of TGF-β signaling and upregulation of MYC pathway. Moreover, our study provides the molecular insights of *Smyca* function in cancer progression.

Keyword: lncRNA, cancer progression, MET, *Smyca*, TGF-β pathway, MYC

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目　錄

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I. Introduction

1. EMT and its function in cancer progression

1.1 Overview of EMT

Epithelial-mesenchymal transitions (EMTs) is a crucial cellular program occurring during some biological processes and are classified into three types. Type 1 EMT allows primitive epithelial cells transitioning to motile mesenchymal cells which have the potential to subsequently undergo a mesenchymal-epithelial transition (MET) to generate secondary epithelia during embryonic development. Type 2 EMT is associated with adult tissue regeneration and wound healing which involves secondary epithelial or endothelial cells transitioning to resident tissue fibroblasts. Type 3 EMT occurs in the cancer process, which epithelial carcinoma cells transitioning to metastatic tumor cells to migrate through the circulatory system and eventually cause tumor metastasis by MET [1-3].

During the physiological process of EMT, epithelial cells lose their cell polarity and cell–cell adhesion, and attain migratory and invasive properties to become spindle-shaped mesenchymal morphology [2, 4]. In the perspective of molecular level, the hallmarks of EMT include reduced epithelial gene expression, such as claudin and occludin, abolished function of cell junction proteins, such as diffusion of zonula occludens 1 (ZO1) and E-cadherin, and elevated expression of mesenchymal-related genes such as vimentin, fibronectin. In addition, cells with EMT features often express matrix metalloproteinases (MMPs), such as MMP2 and MMP9, to degrade and invade their basal extracellular matrix (ECM) [5, 6].

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A variety of molecular processes activated by pleiotropic intrinsic and extrinsic factors are engaged in EMT from initiation to completion. These processes include transcriptional control, epigenetic modifications, reorganization and expression of cytoskeletal proteins, and specific microRNA modulation. Notably, a group of EMT-inducing transcription factors (Snail1, Snail2/Slug, Twist, and Zeb1/2) can induce the expression of genes required for mesenchymal properties and meanwhile repress the expression of genes that are required for the epithelial phenotype. The expression of these EMT-inducing transcription factors are regulated by various signaling pathways, including transforming growth factor β (TGF-β) [7], platelet-derived growth factor (PDGF), fibroblast growth factor (FGF) [8], epidermal growth factor (EGF) [9], hepatocyte growth factor (HGF), bone morphogenetic protein (BMP) [10], Sonic Hedgehog (Shh), integrin, Wnt/beta-catenin [11] and Notch signaling [12]. These signaling pathways are defined as EMT inducers that trigger transcription factors to activate the expression of EMT-associated genes via intracellular kinase cascades. During cell reprogramming, some of these signals may become predominant to drive EMT at specific stages.

1.2 EMT in cancer progression

As introduced above, Type 3 EMT plays an important role in several stages of cancer progression which cover tumor initiation, tumor growth, tumor invasion and metastasis. Previous studies indicate that EMT activation has been associated with cancer stemness, tumor angiogenesis, tumor microenvironment reprogramming, all of which contributed to metastasis.

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For stemness, growing evidence has demonstrated that EMT can promote the generation of cancer stem cells (CSCs). For example, induction of EMT in transformed epithelial cells through ectopic expression of Snail and Twist, leads to the expression of a $CD44^{high}/CD24^{low}$ antigenic phenotype, a feature of breast cancer CSCs, and stem-like traits [13, 14]. Besides, EMT-inducing transcription factor Zeb1 and EMT-inducer TGF-β also contribute to acquisition of stem cell-like properties[13, 15].

In terms of tumor angiogenesis, vascular endothelial growth factor (VEGF) and EGF are the main factors that induce proliferation and differentiation of endothelial cells, increase the vascular permeability, and promote the modification of extracellular matrix [16]. In clinical studies, overexpression in TWIST2 levels and loss of E-cadherin expression are positively correlated with VEGF and EGFR levels, and patients with this condition have been associated with poor prognosis [17]. It has been demonstrated that VEGF activation in combined with extrinsic factors, such as hypoxia, Notch signaling, and p38/JNK-ATF-2 signaling, can induce EMT in angiogenic tumor endothelial cells [18-21]. Additionally, both of EMT and angiogenesis are key factors for initiating tumor metastasis.

Tumor metastasis can be divided as five steps: invasion, intravasation, survival in circulation, extravasation, and colonization. EMT plays an essential role in most steps of metastasis. For example, EMT endows stem-like features to transformed epithelial cells, and it degrades extracellular matrix during tumor invasion. EMT also promotes vascular leakage during

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intravasation and extravasation. Since circulating tumor cells (CTCs) show a partial EMT activation [22], which also means that it exists a mix of epithelial and mesenchymal phenotypes in the circulation, tumor cells can conduct mesenchymal-to-epithelial transition (MET), a reverse mechanism of EMT, while colonizing to the distant organs [23, 24].

In summary, EMT participates in all stages of cancer progression by taking advantage in the crosslink with intrinsic and extrinsic factors in cell programs, and these features of EMT also lead to poor outcomes in cancer therapies.

1.3 EMT and resistance to cancer therapies

Chemotherapy and immunotherapy are common treatments for cancer, but these therapies lose their effectiveness on patients with high tumor malignancy caused by EMT, which develops resistance to the cytotoxic effect of antitumor drugs and immune checkpoint blockers.

Among chemotherapeutic drugs, cisplatin and doxorubicin are widely used in several types of cancers including lung, colorectal, head-and-neck, breast and ovarian cancers, as well as pediatric cancers. Cisplatin is an alkylating agent which kills tumor cells by damaging DNA and inhibiting DNA synthesis. However, recent evidence indicate that the translation initiation factor eIF4E induces Snail expression to trigger EMT as well as cancer metastasis, which eventually leads to resistance to cisplatin in nasopharyngeal carcinoma (NPC) [25]. Similarly, doxorubicin has ability to insert within DNA base pairs, causing breakage of DNA strands and inhibition of both DNA and RNA synthesis [26]. MicroRNAs (miRNAs) have been demonstrated as EMT inducers which cause

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resistance in doxorubicin. For examples, miR-93 interacts with PTEN to promote EMT, thereby causing resistance of doxorubicin in breast cancer cells [27]. miR-21 can induce EMT and gain gemcitabine resistance by PTEN/AKT pathway in breast cancer [28, 29].

Although immunotherapy has been success in treating different types of cancer by blocking immune checkpoint receptors such as PD-1 and CTLA, relapse still occurred [30]. Activation of EMT is one of the major reasons of immunoresistance and immunosuppression. For instance, EMT upregulates expression of PD-L1 in non-small cell lung cancer (NSCLC) and directly results in exhausted CD8+ T cell and immunosuppression [31]. Snail-induced EMT impairs dendritic cells, induces regulatory T cells which can accelerate cancer metastasis via Thrombospondin-1 (TSP1) production in melanoma [32]. Moreover, human breast cancer cells MCF7 undergoing EMT to acquire resistance to cytotoxic T lymphocytes-induced cell death [33].

2. Long noncoding RNA (lncRNA)

2.1 Overview of long noncoding RNA

As the development of RNA sequencing (RNA-seq) technologies and transcript mapping (such as ENCODE [34] and FANTOM [35]), studies have revealed that RNAs that can be translated into protein only occupy a small fraction. In contrast, the vast majority of expressed transcripts lack protein-coding potential, and one group of these RNAs is long noncoding RNAs (lncRNAs) which is defined as non-coding RNAs longer than 200 nucleotides.

Similar to messenger RNA (mRNA), most of lncRNAs are

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transcribed by RNA polymerase II and have 5'-end 7-methylguanosine (m7G) caps and 3'-end poly(A) tails. The common lncRNAs classification is based on their biogenesis loci of nearby encoded protein genes, according to which lncRNAs can be subdivided into intergenic lncRNAs, antisense lncRNAs, sense lncRNAs, intron region lncRNAs, circular RNAs (circRNAs) and enhancer lncRNAs (eRNAs) [36].

LncRNAs are involved in a wide range of cellular mechanisms, from transcriptional regulation to posttranslational modifications. In the nucleus, lncRNAs participate in transcriptional and post-transcriptional regulation, which include recruitment of chromatin modifiers, administration of transcription factors, regulation of splicing, regulation of chromosome looping, and function of sub-nuclear structures [37-39]. In the cytoplasm, lncRNAs can control translation, mRNA degradation, miRNA sequestration and mRNA decay [40-46].

Early mechanistic studies of lncRNAs were mainly associated with epigenetic regulation, such as *H19* [36] and *Xist* [37-39]. In recent years, lncRNAs have been attracting immense research interests since lncRNAs involved in various biological responses including gene regulation, metabolism, pathogenic infection, and tumor progression [47-49].

2.2 Clinical significance of long noncoding RNA in cancer biology

Recent clinical research demonstrated that lncRNA can be applied to cancer diagnosis, prognosis and therapy. First, numerous studies have shown that lncRNAs can become potential biomarkers in many types of cancer, according to its specific expression in lesions. For instance, lncRNA *PCA3* has been permitted for the diagnosis of prostate cancer.

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PCA3 diagnosis has high sensitivity and excellent specificity comparing to serum prostate-specific antigen (PSA) which has low specificity in distinguishing benign and malignant tumors [50-52]. Besides, more and more lncRNAs have implicated in potential diagnostic and prognostic markers, such as *HOTAIR* for diagnosis of breast cancer [53] and *MALAT1* for gastric, colorectal and liver cancers [37, 54-56].

