Modulating the behaviour of pancreatic tumour cells

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Chapter 3: LIMK1 and LIMK2 are important for metastatic behaviour and tumour cell-induced angiogenesis of pancreatic cancer cells.

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Abstract

The two LIM kinases, LIMK1 and LIMK2, are members of the PDZ/LIM family. These serine/threonine protein kinases are involved in actin cytoskeleton reorganization through phosphorylation and inactivation of ADF (actin depolymerizing factor)/cofilin. Different subcellular localizations of LIMK1 and LIMK2 suggest different functions. Whereas LIMK1 is implicated in microtubule disassembly in endothelial- and cancer cells, activation of LIMK2 plays a role in cell cycle progression. To compare the role of the two LIM kinases in cancer related processes, we used a cell-based *in vitro* migration assay, as well as two zebrafish xenograft assays. We analyzed here the metastatic behaviour and tumour cell-induced neovascularization of pancreatic cancer cells in which both LIM Kinase genes were silenced by siRNAs. Both LIMK1 and LIMK2 single knock down led to a reduction of invasion and metastatic behaviour in the zebrafish xenograft metastasis assay. Interestingly, the double knock down completely blocked invasion and formation of micrometastasis *in vivo*. Moreover, in the zebrafish xenograft angiogenesis assay, we observed a reduction of pancreatic cancer cell-induced angiogenesis for both of the LIMK1 and the LIMK2 knock downs. Our results demonstrate similar functions for the two LIM kinases in pancreatic cancer cells and suggest an important role for both, LIMK1 and LIMK2, in tumour progression and metastasis formation.

Introduction

Tumourigenesis involves interactions of cancer cells with each other, neighboring cells and their microenvironments, such as stromal cells or the blood vessels that supply oxygen and nutrients. Cancer cells often induce surrounding endothelial cells to build new vessels in order to balance this supply with the demand of a growing tumour. Blood vessels (as well as lymphatic vessels) are further used by metastasizing cancer cells for their long and short distance traveling. The complex mechanisms that regulate these "nomadic" cancer cells and governs the formation of metastases involves intravasation and extravasation in and out of blood- or lymphatic vessels. In order to successfully form a micrometastasis, cancer cells first need to take advantage of the vasculature and/or the lymphatics, and then after extravasation, have to settle and survive in an often hostile microenvironment. The importance to understand and prevent metastasis formation is underlined by the fact that over 90% of all cancer related deaths are due to secondary tumours arising from metastasizing cancer cells.

The zebrafish has emerged as a novel *in vivo* cancer model to study both tumour-induced neovascularization (Nicoli, S., Presta M., 2007; Nicoli S., Ribatti D., 2007) and the formation of micrometastases of xeno-transplanted cancer cells (Haldi M., et al, 2006; Topczewska JM., et al,2006) and tumour fragments (Marques I., et al, 2009). In the transparent zebrafish embryos, invasion/ migration of tumour cells, their circulation in the vascular system, as well as the formation of micrometastasis and the tumour-induced neovascularization can all be followed with high resolution in real-time (Marques I., et al, 2009). Importantly, these zebrafish models allow to quantitate both metastatic behaviour of transplanted tumour cells and tumour-cell induced neovascularization (Nicoli, S., Presta M., 2007; Marques I., et al, 2009). In this study we use these models to characterize and compare the role of the two LIM Kinases in tumourigenesis of human pancreatic cancer cells.

The LIM Kinases Leucine-rich immunoglobulin-like motif LIMK) are Serine/Threonine Kinases, which regulate actin polymerziation via phosphorylation and inactivation of ADF/cofilin (reviewed in (te Velthuis AJ., et al, 2007)). Actin dynamics contribute to cell motility, morphogenesis, division, differentiation, apoptosis and cancer. Given the importance of the cytoskeleton for most cellular functions, it is not surprising that the LIMKinases are ubiquitously expressed during development, and that disruption of normal LIMK expression and/or signaling is associated with numerous disorders. For example, abnormal expression of LIMK1 in humans has been associated with Williams syndrome (a severe mental disorder with profound deficits in visuospatial cognition) and Alzheimer's disease (Heredia L., et al, 2006; Tassabehji M., et al, 1996). Two LIM Kinase genes are encoded in the human genome. The human LIMK1 gene is located on chromosome 7 (7q11.23) and the LIMK2 gene

is located on chromosome 22 (22q12) (Mao X JT., et al, 1996; Okano I., et al,1995). Both genes encode for proteins with two N-terminal LIM domains, a C-terminal kinase domain and a PDZ domain in between. In addition to their role in regulating the actin cytoskeleton, LIMK1 has also been shown to play a role in microtubule disassembly (Gorovoy M., et al, 2005).

