Tie1 deficiency induces endothelial–mesenchymal transition

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Endothelial–mesenchymal transition (EndMT) has a significant role in embryonic heart formation and in various pathologies. However, the molecular mechanisms that regulate EndMT induction remain to be elucidated. We show that suppression of receptor tyrosine kinase Tie1 but not Tie2 induces human endothelial cells to undergo EndMT and that Slug deficiency reverts this process. We find that Erk1/2, Erk5 and Akt cascades control Slug promoter activity induced by Tie1 deficiency. Interestingly, EndMT is present in human pancreatic tumour. We propose that EndMT associated with Tie1 downregulation participates in the pathological development of stroma observed in tumours.

Keywords: angiogenesis; endothelial–mesenchymal transition; pancreatic cancer; Slug; Tie1

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INTRODUCTION

Endothelial–mesenchymal transition (EndMT), a form of epithelial–mesenchymal transition, is characterized by loss of endothelial cell markers, such as CD31 (or PECAM), gain of mesenchymal markers, such as α -smooth muscle actin (α SMA), and acquisition of invasive and migratory properties [1,2]. EndMT is involved in a variety of pathological settings, including tissue fibrosis and cancer [1,3,4], and is an important source of cancer-associated fibroblasts (CAFs) [4], which are known to facilitate tumour progression [5].

Angiogenesis, the formation of new blood vessels sprouting from pre-existing vasculature, occurs in several diseases and its

importance in solid tumour growth and metastasis has been demonstrated in several studies. The angiopoietins/Tie2 receptor pathway has been described as an endothelial cell-specific proangiogenic system playing a critical part in promoting vessel maturation, as well as vascular destabilization [6,7]. Several studies have shown that interfering with the Tie2 pathway results in defective angiogenesis or/and tumour regression [7,8]. Tie1 is an endothelial receptor essential for development and maintenance of the vascular system and its genetic deletion results in embryonic lethality due to extensive haemorrhage, oedema and defective microvessel integrity [9]. Tie1 remains mainly an orphan receptor as no ligand for Tie1 has been clearly identified. However, Saharinen *et al* [10] showed that Tie1 phosphorylation can be induced by overexpression of several angiopoietin proteins and that this activation is amplified via Tie2. Despite the fact that Tie1 can interact with Tie2, its intrinsic biological role remains elusive.

Here we show that Tie1 deficiency induces EndMT in human endothelial cells and that EndMT is present in human pancreatic tumours. These results might suggest not only a role for Tie1 deficiency in promoting tumorigenesis by the formation of CAFs, but also have implications for therapeutic targeting of endothelial-derived mesenchymal cells.

RESULTS AND DISCUSSION

Tie1 deficiency induces endothelial–mesenchymal transition

We used a short-interfering RNA (siRNA)-based approach to characterize the function of Tie1 (supplementary methods online). Two independent siRNAs were transfected in human microvascular endothelial cells (primary HMVECs and HMVEC cell line; Fig 1A and supplementary Fig S1A online). Tie1A or Tie1B or Tie2 siRNA robustly suppressed Tie1 or Tie2 messenger RNA and protein expressions. Strikingly, 48–72 h after Tie1 siRNA transfection, but not after Tie2 siRNA transfection, the cells underwent a morphological change, from epitheloid to elongated and spindle-shaped fibroblast-like appearance (Fig 1B and supplementary Fig S1B online), suggesting an EndMT. Tie1 deficiency strongly

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◀ **Fig 1** | Tie1 silencing in pHMVECs induces endothelial–mesenchymal transition. (A) Tie1 (left panel) or Tie2 (right panel) siRNA suppresses mRNA and protein expression. pHMVECs were transfected twice with Tie1A or Tie1B or Tie2 or Ctrl siRNA. mRNA and protein levels were quantified by real-time PCR and western blotting, respectively. (B) Tie1 silencing, but not Tie2, induces morphological changes. (C) Tie1 silencing decreases the mRNA (left panels) and protein (right panels) expressions of vascular endothelial markers (CD31, VE-cadherin, CD34, FVIII) and increases the mRNA (left panels) and protein (right panels) expressions of mesenchymal markers (α SMA, S100A4, Col1a1, SM22 α , N-cadherin). (D) Tie2 silencing does not modify the expression of vascular endothelial and mesenchymal markers. TBP was used as internal Ctrl for real-time PCR and β -tubulin as loading Ctrl for western blotting analysis. Results are from triplicates of three different experiments. Significant differences are indicated by solid lines (* $P < 0.03$, ** $P < 0.003$ by t -test). α SMA, α -smooth muscle actin; Col1a1, collagen type I $\alpha 1$; Ctrl, control; FVIII, coagulation factor VIII; mRNA, messenger RNA; pHMVEC, primary human microvascular endothelial cell; siRNA, short-interfering RNA; TBP, TATA-box binding protein.