Most of cancer-associated lncRNAs serve not only as tumor biomarkers but therapeutic targets. For instance, *H19* is an oncogenic lncRNA which is upregulated in a variety of cancers. Given the highly activated nature of *H19* promoter in cancers, diphtheria toxin driven by *H19* promoter is constructed as an anti-cancer strategy. When applied to various cancer types such as pancreatic cancer, ovarian cancer, glioblastoma and HCC cells, this construct can result in selectively killing of tumor cell lines and inhibit tumor growth *in vitro* and *in vivo* [57]. In addition, lncRNAs can be targeted to inhibit their expression by several ways including the usage of lncRNA-specific siRNAs, antisense oligonucleotide (ASO), gapmers, and ribozymes, blockade of lncRNA transcription or function. By using these methods, modulation of cancer-associated lncRNA expression may improve the therapeutic sensitivity of tumors and also be applied into combination therapy.

2.3 Long noncoding RNA in regulating EMT

Emerging studies support the role of lncRNAs in the regulation of tumor progression and metastasis through the regulation of EMT. LncRNAs can be classified into two groups: EMT promotion (pro-EMT), EMT antagonist (anti-EMT).

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Regarding to pro-EMT lncRNAs, the expression levels of many pro-EMT lncRNAs correlate with EMT inducers or EMT biomarkers. For instance, lncRNA-*ATB* plays multiple roles in regulation of miR-200 family members, ZEB1/2, TGF-β, STAT3 in several types of tumor cells [58-61]. Overexpression of *HOTAIR* leads to the activation of Wnt-Notch signaling in malignant cervical cancer cells and suppression of H3K27, thereby supporting EMT in gastric cancer [62, 63]. Moreover, *H19* not only simultaneously responds to several EMT inducers, such as HGF, hypoxia inducible transcription factor 1 (HIF1) and TGF-β, but also serves as a ceRNA for anti-EMT miRNAs [64, 65].

With respect to anti-EMT lncRNAs, these lncRNAs act as tumor suppressors by inhibiting EMT, invasion, metastasis, proliferative activity of tumor cells. For example, lncRNA *GAS5* can suppress EMT in osteosarcoma by directly inhibiting the function of miR-221 and promoting tumor suppressor gene aplasia Ras homologue member I (ARHI) expression [66]. *DREH* can inhibit EMT through direct interaction with and downregulation of vimentin to change vimentin intermediate filament structure [67]. *TUSC7* was found to be downregulated in several cancers. In HCC, *TUSC7* serves as a ceRNA to inhibit EMT promoter miR-10a and then reduce the capacity of EMT [68-70].

In summary, the common feature of all of EMT-related lncRNAs is its ability of regulating EMT biomarkers or EMT inducers. Furthermore, it's worth noting that a majority of EMT-related lncRNAs directly or indirectly affect TGF-β/Smad pathway, which implies TGF-β/Smad pathway may be the main factor in driving EMT by utilizing the

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advantages of lncRNAs.

3. TGF-β signaling pathway

3.1 Overview of TGF-β signaling pathway

TGF-β signaling pathway plays a critical role in many cellular processes, including cell growth, differentiation, apoptosis, angiogenesis, metastasis and immune response [71-73]. The mechanism of TGF-β signaling pathway is initiated through the binding of TGF-β to TGF-β receptor 2 (TβR2), which in turns recruits and activates TGF-β receptor 1 (TβR1) by phosphorylation. Subsequently, the activated TβR1 transduces signals through downstream effectors which can be classified as canonical and non-canonical pathways.

For TGF-β canonical pathway, Smad transcription factors are the main mediators which are generally divided into three distinct groups: receptor-regulated Smads (R-Smads), common mediator Smads (Co-Smads) and the inhibitory Smads (I-Smads) [74]. The R-Smads consist of Smads 1, 2, 3, 5, 8/9 and are phosphorylated in response to TGF-β and bone morphogenetic proteins (BMP). The only known mammalian Co-Smad is Smad4 which form complex with R-Smads to recruit co-regulators. I-Smads which comprise of Smad6 and Smad7, antagonize TGF-β signaling by competitive binding with Smad4, and recruitment of ubiquitin ligases to drive TβR1 degradation. In TGF-β canonical pathway, activated TβR1 phosphorylates intracellular R-Smads (Smad2 and Smad3) on the serine residues of the carboxyl terminus. These phosphorylated R-Smads then recruit Co-Smad (Smad4) to form a heteromeric complex which translocates to the nucleus for regulation of TGF-β target genes

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transcription [75-77].

TGF-β non-canonical pathways, which is known as Smad-independent pathway, can lead to the activation of other well-known intracellular pathways, such as phosphatidylinositol-3-kinase (PI3K), JNK/p38, mitogen-activating protein kinases (MAPK) and EGFR) through the activation of TβRI. Activation of these non-canonical pathways can modulate signaling responses to increase the diversity of TGF-β signaling [78-80].

To summarize, TGF-β signaling exerts a wide variety of cellular functions via activation of canonical and non-canonical pathways, and plays a pivotal role in biological processes such as embryonic development and tissue homeostasis. Thus, dysregulation of TGF-β signaling may cause many diseases such as pathological fibrosis, autoimmune disorders and cancer [81, 82].

3.2 The functions of TGF-β pathway in cancer

TGF-β signaling is considered to play a dual role in cancer, acting both as a tumor suppressor and tumor promoter depending on cellular context and cancer stages [83, 84]. TGF-β signaling pathway serves as tumor suppressor in normal and early-stage cancer cells, whereas it serves as tumor promoter in late-stage cancers.

As a tumor suppressor, TGF-β signaling can induce cell-cycle arrest in G1 phase by enhancing expression of the cyclin-dependent kinase inhibitors (CDKIs) $p15^{INK4B}$, $p21^{CIP1}$ and $p27^{KIP1}$ and suppressing the expression of members of the Id family inhibitors [85, 86]. Meanwhile, anti-proliferative responses can be induced by repressing the expression

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of the proliferation-inducing transcription factors c-Myc [87]. Besides, TGF-β signaling can induce a number of apoptotic responses by variously molecular mechanisms. For instance, promoting expression of the transcription factor E2F1 leads to the formation of a transcriptionally active E2F1-pRb-P/CAF complex on multiple TGF-β pro-apoptotic target gene promoters [88]. The immediate early response to TGF-β-induced apoptosis is conducted by death-associated protein kinase (DAPK), which is transcriptionally activated from Smad-binding elements (SBE) by actions of TGF-β-induced Smads, and acts as upstream of TGF-β-induced mitochondrial damage to assist the release of cytochrome c from mitochondria [89]. The adapter protein Daxx also engages in TGF-β-induced apoptosis by facilitating TGF-β-induced JNK activation which mediates the Fas-apoptotic signaling pathway [90].

On the contrary, TGF-β signaling serves as tumor promoter in malignant cells. Tumor cells mainly use two strategies to escape tumor-suppressive effects of TGF-β: one is inactivating the component of TGF-β signaling by genetic and epigenetic alterations of its receptor complex (TGF-β receptors) [91-93] or signaling mediators (Smads) [94-96], which often occurs in ovarian, gastric, colorectal, pancreatic and head and neck cancers; the other is switching TGF-β into oncogenic factor by selectively impairing the anti-tumor response, which often occurred in gliomas, melanomas, breast and prostate cancers [97]. In the latter strategy, tumor cells hijack and utilize TGF-β signaling to benefit tumor growth and metastasis without affecting the components of the TGF-β signal transduction. For instance, tumor cells carry high active expression of

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PI3K–Akt pathway, and it suppresses TGF-β-induced p21 expression, thereby blocks the TGF-β cytostatic response [98]. In addition, TGF-β elicits strong immunosuppression by inhibiting the antitumor functions of CD4+ or CD8+ T cells, inducing apoptosis in B cells and blocking the production of IFN-γ by natural killer (NK) cells [99-101]. TGF-β contributes to angiogenesis and vascularization surrounding the tumor by increasing VEGF and CTGF expression in epithelial cells and fibroblasts [102, 103]. TGF-β signaling also directly or indirectly promotes EMT, stemness and other cancer-related features to improve tumor growth and metastasis [104-107]. Owing to our study involved in TGF-β-induced EMT, the next section will focus on the role of TGF-β signaling pathway in EMT and EMT-related functions.

3.3 TGF-β signaling in EMT and EMT-related functions

In malignant tumors, TGF-β signaling induces EMT by several ways including transcriptional and post-transcriptional regulation. The regulatory core of TGF-β-induced EMT is the nuclear reprograming which covers a set of transcription factors (e.g. Twist, E47, Snail, Slug, ZEB1, ZEB2, HMGA2 and FOXC2). These transcription factors either function as transcriptional repressors of epithelial genes and promoters of mesenchymal genes [108-112]. On the other hand, TGF-β has been shown to promote EMT by acting as a multifunctional regulator, including the control of epigenetic modification by induction of DNA methyl-transferase DNMT1 [113], posttranslational modifications of histone 3 [114]), expression of EMT-related miRNAs (downregulation of miR-200 family [115]) and mRNA translation by phosphorylation of

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ribonucleoprotein E1 (hnRNP E1) [116].

Given that EMT is highly associated with other tumorigenic functions in cancer such as stemness and metastasis, TGF-β can also promote these EMT-related functions. There is evidence that TGF-β can induce the expression of CSC marker CD133 in liver cancer cells [117]. Besides, TGF-β-induced EMT has been shown to generate cancer stem cells (CSCs) through autocrine and paracrine loops in breast cancer [118]. Moreover, research has shown that tumor-associated macrophages (TAMs) secreted more TGF-β than other type of macrophage, which promotes CSC-like properties via TGF-β-induced EMT in hepatocellular carcinoma (HCC) [119]. In addition, TGF-β induces the expression of cyclooxygenase-2 (COX2), epidermal growth factor receptor (EGFR), and angiopoietin-like 4 (ANGPTL4) to promote metastasis [120].