In mice, ablation of LIMK1 leads to abnormalities in dendritic spine morphology and in synaptic function (Meng Y., et al, 2002). In contrast to *Limk1* null mice, *Limk2* knockout mice do not exhibit any impaired neuronal morphology or other phenotypic abnormalities in postnatal growth and development, except for impairment in spermatogenesis (Takahashi H., et al, 2002). However, when a double knockout was applied, the neuronal phenotype appeared more pronounced than in the genetic deletion of *Limk1* alone (Meng Y., et al, 2004). For both LIM Kinases, cell type specific functions have been suggested and their cellular localization was found to differ in vertebrates (Acevedo K., et al, 2006; Ott EB., et al,2007). While LIMK1 is found in focal adhesions, LIMK2 is shown to be localized in endosome-like punctae (Acevedo K., et al, 2006). Recently, it was demonstrated that activation of LIMK2, but not LIMK1, by ROCK (Rho-associated, coiled-coil containing protein kinase) in 3T3 cells led to increased levels of cyclin A and cell cycle progression (Croft DR., Olson MF., 2006). Increased cell cycle progression was also evident in MDA-MB-435 cancer cells overexpressing LIMK1 (Rozita Bagheri-Yarmand AMAASRK, 2006). Thus both LIM Kinases have been shown to play a role in cell cycle progression, but in a cell-type specific manner (Sumi T., et al, 2002).

To date, robust evidence suggests that LIMK1 activity is important for cancer cell metastasis. High expression levels of LIMK1 are observed in invasive breast and prostate cancer cell lines and in malignant human prostate tumours (Wang W., et al, 2006). Overexpression of LIMK1 increases the invasiveness of non-invasive breast and prostate cancer cells *in vivo* and *in vitro*; while expression of dominant-negative kinase-dead LIMK1 or LIMK1 anti-sense RNA greatly reduced the invasiveness of breast and prostate cancer cells, respectively (Wang W., et al, 2006). The mechanism by which LIMK1 increases the invasiveness of cancer cells is not yet clear. It may be due to increased cell cycle progression, increased motility and also as recently suggested to increased expression levels of the serine protease urokinase type plasminogen activator (uPA) and its receptor, known to be involved in metastasis (Rozita Bagheri-Yarmand AMAASRK, 2006). These latter findings suggest an important role for LIMK1 signaling in breast cancer tumour growth, angiogenesis and invasion and a regulatory connection between LIMK1 and the uPA system (Rozita Bagheri-Yarmand AMAASRK, 2006). Whether all these mechanisms are important in tumour progression or if only a few play a part, is not known and needs to be determined in the future. Notably, in contrast to LIMK1, the role of LIMK2 in tumour progression and metastatic behaviour of cancer cells has not been described so far.

In this study, we have investigated the function of LIMK1 in metastatic behaviour of pancreatic cancer cells and compared it to the role of LIMK2. We further tested the hypothesis that both LIM Kinases might play a role in tumour cell-induced neovascularization.

Materials and Methods

Animal care and handling

Tg(fli1:eGFP) zebrafish were kept at 28°C in aquaria with day/night light cycles (10h dark versus 14h light periods). The developing embryos were kept in an incubator at constant temperatures (which were varied according to the experimental requirements).The zebrafish were handled in compliance with local animal care regulations and standard protocols of the Netherlands.

