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MECHANISMS OF CYR61 MEDIATED BREAST CANCER METASTASIS TO THE LUNG

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et de médecine

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MECHANISM OF CYR61 MEDIATED BREAST CANCER METASTASIS TO THE LUNG

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pour La Doyenne
de la Faculté de Biologie et de Médecine

Prof. Chin-Bin Eap

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Summary

CYR61 (Cysteine-rich angiogenic inducer 61) is a matricellular protein that regulates cell proliferation, adhesion, migration and cell survival through interaction with various types of integrin cell adhesion receptors. At tissue level it is implicated in the regulation of embryonic development, wound healing and angiogenesis. CYR61 has also been involved in cancer progression, however its role appears to be diverse and complex depending on the cancer type and stage. Its contribution to metastasis formation is still unclear. Previous findings reported by our laboratory demonstrated that CYR61 cooperates with $\alpha_v\beta_5$ integrin to promote invasion and metastasis of cancers growing in a pre-irradiated microenvironment.

In this work, we used an orthotopic model of breast cancer to show for the first time that silencing of CYR61 in breast cancer cells suppresses lung metastasis formation. Silencing of MDA-MB-231 reduced both local growth and lung metastasis formation of tumor cells implanted in a pre-irradiated mammary fat pad. CYR61 silencing in tumors growing in non-irradiated mammary fat pads did not impact primary tumor growth but decreased lung metastasis formation. The effect of CYR61 on spontaneous lung metastasis formation during natural cancer progression was further examined by using an experimental model of metastasis. Results from these experiments indicate that CYR61 is critically involved in promoting cancer cells entry into lung parenchyma rather than later steps of colonization. *In vitro* experiments showed that CYR61 promotes tumor cell spreading, migration and transendothelial migration. CYR61 also supported colony formation under anchorage-independent condition and promotes resistance to anoikis through the involvement of $\beta1$ and $\beta3$ integrin. These results indicate that CYR61 promotes lung metastasis of breast cancer by facilitating extravasation into lung parenchyma through enhanced motility, transendothelial migration and resistance to anoikis.

Résumé

CYR61 (Cysteine-rich angiogenic inducer 61) est une protéine matricellulaire qui régule la prolifération, l'adhérence, la migration et la survie des cellules par son interaction avec différents types de récepteurs d'adhésion cellulaire de la famille des intégrine. Au niveau des tissus, CYR61 est impliquée dans la régulation du développement embryonnaire, de la cicatrisation et de l'angiogenèse. CYR61 a également été impliquée dans le cancer, mais son rôle semble être divers et complexe en fonction du type du cancer et de son stade. Son rôle dans la formation des métastases n'est pas encore clair. Des résultats antérieurs rapportés par notre laboratoire ont montré que CYR61 coopère avec l'intégrine avß5 pour favoriser l'invasion et la métastase de tumeurs se développant dans un micro-environnement pré-irradié.

Dans ce travail, nous avons utilisé un modèle orthotopique de cancer du sein pour démontrer pour la première fois que l'extinction (*silencing*) du gène CYR61 dans le cancer du sein réduit la formation de métastases pulmonaires. L'extinction de CYR61 dans la lignée cellulaire de cancer du sein humain MDA-MB- 231 réduit à la fois la croissance local ainsi que la formation de métastases pulmonaires à partir de cellules implantés dans les coussinets adipeux mammaires pré-irradié. L'extinction de CYR61 dans des tumeurs grandissant dans les coussinets adipeux mammaires non irradiées n'a pas d'incidence sur la croissance tumorale primaire mais réduit la formation des métastases pulmonaires. Par la suite nous avons examiné l'effet de CYR61 sur la formation de métastases pulmonaires en utilisant un modèle expérimental de métastase. Les résultats de ces expériences indiquent que CYR61 est impliquée de manière cruciale dans les étapes précoces de la formation de métastases, plutôt que dans les étapes tardives de colonisation du poumon. Des expériences *in vitro* ont montré que CYR61 favorise l'étalement, la migration et la transmigration endothéliale des cellules tumorales. CYR61 favorise également la formation de colonies dans des conditions indépendante de l'ancrage et la résistance à l'anoïkis par l'engagement des intégrines β1 et β3. Ces résultats indiquent que CYR61 favorise les métastases pulmonaires du cancer du sein en facilitant

l'extravasation dans le parenchyme pulmonaire grâce à la stimulation de la motilité, de la migration transmigration endothéliale et de la résistance à l'anoïkis.

Introduction

1. Cancer as a major cause of deaths

Cancer is one of the major causes of death throughout the world. From the reports of the World Health Organization (WHO) and of the International Agency for Research on Cancer in 2008, cancer claimed 14% of adult lives aged between 30 to 70, which corresponds to about 20% of all deaths [1]. The latest GLOBOCAN 2012 report further reported an increasing incidence of cancer and deaths caused by cancer, with the numbers of 12.7 million and 7.6 million, respectively in 2008, rising to 14.1 million and 8.2 million in 2012 [2].

According to this report, the percentage of death caused by cardiovascular diseases, diabetes, and chronic respiratory conditions dramatically decreased with the economical development, while death caused by cancer showed no significant reduction in high-income area. This observation calls for an urgent need to improve our current knowledge of cancer initiation and progression as well as develop novel clinical therapies.

2. Cancer development and progression

Cancer arises from normal tissues through a complex, multistage process. In 2000, six hallmark capabilities of cancer cells were proposed by Hanahan and Weinberg in order to describe comprehensively how normal cells become transformed and progressively acquire a cancerous phenotype. These hallmarks include self-dependence on growth signaling, insensitivity to anti-growth signals, unlimited replication potential, escape from cell death, induction of angiogenesis, and activation of mechanisms of local invasion and distant metastasis formation [3]. These traits depict how cancer cells generate a locally growing tumor and develop the ability to migrate to secondary organs. Moreover, results from further investigations during the following decade have suggested two additional capabilities of cancer cells: reprogramming cellular metabolism and escaping from immune surveillance [4].

Reprogrammed cellular metabolism provides cancer cells with an alternative way to acquire energy and macromolecules required for rapid proliferation [5]. At the same time, cancer cells also develop diverse escape mechanisms to avoid attack and clearance from immune system [6,7].

Underlying these hallmarks are two enabling characteristics. First, heritable changes in DNA sequence (i.e. mutations,) epigenetic modifications and genomic instability allow cancer cells to cumulate alterations in gene expression, which in turn favor their own expansion. This is achieved by activating oncogenes, inactivating tumor suppressor genes, abrogating genome maintenance / DNA repair systems and accelerating the generation of further mutations [8,9].

The second characteristic is tumor-promoting inflammation. Besides the ability to escape from detection and clearance by the immune system, cancer cells can in turn utilize the immune system to their advantage by recruiting specific immune cells to the tumor tissues, mostly innate immune cells, and educate them to their needs. These tumor-promoting immune cells provide cancer cells with communication molecules (i.e. cytokines, growth factors, chemokines), matrix proteins and matrix degrading enzymes that facilitate cancer cell growth, survival, invasion and dissemination [10-12].

3. Tumor microenvironment

Besides the tumor-promoting immune inflammatory cells, there are also other types of stromal cells, which have been shown to promote cancer progression. They include cancer-associated fibroblasts, endothelial cells, pericytes, and progenitor cells as the origin of stroma cells [13,14]. Cancer cells and stromal cells interact reciprocally by direct physical interaction or though the release of soluble factors, matrix proteins and matrix-degrading enzymes, further modifying the whole microenvironment and promoting tumor progression.

Cumulating data indicate that cancer cells actively recruit cells from neighboring tissue or distant sites, in particular from the bone marrow, to constitute a microenvironment that favors cancer development. In turn, tumor microenvironment-derived stimuli affect cancer cells by modulating their gene expression and behavior.

4. Metastasis

Cancer cells can leave the primary tumors to form secondary tumors at distant sites, so called metastases. Compared with the bulk of cells in the primary tumor, metastatic cells have a stronger ability to migrate to and adapt to conditions present in the novel tissue environment. Metastatic cancer cells are also more resistant to traditional chemotherapy drugs, which makes it more difficult to target them [15]. Eventually, metastasis is the primary cause of death for about 90% of cancer patients [16]. In spite of its clinical relevance, our knowledge of metastasis formation is still incomplete. The conceptual understanding of molecular mechanisms of metastasis, as a basis for the development of novel therapeutic strategies, still needs to be improved. Metastasis, like the process of cancer progression, also develops as a multistep process (Fig.1) [17].

4.1. Local invasion

The ability to migrate and invade into surrounding tissue is a critical characteristic of the transition from a benign tumor to a malignant cancer. In general, tumor cells have to first disassemble junctional complexes between neighboring cells, digest underlying matrix substrates and connective tissues at the outer layer of the tumor mass, acquire the ability to rearrange the cytoskeleton and migrate following chemotactic gradients or mechanical flow. Several mechanisms have been discovered for cancer cells to undergo local invasion. Considering that 90% of cancers are derived from poorly motile epithelial tissues, the most prevailing model is the so-called “epithelial to mesenchymal transition” (EMT). This model describes the genetic,

morphological and functional alterations which turn tightly-bound epithelial cells into motile and more aggressive mesenchymal-like cells [18].

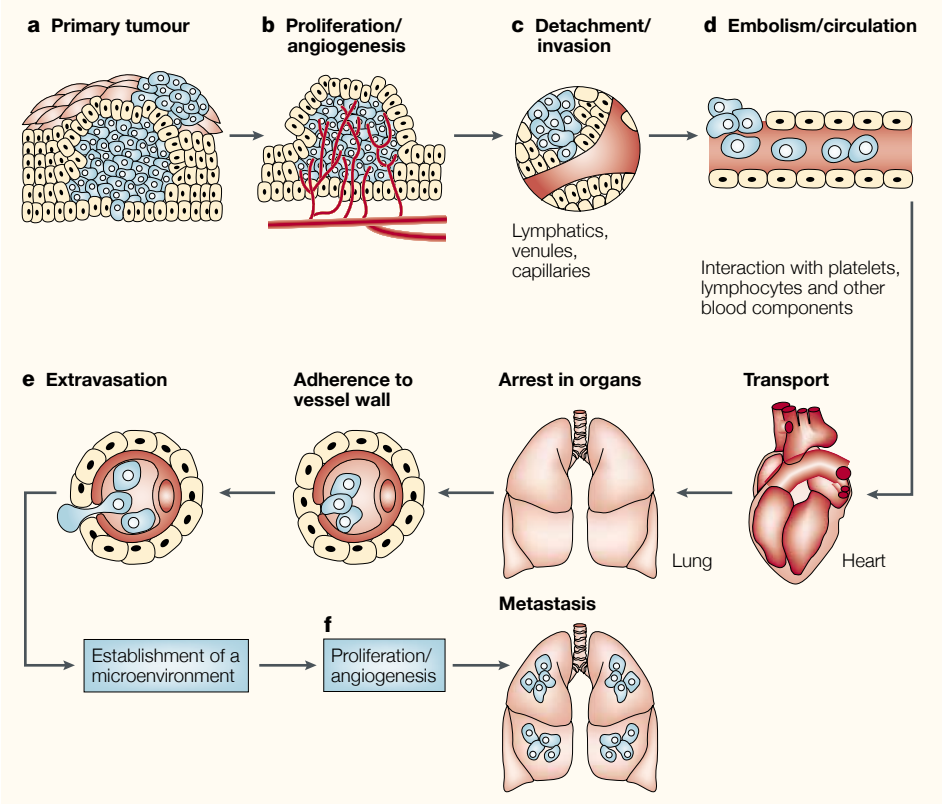

Figure 1. Schematic illustration of metastasis cascade. From [17]

The development of metastasis could be dissected into multiple steps. With expanding primary tumor masses (a), tumor cells initiate angiogenesis (b) to enrich their supply of oxygen and nutrients. Some tumor cells acquire higher mobility, digest the surrounding matrix to start local invasion (c), and then enter into circulation through blood/lymphatic vessels (d). Disseminated tumor cells inside circulation interact with platelets and lymphocytes, and have to escape from immune surveillance and detachment-induced cell death (anoikos) (d). Tumor cells arrive at the secondary organs then penetrate the vessel wall (extravasation) (e), and adapt to the local microenvironment to eventually develop metastasis (colonization) (f).

4.1.1. EMT in cancer progression

EMT is defined as a transition process by which epithelial cells lose intercellular junctions, diminish apical-lateral polarity, repress expression of epithelial markers (i.e. E-cadherin, ZO-1), upregulate mesenchymal markers (i.e. N-cadherin, vimentin, fibronectin), reorganize cytoskeleton and acquire

spindle-shaped morphology as well as migratory ability. To date, TGFβ has been recognized as the most potent molecule in orchestrating EMT [19,20]. Globally, many signaling pathways can contribute to EMT. They include Wnt-, Notch-, Hedgehog-, Ras-, NFκB- dependent pathways. Many growth factors frequently utilized by cancer cells to escape from growth control or acquire malignant traits, such as invasion and metastasis, are also implicated in the regulation of EMT (i.e. EGF, FGF, HGF, IGF, PDGF) [21].

EMT has been originally recognized as an important step during early embryogenesis, such as gastrulation and neural crest cell migration. In adulthood, EMT may occur during tissue repair, wound healing or fibroblast generation from epithelial cells [22]. During cancer progression, EMT confers to tumor cells the capability of breaking cell-cell adhesion to acquire motility. Some EMT-associated genes have been identified to contribute to intravasation and extravasation [21]. However, it is still controversial whether EMT takes place during later stages of metastatic cascade, and whether tumor cells could really reach target organs to form metastasis with a mesenchymal phenotype. The main argument challenging this notion comes from histopathological evidence that metastases often display epithelial morphology similar to their primary tumors, and that one of the epithelial markers, cytokeratin, is often used for detecting metastatic carcinoma cells within distant organs. To solve this apparent contradiction, three models were further proposed: (1) incomplete EMT, (2) reversibility of EMT at metastatic site (i.e. mesenchymal-epithelial transition, MET), and (3) collective migration of cells retaining epithelial morphology [23]. These hypotheses highlight the importance to understand epithelial plasticity and reversible transition between epithelial and mesenchymal phenotypes during metastasis.

4.2. Angiogenesis and intravasation

The concept of tumor angiogenesis is based on the observation that growing tumors can induce the formation of neo-vessel to improve the supply of oxygen and nutrients, compared to passive diffusion. Blood flow also brings

diverse signaling molecules to tumors, which might trigger the cancer cells to invade through basement membrane and endothelial layer of vessel wall to penetrate into the circulation system. In addition to blood vessel systems, recent studies have also recognized the lymphatic vessel system as an alternative route for cancer cells to leave the primary tumor and to metastasize to distant organ.