reduced the mRNA and protein expressions of the vascular endothelial markers in particular VE-cadherin, necessary for maintaining endothelial integrity [11], and reciprocally, augments expression of the mesenchymal markers, especially S100A4, a CAF marker that promotes invasion and metastasis in cancer cells [12] (Fig 1C and supplementary Fig S1C online). Similar results were obtained by transfecting bovine aortic endothelial cells with Tie1 siRNA (supplementary Fig S2A,B online). Tie2 deficiency did not change expression of the vascular endothelial and mesenchymal markers (Fig 1D and supplementary Fig S1D online).

Tie1 deficiency-induced EndMT is Slug dependent

EndMT is regulated by multiple signalling pathways, including TGF- β and Notch leading to an increased expression of the *Snail* gene family [1]. The *Snail* family members, *Snail* and *Slug*, encode zinc finger-containing transcriptional repressors believed to downregulate VE-cadherin, thereby disrupting adherent junctions. We investigated whether expression of these transcriptional factors was altered when EndMT was induced in HMVECs. Reverse transcription–PCR and western blotting analysis revealed a similar expression level of Snail in Tie1-downregulated HMVECs and cells transfected with control siRNA (Fig 2A). On the contrary, the Tie1 siRNA strongly increased Slug mRNA and protein expressions (Fig 2A). Using Slug siRNA transfection, we then tested the effects of Slug deficiency on Tie1 siRNA-induced EndMT. Slug deficiency prevented the morphological changes induced by Tie1 silencing (Fig 2B) and strongly repressed Slug mRNA and protein expression (Fig 2C). Strikingly, the ability of Tie1 deficiency to downregulate endothelial markers and to induce mesenchymal markers was abrogated or partially reverted when Slug was knocked down (Fig 2C).

Tie1 deficiency promotes a motile phenotype

During EndMT, endothelial cells acquire a mesenchymal phenotype characterized by acquisition of invasive and migratory properties [1]. To determine whether Tie1 deficiency promotes a motile phenotype in endothelial cells, we evaluated the effects of Tie1 siRNA on endothelial cell migration and adhesion. We found that Tie1 siRNA induced a strong increase in cell migration when compared with control siRNA (Fig 2D and supplementary Figs S2C and S3A online). In addition, Tie1 siRNA induced a decrease in cell adhesion (supplementary Figs S2D and S3B online). Because platelet-derived growth factor (PDGF) is a known chemotactic factor for mesenchymal cells [13], we analysed the effect of Tie1 siRNA on mesenchymal migration of cells towards PDGF (Fig 2D and supplementary Fig S3A online). In fact, PDGF did not induce cell migration, compared with control. By contrast, in the presence of Tie1 siRNA, migration was strongly increased

upon exposure to PDGF and was accompanied by an increased expression of PDGF receptor β (Fig 2D and supplementary Fig S3A online). Inhibition of Slug expression by Slug siRNA reverted the migration induced by Tie1 deficiency and PDGF. Therefore, we demonstrate here that Slug expression is required for promoting migration of transformed endothelial cells towards PDGF.

Tie1 deficiency activates the human Slug promoter

PI3K/Akt and MAPK pathways are upregulated by epithelial–mesenchymal transition or EndMT activation in cells [2]. We found that inhibition of Tie1 expression by its siRNA increased Erk1/2 and Akt phosphorylation compared with control siRNA (Fig 3A and supplementary Fig S2E online). Studies have shown that the Erk5 pathway is critical for normal cardiovascular development and vascular integrity [14,15] and that it can control Slug expression [16]. Therefore, we looked at the effects of Tie1 siRNA on Erk5 phosphorylation (Fig 3A and supplementary Fig S2E online). Interestingly, Tie1 deficiency strongly increased Erk5 phosphorylation. To identify the relative contributions of the Erk1/2, Erk5 and Akt cascades, MAPK and Akt inhibitors, U0126 and LY-294,002, respectively, were used (Fig 3A). Results show that, indeed, Erk1/2, Erk5 and Akt phosphorylations induced by Tie1 removal are strongly decreased by exposure to U0126 and LY-294,002. When used alone or in combination, these inhibitors induced a drastic decrease in Slug expression induced by Tie1 removal (Fig 3B). To characterize the regulatory mechanisms underlying the transcription of the *Slug* gene by Tie1 deficiency, we analysed the promoter of the human *Slug* gene. Reporter assays using the 1.1-kb fragment upstream from the first human *Slug* exon [17] showed that Tie1 deficiency activated the human Slug promoter (six- to seven-fold increase), and that activation was blocked by MAPK and Akt inhibitors (Fig 3C). To further analyse the involved pathways, HMVECs were transfected with siRNAs against Erk1, Erk2 or Erk5. Erk1/2 or Erk5 deficiencies induced a decrease in *Slug* activation induced by Tie1 removal, Erk5 siRNA being the most potent (Fig 3D). To our knowledge, it is the first time that Erk5 is involved in the EndMT.