Cell culture

The two human pancreatic cancer cell lines, PaTu 8988t (Elsässer HP LU., et al, 1992) were cultured in DMEM high glucose, with 10% FCS and 1:100 Pen/Strep and Panc-1 (Lieber M MJ., et al, 1975) were cultured in RPMI with 15% FCS and 1:100 Pen/Strep. PaTu 8988t were derived from a liver metastasis over a decade ago (Elsässer HP LU., et al, 1992) and we had earlier described the cell line in detail and its behaviour in an *in vitro* wound healing assay and in the zebrafish xenotransplantation assay (Marques I., et al, 2009).

siRNA treatment of pancreatic cancer cells and control PCR

The siRNAs for silencing human LIMK1 and human LIMK2 were purchased from Santa Cruz Biotechnology (LIMK-1: sc-35810 and LIMK-2: sc-35812). The EndoPorter system (Gene Tools, LLC) was used to deliver siRNAs into pancreatic cancer cells. To test for unspecific effects, a control siRNA (Control siRNA-A sc-37007; Santa Cruz Biotechnology) was used, which contains a scrambled sequence that will not lead to the specific degradation of any known cellular mRNA. All siRNAs were used at a final concentration of 50 nM. The efficiencies of LIMK-1 and LIMK-2 siRNAs were tested by an RT-PCR, which we described previously including total RNA preparation (van der Meer DLM, et al, 2006). The annealing temperature was set to 55° C and the extension temperature to 72° C. For the amplification, the commercially available primer pairs of human LIMK1 and LIMK2 were used (LIMK1 primer pair: sc-35810-PR fragment size = 419 bp and LIMK-2 primer pair: sc-35812 fragment size = 594 bp; both Santa Cruz Biotechnology).

Cell staining, transplantation and incubations

Cells were stained with CM-Dil (red fluorescence) (Vybrant, Invitrogen). Cells were seeded in 6-well plates, grown to confluency and then trypsinized. Subsequently, cells were washed with 67% DPBS (GIBCO, Invitrogen), transferred to 1.5 ml Eppendorf tubes and centrifuged 5min, at 1500rpm. Cells were re-suspended in DPBS (Dulbecco's phosphate buffered saline) containing CM-Dil (4ng/ul concentration). Cells stained with CM-Dil were incubated 4 min at 37^{o}C and then 15 min at 4^{o}C.

Subsequently, cells were centrifuged 5 min at 1500 rpm, the supernatant discarded and cells re-suspended in 100% FCS, centrifuged again and washed 2 times with 67% DPBS. Cells were now suspended in 67% DPBS for injection into the embryos. 2 dpf zebrafish embryos were dechorionated and subsequently anesthetized with tricaine (Sigma). Using a manual injector (Eppendorf; Injectman NI2), the cell suspension was loaded into an injection needle (15 µm internal- and 18 µm external diameter) and cells were injected in Tg(fli1:eGFP) zebrafish embryos. After injection, embryos were incubated for 1h at 31^{o}C and checked for cell presence at 2 hours post transplantation (hpt). Fish with fluorescent cells outside the implantation area at 2 hpt were excluded from further analysis. All other fish were incubated at 35^{o}C for the following days.

In vitro *migration assay ("scratch"-assay)*

The *in vitro* migration assay was carried out as previously described (Liang C-C., et al, 2007). Cells were grown to confluency in 6-well dishes. Then the cell monolayer was scraped in a straight line with a 200 µl pipette tip. Pictures of the scratch were taken under a Leica stereomicroscope at 0h, 24h and 48h and the gap was measured and gap closure was calculated in percentage to the initial gap (scratch).

Zebrafish xenograft angiogenesis model (Tumour-cell induced neovascularization assay)

The tumour-cell induced neovascularization assay was in general performed as previously described (Nicoli, S., Presta M., 2007; Nicoli S., Ribatti D., 2007). In detail, 48 hpf zebrafish embryos were dechorionated and anesthesized using 0.042 mg/ mL Trincaine (Sigma, St. Luis, MO) and put on a 1,8 % Agarose dish for injections. Cells were prepared as described above, then suspended in 20 µl 12,0 mg / ml Matrigel solution (Cultrex R Basement Membrane Extract, R&D systems, Minneapolis, USA) and kept on ice for a short period until injection. The CellTram Oil injector (Eppendorf, Hamburg, Germany) was loaded by using a manual injector (Eppendorf; Injectman NI2). An aliquot of 5 µl of the cell suspension in Matrigel was loaded into an injection needle, and embryos were injected as described in more detail in previous studies (Nicoli, S., Presta M., 2007; Nicoli S., Ribatti D., 2007). After injection, embryos were incubated at 35°C for 24 h. Embryos were checked and photographed using the Biorad confocal microscope 1024ES (Zeiss).