4.3. Survival in the circulation

Before reaching the target organs, intravasated and circulating cancer cells have to escape from anoikis (i.e. suspension-induced cell death), mechanical damages by shear stress, and attack from the immune system. Investigations on these circulating tumor cells (CTC) are relatively rare compared to studies on the other steps of metastasis, mostly due to the limitation of detection techniques for CTCs. However, it has been shown that tumor cells in the circulation can associate with platelets and benefit from their protection from shear stress damage and immune surveillance [24]. Moreover, platelets also become activated through interaction with tumor cells and release growth factors or signaling molecules to induce growth pathways or to sustain EMT programs in cancer cells [25,26]. On the other hand, several molecular mechanisms used by cancer cells to escape from anoikis have also been proposed, including caspase-dependent intrinsic/extrinsic pathways regulated by Bcl-2 family proteins [27], and a novel caspase-independent pathway mediated by the balance between Bit1 and TLE-1 [28,29].

4.4. Extravasation

When reaching the target organ, circulating cancer cells have to adhere to the inner endothelial layer and then penetrate the vessel wall to reach the underlying parenchyma. Like the process of intravasation, the mechanisms adopted by cancer cells to extravasate are not yet well understood.

4.5. Colonization

In the secondary organ, there are still several critical hurdles that the extravasated cancer cells have to overcome before they could eventually develop metastases. First of all, cancer cells are residing in a new microenvironment composed of ECM proteins and stroma cells different from those at the primary site. It has been proposed that the different microenvironment might be responsible for the death of most of the disseminated cancer cells in a process that may resemble anoikis. Therefore, these cancer cells have to maintain, at least initially, their survival and self-renewal capacity without the support from the original microenvironment. Besides evading the recognition and clearance from immune system, they also activate and differentiate immune/inflammatory cells into tumor-promoting cells. For example, bone marrow derived cells (BMDC) have now been proposed to play a supportive role at the metastatic site. The characterization of cancer-promoting type 2 macrophages (M2) and neutrophils revealed that these immune/inflammatory cells could be further educated by metastatic cancer cells to support their survival and stemness features upon dissemination. Studies demonstrated that some primary tumors can promote the recruitment of BMDC to pre-metastatic sites before the arrival of metastatic cancer cells, thereby preparing a better soil for cancer cells to seed and form metastasis [30,31].

4.6. From micro-metastasis to macro-metastasis

Not all newly formed micro-metastases are able to form macro-metastases. Some micro-metastases become dormant and some eventually disappear. These dormant micro-metastases can stay latent from months to decades. How dormant cells adapt to the microenvironment and reactivate under such quiescent status is not well understood. Although genome instability is a hallmark of cancer, reactivation of dormant cells driven by genetic mutation is unlikely to occur in the absence of sustained replication [32]. Therefore, it is proposed that dormant disseminated cancer cells acquire proliferative capacities through signals generated from the interaction with the

microenvironment. For instance, the matrix proteins tenascin-C and periostin, secreted by metastatic cancer cells and stromal fibroblasts, were shown to promote outgrowth of micro-metastases through Notch, Wnt and integrin pathways eventually leading to the formation of macroscopic lesions [33-35]. In the end, these macro-metastases will become novel sources of cancer cell spreading into the circulation to form tertiary metastases or seed back to the primary tumor site [31].

Each step of the metastatic cascade could be potentially rate limiting during the process of metastasis formation. In fact, formation of metastasis is extremely inefficient. Results from injecting intravenously radiolabeled melanoma cells to mice showed that only 0.1% of injected cells were still alive 24 hours after injection, and less than 0.01% of the injected cells could successfully develop lung metastasis [36]. Another study of melanoma metastasis to liver showed that although 80% of injected cells extravasated into the liver by day 3, only 2% of the cells were able to initiate the formation of micro-metastases. On day 13, most of the micro-metastases failed to maintain cell growth and only 0.018% of injected cells could finally generate macro-metastases [31,37].

5. Metastasis organ tropism - Seed and soil theory

The multistep progression of metastasis suggests that cancer metastasis is a highly selective process rather than a random event. Based on both clinical and experimental observations, it appears that some tumor types tend to metastasize to specific organs. This phenomenon has been referred to as “metastasis organ tropism”. For example, breast cancer cells tend to metastasize to lung, bone, liver and, in a more advanced stage, to brain [38]. Bone metastasis is frequently diagnosed among prostate cancer patients [39] and brain metastasis in lung cancer patients [40]. In contrast, melanoma metastatizes to multiple organs, in particular to skin, lung, brain and liver [41].

The concept of organ tropism has been originally proposed by Stephan Paget in 1889 and published as the “seed and soil” hypothesis [42]. Based on autopsy of female breast cancer patients, he found a tendency of metastasis to seed to liver, bone, and ovary instead of spleen, which contradicted the theory that metastasis simply develops following the mechanical arrest of cancer cells in vessels based on the anatomy of the blood supply. In his theory, tumor cells behave like “seeds”, which can only form metastasis if they disseminate in the appropriate “soil”, standing for selected organs in this metaphor [43].

To date, this theory has been widely accepted and supported by numerous experimental data. In particular, Filder and co-workers have shown that inoculating B16 melanoma cells into mice generated metastatic lesions in pulmonary and ovarian tissues engrafted into muscle, while implanted renal tissue showed no metastatic lesion [44]. Molecular mechanisms responsible for this tropism have also been unraveled. In breast cancer, the interaction between cancer-expressed chemokine receptors (CXCR4/CCR7) and distant organ-expressed chemokine ligand (CXCL12/CCL21) promotes metastasis through facilitating chemotaxis, arrest and invasion to lymph nodes and lung, respectively. In the case of melanoma metastasis, interaction between the chemokine receptors CCR10 and the chemokine CCL27 seems to play a critical role [45].

6. The pre-metastatic niche

With the conceptual advances on tumor microenvironment, the effect of the stroma on metastasis formation in secondary lesion has also been highlighted. It is proposed that tumors are able to modify tissue components in distant organ to create a more metastasis-supportive microenvironment before disseminated tumor cells (DTCs) physically arrive at the site of metastasis. Kaplan et al. showed that tumor cells could prepare such pre-metastatic niche in advance by activating the expression of fibronectin at the secondary site and directing $VEGFR1^{+}/VLA4^{+}$ BMDCs toward this location, thereby determined which organ would be colonized by metastasis, in this

case the lung [46]. Moreover, S1PR1-STAT3 signaling is also crucial for tumor-promoting myeloid cells to mobilize to and to colonize the prospective metastatic site. This signaling cascade could further regulate proliferation and survival of other metastasis-promoting stroma cells [47].

In addition, ECM remodeling enzymes are also conditioned by tumor or "activated" stroma cells. Lysyl oxidase (LOX) released by hypoxic breast cancer cells was shown to accumulate at pre-metastatic niche and crosslink collagen in order to facilitate the entry for tumor-promoting myeloid cells [48]. Endothelial cells, MAC1 $(CD11b)^+$ macrophages and $VEGFR1^+$ BMDCs in lung facilitate lung metastasis formation through the expression of matrix metalloproteinase-9 (MMP-9), which could process the ECM and release growth factors and chemokines in the lung microenvironment. [46,49].

7. Linear progression model and parallel progression model

It is generally accepted that the acquisition of metastatic traits and capabilities requires additional genetic alterations beyond those responsible for malignant cellular transformation [15]. Many models have been proposed to describe the development of genetic changes during tumor progression toward metastasis formation [38]. These models can be reduced into 2 fundamental models: the linear progression and parallel progression models (Fig. 2) [32].

As a prevailing model, linear progression model describes metastasis as the end product of the sequential accumulation of genetic and epigenetic alterations during primary tumor development. Therefore, metastasis inherits malignant traits from primary tumor and the prognosis and targeting therapies are selected based on the analysis of the primary tumor [32]. This model also predicts that metastasis is a rather late event during natural tumor development and the risk of its occurrence correlates with primary tumor size.

However, the detection of disseminated tumor cells (DTCs) in ectopic sites during early tumor progression and the advent of whole genome analysis of

single tumor cells have suggested a second theory of metastasis - the parallel progression model. In this model, tumor cells escape from the primary tumor before they acquire full oncogenic capacities and further gather malignant hallmarks upon dissemination independently of primary tumor evolution [32,50]. Analysis of paired DTCs and primary tumor from breast, prostate and esophagus cancer patients showed that DTCs have less or different genetic aberrations than primary tumor cells [51-53]. This discordance also exists among DTCs isolated from different sites. Divergence of oncogenic mutation sites between primary tumors and metastases, including KRAS and EGFR, further support this model of parallel progression [32]. According to this notion, therapeutic strategies should be reconsidered. Besides the targeting therapy selected based on primary tumor subtype, alternative combined therapies may be adopted depending on the genetic alterations of DTCs, since these early disseminated tumor cells may already develop different survival strategies within the secondary microenvironment compared to primary tumors [32]. Such approach would require a systematic analysis of DTCs (Fig.2).

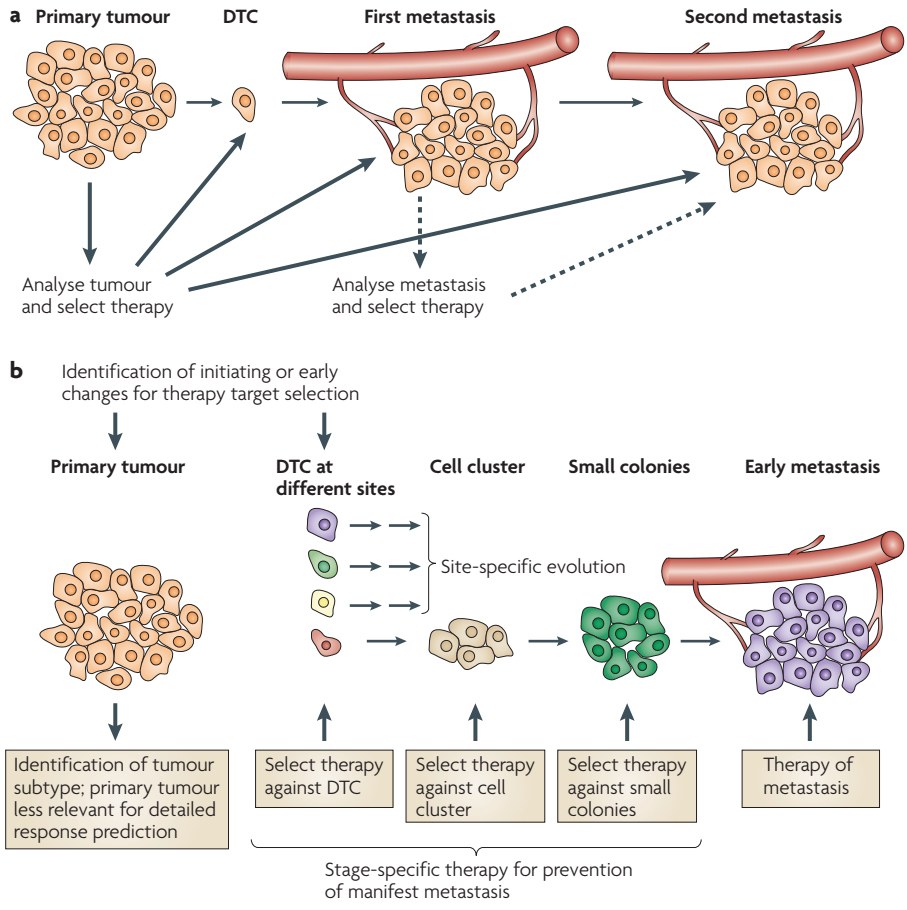

Figure 2. Clinical therapeutic strategies depending on different progression models. From [32].

(A) In linear progression model, it is hypothesized that disseminated tumor cells (DTCs) and metastatic cell colonies are generated from the most aggressive clones among the primary tumors and disseminate during late stages of cancer progression. Additional genetic mutations are not necessary to develop metastasis. Therefore, the analysis of primary tumor is sufficient for choosing therapies targeting metastases.

(B) From the parallel progression theory, DTCs are considered to leave the primary tumor and form metastasis during early stages of tumor development. In this case these tumor cells could gather distinct mutations in parallel of primary tumor progression. On the basis of independent progression, additional analysis on both DTCs and metastatic colonies may be necessary in order to select the most appropriate therapies for treating metastases.

Regardless of the progression models, high genomic instability and mutations occurrence allow cancer cells to keep their diversity among the whole population as well as the plural and dynamic interactions within the microenvironment. Therefore, more investigations still need to be conducted to unravel the mode of progression of cancer metastasis as well as the role of the microenvironment.

8. CYR61 (Cysteine-rich angiogenic inducer 61)

8.1. Structure of CYR61

CYR61 (Cysteine-rich angiogenic inducer 61) was first identified as an immediate-early gene induced by growth factor stimulation [54]. CYR61 belongs to the CCN (CYR61, CTGF and NOV) family of matricellular proteins and shares structural homology with other family members. There are six members in the CCN family: CYR61 (CCN1), connective tissue growth factor (CTGF, CCN2), nephroblastoma overexpressed protein (NOV; CCN3), and Wnt-inducible and secreted protein-1-3 (WISP-1-3; CCN4-6) [55,56].

CYR61 consists of 381 amino acids with an N-terminal signal peptide for secretion [54]. Following the signal peptide are four conserved domains homologous to insulin-like growth factor-binding protein (IGFBP), von Willebrand factor type C repeats (VWC), thrompospondin type 1 repeat (TSP), and a carboxyl-terminal (CT) domain containing a cysteine knot motif [55]. Among the four domains, VWC, TSP and CT homology domains have been described to contain integrin binding sites (Fig. 3) [57].

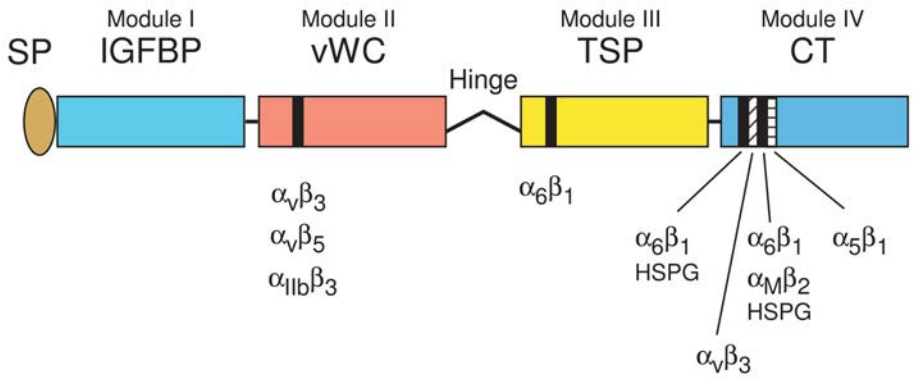

Figure 3. Schematic structure of CCN proteins. From [57].

SP: N-terminal secretory signal peptide; IGFBP: insulin-like growth factor-binding protein; VWC: von Willebrand factor type C repeat; TSP: thrompospondin type 1 repeat; CT: carboxyl terminal domain containing a cysteine-knot motif (absent in CCN5); HSPG: heparin sulfate proteoglycan. Black and hatched bars indicate integrin or HSPG binding sites. Binding integrins are indicated below.