Hence, excessive TGF-β secretion, which often occurred in many tumor types, can induce EMT and then initiates the cascade towards metastasis. This also implies that TGF-β acts a potent EMT inducer throughout the progression of malignant cancer.

3.4 TGF-β signaling and long non-coding RNAs in cancer

To facilitate cancer progression, TGF-β signaling can synergize with various oncogenic stimuli. One common type of oncogenic stimuli is lncRNAs, which can serve as effectors or regulators of TGF-β signaling, or even forms feedback loops with TGF-β signaling [121].

As effectors of TGF-β signaling, lncRNAs are instructed to mediate various cellular responses of TGF-β in cancer progression. For instance, TGF-β upregulated lncRNA-*HIT* which can enhance EMT, migration, and

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invasion by specifically inhibiting E-cadherin expression in NMuMG mouse mammary epithelial cells [122]. On the other hand, TGF-β signaling can negatively regulate some anti-EMT lncRNAs such as. lnc-*Spry1* which suppresses EMT by affecting the alternative splicing of fibroblast growth factor receptors (FGFRs) [123].

As regulators of TGF-β signaling, lncRNAs controls several components of TGF-β signaling pathway, and adjust the amplitude of its response in tumor progression. For instance, lnc-*LFAR1* serves as pro-EMT lncRNA to further promote cell proliferation, invasion and migration in the intrahepatic cholangiocarcinoma cell line QBC939 by reinforcing TGF-β signaling with elevation of *TGF-β1*, *Smad2*, and *Smad4* mRNA levels [124]. LncRNA *NEAT1* acts as ceRNA for *miR-139-5p* to protect *TGF-β1* mRNA from *miR-139-5p*-induced degradation. In other words, LncRNA *NEAT1* acts as an activator of TGF-β signaling [125].

LncRNAs that act as effectors of TGF-β signaling can also be regulated by TGF-β signaling, thus forming feedback loops with TGF-β signaling pathway to enhance or diminish the signal output, respectively. These feedback loops can be classified as positive or negative feedback loops. In the positive feedback loop, lncRNAs (e.g. *lincRNA-p21* [126], *MALAT1* [127], lncRNA-*ATB* [128] and *PCAT7* [129]) are transcriptionally upregulated by TGF-β. Furthermore, these lncRNAs can engage in the enhancement of TGF-β signaling output. For instance, TGF-β induces *PCAT7* by the transcriptional complex of Smad3 with Sp1. *PCAT7* in turns enhances TGF-β signaling by sponging *miR-324-5p*, thereby increasing *TβR1* expression in prostate cancer [129]. Conversely,

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some TGF-β-induced lncRNAs (e.g. *TGFβ2-AS1* [130] and lnc-*TSI* [131]) play inhibitory roles on TGF-β responses, and lead to a negative feedback loop. Taken *TGFβ2-AS1* for an example, *TGFβ2-AS1* is upregulated via TGF-β, but it causes epigenetic silencing of TGF-β target genes by interacting with the PRC2 complex in lung adenocarcinoma cells and human immortalized keratinocytes [130].

In summary, lncRNAs and TGF-β signaling not only regulate each other, but also form the feedback loops that lead to increase or reduce amplitude of TGF-β response.

4. MYC

4.1 Overview of MYC

The *myc* gene was first identified in the avian myelocytomatosis retrovirus as the oncogene capable of inducing myeloid leukemia in chicken [132]. Since then, studies have shown that MYC contributes to the carcinogenesis of many cancer types. The MYC family consists of three paralogs, *c-myc* (c-Myc), *l-myc* (MYCL) and *n-myc* (MYCN), all of which are basic helix-loop-helix leucine zipper (bHLHLZ) DNA binding proteins. c-Myc is common and highly abundant in proliferating cells, whereas MYCL and MYCN have more restricted expression at distinct stages of cell and tissue development [133, 134].

The structure of MYC family proteins can generally be divided into three major domains: the N-terminal domain which is also referred to as the transcriptional activation domain (TAD), the central region involving in nuclear localization, and the C-terminal domain harboring basic HLH-Zip domain for dimerization with MYC-associated protein X (MAX) as

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well as binding to DNA. In addition, the N-terminal domain of MYC has been reported to form complexes with many factors including transformation/transcription domain-associated protein (TRRAP), GCN5 and TBP, which increase the diversity of structured folding of Myc transcriptional regulatory domain [135-138].

MYC proteins mainly function as transcription factors by forming a stable heterodimer with MAX and this complex has a specific DNA-binding activity to the E-boxes (5'-CACGTG-3'). By binding to the E box, MYC-MAX heterodimer can activate gene transcription through the recruitment of TRRAP that further recruits the histone acetyltransferase GCN5 [139, 140]. By contrast, MYC can also form a heterodimer with MIZ-1, which is recruited to MYC target sites to repress Myc-mediated transcription [141]. Finally, the MYC partner MAX can form a heterodimer with MAX dimerization protein (MXD) family proteins through their bHLH-Zip motif. In this way, MXD proteins competes with MYC for MAX binding. Since MAX binding to MXD proteins suppress cell growth, MXD is considered as part of the MYC/MAX/MXD network for cell growth control [142, 143].

Notably, MYC family proteins contain several highly conserved domain called MYC boxes, including MB0, MBI, MBII, MBIIIa, MBIIIb, and MBIV. These doamins contribute to MYC cellular responses and regulation, such as transcriptional regulation, protein degradation, transforming activity, transcription and apoptosis [144-148]. MYC proteins act as transcriptional modulators to control several different cellular processes including cell proliferation, cell cycle, differentiation,

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angiogenesis, metabolism, DNA repair, protein translation [137, 138, 149, 150]. Deregulation of MYC causes many diseases including polycystic kidney disease (PKD) [151], chronic gastrointestinal disorders [152] and cancers [153].

4.2 MYC in tumor progression

Excessive expression of MYC is found in many tumors, which dramatically disrupts the balance between activation and repression of the oncogenic MYC/MAX/MXD1 network, and tends to form MYC-MAX complexes which drive E-boxes to promote downstream gene transcription [133, 154].

Non-proliferating cells express certain E-box-driven genes to maintain metabolic homeostasis, while tumor cells upregulate these genes and rewire metabolism to meet requirements of rapid growth and proliferation through deregulation of MYC [155]. Oncogenic MYC regulates almost every aspect of cellular metabolism. For glucose metabolism, MYC directly activates both glycolytic genes (e.g. Glucose transporter gene *SLC2A1*, *hexokinase II* (*HK2*), *enolase 1* (*ENO1*) and *lactate dehydrogenase A* (*LDHA*)) and monocarboxylate transporter genes (*MCT1* and *MCT2*) to enhance glucose uptake and glycolysis flux within tumor cells by binding the E-box sequence [156, 157]. Besides, MYC also plays an essential role in regulation of amino acid and lipid metabolism in cancer via activation of essential amino acid transporters (SLC7A5, SLC43A1, and SLC1A5) [158] and stimulation of fatty acid/cholesterol synthesis as well as fatty acid oxidation (FAO) [159].

Since c-Myc consistently represses genes of cell adhesion in normal

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cells, tumor cells with c-Myc upregulation can enhance this function to promote EMT [160]. For instance, oncogenic c-Myc can induce EMT via transactivation of Bmi-1 [161], and it also can promote metastasis by activating the expression of miR-9 which leads to E-cadherin downregulation [162]. Furthermore, overexpression of MYC has been reported that it may result in genomic instability *in vitro* [163-166]. To illustrate, some studies indicated that oncogenic MYC induces reactive oxygen species (ROS) which generates mutations upon DNA replication [167], and some supported that MYC triggers chromosomal rearrangements via telomeric fusions [168].

As a transcription factor, MYC has ability to administrate a variety of genes that would affect cellular homeostasis. In tumor cells, MYC functions as oncogenic role that support nutrients for tumor growth by driving metabolic activities, and it also can synergize with other pathways to facilitate cancer progression [169, 170].

4.3 Signaling interplay between MYC and TGF-β

As described above, TGF-β inhibits cell growth via downregulation of growth-promoting transcription factor MYC. However, studies demonstrated that TGF-β-induced growth inhibition is out of action in tumor cells which require MYC to fuel rapid cell growth [171]. Thus, researchers have begun to elucidate the mechanisms involved in interplay between MYC and TGF-β.

TGF-β induces cell cycle arrest in G1 phase by upregulating cyclin-dependent kinase (CDK) inhibitors p15 and p21, then suppresses c-Myc via directly binding of Smad3 to TGF-β inhibitory element (TIE) of the *c-*

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myc promoter [172]. Conversely, oncogenic MYC downregulates these CDK inhibitors through direct interaction with the Zn-finger transcription factor Miz-1 which respectively binds to the initiator sequence of the *p15* promoter [173] and *p21* core promoter [174]. Besides, in tumor cells, hyperactive PI3K-AKT pathway blocks the tumor-suppressive effect of TGF-β by directly inhibiting Smad3 function, then reduces MYC/Smad3 interaction which inhibits the function of MYC [175, 176].

Based on the aforementioned mechanisms, MYC and TGF-β signaling are mutually antagonistic at the cellular and molecular levels. The dominance of cell growth is determined by cellular conditions: normal cells prefer TGF-β-induced growth arrest, while tumor cells prefer upregulated MYC which promote cell growth. Particularly, tumor cells can administrate MYC and TGF-β signaling for enhancing adaptability in response to environmental stresses.