Imaging

Confocal pictures were taken with the Biorad Confocal microscope 1024ES (Zeiss microscope) combined with a Krypton/Argon laser. For light-microscopy images of pancreatic cells, a Leica MZ16FA stereomicroscope and a Leica DFC 420C camera was used.

Results

LIMK1 and LIMK2 are important for migration of human pancreatic cancer cells *in vitro*

Despite their well-documented role in regulation of actin polymerization and microtubule disassembly (Bernard O., 2007), studies comparing other biological functions of LIMK1 and LIMK2 are limited. In contrast to the well described role of LIMK1 in cell migration/invasion e.g. of breast cancer cells (Ding Y., et al, 2008), nothing is known of a role for LIMK2 in these processes.

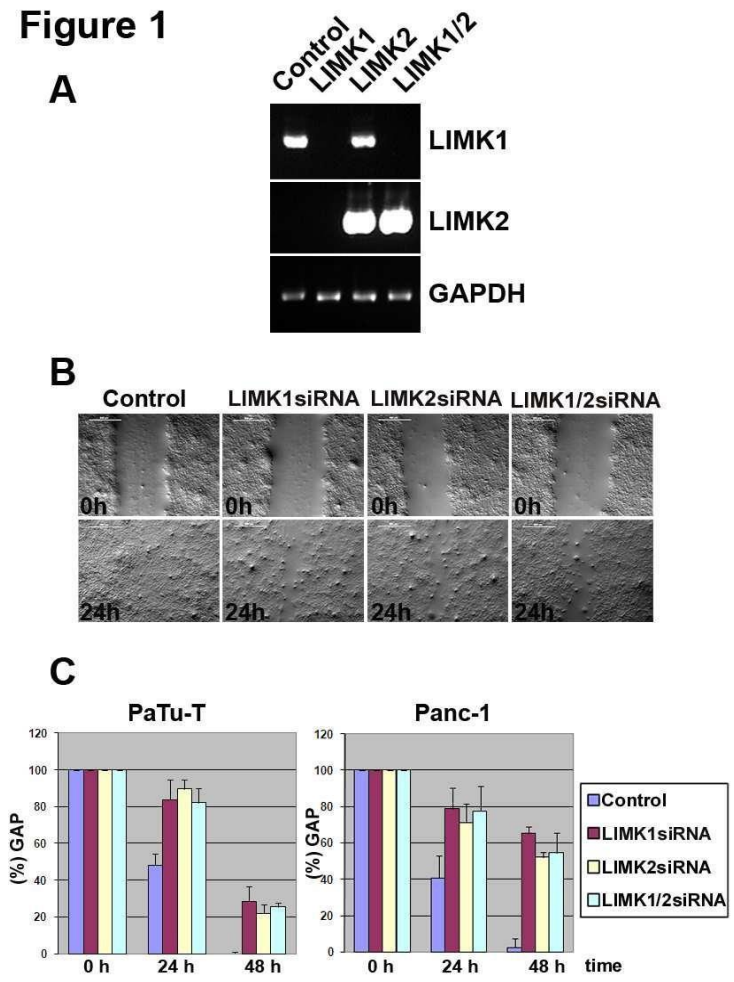

Figure 1: Semi-quantitative RT-PCR and i*n vitro* migration assay of pancreatic cancer cell lines. (A) Semi quantitative RT-PCR shows that LIMK1 and LIMK2 are expressed in PaTu 8988t and in Panc-1 cells and gene specific siRNAs for LIMK1 and LIMK2 silence the respective genes by abolishing mRNA expression. Control GAPDH expression was analyzed by RT-PCR of the same RNA samples. This loading control is shown in the lower panel. LIMK1 specific primers were used in the RT-PCR shown in the top panel and LIMK2 specific amplification is shown

in the middle panel. (B) An *in vitro* migration assay ('scratch assay') shows differences in migration of PaTu 8988t cells treated with control siRNA (Control), LIMK1 siRNA , LIMK2 siRNA or LIMK1 plus LIMK2 siRNA (LIMK1 and 2). Gap closure (gap width) over time is shown in percentages compared to the 0h time point (set to 100%). Similar results were obtained in three independent experiments and error bars show Standard Deviations between the three experiments. On average on day 1 (24 h) for LIMK1 16.4%, LIMK2
10.3%, LIMK1/2 18% and the control 51.8% of the gap was closed. On day 2 (48 h) for LIMK1 53.6%, LIMK2 10.3%, LIMK1/2 18% and the control 51.8% of the gap was found closed. Similar results were obtained in three independent experiments.