8.2. Regulation of CYR61 expression

As an immediate-early gene, the expression of CYR61 RNA is activated within minutes of serum stimulation and lasts until several hours [58]. Transcription of these immediate-early genes does not require *de novo* protein synthesis, and could therefore respond to external stimulation within a

short time [54]. Expression of CYR61 is regulated by a broad range of stimuli, including: growth factors (i.e. PDGF, FGF) [54], inflammatory cytokines (i.e. IL-1β, TNFα) [59], phospholipids (i.e. S1P, LPA) [60,61], TGFβ [62], phorbol ester 12-O-tetradecanoyphorbol-13-acetate [54], thrombin [63], estrogen [64], prostaglandin E2 [59], vitamin D3 [65], angiotensin II [66] and even UV light and mechanical forces [67,68], implying diversified roles of CYR61 among cell-environment interactions [69].

8.3. Cellular functions of CYR61

Several integrins have been reported to interact with CYR61, such as $\alpha_2\beta_1$, $\alpha_6\beta_1$, $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$. In addition, CYR61 also binds to cell surface heparin sulfate proteoglycans (HSPG) [70]. It is generally considered that CYR61 modulates cellular functions by binding to different integrins in different cell types. For example, CYR61 enhances endothelial cell adhesion through interaction with $\alpha_v\beta_3$ integrin [71], and also mediates adhesion of fibroblasts and smooth muscle cells through $\alpha_6\beta_1$ and HSPG [72,73], platelets through $\alpha_{2b}\beta_3$ [74], monocytes and macrophages through $\alpha_M\beta_2$ [75,76]. In addition, by interacting with different integrins, CYR61 was shown to elicit distinct cellular responses in the same cell type. In fibroblasts, CYR61 regulates cell adhesion, senescence and apoptosis through $\alpha_6\beta_1$ and HSPG [72,77-79], migration through $\alpha_v\beta_5$ [80], proliferation through $\alpha_v\beta_3$ [80] ; while in activated endothelial cells, $\alpha_v\beta_3$ integrin mediates most of the CYR61 cellular functions including cell adhesion, migration, survival and tube formation [71,81,82]. All these investigations strongly suggest that the cellular functions of CYR61 depend on the expression profile of cell surface receptors that present in diverse cell types within distinct contexts. Such complexity also renders CYR61 a regulatory role in different physiological events, such as embryonic development, chondrogenesis, angiogenesis, wound healing, fibrosis and cancer progression [55,69,83].

8.4. CYR61 in tumorigenesis and cancer progression

Studies in different cancers also present diversified roles of CYR61 during cancer progression.

8.4.1. Different effects of CYR61 on different types of cancer

Aberrant up-regulation of CYR61 mRNA is found in 39% of breast tumor samples and is associated with tumor size, more advanced stages and lymph node involvement [84]. Another study using immunohistochemical staining on breast cancer tissue arrays confirmed the correlation between CYR61 protein expression and those clinical factors [85]. Elevated CYR61 expression was found from the early proliferative step and was kept throughout the whole progression process [86].

Similarly, in gastric cancer it was proposed that high CYR61 expression level correlated with tumor grade, lymph node metastasis and recurrence [87]. In ovarian cancer, glioma, squamous cell carcinoma, osteosarcoma and pancreatic ductal adenocarcinoma, overexpression of CYR61 was found and correlated with tumor grade or survival of patients [88-95]. Higher level of CYR61 was also found in prostate cancer tissue compared to paired normal tissue around cancer lesion [96]. However, CYR61 expression also correlated with less recurrence for patients with radical prostatectomy [97].

For colorectal cancer, higher expression of CYR61 was found during cancer initiation and development, and was reduced in more advanced stage [98].

On the other hand, CYR61 plays a tumor-suppressive role in non-small-cell lung cancer (NSCLC) [99,100]. The expression level was downregulated following cancer progression, and was correlated with more advanced stage, poor prognosis and poor survival rate of patients and [100-102]. Controversial correlation were proposed in endometrial cancer and hepatocellular carcinoma [103-106].

8.4.2. CYR61 effects from experimental models

- Breast cancer

Diverse cellular effects of CYR61 were also reported from cell-based functional experiments. In breast cancer cell lines, CYR61 level is significantly elevated in more malignant cells (i.e. MDA-MB-231, HS578T) among the others [84,107]. Ectopic expression of CYR61 caused estrogen-independent growth of the estrogen-dependent beast cancer cell line MCF-7 [108]. MCF-7 cells with stable CYR61 expression acquired enhanced the ability to form colonies under anchorage-independent conditions and promoted the development of vascularized tumors in nude mice through the activation of $\alpha_v\beta_3$ integrin [108,109], indicating the promoting role of CYR61 in tumorigenesis. In addition, CYR61 protected cells from apoptosis induced by various anti-cancer drugs through integrin $\alpha_v\beta_3$-mediated ERK1/2-MAPK pathway and NF-κB-mediated upregulation of XIAP (inhibitor of apoptosis protein) expression [110,111]. Experiment in which selected domains of CYR61 were deleted, demonstrated critical roles for the second and fourth modules in migration and invasion mediated by CYR61 [86].

- Gastric cancer

In gastric cancer, CYR61 promoted cancer cell migration and invasion through integrin $\alpha_v\beta_3$/NF-κB/COX2 and HIF1-α/PAI-1 pathways [87,112]. CYR61 also up-regulated the expression of CXCR1/CXCR2 through integrin $\alpha_v\beta_3$/Src/PI3K/AKT pathway to facilitate transendothelial migration and intravasation of gastric cancer cells [113]. Through integrin $\alpha_2\beta_1$, CYR61 promoted dissemination and nodule formation of gastric cancer cells in the peritoneal cavity of mice [114].

- Ovarian cancer

In ovarian cancer cell lines, CYR61 overexpression promoted cell proliferation and resistance to the anti-cancer drug cisplatin [88]. The chemoresistance is achieved by inhibiting caspase 3 activity [115]. In addition, CYR61 also confers

resistance to apoptosis induced by carboplatin, a cisplatin analogue, by regulating the expression of Bcl-2 family proteins, NF-κB, and p53 through the activation of the AKT pathway [116].

- Glioma

In glioma, CYR61 acts as a downstream mediator of GPCR signaling pathways, including thrombin receptor/RhoA-induced cell proliferation, and S1PR-mediated cell invasion [63,117]. CYR61 was also required for HGF-regulated cell growth and migration [90]. Overexpression of CYR61 promotes cell growth through PI3K/AKT-induced GSK3β phosphorylation and the β-catenin/TCF/Lef pathway, and generated larger and more vascularized tumors [89].

- Squamous cell carcinoma

Ectopic expression of CYR61 in human oral squamous cell carcinoma (SCC) enhanced cell growth, cell motility and *in vivo* tumor development [92]. By using blocking antibodies and chemical inhibitors, Chuang *et al.* showed that CYR61-improved cell migration was mediated through integrin ($\alpha_v\beta_3$/ $\alpha_6\beta_1$)/FAK/ERK and NF-κB-induced expression of MMP-3 [118]. Interestingly, secreted CYR61 accelerated SCC collective cell migration and invasion through both autocrine and paracrine routes, providing a model of invasive sheet formation at the tumor border [119].

- Osteosarcoma

CYR61 expression was found correlated with tumor grade in human osteosarcoma. Consistently, *in vitro* apoptosis, migration, invasion and *in vivo* metastasis were also related to CYR61 expression levels. It is noteworthy that its contribution to enhanced metastasis was mainly due to increased size of metastatic nodules [95,120]. Recently, an intracellular binding protein of CYR61, Caprin-1, was identified in cytoplasmic stress granules. Overexpression of

Caprin-1 resulted in enhanced cell growth, inhibition of apoptosis, increased *in vivo* tumor growth and lung metastasis [121]. However, it is still not clear whether CYR61 cooperates with Caprin-1 during tumor progression.

- Pancreatic cancer

Haque *et al.* showed that CYR61 enhanced cell growth, cell migration and tumorigenicity of pancreatic cancer cell lines by promoting EMT and stemness [94]. Through the interaction with integrin $\alpha_v\beta_3$, CYR61 activated Notch-1 signaling and inhibited degradation of Notch intracellular domain, thereby trans-activated Sonic Hedgehog (SHh) pathway and enhancing cell migration [122].

- Prostate cancer

Like in breast cancer cell lines, high level of CYR61 was found in PC3 and Du145 compared to the less aggressive cell lines LnCap and 22RV1, consistent with the observations in human prostate cancer [96,123]. CYR61 supported cell proliferation, colony formation in soft agar and subcutaneous tumor growth of prostate cancer, and sensitized cells to TRAIL-elicited apoptosis through PKC activation [123,124]. CYR61 promoted cell migration by regulating Rac1 and focal adhesion formation, and conferred capability to form metastatic lesions in lung [123].

- Colorectal cancer

By modulating expression level in colon cancer cell line, CYR61 was shown to regulate cell invasion and lung metastasis formation through cooperation with integrin $\alpha_v\beta_5$ [125].

- Lung cancer

Besides the tumor-promoting role observed in most types of cancer, CYR61 was also shown to act as a tumor suppressor in lung cancer. The suppressive mechanism of CYR61 to tumor growth *in vitro* and *in vivo* involves β-catenin/c-Myc/ p53-regulated cell cycle arrest [99,126]. However, the role of CYR61 in other cellular functions was not addressed.

- Endometrial cancer

Although Chien *et al.* showed that CYR61 expression suppress tumor growth through expression of pro-apoptoitc proteins and elevated caspase-3 activity, there was another report claiming that CYR61 expression, together with lymph node metastasis, could be used to categorize cancer patients and predict 5-year survival rate. Therefore, more investigations need to be done to confirm the role of CYR61 in endometrial cancer [103,104].

- Hepatocellular carcinoma

The role of CYR61 in hepatocellular carcinoma is full of controversy from both clinical and laboratorial data. While Feng *et al.* showed that expression of CYR61 reduced cell growth rate through p53 regulation [106], increased cell adhesion and slowed down cell migration and invasion, another report suggested a growth promoting role of CYR61 both *in vitro* and *in vivo* [105].

8.5. CYR61 in cancer metastasis

Among all the reports about CYR61 in different types of cancer, there are only a few investigations about the role of CYR61 during metastasis development. In most of studies, the pro-metastatic ability was proposed based on the ability of CYR61 to enhance invasion *in vitro*. One study based on the orthotopic intratibial injection of osteosarcoma cells demonstrated enhanced spontaneous lung metastasis formation by CYR61-overexpressing cells [95]. Another study reported that a monoclonal CYR61 antibody could block lymph

node metastasis in a footpad tumor inoculation model [127]. By injecting tumor cells through the tail vein, Sun *et al.* demonstrated that overexpression of CYR61 enhanced prostate cancer cell metastasis while downregulation of CYR61 suppressed the growth of metastatic foci in bone, lung and the peritoneal cavity [123].

We have previously reported that tumors growing in a pre-irradiated stroma were selected for tumor cell variants with stronger invasive and metastatic abilities compared to tumors growing in a non-irradiated environment. This effect was associated with the development of strong tumor hypoxia secondary to radiation-impaired angiogenesis. CYR61 was upregulated in invasive and metastatic cells, and was found to contribute to invasion and metastasis through cooperation with $\alpha_v\beta_5$ integrin [125]. However, the detailed regulatory mechanism by which CYR61 promotes metastasis was not addressed.

Aim of the study

The aim of my thesis work was to investigate the role of CYR61 in breast cancer metastasis formation and to unravel the possible cellular and molecular mechanisms involved.

The rationale for the study were, firstly, the previous observation of the laboratory that CYR61 promotes invasion and metastasis of cancers growing in a pre-irradiated microenvironment and, secondly, the shorter disease free and overall survival of breast cancer patients expressing elevated levels of CYR61 in their primary tumors.

To address this question I have used a combination of *in vivo* and *in vitro* cellular and molecular approaches, in particular an orthotropic model of metastatic breast cancer.

Results

Silencing of CYR61 expression in human breast cancer cell line MDA-MB-231

To investigate the role of CYR61 in breast cancer metastasis, we first analyzed CYR61 expression level in four human breast cell lines: MCF7, MCF10A, MDA-MB-231 and MDA-MB-468. Among the four cell lines, MCF10A is a cell line isolated from a fibrocystic patient and has no tumorigenic ability. MCF7, MDA-MB-468 and MDA-MB-231 are tumorigenic, but only MDA-MB-231 efficiently forms metastasis and has therefore been frequently used for the study of metastasis development in breast cancer.

Consistent with other studies, we found that MDA-MB-231 showed the highest CYR61 protein level among all tested cells lines (Fig. 1A). Therefore, we knocked down CYR61 expression in MDA-MB-231 cells by stable shRNA expression (Fig. 1B). To further consolidate our results, we established additional MDA-MB-231 cell lines with silenced CYR61 expression by using an inducible shRNA system. Two different sequences of shRNAs targeting *CYR61* were tested and the combination of these 2 shRNAs was then used to obtain the highest silencing efficiency. After puromycin selection, resistant cells were treated with 2µg/mL of doxocyclin (Dox) to induce the expression of shRNA. Time course induction experiments were done in order to characterize and validate the silencing efficiency (Fig. 1C). Total RNA was extracted from MDA-MB-231 cells at different time points and the level of CYR61 mRNA following Dox treatment was determined by qPCR (Fig. 1C). CYR61 mRNA level started to decrease 1 day after initiation of treatment compared with non-silencing control (NS), and reached the lowest values from the 3rd day of Dox treatment on. The level of CYR61 protein started to show a dramatic decrease from Day 3 on upon Dox treatment (Fig. 1D). The silencing effects of both constitutive and inducible shRNA systems were maintained for at least few months (data not shown).

***In vivo* studies of the capability of CYR61 to form metastasis:**

Silencing CYR61 in primary tumors grown in pre-irradiated mammary fat pads reduces spontaneous lung metastasis formation

We have previously reported that tumors growing subcutaneously in pre-irradiated stroma express higher level of CYR61 compared to tumors growing in normal, non-irradiated stroma and that are more metastatic. We then showed that CYR61 is directly involved in enhanced metastasis formation of tumors growing in a pre-irradiated stroma [125]. To better mimicking the tumor microenvironment and to mirror a clinically-relevant condition, we used an orthotopic model of breast cancer and injected NS and CYR61 knockdown (KD) MDA-MB-231 cells into pre-irradiated mammary fat pads (MFP) of immune deficient NSG mice. One month after injection, mice were euthanatized and the primary tumors and lungs were fixed and embedded in paraffin for section (Fig. 2A). Immunohistochemical staining of human vimentin was used to determine the formation of metastasis in lung (Fig. 2B).

Consistent with our previous findings, CYR61 expressing cells developed more metastatic colonies in lung (Fig. 2B, quantification in Fig. 2D). With CYR61 expression, NS cells formed bigger tumor masses than in the CYR61 KD group (Fig. 2C). Moreover, bigger metastases were found in the lung sections of NS group of mice (Fig. 2B). The results suggest that CYR61 provides survival advantages for cancer cells growing in pre-irradiated orthotopic microenvironment and increased ability to metastasize to the lung.