4.4 Crosstalk between MYC and lncRNA in cancer

A large numbers of MYC-regulated lncRNAs have been identified via RNA sequencing (RNA-seq) [177] and CRISPR interference (CRISPRi) [178]. LncRNAs and MYC depend on each other closely, and some lncRNAs regulated by MYC also can mediate MYC signaling [179].

MYC-regulated lncRNAs have been shown to promote tumor progression by various mechanisms. Some MYC-activated lncRNAs act as ceRNA to sponge miRNAs; some stabilize target mRNAs by cooperating with RNA-binding proteins; and others directly target and enhance specific protein activity. For examples, MYC-activated *LINC00176* acts as ceRNA to bind to the tumor suppressor miRNAs miR-

doi:10.6342/NTU202101996

9 and miR-185, thereby promoting cell proliferation in HCC [180]; MYC-activated lncRNA *LAST* stabilizes *CCND1* mRNA with cellular nucleic acid-binding protein (CNBP) that binds to the 5'-UTR of *CCND1* mRNA to protect the mRNA from nuclease degradation, and this action facilitates tumorigenesis [181]; LncRNA *IDH1-AS1* leads to attenuation of glycolysis by promoting homodimerization of IDH1 and enhancing its enzymatic activity. MYC transcriptionally represses lncRNA *IDH1-AS1*, so that it can collaborate with HIF-1α to activate aerobic glycolysis in tumor cells [182].

Conversely, lncRNAs also can regulate MYC through various mechanisms from transcriptional mediation to protein modification. For transcriptional level, lncRNAs can regulate *myc* transcription by influencing its chromatin structure (e.g. lncRNA *CCAT1-L* [183]) or recruiting transcription factors (e.g. lncRNA *MYMLR* for binding Poly-C-binding protein 2 (PCBP2) [184], and Lnc*CMPK2* for binding FUBP3 [185]). For translational regulation, lncRNAs can maintain high MYC levels by sequestering miRNAs which target *myc* mRNA (e.g. *CCR492* antagonizing let-7 family [186], and *SNHG3* sponging miR-182 [187]), or regulate *myc* mRNA abundance by affecting the interaction between *myc* mRNA and its RNA binding proteins (e.g. Linc-*RoR* stabilizing *myc* mRNA by interacting with AUF1 to prevent it from binding to *myc* mRNA [188]). For protein level, lncRNAs can affect MYC protein stability or activity. Taken lncRNA *PVT1* for example, *PVT1* is able to stabilize the MYC protein by reducing its phosphorylation at threonine 58 (Thr58) to protect it from proteasome-dependent degradation [189].

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Furthermore, feedback regulation has also been discovered between MYC and lncRNAs, in a similar way as TGF-β and lncRNA. For example, ovarian adenocarcinoma-amplified lncRNA (*OVAAL*) which transcriptionally induced by MYC, upregulates MYC protein levels by enhancing binding between STK3 and RAF1 with serine/threonine-protein kinase 3 (STK3), thus *OVAAL* forms a positive feedback loop with MYC [190]. MYC transcriptionally inhibits the lncRNA *FGF13-AS1* which causes MYC mRNA degradation by disrupting the interaction between IGF2BP1 and *myc* mRNA, thus it exists a negative feedback loop between MYC and *FGF13-AS1* [191].

5. Smyca

5.1 The discovery of *Smyca*

LOC284454 is a cancer-associated long noncoding RNA and was re-named as *Smyca* (Smad/Myc coactivator) by our group based on its function. From NCBI BLAST analysis, *LOC284454* is located on human chromosome 19. It contains only one exon which is shared with microRNA cluster miR-23a~27a~24-2. Through the cleavage of this 2.2 kb primary transcript by nuclear RNase III Drosha, *Smyca* is separated from the upstream microRNA cluster (miR-23a~27a~24-2) to be a stable, unspliced and polyadenylated transcript with a length of 1.77 kb. Besides, evidence has demonstrated that sequence of miR-23a~27a~24-2 is highly conserved in all the mammalian species, but the sequence of *LOC284454* is highly conserved only in primate genomes, which implies that *LOC284454* has a functional role specially in primates [192].

5.2 *Smyca* in cancer

doi:10.6342/NTU202101996

The first study for *Smyca* identified it as a DDX5/p68 associated lncRNA. From real-time qPCR analysis in a small numbers of tumor samples compared to normal samples, they found that *Smyca* is significantly downregulated in breast, prostate, uterus and kidney cancer, while it is upregulated in colon and ovarian cancer. In addition, ectopically overexpressed *Smyca* affects the expression of some oncogenes in HEK293T cells, and downregulates focal adhesion genes as well as migration pathway genes in some breast cancer cells (MCF7 and T47D). Although *Smyca* is characterized as a cancer associated lncRNA, its functional role in cancer cells still need to further investigate [192].

Next, Chunmei et al. demonstrated a tumor-promoting role of *Smyca* in cancer. From analysis of GEO database, they found *Smyca* is highly expressed in serum of patients with nasopharyngeal carcinoma (NPC), oral cancer and thyroid cancer [193], and upregulation of *LOC284454* leads to poor prognosis in NPC. Besides, *Smyca* can promote the migration and invasion of NPC cells presumably by regulating the expression of certain proteins in Rho/Rac signaling pathway [194]. Moreover, Huimin et al. also supported the oncogenic role of *Smyca* by using integrative analysis. The result showed that *LOC284454* regulated by copy number variations (CNVs) contributes to poor prognosis in colorectal cancer [195]. In summary, emerging studies have shown that *LOC284454* involves in cancer progression and may serve as prognostic and diagnostic markers, but how *LOC284454* engages in cancer progression and its other functional mechanisms still need to be elucidated.

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II. Materials and methods

Cell culture

MDA-MB-231, Hs-578T, and M10 cell lines were cultured in Dulbecco's modified eagle medium (DMEM) containing 10% FBS at 37℃ in humidified incubator with 5% CO_2.

Plasmids

To establish stable *Smyca* knock-down clones, *Smyca* shRNAs were cloned into the pLKOAS1010 vectors (RNAi core, Academia Sinica, Taipei, Taiwan). To overexpress *Smyca*, the full-length transcript was amplified from the cDNA of BT474 cells and then cloned into transiently expressing pRK5F vector and lentivirus-based pLAS5W vector (RNAi core, Academia Sinica, Taipei, Taiwan).

Lentivirus transduction

To generate stable *Smyca* overexpressing and knockdown cell lines, lentivirus infection was used. First, prepare high titer of lentivirus stocks generated from HEK 293FT cells that co-transfected with the 14 μg pCMV-Δ8.91 packaging plasmid, 2 μg pMD.G envelope plasmids, and 14 μg *Smyca* or its shRNAs expressing constructs. At least 12 hours after transfection, the medium was refreshed for virus production. The virus supernatants were harvested after 48 hours of infection, and filtered by 0.45 μm syringe filter. To conduct efficient infection, breast cancer cells (MDA-MB-231 and M10) were infected with viral stock that was supplemented with 8 μg/ml polybrene. After 48 hours of infection, the infected cells were selected by appropriated antibiotic. The shRNA target sequences are presented in Table 1.

Table 1. The shRNA target sequences

shRNA	Target sequence
shLuc	CTTACGCTGAGTACTTCGAGTG

doi:10.6342/NTU202101996

shControl	CAACAAGATGAAGAGCACCAA
sh*Smyca*#1	GCATAGATAGGTGGGTGAGTG
sh*Smyca*#2	GCTGATGCTTGGAGCAGAGAT
sh*Smyca*#3	GGGCATGGAACAAGTTCCTTGTG
sh*Smyca*#4	GGCATGGAACAAGTTCCTTGT

Quantification of miRNA-23a, miR-24-2 and miR-27a

Total RNA was extracted by using Trizol (Invitrogen), and reverse transcription was carried out with Transcriptor first strand cDNA synthesis kit (Roche, USA). Reverse transcription reactions (10 μl) consisted of 20 ng RNA, 1x reverse transcriptase buffer, 5 units reverse transcriptase, 25 nM stem-loop RT primer, 0.5 mM dNTP mix, 10 mM DTT, 10 units RNase inhibitor. The reactions were incubated for 30 minutes at 16℃, followed by pulsed RT of 60 cycles at 30℃ for 30 seconds, 42℃ for 30 seconds, and 50℃ for 1 second. Reactions were terminated by incubating for 5 minutes at 85℃ to inactivate the reverse transcriptase. Gene expression levels were measured by real-time qPCR using Universal ProbeLibrary probe assay. For Universal ProbeLibrary probe assay, final reactions (20 μl) contained 0.5 μM of each forward and reverse primer, 0.1 μM Universal ProbeLibrary probe #21, 1x Light Cycler® TaqMan® Master, and cDNA (1 μl). PCR reactions were subjected to Light Cycler 480 II PCR detector system (Roche, Indianapolis, USA). The condition for qPCR reactions were as follow: 95℃ for 10 minutes, followed by 45 cycles of 95℃ for 5 seconds, 60℃ for 10 seconds, and 72℃ for 1 second. The quantification of each microRNA was achieved by expressing the abundance of each microRNA gene relative to that of miR-24 as an internal control gene. The sequences of stem-loop RT primer are presented in Table 2, and specific primer sequences of PCR are presented in Table 3.