Both LIM Kinases had been shown to be expressed in the human pancreas, with LIMK2 showing a relatively high abundance compared to several other tissues (Smolich B., et al, 1997). We found that LIMK1 and LIMK2 are both expressed in the pancreatic cancer cell line, PaTu 8988t (Figure 1A).

In this study, we were interested in the diverse and common functions of LIMK1 and LIMK2 in cancer related processes. In an *in vitro* migration assay (also called *in vitro* scratch assay) (Liang C-C., et al, 2007), we investigated the effects of LIMK1 and LIMK2 knock down in two pancreatic cancer cell lines, PaTu 8988t and Panc-1 cells. We had previously described the behaviour of PaTu 8988t cells in an *in vitro* migration assay (Marques I., et al, 2009). We used gene specific siRNAs to silence the LIMK1 and LIMK2 genes in this cell line and in addition to the respective single knock downs, we performed a LIMK1/2 double knock down. Confirmation of gene silencing in PaTu 8988t cells was obtained by semi-quantitative RT-PCR (Figure1A). The results of the *in vitro* migration assay using PaTu 8988t and Panc-1 cells showed that both, LIMK1 and LIMK2 knock down have an inhibitory effect on gap closure over time (Figure 1B and C). The double knock down had no additive effect and inhibition was comparable to that exerted by both single knock downs (Figure 1B and C). These findings suggest a role for both LIM kinases in invasion/migration of pancreatic cancer cells.

LIMK1 and LIMK2 both play a role in metastatic behaviour of human pancreatic cancer cells

In a previous study we have demonstrated that fluorescently labelled PaTu 8988t cells and tumour fragments allow for *in vivo* imaging of metastatic behaviour (Marques I., et al, 2009). PaTu 8988t cells rapidly invaded the zebrafish body, translocated into the vasculature and colonized at distant sites in the zebrafish larvae (5 days post fertilization (dpf)) forming micro-metastases (Marques I., et al, 2009). The sensitivity of the zebrafish tumour xenograft metastasis model allows observation of individual cells and their daughter cells *in vivo* and thereby enables the quantification of metastatic behaviour *in vivo*. To study the role of the LIM Kinases in metastatic behaviour, we labelled two pancreatic tumour cell lines with a carbocyanine dye (CM-DiI) and ectopically transplanted them in the yolk sac of 2 dpf zebrafish embryos. In order to achieve optimal visualization, we utilized the

transgenic zebrafish line, Tg(flil:eGFP) (Lawson ND., et al, 2002), which expresses **GFP** under the *flil* promotor (an early endothelial marker) and therefore exhibits a green fluorescent vasculature (Lawson ND., et al, 2002).