CYR61 silencing in primary tumors reduces spontaneous lung metastasis formation

These results raised the question of whether CYR61 might also promote metastasis formation during the natural course of breast cancer progression. To address this question we orthotopically injected NS and CYR61

knockdown (KD) MDA-MB-231 cells into non-irradiated MFP of immune deficient NSG mice to generate primary tumors that spontaneously metastasize to the lung. One month after injection, mice were euthanatized and the primary tumors and lungs were fixed and embedded in paraffin for sectioning (Fig. 3A). Immunohistochemical staining of CYR61 in primary tumors confirmed the efficiency of knockdown *in vivo* (Fig. 3B). Human vimentin staining was used to determine the formation of metastasis in lung (Fig. 3D).

Although CYR61 was reported to promote cell proliferation and tumor growth in some experimental models as well as in our irradiation model, the size of primary tumors showed no difference between NS and KD groups (Fig. 3C). However, mice in the NS group developed significantly more metastasis colonies in the lung as detected by quantification of human vimentin staining (Fig. 3D, in Fig. 3E). The metastasis index, which reflects metastasis formation relative to primary tumor size, further confirmed that CYR61 KD cells lost partially their ability to form spontaneous lung metastasis (Fig. 3E).

CYR61 down-regulated cells are less metastatic in an experimental model of metastasis

The development of distant organ metastasis can be dissected into various steps: local invasion and escape from the primary site, entry into the blood circulation, survival in suspension and escape from immune surveillance, extravasation into distant organ, adaption to the new microenvironment and colonization to develop macrometastasis. To understand which one of these steps depends on CYR61, we injected tumor cells into the tail vein to form experimental metastasis, thereby bypassing the need for primary tumor formation, local invasion and intravasation.

Twenty-eight days after tail vein injection of tumor cells, mice were euthanatized and lungs were fixed and embedded in paraffin for sectioning (Fig. 4A). Consistent with the orthotopic injection model, significantly more

lung metastases were observed in the NS group of mice compared with the KD group (Fig. 4B, quantification in Fig. 4C). Collectively, results from both spontaneous metastasis and experimental metastasis models indicate that CYR61 has limited effect on primary tumor growth under normal condition, but promotes breast cancer metastasis to lung. These experiments also indicate that CYR61 facilitates later stages of metastasis formation - survival in circulation, extravasation, or colonization in lung.

CYR61 facilitates breast cancer cell extravasation into lung parenchyma

In order to identify if CYR61 affects cancer cell extravasation into lung parenchyma or the successive colonization step in the new microenvironment, we conducted a short-term tail vein injection experiment, where mice were sacrificed 24 hours after injection. Half-million of NS or KD cells labeled with Green Cell Tracker were injected into tail vein of NSG mice. Twenty-four hours after injection, mice were anesthetized and perfused with 2%PFA/PBS to wash away the cancer cells remained in circulation (Fig. 5A).

From the whole lung image taken under fluorescent microscope, we found that significantly more cancer cells were retained in lungs of the NS group of mice compared with KD cell-injected mice (Fig. 5B). The same result was obtained when we sectioned the lungs to count cancer cell colonies in lung (Fig. 5C, quantification in Fig. 5D). These results suggest that CYR61 facilitates breast cancer cell extravasation into lung parenchyma during the fist 24-hour period after injection, and thereby affected lung metastasis formation.

Delayed down-regulation of CYR61 expression does not prevent lung metastasis formation

To further support the finding that CYR61 promoted lung metastasis formation by facilitating the extravasation step, we took advantage of the inducible

shRNA knockdown system and designed an experiment whereby CYR61 down-regulation *in vivo* was induced 24 hours upon tumor cell injection, a time point when most tumor cells have already entered the lung parenchyma.

Half-million of MDA-MB-231 cells expressing NS or CYR61 shRNA plasmid were injected into NSG mice through the tail vein. By the time of injection, these cancer cells were not yet treated with Dox and the shRNA was not induced. Twenty-four hours after injection, both groups of mice were fed with Dox water to induce the expression of shRNA, and Dox treatment was continued until the mice were sacrificed. Twenty-four days after injection, mice were euthanatized and the lungs were fixed and embedded in paraffin for sectioning (Fig. 6A). Total colony numbers of lung metastasis were counted, and the result showed that delayed downregulation of CYR61 did not impinge on lung metastasis formation (Fig. 6B, quantification in Fig. 6C).

Overall, these *in vivo* data indicate that the expression of CYR61 promotes lung metastasis of breast cancer cells though an effect occurring within the fist 24 hours following their injection into the blood, most likely involving facilitatied tumor cell extravasation into the lung parenchyma.

In vitro studies of cellular functions modulated by CYR61:

To extravasate into secondary organs, cancer cells have to adhere first to the endothelium and then transmigrate through the vessel wall, starting with the endothelial barrier. At the same time, cancer cells have to escape from cell death induced by loss of cell-matrix adhesion signals (anoikis) before they could successfully reach the proper matrix in the new microenvironment.

To further dissect which of these steps are promoted by CYR61 we did *in vitro* validation experiments to analyze the effect of CYR61 on various cellular functions, including cell adhesion, spreading, migration, transendothelial migration, proliferation and colony formation under anchorage-independent condition.

CYR61 affects MDA-MB-231 cell spreading but has minimal effects on cell adhesion to extracellular matrix proteins

Non-silencing (NS) and CYR61 KD cells were trypsinized and plated in matrix-pre-coated 96-well plates for adhesion for 15 minutes, 30 minutes, 45 minutes and 1 hour. Although in some previous publications, CYR61 was described to enhance cell adhesion, we found only limited effect of CYR61 silencing on adhesion. Both cell lines efficiently adhered to collagen I and fibronectin, and cell adhesion reached plateau already in 15 minutes for cells plated on higher matrix concentration (Fig. 7A). Although it seems that NS cells adhere more efficiently to collagen I and fibronectin, differences between the two cell lines were minimal with a tendency of NS cells to adhere better to laminin than KD cells. Overall, CYR61 induced only limited adhesion advantage on adhesion to extracellular matrix, suggesting that this might not be a main mechanism responsible for cancer cell extravasation *in vivo*.

In addition, we also analyzed the spreading ability of both cell lines on tissue culture plates. Cells were seeded on plates in serum-free medium for up to 24 hours. Four hours after plating, NS cells started to extend their protrusions and spread, and this phenomenon became more evident at later time points (Fig. 7B). In contrast, CYR61 silenced cells had a clearly retarded spreading, still observable at 24 hours after plating.

CYR61 promotes MDA-MB-231 cell migration and transendothelial migration

As cell spreading is a considered as a surrogate of cell migration, we performed migration assays to study the effect of CYR61 on cellular mobility, including scratch wound assay, transwell migration and transendothelial migration.

Both NS and KD cells were cultured in complete medium until they reached 100% confluence. Scratches with identical width were made using pipet tips and the media was replaced immediately thereafter in order to remove floating cells. The scratched areas were photographed every hour by timelapse microscopy to monitor cell migration and wound closure (Fig. 8A). The distance between two edges of the scratch was determined and migration velocity was calculated (Fig. 8B). Total 7 spots from 3 different scratches for each cell line were recorded. As the result showed in Figure 8B, NS cells migrated significantly faster than CYR61 KD cells.

To validate the result from the scratch wound assay, we conducted another migration assay by using the Transwell system. Forty thousand cells were seeded in the upper chamber of Transwell inserts and were allowed to migrate to the bottom part for 8 hours. At the end of the experiment, cells that remained in the upper part of the insert were carefully removed with cotton swabs. Cells that migrated to the other side of insert membrane were fixed and stained with crystal violet (Fig. 8C). Three areas per insert were chosen randomly and photos from total 9 areas were used to quantify numbers of migrated cells (Fig. 8D). The results demonstrated that NS cells have stronger migratory ability than CYR61 KD cells, consistent with the results from scratch assay.

Since extravasation across the vessel wall also implicates a transmigration step through the endothelial layer, we tested the ability of cancer cells to migrate through confluent layer of endothelial cells *in vitro*. Human umbilical vein endothelial cells (HUVEC) were grown on the upper part of the Transwell membrane until they reached 100% confluence. Then, NS and CYR61 KD cells expressing RFP were seeded into the upper chamber and were allowed to migrate for 12 hours. At the end of the experiment, cells remained in the upper compartment were carefully removed with cotton swabs. Cells that migrated through the endothelial monolayer and reached the bottom side of the insert membrane were fixed and photographed by fluorescent microscopy (Fig. 8E). Nine areas per insert were chosen randomly and photographs from a total of 27 areas were used to quantify the percentage of area occupied by

migrated cells (Fig. 8F). Results from these experiments demonstrated that NS cells migrated more efficiently across the endothelial monolayer compared to silenced cells.

CYR61 enhances colony formation under anchorage-independent condition but has limited effect on cell proliferation under 2D conditions

When travelling in the blood from the primary tumor to a distant organ, tumor cells do so in the absence of adhesion to extracellular matrices. Lack of cell-matrix interaction triggers integrin-dependent death-promoting signals in normally adherent cells, an event known as anoikis.

To test the ability of NS and CYR61 KD MDA-MB-231 cells to survive and grow under anchorage-independent conditions, we suspended the cancer cells in soft agar and let them grow to form colonies. After two weeks, random areas were photographed (Fig. 9A) and both number and the size of colony formed by each cell line were measured (Fig. 9B). The result showed that NS cells formed significantly more and larger colonies compared to CYR61 KD cells.

However, both cell lines grew to similar rates under normal, 2D culture conditions (Fig. 9C), suggesting that CYR61 protects cells under conditions of limited or absent cell adhesion and could therefore supports cell growth under anchorage-independent conditions.

Expression of CYR61 protects cancer cells in suspension from apoptosis

To collect further evidence whether CYR61 conferred resistance to anoikis, we kept the MDA-MB-231 cells in suspension and then stained them with Annexin V and near-IR dead cell marker dye. Flow cytometry analysis was

conducted to identify apoptotic (Annexin V positive) from viable (double negative) cell populations.

The suspension time course clearly showed that there was an increasing fraction of apoptotic MDA-MB-231 cells among the CYR61 KD population, while in NS population the fraction of apoptotic cells remained low and steady throughout the whole period of the experiment (Fig. 10A-B).

CYR61 protects cancer cells from apoptosis through β1/β3 integrin

Previous studies have shown that CYR61 interacts with various integrins to activate cellular signaling pathways. To investigate whether CYR61 prevented anoikis through engagement of integrins, we first analyzed the surface level of CYR61 (Fig. 10C) and different integrin subunits (Fig. 10D). We confirmed that CYR61 shRNA decreased surface level of CYR61 protein without alternating surface level of integrins.

Then, we treated both cell populations with functional antibodies to specifically block or activate integrins. Addition of a functional blocking anti-β1 integrin antibody in NS cells increased the fraction of apoptotic cells. In contrast, addition of a β3 integrin activating antibody partially rescued apoptosis in CYR61 KD cells (Fig. 10E). These observations suggest that the ability of CYR61 to confer resistance of breast cancer cells to anoikis depends on β1 and β3 integrins.

Discussion

CYR61 promotes breast cancer metastasis to the lung by facilitating extravasation

CYR61 was shown to regulate various cellular functions including cell proliferation, adhesion, and migration. At tissue level, CYR61 was reported to contribute to the development of the vascular system, chondrogenesis, T cell production in thymus, wound healing, fibrosis and cancer progression [70,128-133]. Clinical studies on different types of cancer clearly revealed the complex role of CYR61 in cancer biology. CYR61 promoted cancer progression in most types of cancer, including breast, gastric, ovarian cancer, glioma, squamous cell carcinoma, osteosarcoma and pancreatic ductal adenocarcinoma. In prostate and colorectal cancers, CYR61 is more related to tumor initiation, since the expression level decreased in more advanced stages. On the contrary, a tumor-suppressing role was proposed in non-small cell lung cancer, endometrial cancer and hepatocellular carcinoma. The apparent dual role of CYR61 (i.e. tumor promotion *vs* tumor suppression) was confirmed by many cell-based experiments. It was suggested that the complexity is due to the heterogeneous cellular context in different cell types, including binding to different cell surface receptors, in particular integrins, and activation of diverse signaling pathways in different cell types. Importantly, most of the published experimental reports investigating the *in vivo* role of CYR61 during cancer progression focused on primary tumor development, especially primary tumor growth, but did not address the role of CYR61 on metastasis formation.

Considering that metastasis is responsible for over 90% of cancer-related patients' death, research efforts should focus on the study of the mechanisms of multistage metastasis formation in order to improve cancer therapies. For example, based on the linear progression model of metastasis formation, cancer dissemination occurs late during tumor progression and therefore metastatic lesions are considered to be formed by the same type of tumor

cells as primary lesion and therefore treated based on the characteristics of primary tumors. Thus, Trastuzumab is used to treat metastatic breast cancer based on the expression of its target (ErbB2) in primary tumor cells. However, the recent finding that circulating tumor cells could be released during early stage of tumorigenesis and have therefore much longer time to accumulate mutations independently of the progression of the primary tumor (i.e. the parallel model of metastasis progression) provides a novel perspective to reconsider the timing or strategy of metastasis treatment [32].

We previously showed that CYR61 is upregulated in tumor cells grown in pre-irradiated stroma in a subcutaneous xenograft cancer model (i.e. mimicking postradiation relapses), and that expression of CYR61 promoted tumor cell invasion and metastasis [125]. However, it remained unclear at which step CYR61 plays its critical role in promoting cancer metastasis. With another orthotopical mammary fat pad injection model, we found that 4T1 murine breast cancer tumor cells growing in pre-irradiated stroma developed more lung metastases than in non pre-irradiated fat pads [134]. To understand if CYR61 promotes cancer metastasis under this condition, we adopted a model of human breast cancer by injecting human breast cancer cells (MDA-MB-231) orthotropically in pre-irradiated mammary fat pads. With this model we demonstrated that silencing of CYR61 expression reduced lung metastasis formation. As these relapses are indeed quite rare in patients, we wondered whether CYR61 also plays a role in lung metastasis during the natural course of breast cancer progression. To address this question we used different models of breast cancer metastasis.

First we injected non-silenced (NS) and CYR61 silenced (CYR61 KD) MDA-MB-231 breast cancer cells orthotopically into mammary fat pads of non-irradiated mice (Fig. 3A). Lung metastasis visualized by vimentin staining clearly showed that down-regulation of CYR61 reduced spontaneous metastasis formation (Fig. 3D-F). To avoid the influence of primary tumor mass and evasion from primary site and focus on the later 2 steps - extravasation and colonization, we injected the same number of NS and CYR61 KD cancer cells directly into tail vein (Fig. 4A). The NS group of mice

showed significantly more lung metastases compared to the KD group (Fig. 4B-C). Similar results were obtained from 24-hour tail vein injection (Fig. 5), suggesting that CYR61 promotes metastasis either by facilitating extravasation or promoting cancer cell growth in the lungs. To discriminate between these two hypotheses, we delayed CYR61 silencing by inducing expression of NS and CYR61 shRNA 24 hours after injection. This timeframe was adopted from the initial time course of inducible KD efficiency (Fig. 1C). Result from this experiment showed that delayed CYR61 silencing rescued lung metastasis formation in the KD group of mice, thereby strengthening the hypothesis that CYR61 facilitates cancer cell extravasation rather than lung colonization (Fig 6).