Table 2. The stem-loop RT primer sequences

RT primer	Sequence

doi:10.6342/NTU202101996

miR-23a	GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCA ACGGAAAT
miR-24-2	GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCA ACCTGTGT
miR-24	GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCA ACCTGTTC
miR-27a	GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCA ACGCGGAA

Table 3. The miRNA target sequences of PCR

Gene	Primer	Sequence
miR-23a	Forward	GCGTCC ATCACATTGCCAGGG
miR-24-2	Forward	CGTCG TGCCTACTGAGCTGAA
miR-24	Forward	TTGGG TGGCTCAGTTCAGCAG
miR-27a	Forward	CGTTCCG TTCACAGTGGCTAAG
Universal	Reverse	GTGCAGGGTCCGAGGT

RNA extraction and RT-qPCR

RNA extraction from cultured cells was conducted by Trizol (Invitrogen). Reverse transcription to cDNA (complementary DNA) was carried out with iScriptTM cDNA synthesis kit (Bio-Red, Richmond, CA, USA) according to the manufacturers' protocol. Quantification of gene expression was conducted by real-time qPCR using the FastStart Universal SYBR Green Master reagent (Roche, Indianapolis, USA) then subjected to Light Cycler 480 II PCR detector system (Roche, Indianapolis, USA). All qPCR reactions were incubated 2 minutes at 94℃ followed by 50 cycles of 94℃ for 20 seconds, 60 ℃ for 30 seconds, and 72℃ for 30 seconds. Light Cycler 480 Gene Scanning Software was used for data analysis. GAPDH was served as internal control. The formulation to calculate gene expression levels was as follow: dCt = (Ct of target gene) – (Ct of internal control); ddCt = (dCt of sample) – (dCt of calibrator); relative RNA level

doi:10.6342/NTU202101996

= 2^{-ddCt}. The specific primer sequences of PCR are presented in Table 4.

Table 4. The specific primer sequences of PCR

Gene	Forward (5'-3')	Reverse (5'-3')
GAPDH	TGTTGCCATCAATGACCCCTT	CTCCACGACGTACTCAGCG
Smyca	TTGAATTCCTGGGCACAAGT	CACACCTGAGCCACCTGTAA
MMP2	CACCCTGGAGCGAGGGTAC	CTGATTAGCTGTAGAGCTGAAGGC
MMP9	TGTACCGCTATGGTTACACTCG	GGCAGGGACAGTTGCTTCT
Smad7	CCTTAGCCGACTCTGCGAACTA	TGCATAAACTCGTGGTCATTGG
FN1	CATCGAGCGGATCTGGCCC	GCAGCTGACTCCGTTGCCCA
c-Jun	GGAAACGACCTTCTATGACGA TGCCC	GGCGCGCACGAAGCCCTCGGCGAA CC

Luciferase reporter assay and transfection

$1.5x10^5$-$2.5x10^5$ cells with or without *Smyca* overexpressing were seeded in 6-well plates for 24 hours before transfection. For transfection, the report plasmid was co-transfected with pRK5-Renilla luciferase vector carried out with TransIT-X2® Dynamic Delivery System (Mirus Bio). 18-24 hours after transfection, cells were incubated in refreshed medium for 8 hours, then cultured in serum free medium for 12 hours for serum starvation. After serum starvation, cells were stimulated with 5 ng/ml TGF-β for 12 hours. For luciferase reporter assay, cells were lysed and measured by Dual-Glo™ Assay kit (Promega Corporation). The reporter activity was detected by multimode ELISA reader (Tecan M1000 Pro). Above procedure was conducted in triplicates.

Western blot and Smad2/3 phosphorylation assay

Cells were lysed with 1xRIPA lysis buffer (20 mM Tris-HCl (pH=7.5), 150 mM NaCl, 1% sodium deoxycholate, 0.1% SDS, and 1% NP-40) supplemented with protease inhibitors (1 mM PMSF, 1 μg/ml aprotinin, and 1 μg/ml leupeptin) and phosphatase inhibitors (20 mM sodium fluoride (NaF), 1 mM sodium vanadate (Na_3VO_4), and 4 mM

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sodium pyrophosphate (NaPPi)) on ice. Protein concentration was detected using bradford reagent, and diluted for quantification by sample buffer (50 mM Tris-HCl (pH=6.8), 10% glycerol, 2% SDS, 2.5% β-mercaptoethanol, and 0.02% bromophenol blue). The protein samples were separated on SDS-PAGE and blotted onto PVDF membranes (Millipore) which was activated with methanol. The membranes were blocked with blocking buffer (1xTBST (Tris-buffered saline with 0.1% Tween-20) with 1% w/v non-fat dry milk or 1% w/v BSA) at room temperature for at least 30 minutes. Thereafter, membranes were incubated with the appropriate primary antibody at 4℃ overnight or at room temperature for 2 hours. The membranes were washed three times in TBST, then incubated with appropriate secondary antibody (anti-mouse or anti-rabbit horseradish peroxidase(HRP)-conjugated secondary antibody) at room temperature for 1 hour. After washed with TBST for three times, the protein bands on membranes were visualized with Western Lightning Plus-ECL (PerkinElmer) or Luminata Crescendo Western HRP substrate (Millipore). The images of protein bands on the membranes were captured with hyperfilm (GE Healthcare Life Sciences) in dark room. The primary antibodies included: anti-GAPDH (GTX100118, GeneTex), anti-ZO-1 (GTX108613, GeneTex), anti-E-cadherin (ab40772, Abcam), anti-Twist1/2 (GTX127310, GeneTex), anti-Vimentin (AB1260, Sigma-Aldrich).

For Smad2/3 phosphorylation assay, cells stably expressing control or *Smyca* shRNAs (MDA-MB-231 or Hs-578T) were seeded in 10-cm dishes for 24 hours, then incubated in serum free medium for 16 hours. Thereafter, these cells were stimulated with 5 ng/ml TGF-β for 30 minutes before harvesting cells with 1xRIPA lysis buffer, then conducted the procedure of western blot mentioned above. The primary antibodies included anti-Smad2/3 (3102, Cell Signaling Technology), anti-phospho-Smad2 (Ser465/467)/Smad3 (Ser423/425) (8828, D27F4, Cell Signaling Technology).

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Co-immunoprecipitation (Co-IP) assay

Cells were lysed in 1xNP-40 lysis buffer (50 mM Tris (pH=7.5), 150 mM NaCl, 1% NP-40) supplemented with protease inhibitors and phosphatase inhibitors, and incubated on wave shaker at 4℃ for 20 minutes. After centrifugation, the lysates were pre-cleared with 10 μl of protein A magnetic beads (LSKMAGA10, Millipore) at 4℃ for 30 minutes. After quantification of lysate protein, the lysates containing equal amounts of proteins were incubated with the anti-c-Myc antibody (9402, Cell Signaling Technology) at 4℃ overnight with rotation. 10 μl of protein A magnetic beads were added in each sample to capture immunocomplex, and incubated at 4℃ for 2 hours with rotation. After incubation, the precipitate with protein A bead was washed 5 times with 1ml of 1xlysis buffer containing protease inhibitors and phosphatase inhibitors in each sample, then denatured the samples by boiling the precipitates in 60 μl sample buffer at 95-100˚C for 5 minutes. For immunoblot analysis, the primary antibodies included anti-Max antibody (4739, Cell Signaling Technology) and anti-c-Myc antibody (9402, Cell Signaling Technology), and EasyBlot anti-rabbit IgG (HRP) (GTX221666-01, GeneTex) was used as secondary antibody.

Statistical analysis

Statistical analyses were performed by GraphPad Prism for Windows version 6.0 (GraphPad Software Inc, San Diego, California) and values of data were shown as mean ± S.D. (standard deviation). Two-tailed, unpaired Student's t-test was used to compare between two groups and expressed as P values. P values of statistical significance are presented as *$p<0.05$, **$p<0.01$, and ***$p<0.001$.

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III. Result

1. *Smyca* shRNAs downregulate *Smyca* expression without affecting the expression of miRNAs in the miR-23a~27a~24-2 cluster

Previous studies identified *Smyca* as a cancer-associated lncRNA, but its function and underlying mechanism in cancer progression have not been completely understood [192, 194, 195]. Therefore, we decided to knock down the expression of *Smyca* by shRNAs which specifically target sequences of *Smyca*, for establishing *Smyca* stable knockdown lines in MDA-MB-231 breast cancer cells. RT-qPCR analysis showed that each of the four *Smyca* shRNAs efficiently decreased *Symca* expression (Figure 1A). However, since the microRNA cluster miR-23a~27a~24-2 resides upstream of *Smyca* in the same primary transcript, we needed to exclude the possibility that the expression of miRNAs in the miR-23a~27a~24-2 cluster is also affected by *Smyca* shRNAs. If this occurs, it would cause the problem that we can't distinguish the cellular responses are due to knockdown of *Smyca* or/and these miRNAs. Thus, we used RT-qPCR to check the expression of each miRNA in the miR-23a~27a~24-2 cluster. The result showed that the expression of miR-23a, miR-27a and miR-24-2 were not affected by *Smyca* shRNAs (Figure 1B). Thus, we will use these stable line to assess the effects of *Smyca* in the following experiments.

2. *Smyca* induces MET in breast cancer cells

Epithelial-mesenchymal transition (EMT) is an essential step of tumor metastasis [196]. Previous study in our laboratory found that *Smyca* is expressed at higher levels in mesenchymal-like than epithelial-like breast cancer cells. Furthermore, *Smyca* overexpression in epithelial-like breast cancer cell line MCF7 promotes EMT (Xinxin Liu, Master thesis). We therefore determined whether *Smyca* knockdown in mesenchymal-like breast cancer cells could lead to a transition to epithelial type. To test

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the hypothesis, we detected the expression levels of EMT markers under the effect of *Smyca* knockdown in MDA-MB-231 breast cancer cell lines. We found decreased expression of mesenchymal markers vimentin and twist and increased expression of epithelial markers E-cadherin and ZO-1 in *Smyca* knockdown MDA-MB-231 cell lines (Figure 2). This result indicated that downregulation of *Smyca* induces an MET in MDA-MB-231 cell lines.