Figure 2 A

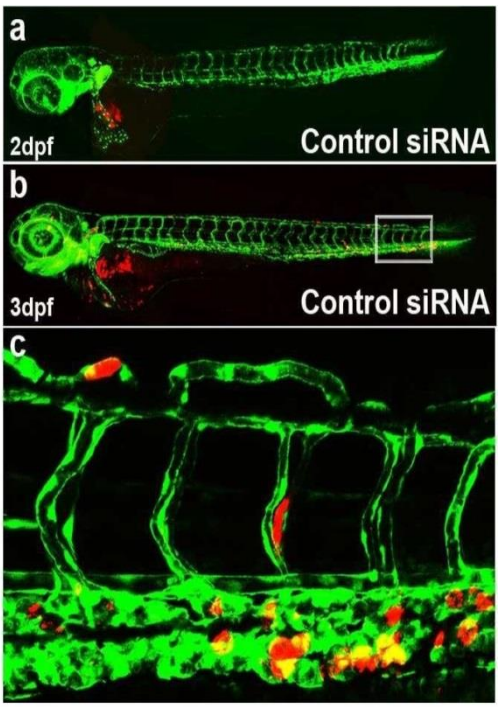

Figure 2 B

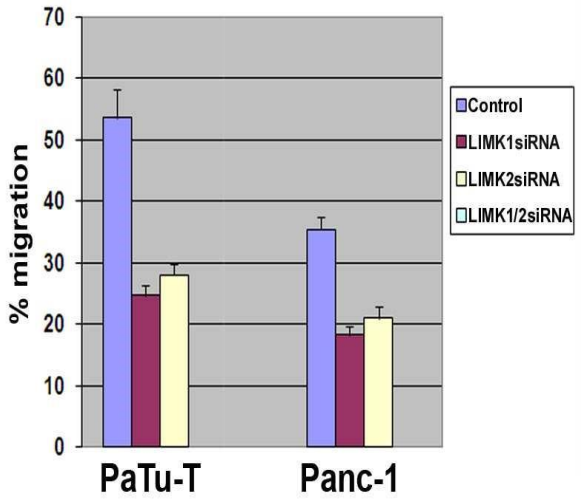

Figure 2:The zebrafish tumour xenograft metastasis assay.

(A) Shown are example pictures taken by confocal microscopy of xeno-transplanted PaTu 8988t cells either treated with the control siRNA (a and b) or the LIMK1/2 siRNAs (d and e). The pictures show the zebrafish directly after transplantation (2 dpf) and 24 hours later (3dpf). In the control, cancer cells have invaded the embryo and migrated to distant sites (b). This behaviour is blocked by the double knock down (e). The area marked with a square (b) is shown in an enlargement (c) and here, cancer cells are visible in the vasculature (c). (**B**) Shown are results of the Zebrafish tumour xenograft metastasis assay performed in two different pancreatic cancer cell lines. Two independent experiments are combined for each treatment and cell type. The standard deviations (SD) are shown in brackets. The results are also represented in the graph in Figure 2 and all experiments are shown in Supplemental Table 1 (Vlecken and Bagowski, 2009).

We used once again gene specific siRNAs to silence the LIMK1 and LIMK2 genes individually as well as in combination in the LIMK1/2 double knock down and investigated the effects in the zebrafish xenotransplantation model. Our results, obtained in two different pancreatic cancer cell lines, showed that both LIMK1 and LIMK2 knock down lead to inhibition of metastatic behaviour (Figure 2). These results were not identical but similar to the effects we observed in the *in vitro* migration assay using PaTu 8988t cells (Figure 1B and C). However, in contrast to the *in vitro* migration assay where the double knock down had no additional inhibitory effect, it completely abolished metastatic behaviour in the zebrafish *in vivo* model (Figure 2 A and B). This was observed for both pancreatic cancer cell lines investigated. These findings indicate significant qualitative and quantitative differences between the two assays and they demonstrate an important role for both LIM Kinases in metastatic behaviour of pancreatic cancer cells.

Knock down of LIMK1 and LIMK2 inhibits tumour cell-induced neovascularization

Tumour-induced angiogenesis and the recruitment of new blood vessels by solid tumours is an essential component of the process leading to the formation of metastasis. The tumour-induced newly built vessels provide the principal route by which tumour cells exit the primary tumour site and enter the circulation. Recently, it had been shown that human MDA-MB-435 cancer cells xeno-transplanted into athymic nude mice showed not only increased tumour growth but also promoted tumour angiogenesis, when they overexpressed LIMK1 (Rozita Bagheri-Yarmand AMAASRK, 2006). For LIMK2 similar effects had not been described.

Recently, a method has been established to study tumour angiogenesis in zebrafish (*Danio rerio*) based on the injection of pro-angiogenic mammalian tumour cells into the perivitelline space of

zebrafish embryos (Nicoli, S., Presta M., 2007; Nicoli S., Ribatti D., 2007). This zebrafish tumour xenograft angiogenesis assay allows observation of tumour cell-induced angiogenesis in real time.