The first direct evidence that CYR61 promotes pulmonary metastasis

Our work presents the first direct experimental evidence that CYR61 promotes breast cancer metastasis to the lung. Lin *et al.* who used also MDA-MB-231 cells showed that a CYR61 antibody could block lymph node metastasis [127]. However, they inoculated cancer cells into the footpad, a model that does not recapitulate the biological environment for breast cancer development and metastasis. Moreover, although lymph node infiltration is generally considered as an indicator of cancer cell spreading out of primary site, whether these infiltrated cells could actively develop overt metastases at distant sites is still under debate. In fact, clinical and experimental evidence indicate that cancer cells may remain inert in lymph nodes. The correlation between lymph node infiltration and patient prognosis or survival rate also needs to be further analyzed. Evidence so far indicates that removal of metastatic lymph nodes does not significantly impact distant metastasis formation and survival. In our experiment, lymph node metastasis was found in both groups of mice and was independent of CYR61 level or lung lesion numbers (data not shown).

Besides lung, bone is another major target organ of breast cancer metastasis. However, we found only sporadic cases of bone metastasis in mice with severe lung metastasis in both groups. Considering that the MDA-MB-231 cells already express extremely high level of CYR61, we conclude that CYR61 may not play a critical role during bone metastasis formation, and that alternative mechanisms and molecules are required. For instance, a gene set favoring bone metastasis formation was identified by comparing parental MDA-MB-231 cells and cell lines derived from bone metastasis [135]. In this study, overexpression of IL-11, CTGF, CXCR4, and MMP-1 dramatically increased rate and incidence of MDA-MB-231 cell bone metastasis. Interestingly, CTGF also belongs to the CCN family and shares many features with CYR61. Like CYR61, CTGF is known to modulate diverse cellular functions also mainly through integrins. The functions of these two proteins may be overlapping or redundant by binding to the same integrin receptors.

CYR61 enhances MDA-MB-231 cell motility but not adhesion

After having demonstrated that CYR61 promotes MDA-MB-231 breast cancer cell metastasis to the lung by facilitating extravasation, we aimed to investigate more in detail how CYR61 promotes cancer cell extravasation. The process of extravasation could be further dissected into 3 parts: (1) tumor cell survival in the circulation, (2) tumor cell adhesion on endothelial cells, and (3) tumor cell transendothelial migration. Each step presents a hurdle for cancer cells in order to reach lung parenchyma.

Many papers have demonstrated that CYR61 enhances cell adhesion on ECM proteins. However, we found very limited effect on MDA-MB-231 cells cell adhesion to ECM proteins by downregulating CYR61 expression (Fig. 7). Consistent with others' finding, we found that CYR61 silencing decreased cell motility in scratch wound and transwell migration assays. Moreover, silencing CYR61 decreases cancer cell transmigration across an endothelial cell monolayer *in vitro* (Fig. 8).

CYR61 promotes anchorage-independent growth and confers resistance to anoikis

Result from anchorage-independent colony formation assay using NS and CYR61 KD MDA-MB-231 cells showed that CYR61 promotes the formation of more and larger colonies under 3D non-adhesive conditions (soft agar assay), while cell growth on normal culture dishes was not different (Fig. 9). These data indicate that CYR61 favors cell survival under anchorage-independent conditions. A pro-survival effect was further investigated by keeping both NS and CYR61 KD cells in suspension and detecting apoptosis by Annexin V staining (Fig. 10). We found a clear time-dependent increase of the fraction of apoptotic cells in CYR61 KD cells, while in NS cells the fraction of Annexin V positive cells remained low. This observation is consistent with other lab's finding that MDA-MB-231 cells are largely resistant to anoikis [136]. Moreover, incubation of NS cells in suspension with anti-β1 integrin blocking antibody increased the percentage of apoptotic cells, while incubation of CYR61 KD cells in suspension with anti-β3 integrin activating antibody partially reduced the fraction of apoptotic cells, suggesting that integrin ligation protects cells from anoikis and that integrin activation partially compensate for the loss of CYR61. Thus CYR61 confers MDA-MB-231 cells resistance to anoikis through integrin β1 and β3.

Anoikis is a form of apoptosis induced when adherent cells are detached from their substrate and lose their adequate cell-matrix interaction. Along the metastatic cascades, cancer cells detach from the ECM and float in blood or lymph. Resistance to anoikis therefore protects malignant cells from dying while in suspension and thereby facilitates their dissemination toward secondary sites [27,28]. Three pathways regulating anoikis have been proposed: (1) intrinsic apoptotic pathway, (2) extrinsic apoptotic pathway, and (3) caspase-independent death signaling [28]. The first two pathways rely on the BCL family proteins and mitochondria-dependent caspase activation. The key

regulator of the third pathway - Bit1 - is released from mitochondria to the cytosol when integrin-matrix interaction is disrupted. Cytosolic Bit1 stimulates the relocation of TLE1/AES heterodimer complex from nucleus to cytosol. TLE1 is a transcription regulator of many anoikis-related genes. Although the downstream genes are not characterized yet, TLE1 has been shown to enhance Bcl-2 activity and upregulate expression of anti-apoptotic proteins – HSP70 and thymosin-β [29]. TLE1 proteins translocated into the cytosol therefore loose their transcriptional function. Cytosolic TLE1 are targeted to the proteasome for degradation [28,29]. Interestingly, the expression pattern of Bit1, an effector molecule of anoikis, is opposite to the expression pattern of CYR61 both *in vivo* and *in vitro*. In invasive breast tumors, lower expression of Bit1 was found when compared with normal or ductal carcinoma *in situ*. Besides, reduced level of Bit1 was also shown in the more aggressive MDA-MB-231 cells compared to MCF-7 cells. Silencing of Bit1 expression resulted in an increased amount of pulmonary metastases without affecting primary tumor growth [136]. In addition, increased ERK2 phosphorylation was found in Bit1 KD cells, due to the downregulation of ERK2 phosphatase activity. ERK phosphorylation was proposed to support cell survival under non-adhesive conditions and to protect cells from anoikis [136,137]. In our CYR61 KD MDA-MB-231 cells, ERK2 phosphorylation showed no difference with NS cells in adherent condition, but was downregulated when cells were kept in suspension (data not shown). This data suggested the hypothesis that in MDA-MB-231 cells losing cell-matrix contact, ERK becomes de-phosophorylated, while CYR61 reduces ERK2 de-phosophorylation possibly through the negative regulation of Bit1.

Although there is no publication showing that CYR61 modulates ERK phosphatase activity or expression, findings from Mitsushima *et al.* showed that the focal adhesion (FA) protein vinexin β prevents ERK2 de-phosophorylation by suppressing MAP kinase phosphatase-3 (MKP-3) when keeping cells in suspension [138]. In addition, the other CCN family member, CTGF, has been reported to upregulate MKP-1 and enhancing cell survival of activated mesangial cells by inactivating p38 MAPK [139]. Taken together these

observations suggest that CYR61 protects cells in suspension from apoptosis (anoikis) by interacting with integrins and sustaining ERK phosphorylation through the inhibition of MKP.

CYR61 controlled cell death and cancer therapeutic perspectives

Besides conferring resistance to anoikis, CYR61 has also been reported to promote cell survival and resistance against the cytotoxic effects of chemotherapy drugs. In the breast cancer cell line MCF-7, overexpression of CYR61 inhibited p53 accumulation induced by Taxol treatment, which could be reversed by inhibiting integrin $\alpha_v\beta_3$ ligation or ERK1/2 signaling [110]. Lin et al further showed that CYR61 mediates resistance to apoptosis induced by multiple chemotherapy drugs through an integrin/NF-κB/XIAP (inhibitor of apoptosis protein) pathway in MCF-7 cells [111]. However, in fibroblasts CYR61 played an opposite role by regulating TNFα- and Fas-mediated apoptosis through ROS production, p38 MAPK activation, cytochrome c release and caspase-dependent apoptosis [78,79,140]. These controversial data highlight the complex and pleiotropic functions of CYR61 and call for further work to better understand how CYR61 regulates cell survival. One possibility is that by interacting with different membrane receptors (i.e. integrins), CYR61 elicits distinct signaling events differentially affecting cell survival. On the other side, cancer cells may have already up-regulated anti-apoptotic genes or deregulated pro-apoptotic pathways which protect them from traditional cell death programs. In more aggressive tumors and cancer cell lines, they showed even resistance to anoikis with CYR61 expression [28,136]. A better understanding of CYR61-regulated cell survival programs, might help to inhibit metastasis formation by impeding anoikis resistance, and at the same time counteract the development of resistance to chemotherapy.

Figures

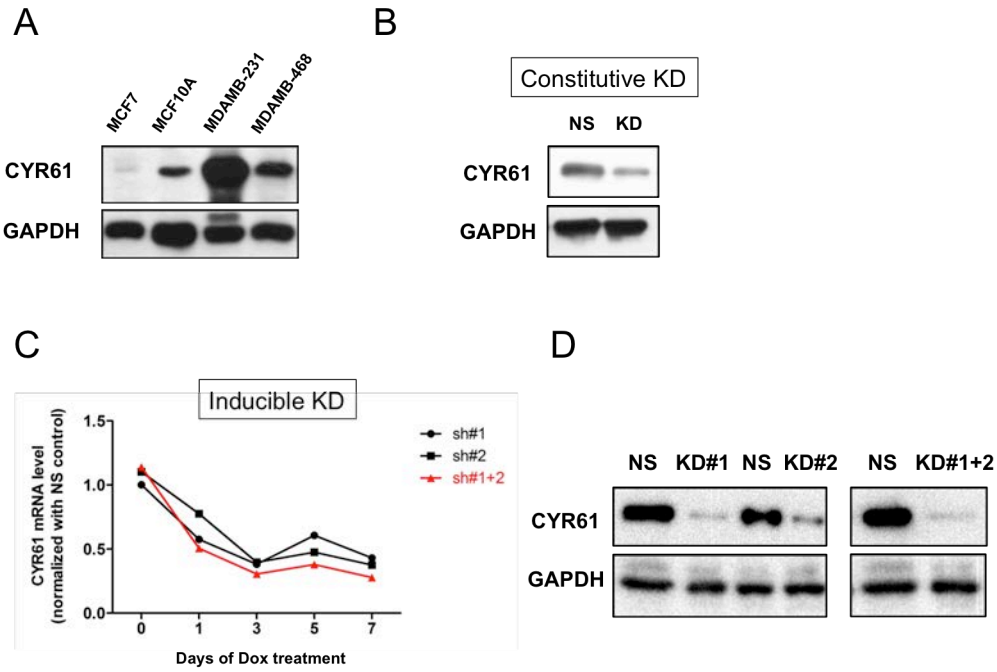

Figure 1. Silencing CYR61 expression in MDA-MB-231 cells. (A) Representative Western blot showing endogenous CYR61 protein levels in MCF7, MCF10A, MDA-MB-231 and MDA-MB-468 cells. (B) Western blot demonstrating constitutive silencing efficiency of CYR61 in MDA-MB-231 cells using a CYR61 targeting shRNA. NS: non-silencing, KD: CYR61 knockdown. (C) CYR61 mRNA level relative to NS control after doxocycline (Dox) treatment showing the efficiency of inducible shRNA targeting *CYR61* in MDA-MB-231 cells. (D) Representative Western blot of CYR61 protein showing that the inducible shRNA efficiently silence CYR61 protein expression in MDA-MB-231 cells.

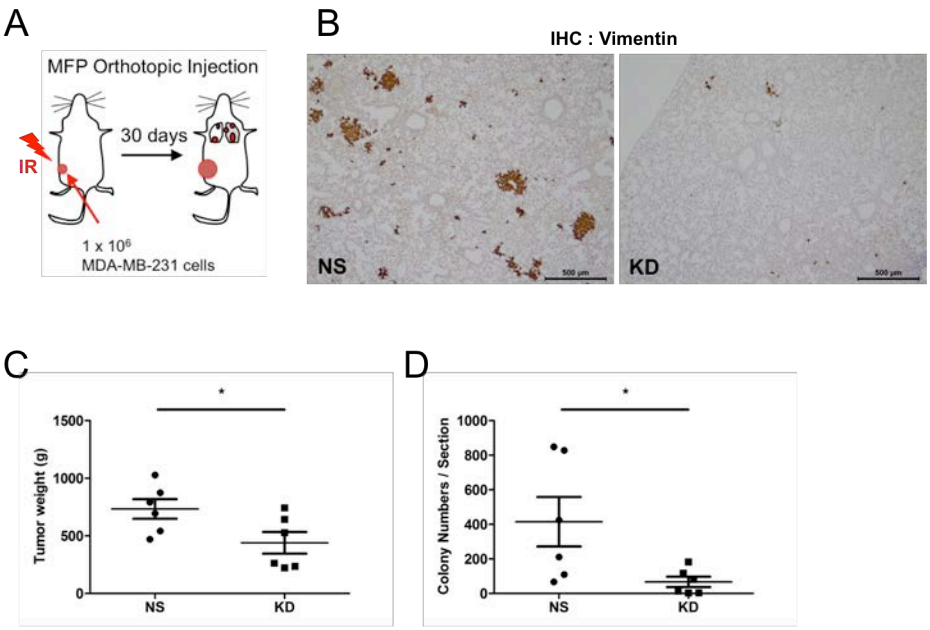

Figure 2. Silencing CYR61 in primary tumors grown in pre-irradiated mammary fat pads reduces tumor growth and spontaneous lung metastasis formation. (A) Schematic illustration of pre-irradiation orthotopic injection breast cancer model. MFP: mammary fat pad. IR: irradiation. (B) Immunohistochemical staining of human vimentin to reveal lung metastases. Scale bar: 500µm. (C) Primary tumor volume by weight (g). N=6 for both groups; *: $p<0.05$. (D) Quantification of lung metastasis colony numbers. N=6 for both groups; *: $p<0.05$.