3. The modulation of *Smyca* influences TGF-β downstream genes

The next question is how *Smyca* regulates EMT. Through RNA-seq analysis followed by bioinformatics analysis, our lab found that *Smyca*-regulated gene signature correlates TGF-β-regulated gene signature (Hsin-Yi Chen, unpublished data). To investigate whether *Smyca* regulates TGF-β-induced gene expression, we used normal mammary epithelial cells M10 overexpressing *Smyca* or control vector (Figure 3A) and treated with TGF-β. Then, we detected the expression of TGF-β downstream genes in these cells by RT-qPCR. In control cells, the expression levels of several Smad target genes, including *MMP9* and *MMP2*, were increased by TGF-β treatment. Notably, in *Smyca* overexpressing cells, these induction effects were significantly higher than the control group (Figure 3B). Next, we tested whether *Smyca* knockdown impairs TGF-β-induced gene expression. We found that TGF-β induced a higher level of *c-Jun* in MDA-MB-231 cells expressing control shRNA than those with *Smyca* shRNAs (Figure 4). Since MMP9 and MMP2 are known as hallmarks of EMT [5, 6], we propose that *Smyca* regulate EMT via TGF-β signaling pathway which serves as an important inducer of EMT. Besides, *Smyca* positively regulates the expression of TGF-β downstream genes.

4. *Smyca* promotes Smad-induced transactivaton without affecting Smad

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expression and phosphorylation

Next, we wanted to know which step in the TGF-β pathway is affected by *Smyca*. Luciferase reporter assays showed that the TGF-β-induced an activation of Smad-responsive reporter such as SBE 4x-Luc in control group. Meanwhile, the induction effects on *Smyca* overexpression group were significantly higher than control group (Figure 5). However, *Smyca* could not affect the expression or activation (phosphorylation) of Smads in *Smyca* knockdown cell lines (MDA-MB-231 and Hs-578T) under TGF-β treatment (Figure 6). These finding is consistent with the finding that *Smyca* is mainly expressed in nucleus (Xinxin Liu, Master thesis). Thus, *Smyca* acts as a positive regulator of TGF-β signaling by promoting Smad-mediated transactivation.

5. The positive feedback loop between *Smyca* and TGF-β signaling

Previous studies from our lab revealed that the expression of *Smyca* can be induced by TGF-β in several breast cancer cell lines (Appendix 1A, Xinxin Liu, Master thesis), and the induction effects of TGF-β treatment was attenuated with downregulated Smads in MDA-MB-231 cells (Appendix 1B, Xinxin Liu, Master thesis). Therefore, we proposed that TGF-β signaling engages in the activation of *Smyca*, and this process depends on Smads. Previously, our lab also found that *Smyca* can promote TGF-β-induced Smad3/4 complex formation by interacting with both Smad3 and Smad4 (Hsin-Yi Chen, unpublished data). We thus investigated whether Smyca can contribute to a positive feedback regulation of TGF-β signaling. RT-qPCR analysis showed that *Smyca* overexpression led to an increased amplitude and duration of TGF-β-induced gene expression, compared to the control group (Figure 7). Hence, TGF-β can induce *Smyca* expression through canonical pathway, and *Smyca* further promotes and extends TGF-β signaling. Altogether, our study indicates that *Smyca* is involved in a positive feedback

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loop to enhance TGF-β signaling.

6. *Smyca* does not affect Myc-Max binding

In addition to regulating TGF-β signaling, *Smyca* also promotes Myc signaling. Our lab found that Smyca binds Myc to promote its ability to induce gene expression (Hsin-Yi Chen and Shu-Jou Chan, unpublished data). Since MYC requires the formation of a stable heterodimer with MAX to induce gene expression, we decided to study whether *Smyca* regulates the transcription of Myc target genes by promoting Myc-Max complex formation [139]. Through immunoprecipitation analysis on *Smyca* knockdown or *Smyca* overexpressing MDA-MB-231 cells, we found that Myc-Max binding could not be affected by *Smyca* upregulation or downregulation (Figure 8). These data suggest that *Smyca* uses other mechanism to promote Myc-induced gene expression.

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IV. Discussion

We show that *Smyca* shRNA does not affect the expression of miRNAs in the miR-23a~27a~24-2 cluster, even though they are derived from the same primary transcript. This finding is conceivable based on the action place of RNA interference. The cellular processes of shRNAs are similar with miRNAs biogenesis. In the nucleus, shRNAs are first processed into pre-shRNA via the RNase III enzyme Drosha and the double-stranded RNA binding domain protein DGCR8. Subsequently, the pre-shRNA is exported to cytoplasm by Exportin 5, and turns into siRNA via Dicer cleavage. In the cytoplasm, this siRNA is incorporated into the RNA-induced silencing complex (RISC), then remains its antisense strand which guides RISC to the target mRNA that has complementary sequence for silence, while its sense strand is degraded [197]. Thus, the action place of RNA interference is in the cytoplasm. The primary transcript of *Smyca* which harbors microRNA cluster miR-23a~27a~24-2, is cleaved by Drosha, which separates the sequence of *Smyca* and miR-23a~27a~24-2 [192]. Since this process occurs before nuclear export of the cleavage products, shRNAs, which is designed by the sequence of *Smyca*, can only can target the mature transcript of *Smyca* in the cytoplasm, whereas the miR-23a~27a~24-2, which is already separated from *Smyca*, can evade the effect of shRNAs.

Our lab found that *Smyca* is mainly expressed in nucleus (Xinxin Liu, Master thesis). So, how could *Smyca* be targeted by shRNAs? We postulate that *Smyca* shuttles between cytoplasm and nucleus, which is consistent by a small fraction of *Smyca* in the cytoplasm. Once in the cytoplasm, it is silenced by shRNAs. However, since the majority of *Smyca* resides in the nucleus, the silencing efficiency is low. To establish stable *Smyca* knockdown lines with good efficiencies, Dr. Hsin-Yi Chen used double lentivirus

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infection to improve the knockdown efficiency.

Previous studies have demonstrated that EMT contributes to cancer metastasis by promoting cell migration and invasion [196]. In our study, we found that the mesenchymal-like breast cancer cells, MDA-MB-231, induce MET under the effect of *Smyca* knockdown (Figure 2). Moreover, we found downregulation of cell migration and invasion ability in *Smyca* knockdown MDA-MB-231 cell lines (Xinxin Liu, Master thesis). In line with other findings, other groups demonstrated that *Smyca* promotes migration and invasion of nasopharyngeal carcinoma cells [194] and hepatocellular carcinoma cells [198]. However, Monalisa et al. reported the opposite result that ectopic overexpression of *Smyca* in MCF7 and T47D breast cancer cell lines decreases cell migration and proliferation. The cause of the discrepancy between this report and all other studies is unclear. However, in the study by Monalisa et al., they evaluated the migration ability by wound healing assay, but *Smyca* overexpression cells display a decreased proliferative rate compared with control cells. Thus, the lower ability of wound healing in *Smyca* overexpression cells might be due to the decreased proliferative rate [192].

LncRNAs play important roles in cancer progression via diverse molecular functions, and these lncRNAs can be classified as nuclear and cytoplasmic lncRNAs [199]. Nuclear lncRNAs engage in transcriptional and post-transcriptional regulation by functioning as decoy, scaffold, and guide [200], while cytoplasmic lncRNAs can control translation, mRNA degradation, miRNA sequestration and mRNA decay [201]. We found that *Smyca* regulates EMT through positive regulation of TGF-β signaling pathway in breast cancer cells. However, what is the underlying mechanism for this regulation? Studies in our lab found that *Smyca* can promote TGF-β-induced Smad3/4 complex formation by

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interacting with both Smad3 and Smad4 (Hsin-Yi Chen, unpublished data). In addition, chromatin isolation by RNA purification (ChIRP) and chromatin immunoprecipitation (ChIP) analyses revealed that *Smyca* is loaded to the chromatin of many Smad target loci, thereby enhancing the recruitment of Smad3/4 (Hsin-Yi Chen, unpublished data). According to these results, we conclude that *Smyca* functions as a RNA scaffold that directly binds to Smad3 and Smad4 for stabilizing the Smad3/4 complex and a guide that brings Smad3/4 complex to the corresponding sites of chromatin. Nevertheless, we do not exclude the possibility that Smyca may help the recruitment of other Smad partners [202], such as transcription factors (e.g. AP-1, IRF-7) or co-activators (e.g. p300, CBP), or chromatin modifiers to Smad target loci to potentiate the transcription function of Smad. Future study will be needed to test these possibilities.

Emerging studies have suggested that some oncogenic lncRNAs can drive positive feedback regulations which not only maintain active transcription of themselves, but also prolong some distinct pathways that contribute to cancer progression. For example, the positive feedback loop between lncRNA *NEAT1* and STAT3 promotes cell proliferation and cell cycle progression of breast cancer cells, since *NEAT1* acts as a ceRNA that sponges miR-124 (a tumor suppressor that inhibits STAT3), and the constitutively activated STAT3 serves as a transcriptional factor to promote *NEAT1* transcription via binding to *NEAT1* promoter region [203]. The lncRNA *PVT1* facilitates gastric cancer tumor growth and metastasis by directly binding and stabilizing FOXM1 protein (a critical proliferation-associated transcription factor), and FOXM1 in turns transactivates the promoter activity of *PVT1* [204]. Besides, some oncogenic lncRNAs can simultaneously involve in various pathways for tumor progression, such as lncRNA *PVT1*. In addition to FOXM1, c-Myc protein also serves as another transcription factor to

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enhance *PVT1* transcription, and *PVT1* prevents c-Myc from degradation via blocking the phosphorylation site (Thr58) of c-Myc, thus form an another positive feedback loop to maintain excessive c-Myc for promoting tumor growth [205]. On the basis of these studies, we uncovered the positive feedback loop between *Smyca* and TGF-β signaling pathway in breast cancer cells, which leads to prolonged TGF-β signaling transduction by promoting Smad3/4 complex formation via *Smyca*, and constitutive expression of *Smyca* that transactivated through TGF-β/Smad signaling stimulation (Figure 9).