Here we describe how we used this method to study the role of the two LIM Kinases in tumour cell-induced angiogenesis. We injected two pro-angiogenic human pancreatic cancer cell lines, PaTu 8988t (PaTu 8988t) (Elsässer HP LU., et al, 1992) and Panc-1 (Lieber M MJ., et al, 1975), into the perivitelline space of zebrafish embryos at 48h post-fertilization. These pro-angiogenic tumour cell grafts induce a neovascular response originating from the developing subintestinal vessels. In both pancreatic cancer cell lines, we compared the effects of control siRNA treatment to LIMK1 and LIMK2 gene specific siRNA treatment. We also performed a LIMK1/2 double knock down. Successful gene silencing was controlled by RT-PCR in PaTu 8988t cells (Figure1 A). Our results revealed that both LIMK1 and LIMK2 knock down inhibit tumour cell-induced angiogenesis (Figure 3). The double knock down displayed a slightly additive inhibitory effect but did not lead to complete inhibition of tumour-induced angiogenesis (Figure 3). To the best of our knowledge, this is the first indication that LIMK2 could play a role in tumour angiogenesis.

Figure 3

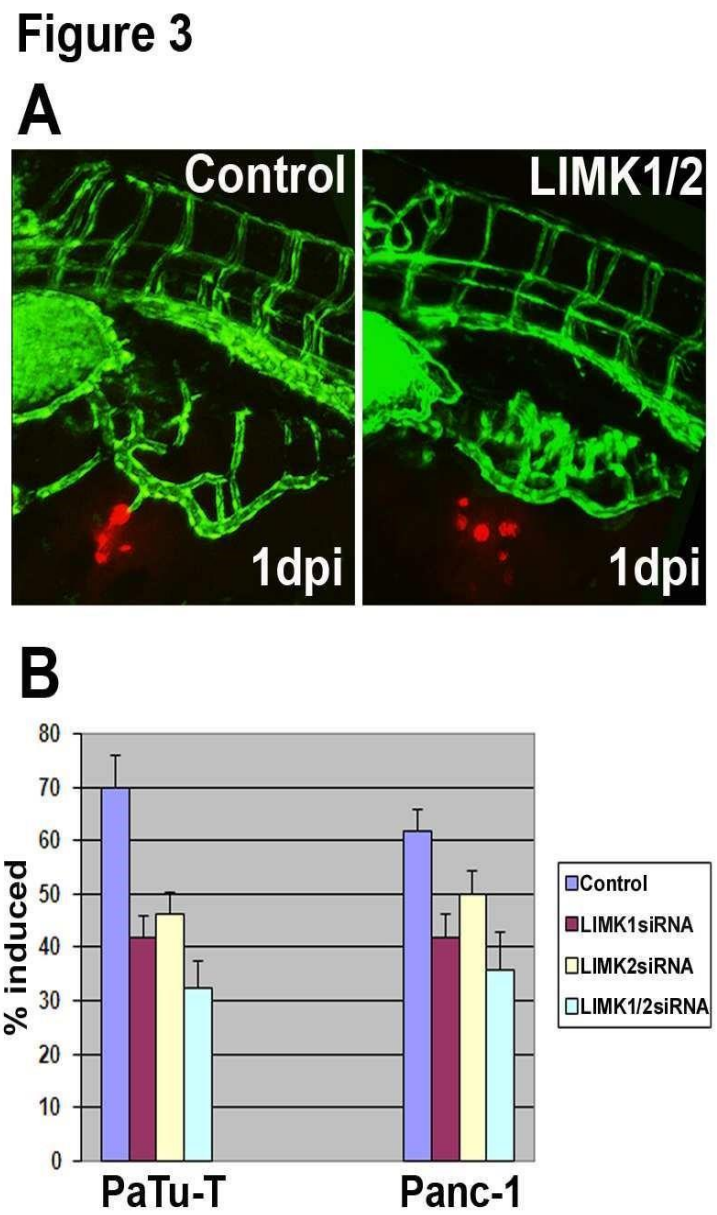

Figure 3: The zebrafish tumour xenograft angiogenesis assay. (A) Images shown are examples taken by confocal microscopy of PaTu 8988t cells 24 hours after cells were injected in the perivitelline space of zebrafish embryos. PaTu 8988t cells were either treated before implantation with the control siRNA (left image) or the LIMK1/2 siRNAs (right image). The picture on the left shows the induction of a new vessel induced by the tumour cells leading from the SIV to the PaTu 8988t cell cluster. This is inhibited by the double knock down shown in the right image. (**B**) Shown are the results of the Zebrafish tumour xenograft angiogenesis assay performed in two different pancreatic cancer cell lines. Two independent experiments are combined for each treatment and cell type and the number of zebrafish used for each individual treatment was 50. Standard deviations (SD) are shown in brackets and the results are also represented in Supplemental Table 1 (Vlecken and Bagowski, 2009.