The EndMT is present in human pancreatic tumours

Previous studies have shown that EndMT contributes to the accumulation of fibroblasts during heart development and fibrosis [1,3,4]. Zeisberg *et al.* showed in two different mouse models of carcinogenesis (Rip-Tag2 spontaneous pancreatic carcinoma and B16F10 melanoma) that EndMT is an important source of CAFs [4]. EndMT was characterized by the simultaneous expression of CD31 with S100A4 or α SMA. In all, 40% of S100A4⁺ CAFs were found to be CD31⁺, as were 11% of α SMA⁺ CAFs. Importantly, the authors provide evidence that, in endothelial cell-specific

LacZ reporter mice, tumours contain fibroblasts that are also *LacZ*, thereby demonstrating their endothelial origin. Here, we explored the hypothesis that, in human pancreatic tumours, endothelial

cells contribute to CAFs production via EndMT and that Tie1 downregulation is associated with EndMT triggering. We chose to study pancreatic cancer because it is characterized by a dense

Fig 2 | Tie1 silencing-induced endothelial–mesenchymal transition is Slug dependent. (A) Tie1 silencing increases Slug expression. HMVECs were transfected with Tie1 or Ctrl siRNA. Slug and Snail mRNA levels and protein expressions were quantified by real-time PCR or western blotting. (B) Slug deficiency prevented the morphological changes induced by Tie1 silencing. (C) Slug siRNA abrogated or partially reverted the modifications induced by Tie1 silencing on the expression of vascular endothelial markers (VE-cadherin, CD31) and mesenchymal markers (Colla1, S100A4). HMVECs were transfected with Tie1 or/and Slug or Ctrl. mRNA level (left panels) and protein expression (right panels) were quantified by real-time PCR or western blotting. TBP was used as internal Ctrl for real-time PCR and β -tubulin as loading Ctrl for western blotting analysis. Results are from triplicates of three different experiments. (D) Tie1 silencing increases pHMVEC migration and Slug siRNA partially reverts this effect. pHMVECs were transfected with Tie1 siRNA or/and Slug siRNA or Ctrl and were allowed to migrate in the presence or absence of PDGF in a modified Boyden chamber assay. Migration scores were measured for three fields. Similar results were obtained in three different experiments. Significant modifications are indicated by solid lines (* $P < 0.03$, ** $P < 0.003$ by t -test). Colla1, collagen type I α 1; Ctrl, control; HMVEC, human microvascular endothelial cell; mRNA, messenger RNA; pHMVEC, primary HMVEC; PDGF, platelet-derived growth factor β ; siRNA, short-interfering RNA; TBP, TATA-box binding protein.