What is the importance for the role of Smyca in contributing to a feedback control of TGF-β signaling? A previous study reported a differential responses of cells to TGF-β treatment at early and late time points. At the late phase, Smad2/3 complex tends to bind AP-1 component JUNB which is itself a TGF-β target. JUNB can redirect Smad2/3 to different target sites on DNA sequence, thereby selectively activating TGF-β-induced genes that promotes EMT and invasion of cancer cells [206]. Based on this finding, we postulate that the ability of *Smyca* to prolong TGF-β signaling would play a role in switching the dichotomous functions of TGF-β signaling in cancer progression into tumor promoting. Besides, Smad7 is known as an inhibitor Smad (I-Smad) for termination of TGF-β signaling [207]. In our study, the expression of Smad7 was enhanced in *Smyca* overexpression group under TGF-β treatment, but the amplitude and duration of TGF-β signaling in *Smyca* overexpression group wasn't reduced as treatment time increased (Figure 7). Thus, we assume that *Smyca* leading effect on TGF-β pathway may outcompete the inhibitory effect of Smad7 on TGF-β/Smad signaling transduction.

As an oncogenic lncRNA, *Smyca* also involves in the other pathway, MYC. Our lab found *Smyca* induces the expression of MYC target genes through direct interaction with MYC

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(Hsin-Yi Chen and Shu-Jou Chan, unpublished data). Given that MYC requires MAX to form a stable heterodimer for induction of target genes expression, we assumed that *Smyca* may promote the interaction of MYC and MAX to upregulate the transcription of MYC target genes [139]. However, we found that MYC-MAX binding could not be affected by *Smyca* upregulation or downregulation (Figure 8). Rather, studies in our lab found that Smyca is loaded onto many Myc target loci and *Smyca* knockdown significantly decreases the association between Myc and its binding site on DNA sequence (Hsin-Yi Chen and Shu-Jou Chan, unpublished data). These data indicate that *Smyca* may serve as a guide to promote Myc binding to its responsive promoters (E-box), thus promoting MYC target genes transcription. Nevertheless, whether *Smyca* can affect the binding of Myc partners including co-activators (e.g. TRRAP, GCN5, p300/CBP) and co-repressors (e.g. HDAC3, MIZ-1, WDR5) [208], warrants for further analysis.

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V. Figures

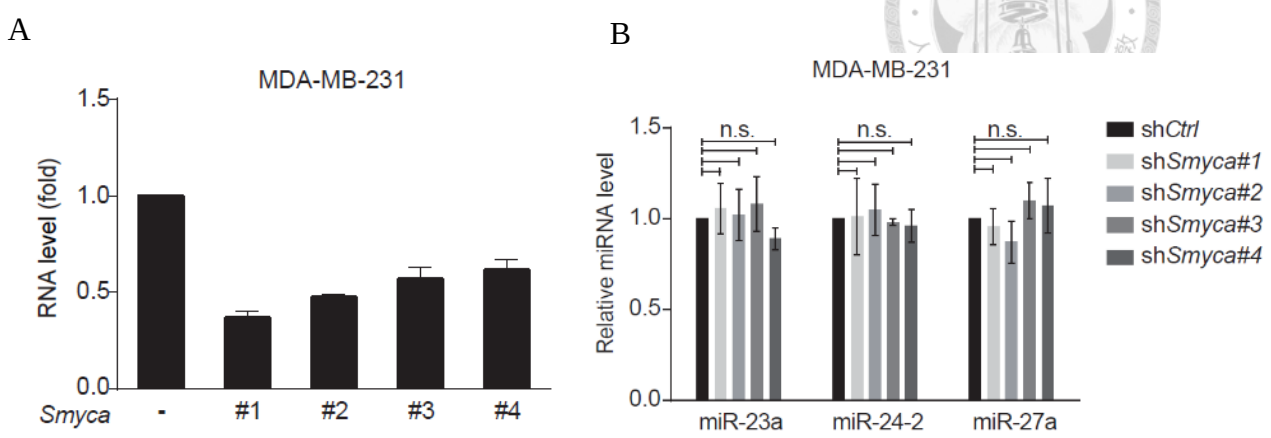

Figure 1. Knockdown of *Smyca* doesn't affect the expression levels of miR-23a~27a~24-2 cluster

(A) RT-qPCR analysis of knockdown efficiency of *Smyca* in MDA-MB-231 cells

Data in both panels are mean ± S.D., n=3. P values are determined by unpaired t-test, n.s. not significant.

(B) The expression levels of indicated miRNAs in the miR-23a~27a~24-2 cluster in MDA-MB-231 cells stably expressing control or *Smyca* shRNAs are analyzed by RT-qPCR

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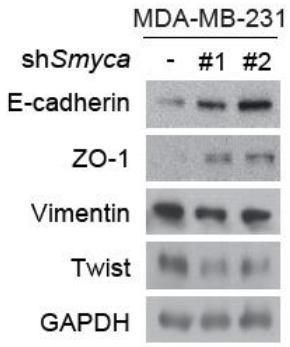

Figure 2. Knockdown of *Smyca* induces MET.

The protein levels of four EMT markers were analyzed by Western blot in MDA-MB-231 cells stably expressing control or *Smyca* shRNAs.

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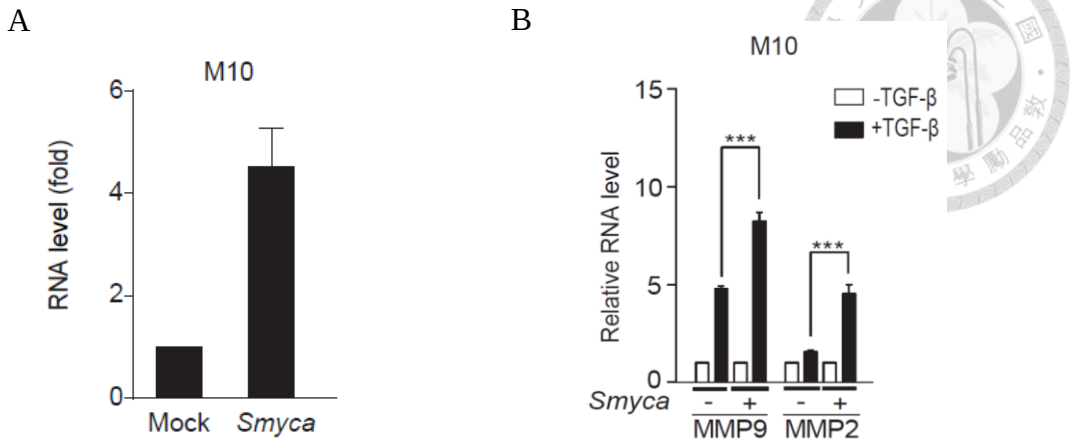

Figure 3. Overexpression of *Smyca* promotes the expression of Smad target genes.

(A) *Smyca* expression levels in indicated M10 stable lines. Data are mean±S.D., n=3.

(B) The expression levels of Smad target genes were analyzed by RT-qPCR in M10 cells stably expressing control vector or *Smyca* and treated with 5 ng/ml TGF-β for 24 h. Data are mean±S.D., n=3, ***p<0.001 by unpaired t-test.

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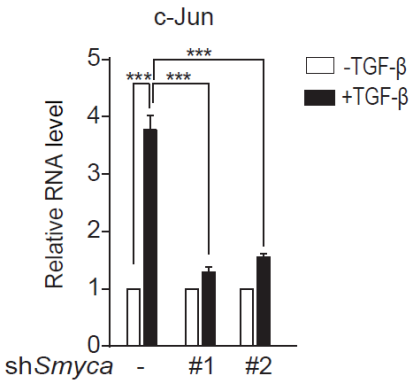

Figure 4. Knockdown of *Smyca* reduces the expression of Smad target gene c-Jun.

The expression levels of c-Jun were analyzed by RT-qPCR in MDA-MB-231 cells stably expressing control or *Smyca* shRNAs and treated with 5 ng/ml TGF-β for 24 h. Data are mean±S.D., n=3, **p<0.01, ***p<0.001 by unpaired t-test.

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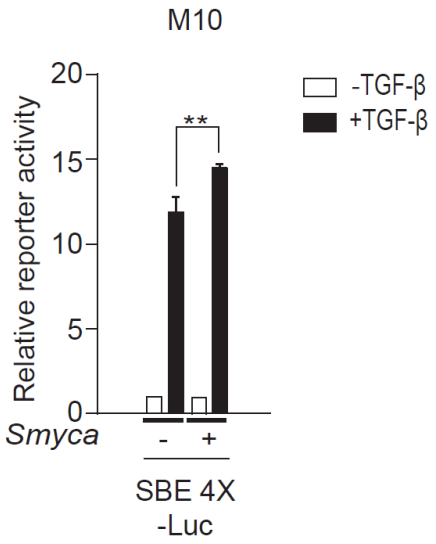

Figure 5. Overexpression of *Smyca* promotes the activity of Smad-responsive reporters.