Discussion

Secondary tumours arising from cancer cell metastasis are the major cause of death in cancer patients, and currently no anti-metastatic drugs are available for therapy. Moreover, the mechanisms that regulate metastatic behaviour remain so far only partially understood. The current models envision metastasis as a highly dynamic multi-step process.

The zebrafish xenograft metastasis model used in this study provides the means to quantitatively study these dynamic processes in high-resolution in real time. Moreover, the zebrafish xenograft angiogenesis model allows further the investigation of tumour cell-induced neovascularization in living embryos. It is important to note, that substantial evidence is accumulating that demonstrates functional conservation of human and zebrafish vascular biology and many pro-angiogenic genes have been described in both fish and men (Bussmann J., et al, 2008; Isogai S., et al, 2003).

We used the two zebrafish xenograft assays here as powerful tools to address and compare the role of the LIM Kinases in metastasis and tumour cell-induced angiogenesis of human pancreatic cancer cells. We discovered here that LIMK1, which has been implicated in tumour cell metastasis in various tumour types, is also important for the metastatic behaviour of pancreatic cancer cells. More importantly, our data gives the first evidence that LIMK2 can play a similar role in metastasis formation. Non-overlapping functions of these two kinases in pancreatic cancer cells are demonstrated by the complete abolishment of metastatic behaviour by the double knock down. Interestingly, this differs from our *in vitro* data, where a double knock down had no additional inhibitory effect. A possible explanation is that the results in the *in vitro* migration assay reflect the contribution of the LIM Kinases to the initial step of invasion/migration and in part to cell proliferation, which are both measured in this assay. Cell proliferation, which has been shown to be regulated by both LIM Kinases (Croft DR., Olson MF., 2006; Rozita Bagheri-Yarmand AMAASRK, 2006), certainly plays a partial role in the gap closure times which were measured in the *in vitro* migration assay. Overall, the results point to additional roles (despite regulation of invasion/migration) of the LIM Kinases in later steps of metastasis formation. Similar non-overlapping functions for LIM Kinases have also been implicated in knock-out mice, where double ablation of LIMK1/2 induced a more pronounced neuronal phenotype than in the genetic deletion of *Limk1* alone (Meng Y., et al., 2004). Although LIMK2 ablation alone did not lead to any impaired neuronal morphology, these results revealed redundancies between the two LIM Kinases (Takahashi H., et al, 2002).

Although LIMK1 had been implicated in angiogenesis induced by xeno-transplanted human MDA-MB-435 cells in nude mice (Rozita Bagheri-Yarmand AMAASRK, 2006). No similar function has been

attributed to LIMK2. In our zebrafish xenograft angiogenesis study, we observed a reduction of pancreatic cancer cell-induced angiogenesis for both, the LIMK1 and the LIMK2 single knock downs. However, the double knock down did not exert a complete inhibition and only had a slightly more pronounced inhibitory effect compared to the single knock downs.

The mechanisms by which LIM Kinases increase the invasiveness of cancer cells and their metastatic behaviour and tumour-induced angiogenesis have not been identified yet. The zebrafish xenograft metastasis and angiogenesis assays are well suited to help in delineating (e.g. by reverse genetics using siRNAs) the responsible mechanisms and will assist in discovering novel anti-metastatic drugs in the not so distant future.

In summary, we find here that in addition to LIMK1, LIMK2 is also very important for metastasis of pancreatic cancer cells. A LIMK1/2 double knock down is necessary to completely block metastatic behaviour *in vivo*. We further show that both LIM Kinases play an important role in tumour cell-induced angiogenesis. Our findings propose that simultaneous targeting of both LIM Kinases might be a practical therapeutic approach to inhibit tumour progression and metastasis.

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Competing interest statement

The authors declare that they have no competing interests.

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