desmoplastic stroma [18]. Strong evidence suggests that CAFs, the predominant stromal cell type, actively stimulate tumour cells, thereby contributing to tumour development and progression [5]. Confocal microscopy on human pancreatic tumours revealed the coexpression of both CD31 and mesenchymal marker in a sub-population of cells: CD31⁺/ α SMA⁺, CD31⁺/S100A4⁺, CD31⁺/N-cadherin⁺, CD31⁺/FAP⁺ and CD31⁺/SM22 α ⁺ (Fig 4A). These double labelling might be identified as intermediate stages of EndMT. A complete conversion cannot be detected because of the loss of endothelial markers in the newly emerged fibroblast-like cells. This EndMT could contribute to the fact that pancreatic tumour are hypovascularized with 80% reduction of the microvascular density compared with normal pancreas [19]. This process seems to be restricted to pancreatic tumour, as CD31⁺/ α SMA⁺ coexpression is never detected in non-tumoural pancreatic tissue (Fig 4B). Triple-labelling experiments on human pancreatic tumours showed that CD31⁺/ α SMA⁺, CD31⁺/S100A4⁺, CD31⁺/N-cadherin⁺, CD31⁺/FAP⁺ and CD31⁺/SM22 α ⁺ coexpression in cells was detectable in association with Tie2 (Fig 4A) but never with Tie1 (Fig 5). Tie1 downregulation in endothelial cells from human pancreatic tumours might be due to the presence in pancreatic tumour of the human *tie-1*AS long noncoding RNAs recently described [20]. The *tie1*-AS long noncoding RNA selectively binds *tie-1* mRNA *in vivo* and induces the downregulation on the endogenous *tie-1* transcript, resulting in specific defects in endothelial cell contact junctions *in vivo* and *in vitro*. An alternative or complementary mechanism might explain the disappearance of Tie1 in endothelial cells: Tie1 could undergo regulated ectodomain cleavage in response to vascular endothelial growth factor overexpression or inflammatory stimuli specific to pancreatic cancer [12,19]. The newly formed endodomain could undergo additional proteolytic processing mediated by γ -secretase to generate an amino-terminally truncated fragment, which is degraded by proteasomal activity [21].

In conclusion, our results provide new insights into the molecular mechanisms controlling tumour–environment interactions: (i) Tie1 downregulation activates signal transduction cascades and induces an EndMT process by the regulation of Slug expression; (ii) Erk5 is a new cascade implicated in this process; and (iii) EndMT could be a source of CAFs in human tumours associated with Tie1 downregulation. It has been shown that the activated stroma of pancreatic cancer is an independent prognostic marker with an impact on patient survival as much as the lymph node status of the cancer [22]. Targeting

endothelial-derived mesenchymal cells might be beneficial in the therapy of some tumours.

METHODS

Cell lines. Primary HMVECs were purchased from Invitrogen and HMVEC cell lines were kindly supplied by Dr Xing Guo (Duke University Medical Center, Durham, NC, USA).

RNA interference. Tie1, Tie2, Slug and control siRNAs were purchased from Eurogentec, and Erk1, Erk2 and Erk5 siRNAs were purchased from Eurofins (supplementary Table S2 online). As control siRNA, we used an non-relevant siRNA (Ctrl). Cells were transfected twice with siRNAs using the calcium phosphate method. Similar results were obtained when endothelial cells were transfected with Tie1A or Tie1B siRNA. For the α SMA expression studies, cells were transfected in medium without hydrocortisone and FGF2 because these compounds inhibit the expression of α SMA [23]. Transfected cells were treated for 24 h with U0126 (10 μ M; Sigma) or/and LY-294,002 (10 μ M; Sigma).

Quantitative real-time PCR. Total RNA was extracted using TRIzol (Invitrogen) and complementary DNAs were prepared using ImProm-IITM kit (Promega, Madison, WI, USA) following manufacturer's instructions. Quantitative PCR was performed in a LightCycler[®] (Roche, Basel, Switzerland). Primer sequences are available in supplementary Table S3 online.

Immunoblotting. The antibodies used are described in supplementary methods online.

Cell migration assay. Endothelial cell migration assays were performed using a 24-well chemotaxis chamber (Transwell, Falcon). Membranes of 8 μ m pores were coated with fibronectin (Sigma) and gelatin in phosphate-buffered saline (PBS). The lower wells contained serum-free medium in the presence or absence of PDGF-BB (20 ng/ml; AbCys S.A.). Cells were allowed to migrate at 37 °C in a 5% CO₂ humidified incubator. After 3 h, the upper side of the filter containing the non-migrated cells was scraped. The filters were fixed and stained with crystal violet. Migration was quantified by counting cells in three random high-power fields ($\times 200$) in each well.

Cell adhesion assays. Wells were coated with 0.65 μ g/ml of fibronectin, non-coated wells are used as negative control. Then wells were washed with PBS and then blocked with PBS containing 2% bovine serum albumin. Cells were plated. After 15 min of incubation at 37 °C, cells were washed with PBS, fixed 15 min with 4% paraformaldehyde (PFA), stained with crystal violet for 30 min and washed.