Luciferase activity assays using M10 cells stably expressing control vector or *Smyca*, transfected with indicated Smad-responsive reporter and treated with 5 ng/ml TGF-β for 12 h. Data are mean±S.D., n=3, **p<0.01 by unpaired t-test.

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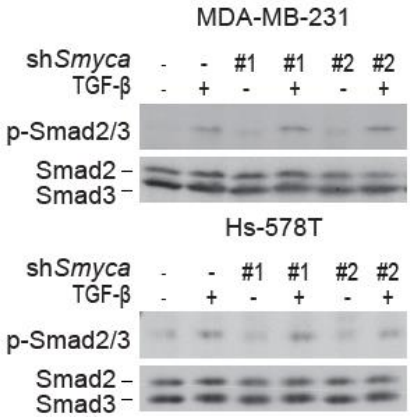

Figure 6. Knockdown of *Smyca* doesn't affect Smad2/3 phosphorylation.

The expression levels of Smad2, Smad3 and p-Smad2/3 were analyzed by Western blot in MDA-MB-231 cells or Hs-578T cells stably expressing control or *Smyca* shRNAs and treated with 5 ng/ml TGF-β for 0.5 h.

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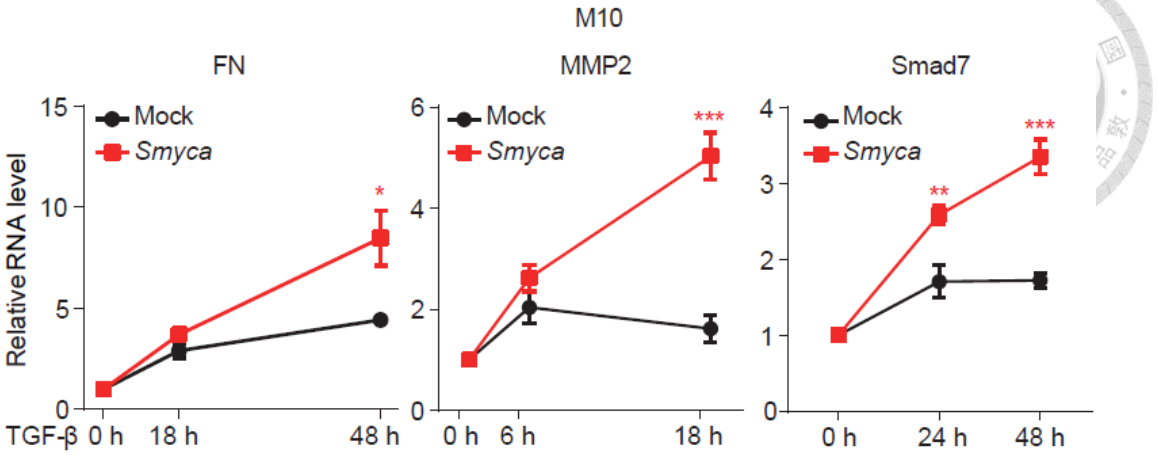

Figure 7. *Smyca* enhances the amplitude and duration of TGF-β induced signaling.

The expression levels of TGF-β induced genes were analyzed by RT-qPCR in M10 cells infected stably expressing control vector or *Smyca* and treated with 5 ng/ml TGF-β for indicated time periods. Data are mean±S.D., n=3, *p<0.05, **p<0.01, ***p<0.001 by unpaired t-test.

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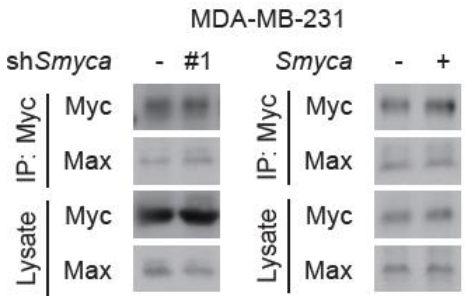

Figure 8. *Smyca* doesn't affect Myc-Max binding.

Immunoprecipitation analysis of *Smyca* knockdown or *Smyca* overexpression MDA-MB-231 cells followed by immunoblot analysis.

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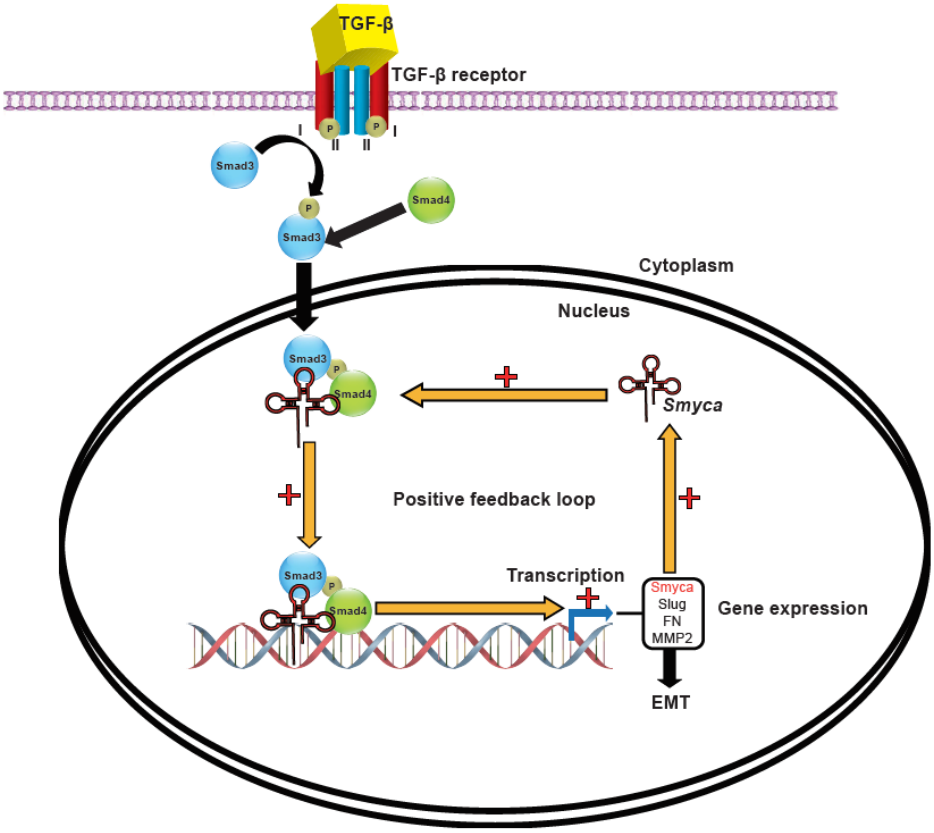

Figure 9. The feedback mechanism for *Smyca* in TGF-β pathway

Smyca plays a key role in positive feedback regulation of TGF-β pathway to promote EMT.

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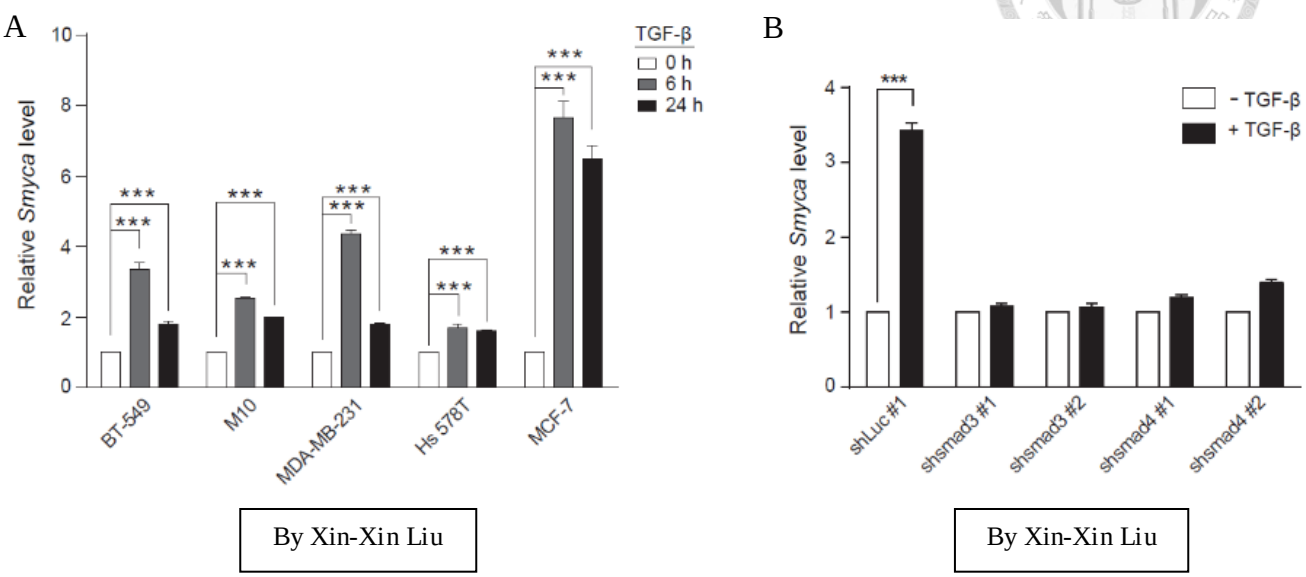
VI. Appendix



Appendix 1. *Smyca* is induced by TGF-β signaling through Smad3/4

(A) RT-qPCR analysis of *Smyca* expression indicated breast cancer cells treated with 5 ng/ml TGF-β for 0, 6, 24 h. Data are mean±S.D., n=3, ***p<0.001 by unpaired t-test.

(B) RT-qPCR analysis of *Smyca* expression in MDA-MB-231 cells stably expressing control, Smad3, or Smad4 shRNAs and treated with 5 ng/ml TGF-β for 0 or 24 h. Data are mean±S.D., n=3, ***p<0.001 by unpaired t-test.

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