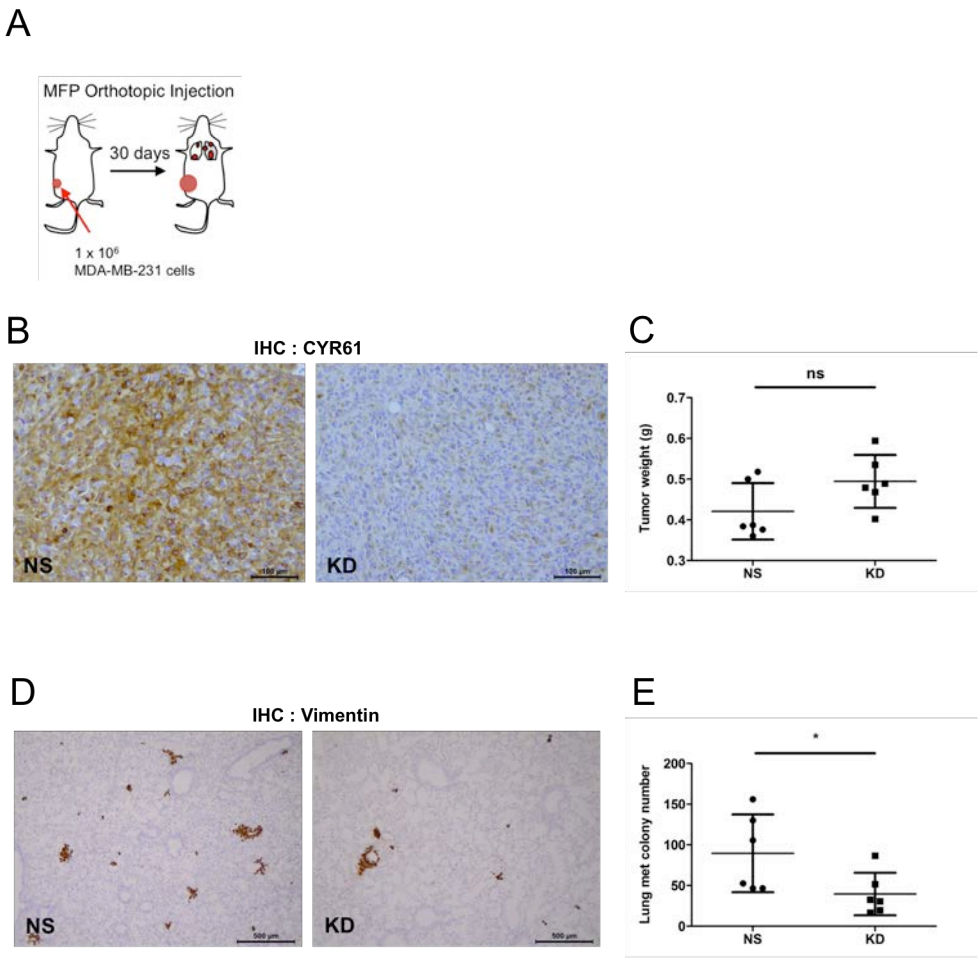

Figure 3. Silencing CYR61 in primary tumors reduces spontaneous lung metastasis formation. (A) Schematic illustration of the protocol of the orthotopic injection model. (B) Immunohistochemical staining of CYR61 showed the knockdown efficiency *in vivo*. Scale bar: 100µm. (C) Primary tumor volume by weight (g). N=6 for both groups; ns: none significance. (D) Immunohistochemical staining of human vimentin demonstrates lung metastases. Scale bar: 500µm. (E) Quantification of lung metastasis colony numbers. N=6 for both groups; *: $p<0.05$.

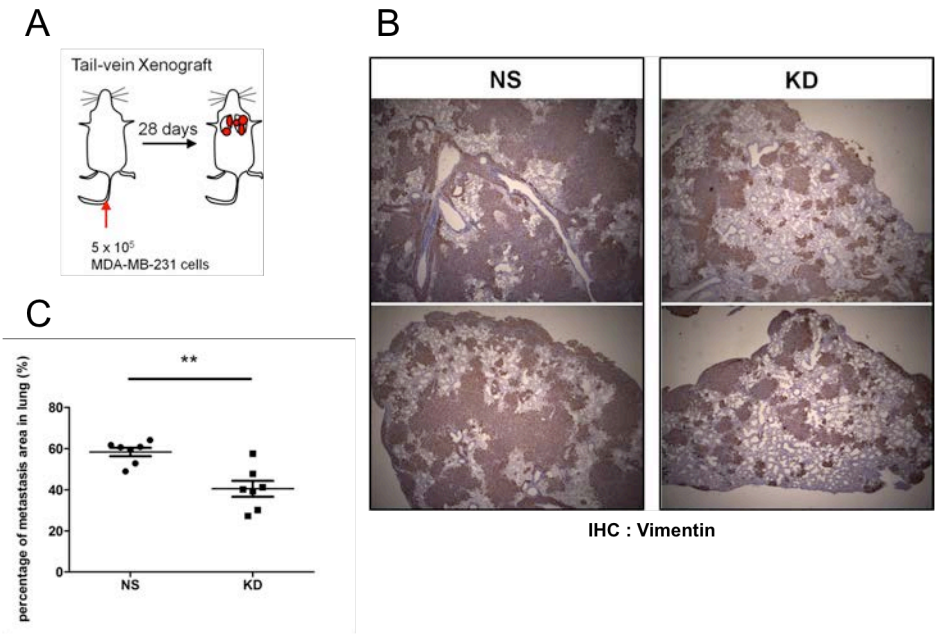

Figure 4. CYR61 down-regulated cells are less metastatic in an experimental model of lung metastasis. (A) Schematic illustration of the protocol of the tail vein injection model. (B) Immunohistochemical staining of human vimentin demonstrates lung metastases. (C) Quantification of lung area occupied by metastatic tumors. N=7 for both groups; **: $p<0.01$.

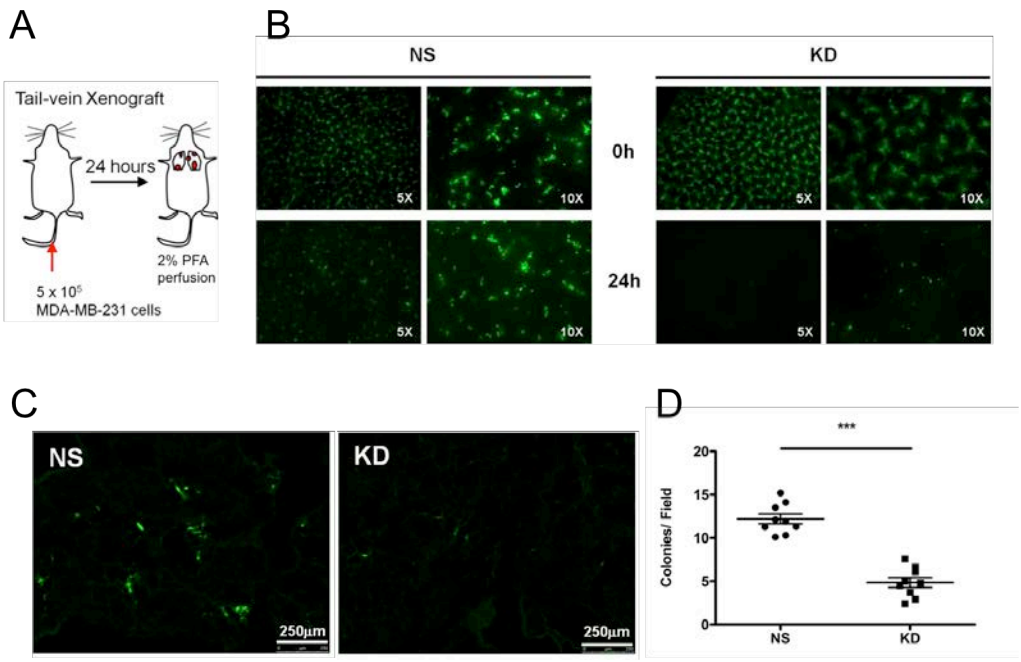

Figure 5. CYR61 facilitates breast cancer cell extravasation into the lung parenchyma. (A) Schematic illustration of the protocol of the 24-hour tail vein injection model. (B) Images of the lung surface taken immediately post-injection (0 hour) show that equal amount of cancer cells labeled with Green Cell Tracker CMFDA were injected. After 24 hours, images of the lung surface demonstrate that significantly more NS cancer cells were present in the lungs. (C) Images from 7µm of lung sections obtained 24 hours post-injection. Scale bar: 250µm. (D) Quantification of cancer cell colony numbers per field from (C). N=9 for both groups; ***: $p<0.005$.

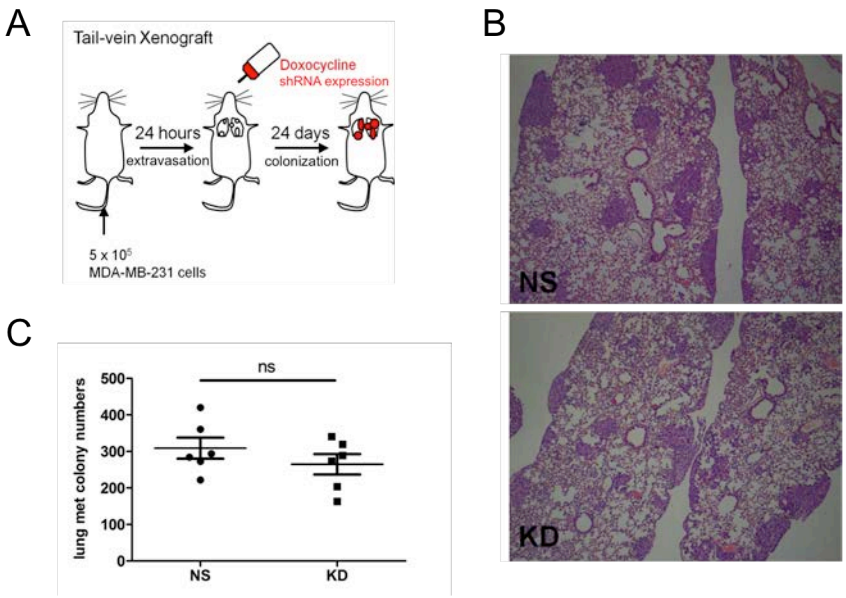

Figure 6. CYR61 does not affect breast cancer lung metastasis formation once cells are extravasated. (A) Schematic illustration of the protocol of delayed down-regulation of CYR61 in the tail vein injection model. (B) HE staining of lungs reveals metastasis formation. (C) Quantification of lung metastasis colony numbers from (B). N=6 for both groups; ns: none significance.

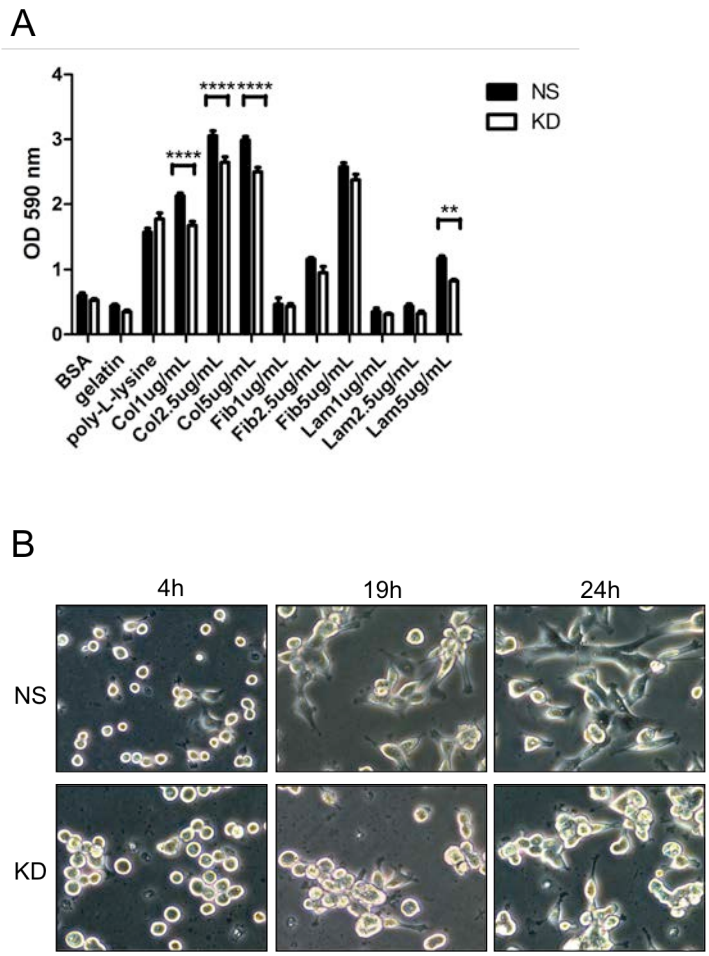

Figure 7. CYR61 affects MDA-MB-231 cell spreading but has minor effects on cell adhesion to extracellular matrix proteins. (A) Silencing CYR61 in MDA-MB-231 cells show minor reduction on adhesion to collagen I and laminin. Adhesion on fibronectin was not affected. Adherent cells were stained with crystal violet (CV) and the absorbance of eluted CV measured at 595 nm was used for quantification. Col: collagen I; Fib: fibronectin; Lam: laminin. **: $p<0.01$; ****: $p<0.001$. (B) CYR61 silenced cells showed retarded spreading under serum-free condition.