Fig 3 | Tie1 silencing increases Erk1/2, Erk5, Akt phosphorylation and Slug expression. Effect of Tie1 silencing on Erk1/2, Erk5, Akt phosphorylation (A) and on Slug mRNA (B, upper panel) and protein (B, lower panel). HMVECs were transfected with Tie1 or control siRNA and were treated for 24 h with MAPK (U0126) or/and Akt (LY-294,002) inhibitors. Protein expression was quantified by western blotting. β-tubulin, Erk1/2, Erk5 and Akt were used as loading controls. TBP was used as internal control for real-time PCR. Results are from triplicates of three different experiments. (C,D) Effect of Tie1 deficiency on the human Slug promoter. HMVECs were treated for 24 h with MAPK (U0126) or/and Akt (LY-294,002) inhibitors (C) or with Erk1/2 and Erk5 siRNAs (D). Thereafter, cells were co-transfected with Tie1 or control siRNA and pGL3-human Slug promoter plasmid. After normalization, the luciferase activity was expressed as fold induction compared with the promoterless plasmid (pGL3-basic vector) expression. In D, as shown in the western blot insets, Erk1/2 or Erk5 siRNA induce decreases in Erk1/2 or Erk5 protein levels. Similar results were obtained in three different experiments. Significant modifications are indicated by solid lines (** $P < 0.003$ by *t*-test). Erk 1/2, 5, extracellular signal-regulated kinase 1/2, 5; HMVEC, human microvascular endothelial cell; MAPK, mitogen-activated protein kinase; mRNA, messenger RNA; siRNA, short-interfering RNA; TBP, TATA-box binding protein.

Cell transfection and reporter assays. At 24 h after transfection with siRNAs, reporter assays were performed. Cells were transfected using the lipofectamin reagent with 2 μg of the pGL3-human Slug promoter plasmid (kindly supplied by Dr Kenji Sobue) or pGL3-basic vector as a normalization control and

co-transfected with 0.2 μg of the pRLuc-C2 (BioSignal Packard). The luciferase activities were measured 24 h after transfection and assessed with the Promega luciferase assay system. After normalization, luciferase activities obtained was expressed as fold induction compared with the expression of the pGL3-basic vector.

Fig 4 | EndMT is present in human pancreatic tumours and not in human pancreas. (A) Confocal microscopy of immunofluorescence triple labelling to endothelial marker (CD31; blue), mesenchymal markers (α SMA, S100A4, SM22 α , FAP, N-cadherin red) and Tie2 (green) were performed in human pancreatic adenocarcinoma tissues. Coexpression of CD31⁺/ α SMA⁺ or CD31⁺/S100A4⁺ or CD31⁺/N-cadherin⁺ or CD31⁺/FAP⁺ or CD31⁺/SM22 α ⁺ are detectable with Tie2 (white arrow). (B) Confocal microscopy of immunofluorescence double labelling to endothelial marker (CD31; green), mesenchymal marker (α SMA, red) were performed on non-tumoural pancreas tissues. Pictures display representative photomicrographs. Scale bars, 10 μ m (inset) or 20 μ m. α SMA, α -smooth muscle actin; EndMT, endothelial–mesenchymal transition; FAP, fibroblast activation protein; N-CAD, N-cadherin.

Confocal microscopy. Pancreatic tissues were obtained at the Department of Pathology, Centre Hospitalier Universitaire Nord (Marseille, France). The characteristics of the tissue samples and patients are described in supplementary Table S1 online. Cryosections of pancreatic adenocarcinoma tissue

were used. Formalin-fixed paraffin-embedded tissue blocks containing non-tumoural pancreatic tissue were collected from an area distant from the tumour without histological evidence of tumoural lesion. The slides were incubated with the antibodies as described in supplementary methods online

Fig 5 | EndMT is not associated with Tie1 expression in human pancreatic tumours. Confocal microscopy of immunofluorescence triple labelling to endothelial marker (CD31; blue), mesenchymal markers (α SMA, S100A4, SM22 α , FAP, N-cadherin; red) and Tie1 (green) were performed on human pancreatic adenocarcinoma tissues. Coexpression of CD31⁺/Tie1⁺ is detectable but never in association with mesenchymal marker. Pictures display representative photomicrographs. Scale bars, 10 μ m (inset) or 20 μ m. α SMA, α -smooth muscle actin; EndMT, endothelial-mesenchymal-transition; FAP, fibroblast activation protein; N-CAD, N-cadherin.

and observed with a Zeiss LSM 510 confocal microscope (Carl Zeiss).

Statistical analysis. Significance of the differences between groups was calculated using a two-tailed Student's *t*-test. Values are given as mean $\pm$ s.e. A *P*-value of <0.03 was interpreted as statistically significant.

Supplementary information is available at EMBO reports online (<http://www.emboreports.org>).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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