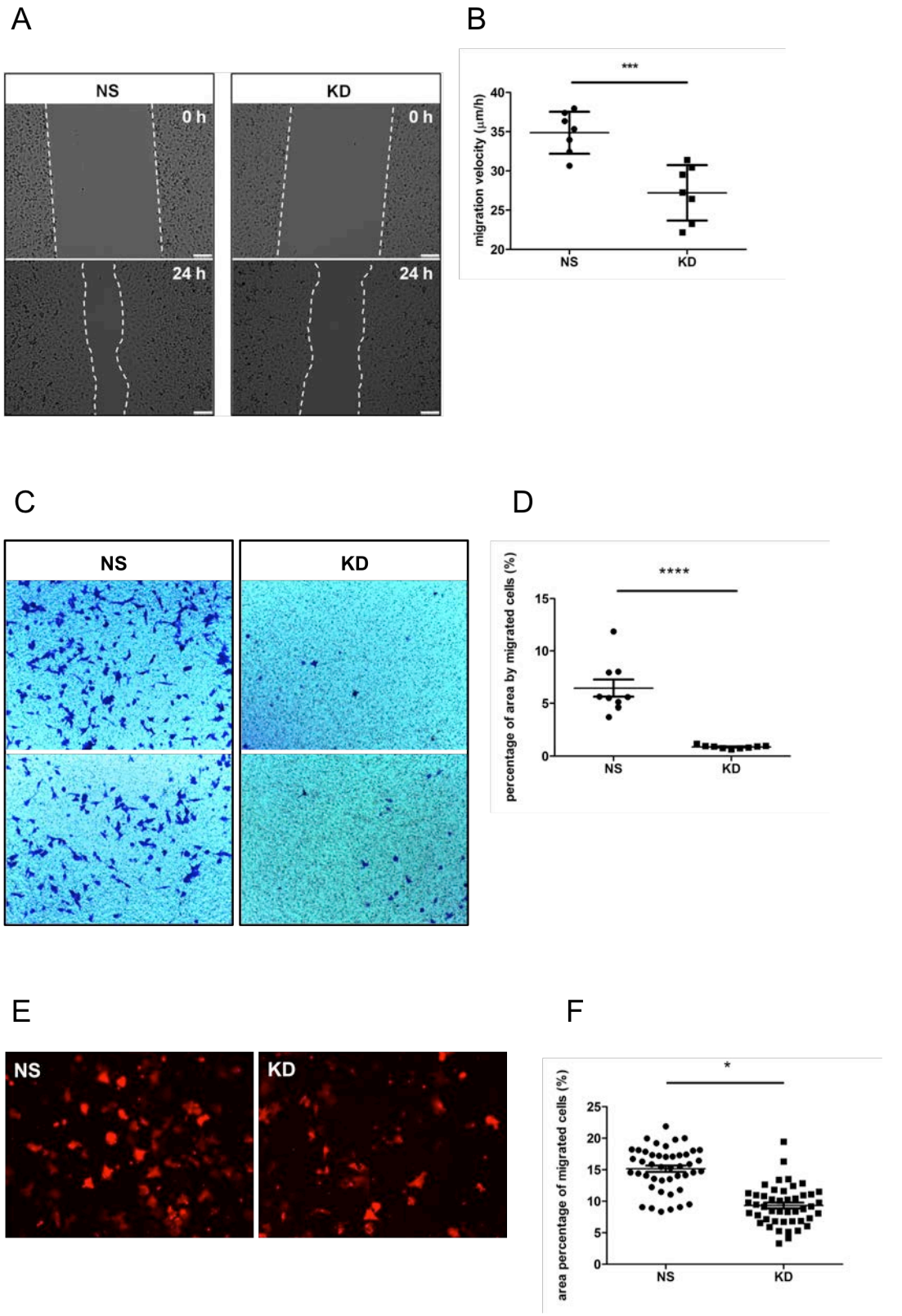

Figure 8. CYR61 enhances MDA-MB-231 cell migration and transendothelial migration. (A-B) CYR61 KD MDA-MB-231 cells migrated

slower in a scratch wound assay. Quantification of migration velocity (μm/hour) is shown in (B). A total of 7 spots from 3 scratches were monitored. ***: $p<0.005$. (C-D) Reduced migration of CYR61 KD MDA-MB-231 cells in a Transwell migration assay. Migrated cells were fixed and stained with crystal violet. Quantification of migrated cells per field is shown in (D). A total of 9 random fields were chosen from 3 transwell inserts. ****: $p<0.001$. (E-F) Reduced migration of CYR61 KD MDA-MB-231 cells in a transendothelial migration assay. RFP-expressing cancer cells that migrated across the endothelial monolayer were photographed and quantified in (F). A total 27 random fields were analyzed from 3 Transwell inserts. *: $p<0.05$.

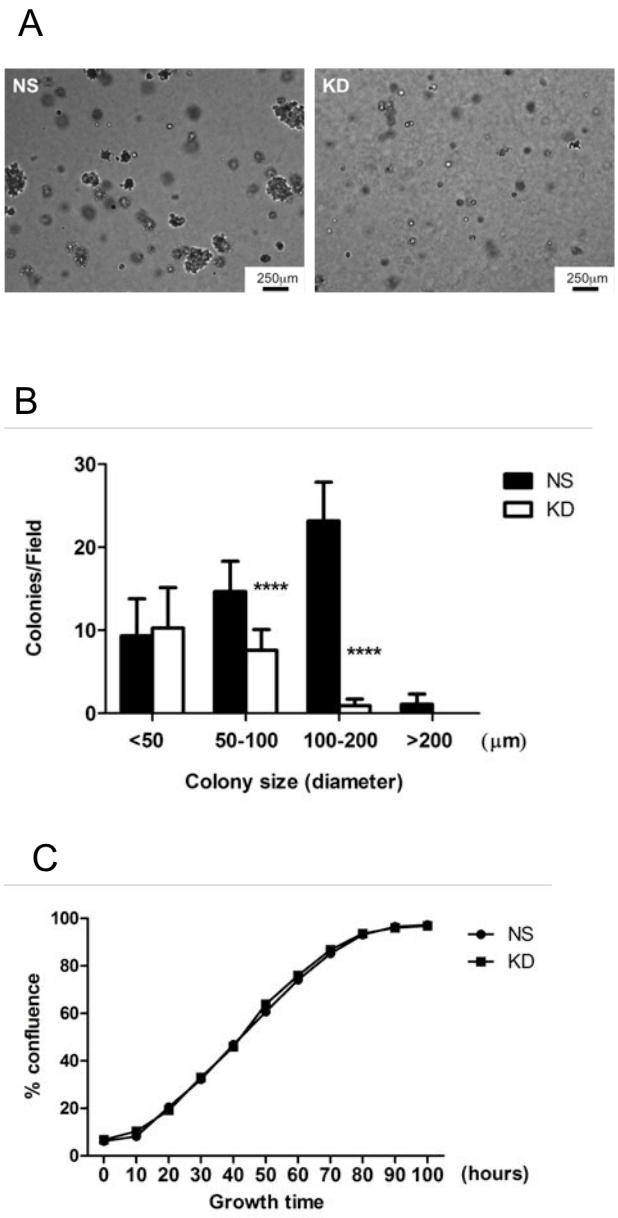

Figure 9. CYR61 enhances colony formation under anchorage-independent conditions. (A) CYR61 silenced MDA-MB-231 cells formed smaller and fewer colonies in soft agar. Scale bar: 250μm. (B) Quantification of colony size and colony number per field from (A). Total 12 random fields from triplicate wells, and 3 focusing depth in each field were analyzed. ****: $p<0.001$. Representative data from one of three independent experiments are shown. (C) 2D proliferation assay showed no difference between NS and CYR61 silenced cells.

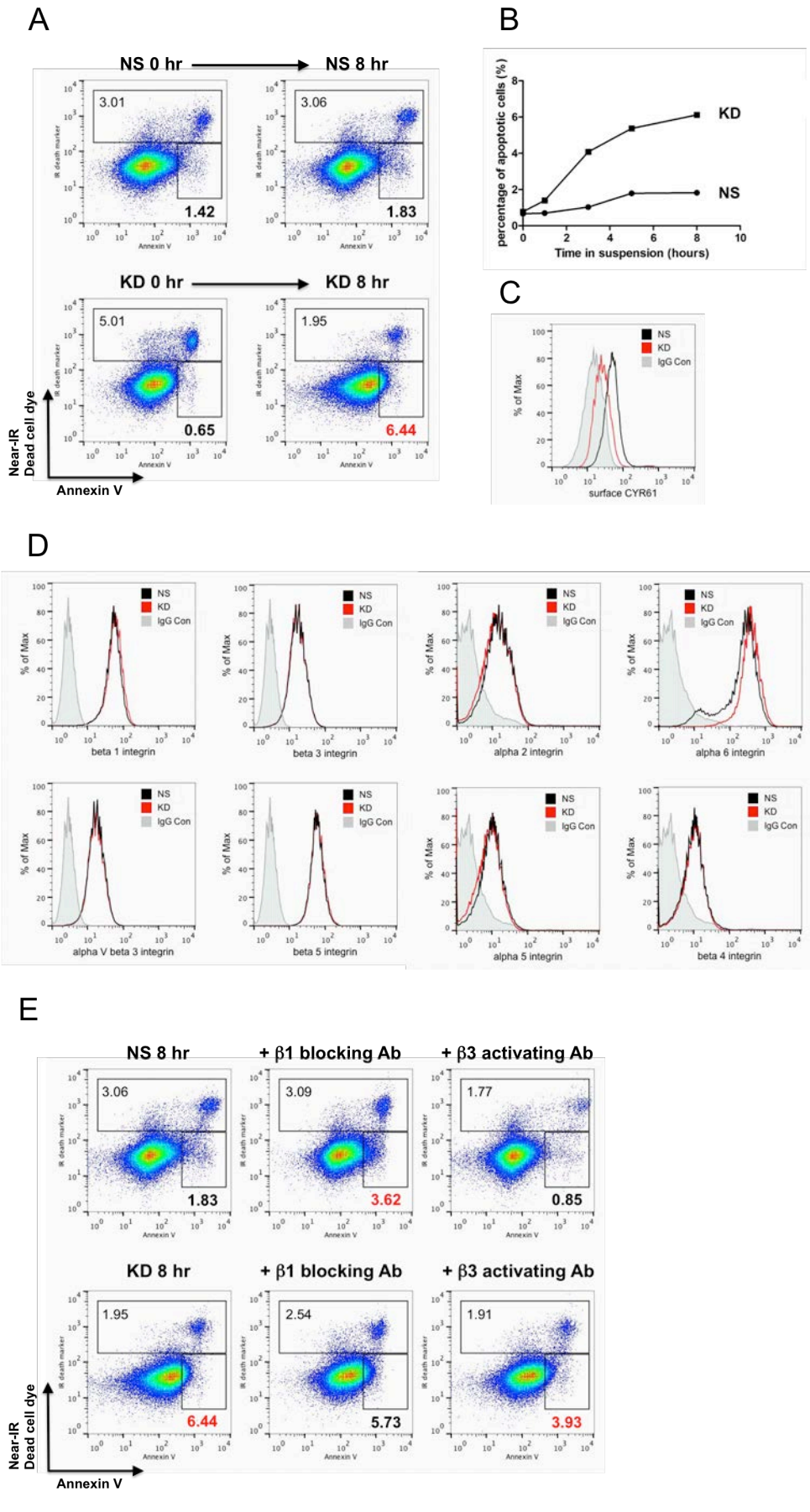
A
NS 0 hr
NS 8 hr
3.01
1.42
3.06
1.83
KD 0 hr
KD 8 hr
5.01
0.65
1.95
6.44
Near-IR Dead cell dye
Annexin V
B
percentage of apoptotic cells (%)
Time in suspension (hours)
KD
NS
C
NS
KD
IgG Con
% of Max
surface CYR61
D
beta 1 integrin
beta 3 integrin
alpha 2 integrin
alpha 6 integrin
alpha V beta 3 integrin
beta 5 integrin
alpha 5 integrin
beta 4 integrin
E
NS 8 hr
+ β1 blocking Ab
+ β3 activating Ab
3.06
1.83
3.09
3.62
1.77
0.85
KD 8 hr
1.95
6.44
2.54
5.73
1.91
3.93

Figure 10. CYR61 confers resistance to anoikis in MDA-MB-231 cells. (A) NS and CYR61 KD MDA-MB-231 cells kept in suspension from 0 to 8 hours were stained with Annexin V-Alexa 488 and near-IR dead cell dye to distinguish viable, apoptotic and dead cell populations. (B) Quantification of the fraction of apoptotic cells of both NS and CYR61 KD populations during suspension time course. (C) CYR61 cell surface level. CYR61 KD cells have reduced CYR61 expression at the cell surface. (D) Integrin expression on NS and CYR61 KD cells. No significant changes in integrin profiles were observed among the two cell lines. (E) Incubation of β1 integrin blocking antibody with NS cells and β3 integrin activating antibody with CYR61 KD cells partially rescue resistance to anoikis mediated by CYR61.

Materials and Methods

Cell culture media, reagents proteins and antibodies

DMEM, DMEM/F12, M199, penicillin/streptomycin and trypsin were purchased from Gibco (NY, USA), and fetal bovine serum (FBS) was from Eurobio (Les Ulis, France). Doxocycline, 3,3'-diaminobenzidine (DAB) tablets, bovine placenta fibronectin and mouse laminin were obtained from Sigma-Aldrich (Buochs, Switzerland). TriPure Isolation Reagent was product of Roche (Mannheim, Germany), and the SuperScript First-Strand Synthesis Kit, Green Cell Tracker CMFDA, Annexin V and LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit were obtained from Invitrogen Life Technologies (Basel, Switzerland). KAPA SYBR FAST Universal 2x qPCR Master Mix kit was purchased from Kapa Biosystems (Boston, USA). Luminata Western HRP Substrate was bought from Millipore (Billerica, MA). Growth factor-reduced Matrigel and rat tail collagen I were purchased from Ceton-Dickinson (Basel, Switzerland). Luciferin was from Biosynth (Staad, Switzerland). Mouse on Mouse (M.O.M.) blocking reagent, Avidin/Biotin blocking kit, Vectastain ABC kit and biotinylated horse anti-mouse IgG were obtained from Vector Laboratories (Burlingame, USA). Vimentin antibody, goat anti-mouse IgG-HRP and goat anti-rabbit IgG-HRP were from DAKO (Glostrup, Denmark), and the CYR61 antibody was from Santa Cruz (TX, USA).

Cell culture

HEK293T, MCF-7, MDA-MB-468 and MDA-MD-231 cells were grown in DMEM supplemented with 10% FBS, and 1% penicillin/streptomycin. Special DMEM/F12 supplemented with 10% horse serum, 20 ng/mL EGF, 0.5 mg/mL hydrocortisone, 100 ng/mL cholera toxin, 10 µg/mL insulin and 1% penicillin/streptomycin was used to grow MCF-10A.

Human umbilical vein endothelial cells (HUVEC) were grown in M199 with 10% FBS, 1% penicillin/streptomycin, 1 µg /mL hydrocortisone, 5 µg /mL

EGF, 5 µg/mL bovine pituitary extract, and 25 unit/mL heparin. Culture plates for HUVEC were pre-coated with 1% gelatin for 30 min. HUVECs between 3rd to 7th passages were used for experiments. For subculture, cells were trypsinized with 0.05% trypsin-EDTA and passaged every 2-3 days. All cultures were kept at 37°C with 5%CO2.

Lentivirus production and transduction

All shRNA vectors (pLKO.1 for constitutive knockdown and pTRIPZ for Tet-On inducible knockdown) were obtained from Open Biosystems (Huntsville, AL). Lentiviruses were produced by co-transfection of shRNA sequence-containing vectors with pSD11 (VSV-G, viral envelope) and pSD16 (packaging construct) into HEK293T cells by calcium phosphate transfection method. Cells were replaced with fresh medium after overnight incubation, and the virus-containing supernatant media were collected, filtered through 0.45µm filters and aliquoted for virus infection.

For transduction, MDA-MB-231 cells were seeded into culture dishes one day before transduction to reach 70% confluence before exposure to the lentivirus. Lentivirus-containing supernatant was supplemented with 10% fresh culture DMEM and 8 µg/mL polybrene and incubated with cells for 6-8 hours. Afterwards, medium was replaced again with fresh culture medium and the cells were kept for 2 days before puromycin selection. Over 90% of cells were successfully transduced and resulted in stable cell lines. To induce the expression of pTRIPZ inducible shRNA system, doxocycline was added to culture medium of puromycin-selected cells with a concentration of 2 µg/mL. Except for specific time course experiment, cells with inducible shRNA system were treated with doxocycline for at least 3 days before conducting experiments. The knockdown efficiency of each shRNA was validated by qPCR and immunoblot for mRNA and protein expression.

Real-time quantitative PCR

Total RNA was isolated from cells lysed with TriPure Isolation Reagent following manufacturer's instruction. RNA concentration was measured by Nanodrop spectrophotometer (Wilmington, USA). For complementary DNA synthesis, up to 5 μg of total RNA was mixed with Superscript First Strand Synthesis Kit for reverse transcription. cDNA were diluted 1:20-1:100 for conducting real-time PCR by using KAPA SYBR FAST Universal 2x qPCR Master Mix kit on the Step One Plus Real-Time PCR System (Applied Biosystems). Dissociation curve of each reaction was checked to determine the purity of PCR end products. Relative Ct values of target genes to house-keeping gene (GAPDH) were calculated to compare the expression level between samples. All qPCR reactions were done in duplicate, and the data from one of three independent repeats was shown. Primer sequences were as following:

CYR61 forward: 5'- ACGCTGGATGTTTGAGTGTG
CYR61 reversed: 5'- TGTAGAAGGGAAACGCTGCT
GAPDH forward: 5'- GGACCTGACCTGCCGTCTAG
GAPDH reversed: 5'- CCACCACCCTGTTGCTGTAG

Immunoblotting

Cells were lysed with 1% NP-40 lysis buffer with protease inhibitors. Twenty μg of total proteins were boiled with 6x sample buffer at 95°C for 5 minutes and resolved by 10% SDS-PAGE with 1x running buffer (25mM Tris, 192mM glycine, 0.1% SDS). After electrophoresis, SDS-PAGE was transferred to Immobilon-P Transfer Membrane (Milipore, Zug, Switzerland) with 1x transfer buffer (25mM Tris, 192mM glycine, 20% methanol) under 250mA for 4 hours. Membranes were blocked by 5% BSA/1x TBSt (10mM Tris, 150mM NaCl, 0.1% Tween 20, pH7.4) at room temperature for 2 hours. Primary antibodies and peroxidase-conjugated secondary antibodies were diluted to 1:1000 and 1:4000 in 1% BSA/1x TBSt, incubated with membranes at room temperature for 1.5 hours. After antibody incubation, the blots were quickly rinsed by 1x

TBSt for three times, and washed by 1x TBSt for three times, 15 minutes per wash time. Blots were incubated with Luminata Western HRP Substrate for 3 minutes, and the chemiluminescent signal was detected by exposing blots to X-Ray films.

In vivo tumor growth and metastasis formation

Eight-weeks-old of NSG female mice (animal facility, CIL, University of Lausanne, Epalinges, Switzerland) were used for all in vivo tumor growth and metastasis formation studies. For orthotopic primary tumor formation, one million of MDA-MB-231 luciferase-expressing cells were resuspended in 40 µL serum-free DMEM and mixed with 10 µL Matrigel were injected into the right 4^{th} mammary fat pad. Formation of spontaneous metastasis was indicated by bioluminescence signal obtained from the Caliper IVIS Lumina II (PerkinElmer, USA) following luciferin i.p. injection. Half million of MDA-MB-231 cells in 100 µL PBS was injected through tail vein to generate experimental metastasis. At the end of the experiments, mice were anaesthetized and perfused with 2% paraformaldehyde (PFA)/PBS. Primary tumors and lungs were fixed by 4% PFA overnight and then embedded in paraffin. Lungs from 24-hour tail vein injection experiment were embedded in OCT for cryosection. All animal experiments were done according to national ethical guidelines and were authorized by the veterinary service of Canton Fribourg.

To quantify metastasis in lung, sections from 2 to 4 different planes with 200 µm distance between each plane were stained by HE or vimentin IHC. Total number of metastasis nodules from the whole section was counted under light microscope (Zeiss, Germany). To determine the lung area occupied by metastasis, five random areas per section were photographed, and the average values for each mouse was calculated. Metastasis index was calculated by normalizing the mean values of metastatic foci number by primary tumor weight.

Immunohistochemistry

Paraffin blocks were cut into 4 μm thin sections. After deparaffinization and dehydration, sections were heated in Tris-EDTA (pH 10) buffer for 20 minutes and slowly cooled down to retrieve antigen epitopes. To quench endogenous peroxidase, sections were incubated with 3% H_2O_2 for 10 minutes. Mouse on Mouse (M.O.M.) blocking reagent was adopted to reduce non-specific binding for vimentin staining. Vimentin antibody and biotinylated goat anti-mouse secondary antibody were diluted 1:200 and 1:800 respectively. Following the incubation of Vectastain ABC kit, the specific immunostaining from antibody-peroxidase complex was visualized with the DAB peroxidase substrate. Sections were then counterstained with hematoxylin and mounted for examination.

Adhesion assay

Collagen I, fibronectin, and laminin were coated at different concentrations (1, 2.5, and 5 μg/mL) on 96-well plates at 37°C for 3 hours followed by blocking with 1% BSA at 4°C overnight. NS and CYR61 KD cells were trypsinized, resuspended in serum-free DMEM, and allowed to adhere on coated plates ($4x10^4$ cells / 100μL / well) for different time period (from 15 minutes to 40 minutes) at 37°C. At the end of adhesion, unattached cells were washed away by rinsing with PBS. Adhered cells were fixed by 4% PFA for 10 minutes and stained with crystal violet. Specific binding to each ECM protein was determined by quantification of absorbance at 595 nm wavelength in a multiwall plate reader (Modulus II microplate reader, Turner Biosystems). Results were analyzed by Prism, and the representative results from three independent experiments are shown.

Spreading assay

Cells were trypsinized and resuspended in serum-free DMEM. Equal amount of NS and CYR61 KD cells were plated under serum-free conditions. Cell

spreading was monitored and photographed by phase contrast microscopy every 2 hours. Representative data from one of the three independent experiments are shown.

Scratch wound migration assay

MDA-MB-231 cells were grown to 100% confluent in 6 well plates. Scratches were made by micro-pipet tips, and the medium was immediately replaced with 5% FBS-containing DMEM to remove suspended cells. The closure of scratches was monitored by timelapse microscopy for 24 hours. In total, seven spots from three scratches were recorded for each cell line, and the migration velocity was presented as migrated distance (μm) per hour. Representative data from one of the three independent experiments was shown.

Transwell migration assay

The Transwell inserts (8μm pore size, Becton-Dickinson, Bedford, MA) were pre-incubated with serum-free DMEM at 37°C for 30 minutes before use. Then, 700 μL DMEM containing 5%FBS was carefully loaded to the lower part of chamber, and $4x10^4$ cells in 200μL serum-free DMEM was added to the upper part of chamber. Cells were allowed to migrate through the membrane for 8 hours at 37°C. Non-migrated cells still in the upper part of chamber were carefully removed by cotton swabs. The membrane was then fixed with 4% PFA for 10 minute to fix the cells migrated on the lower side of the membrane, followed by crystal violet staining for 10 minutes. Three random fields were chosen from each insert and total 9 fields were photographed to quantify the numbers of migrated cells. Representative data from one of the three independent experiments are shown.

Transendothelial migration assay

HUVECs (5000cells / insert) were plated on gelatin pre-coated Transwell inserts (8μm pore size) and cultured for 2 days to reach 100% confluence. Before experiments, medium in the lower chamber was replaced with 700 μL M199 containing 5% FBS, and medium in the upper chamber was replaced with 100 μL serum-free M199. RFP-expressing NS and CYR61 KD MDA-MB-231 cells were trypsinized, and $4x10^4$ cells in 100 μL serum-free DMEM was loaded to each chamber. Transendothelial migration was allowed for 12 hours at 37°C. At the end of the experiment, HUVECs and non-migrated MDA-MB-231 cells in the upper part of the chamber were carefully removed by cotton swabs. The membrane was then fixed with 4% PFA for 10 minute to fix the cells migrated on the lower side of the membrane and migrated cells were visualized under fluorescent microscope. Nine random fields from each insert were photographed. Total 27 fields were analyzed by ImageJ to quantify the area covered by migrated cells. Representative data from one of the three independent experiments are shown.

Proliferation assay

Cell growth rate was calculated from surface area covered by cells optically determined by IncuCyte FLR (Essen Bioscience, USA). MDA-MB-231 cells ($12.5x10^4$ cells / well) were plated in a 6-well plate at 5% initial confluence and cultured until the confluence reached 100%. Total 18 spots from 2 wells per cell line were monitored. Representative data from one of the three independent experiments are shown.

Anchorage-independent cell growth assay

Six-well plates were pre-coated with 0.6% agar in DMEM supplemented with 5% FBS. Cells ($2x10^4$ cells / 2mL / well) mixed with 0.3% agar in DMEM supplemented with 5% FBS were plated on top. DMEM with 5% FBS was added to the wells and replaced with fresh medium every 2 days. After 2

weeks, colonies were stained with 0.005% crystal violet. Twelve random fields from triplicate wells were photographed with 3 different focuses to calculate both colony number and colony size. Colony size is categorized into 3 groups according to the diameter: <50μm, 50-100μm, 100-200μm, and >200μm. Representative data from one of the three independent experiments are shown.

Anoikis assay

Cells were trypsinized and $3x10^5$ cells were kept in FACS tubes in serum-free DMEM for the indicated periods of time. Different integrin blocking or activating antibodies were co-incubated with cells throughout the time being in suspension. At the end of experiment, cells were stained with Annexin V-Alexa 488 and LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit. The apoptotic (Annexin V positive) and dead cell (near-IR dye positive) populations were determined by FACSCalibur flow cytometer (BD, Germany) and analyzed by FlowJo software (TreeStar, USA).

Statistical analysis

Data from all experiments were expressed as mean ± SD. The analysis was performed by unpaired T-test by Prism. P-value < 0.05 was considered statistically significant.

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Conferences and Publications

Conferences:

2014	24th CytoMeet, Bern, Switzerland (Oral presentation)
2013	5th International Conference on Tumor-Host Interaction and Angiogenesis, Monte Verità, Ascona, Switzerland
2013	2nd Research Day in Medicine, Fribourg, Switzerland
2012	2012 SmART Symposium: Vascular Progenitors in Biology and Medicine, Fribourg, Switzerland
2012	1st Research Day in Medicine, Fribourg, Switzerland
2011	New Concepts in Cancer Metastasis, Lisbon, Portugal
2010	4th International Conference on Tumor-Host Interaction and Angiogenesis, Monte Verità, Ascona, Switzerland
2008	Experimental Biology, San Diego, USA
2005	Experimental Biology, San Diego, USA

Publications:

1. **Huang YT**, Lan Q, Rüegg C. CYR61 promotes breast cancer metastasis to lung by facilitating extravasation. *in preparation*.
2. **Huang YT**, Blanquet M, Ponsonnet L, Christofori G, Zaric J, Rüegg C. CYR61 promotes tumor growth and invasion but not angiogenesis in the Rip1Tag2 model. *in preparation*.
3. Lan Q, Lorusso G, Duffey N, **Huang YT**, Rüegg C. Mechanisms of chemotherapy-induced tumor dormancy in metastatic breast cancer. *in preparation*.
4. Huang J**,** Che MI, Hung JS, Lin NY, **Huang YT**, Lin WC, Huang HC, Lee PH, Liang JT, Huang MC. The molecular chaperone Cosmc enhances malignant behaviors of colon cancer cells via activation of Akt and ERK. ***Molecular Carcinogenesis***. 2013 Feb 6. doi: 10.1002/mc.22011. [Epub ahead of print]

5. Huang J, Che MI, **Huang YT**, Shyu MK, Huang YM, Wu YM, Lin WC, Huang PH, Liang JT, Lee PH, Huang MC. Overexpression of MUC15 Activates Extracellular Signal–Regulated Kinase 1/2 and Promotes the Oncogenic Potential of Human Colon Cancer Cells. ***Carcinogenesis***. 2009 Aug;30(8):1452-8.
6. **YT Huang**, SU Chen, CH Chou, and H Lee. Sphingosine 1-Phosphate Induces Platelet/Endothelial Cell Adhesion Molecule-1 Phosphorylation in Human Endothelial Cells Through cSrc and Fyn. 2008. ***Cellular Signaling***, 20(8):1521-7.
7. **YT Huang**, YW Lee, JJ Liao , CI Lin, J. H. Haga, J. Li, and H Lee. Sphingosine 1-Phosphate Induces Platelet/Endothelial Cell Adhesion Molecule-1 Tyrosine Phosphorylation in Bovine Aortic Endothelial Cells through a PP2-Inhibitable Mechanism. 2007. ***Taiwania***, 52(4): 324-331.
8. YW Lee, JJ Liao, **YT Huang**, YT Kuo and Lee H. The Expression Profiles of LPLs Receptors in Different Endothelial Cells. 2006. ***Taiwania***, 51(1):11-24.
9. Liao JJ, **Huang YT**, Lee H. Inhibitory Effects of Sphingosine 1-Phosphate on Proliferation of PC-3 Human Prostate Cancer Cell. 2005. ***Zoological Studies***, 44(2):219-227.

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Education

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2005/9-2007/6 **National Taiwan University** Taipei, Taiwan
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Thesis topic: Studies of the Mechanisms of Sphingosine 1-phosphate Induces Platelet/Endothelial Cell Adhesion Molecule-1 Phosphorylation in Human Endothelial Cells

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Publications

Research papers

1. **Huang YT**, Lan Q, Rüegg C. CYR61 promotes breast cancer metastasis to lung by facilitating extravasation. *in preparation*.
2. **Huang YT**, Blanquet M, Ponsonnet L, Christofori G, Zaric J, Rüegg C. CYR61 promotes tumor growth and invasion but not angiogenesis in the Rip1Tag2 model. *in preparation*.
3. Lan Q, Lorusso G, Duffey N, **Huang YT**, Rüegg C. Mechanisms of chemotherapy-induced tumor dormancy in metastatic breast cancer. *in preparation*.
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6. **YT Huang**, SU Chen, CH Chou, and H Lee. Sphingosine 1-Phosphate Induces Platelet/Endothelial Cell Adhesion Molecule-1 Phosphorylation in Human Endothelial Cells Through cSrc and Fyn. 2008. ***Cellular***

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7. **YT Huang**, YW Lee, JJ Liao , CI Lin, J. H. Haga, J. Li, and H Lee. Sphingosine 1-Phosphate Induces Platelet/Endothelial Cell Adhesion Molecule-1 Tyrosine Phosphorylation in Bovine Aortic Endothelial Cells through a PP2-Inhibitable Mechanism. 2007. ***Taiwania***, 52(4): 324-331.
8. YW Lee, JJ Liao, **YT Huang**, YT Kuo and Lee H. The Expression Profiles of LPLs Receptors in Different Endothelial Cells. 2006. ***Taiwania***, 51(1):11-24.
9. Liao JJ, **Huang YT**, Lee H. Inhibitory Effects of Sphingosine 1-Phosphate on Proliferation of PC-3 Human Prostate Cancer Cell. 2005. ***Zoological Studies***, 44(2):219-227.

Conference Presentation:

1. **Huang YT**. CYR61 promotes breast cancer metastasis to lung by facilitating extravasation. 2014. At the 24th CytoMeet, Bern, Switzerland

Conference Abstracts:

1. **Huang YT** and Rüegg C. CYR61 promotes early steps of breast cancer cell metastasis to the lung. 2013. 5th International Conference on Tumor-Host Interaction and Angiogenesis, Monte Verità, Ascona, Switzerland.
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Scholarship

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