EXPERIMENTAL CELL RESEARCH 316 (2010) 826–835

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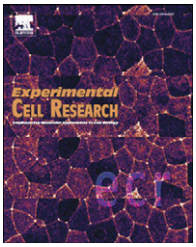

Research Article

Fibroblast nemosis induces angiogenic responses of endothelial cells

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ARTICLE INFORMATION

Article Chronology:
Received 14 August 2009
Revised version received
6 November 2009
Accepted 17 November 2009
Available online 20 November 2009

Keywords:
Angiogenesis
Cell activation
Growth factor
Inflammation
Microenvironment
Tumorigenesis

ABSTRACT

Increasing evidence points to a central link between inflammation and activation of the stroma, especially of fibroblasts therein. However, the mechanisms leading to such activation mostly remain undescribed. We have previously characterized a novel type of fibroblast activation (nemosis) where clustered fibroblasts upregulated the production of cyclooxygenase-2, secretion of prostaglandins, proteinases, chemotactic cytokines, and hepatocyte growth factor (HGF), and displayed activated nuclear factor-κB. Now we show that nemosis drives angiogenic responses of endothelial cells. In addition to HGF, nemotic fibroblasts secreted vascular endothelial growth factor (VEGF), and conditioned medium from spheroids promoted sprouting and networking of human umbilical venous endothelial cells (HUVEC). The response was partly inhibited by function-blocking antibodies against HGF and VEGF. Conditioned nemotic fibroblast medium promoted closure of HUVEC and human dermal microvascular endothelial cell monolayer wounds, by increasing the motility of the endothelial cells. Wound closure in HUVEC cells was partly inhibited by the antibodies against HGF. The stromal microenvironment regulates wound healing responses and often promotes tumorigenesis. Nemosis offers clues to the activation process of stromal fibroblasts and provides a model to study the part they play in angiogenesis-related conditions, as well as possibilities for therapeutical approaches desiring angiogenesis in tissue.

Introduction

The repair process of wounded tissue consists of several often overlapping phases with distinct pathological characteristics [1,2]. Injured tissue reacts initially with bleeding and vasodilation. After the initial hemostatic response that results in clot formation, acute inflammation takes place with inflammatory cells invading the injured tissue and surrounding stroma. The following step is the formation of granulation tissue, which serves as a scaffold for angiogenesis and reepithelialization. Here fibroblasts enter the stage and form a major stromal component [3]. They are known to produce and maintain the extracellular matrix (ECM), and also seem to have an immunological function as ubiquitous sentinel cells capable of transforming into an inflammatory phenotype,

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Abbreviations: BAEC, bovine aortic endothelial cell; CAF, cancer-associated fibroblast; COX, cyclooxygenase; ECM, extracellular matrix; FGF, fibroblast growth factor; HGF, hepatocyte growth factor; HMEC-d, human microvascular endothelial cell-dermal; HUVEC, human umbilical vein endothelial cell; IL, interleukin; MET, mesenchymal–epithelial transition factor; MIP, macrophage inflammatory protein; NF-κB, nuclear factor κB; PCNA, proliferating cell nuclear antigen; PGE, prostaglandin E; RANTES, regulated upon activation, normal T-cell expressed and secreted; SF, scatter factor; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor

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doi:10.1016/j.yexcr.2009.11.012

often with myofibroblastic characteristics. Myofibroblasts also orchestrate wound contraction [4,5].

The process of tissue repair requires intense proliferation, which in turn places demands on the nutrition and oxygen supply in the tissue surrounding the injury. This shortage is corrected by angiogenesis, resulting in neovascularization of the inflammatory microenvironment and granulation tissue. Wound angiogenesis is activated by the innate immune system and proceeds as a tightly controlled course of action, where endothelial cells and mural cells are guided by the microenvironment to sprout, migrate, and form new capillaries [6,7]. Endothelial cells themselves are anchored to basement membranes, and the cells of a functioning blood vessel require constant contact with the ECM. Endothelial cells depend on the microenvironment for proper function, displaying various phenotypes depending on the type of vasculature or the organ they reside in [8].

Angiogenesis is promoted by vascular-endothelial growth factor (VEGF) and its main receptor on endothelial cells is VEGFR2 [9]. Characteristics further attributed to VEGF are promotion of survival and prevention of apoptosis in endothelial cells, as well as increased vascular permeability, all components required in acute inflammation and building of new vasculature.

Hepatocyte growth factor or scatter factor (HGF or SF) is another growth factor with an important role in the tissue repair process. Produced mainly by stromal fibroblasts, it promotes cell motility, proliferation, morphogenesis, and matrix construction [10–12]. HGF is now also recognized as a promoter of angiogenesis and lymphangiogenesis [13,14]. It is secreted in an inactive form, and activated by proteolytic cleavage in damaged tissues [11]. HGF binds to the receptor MET (mesenchymal–epithelial transition factor), mostly present on epithelial cells, but also on endothelial cells [15–18]. MET plays an important role in wound healing, as shown by the obligatory presence of MET in keratinocytes for successful wound reepithelialization [19].

A strong link exists between inflammation and tumorigenesis, a connection recently placed in the center of intense investigation. Many types of cancer arise at sites of chronic inflammation [20] and in the light of recent results, tumors seem to be capable of redirecting tissue healing responses, such as inflammation and wound repair, to promote tumorigenesis. The highly vascularized and growth-factor rich stromal microenvironment often functions to serve the developing tumor. Some types of tumors consist mostly of stromal cells including fibroblasts, and several reports have observed a tumor-promoting effect of activated stromal fibroblasts (CAFs) on tumor cells [21,22]. CAFs also promote invasiveness of otherwise non-invasive cancer cells [23], and are now considered a possible target for cancer therapy [24,25].

We have developed an experimental model for stromal activation. Allowing human fibroblasts to cluster into spheroids leads to proinflammatory activation through upregulation of cyclooxygenase-2 (COX-2), production of prostaglandins E_2, I_2, and $F_{2\alpha}$, extracellular proteinases (plasminogen activation, matrix metalloproteinases), chemotactic cytokines (CXCL8 (IL-8), CCL3 (MIP-1α), CCL5 (RANTES)), and activation of NF-κB. This process of fibroblast activation has been designated as nemosis [reviewed in 26,27–29]. Fibronectin–Integrin interaction mediates initial cluster formation [30]. The activated, nemotic fibroblasts also produce considerable amounts of HGF, which directly promotes invasiveness of MET-expressing cancer cells [31]. Also bone marrow mesenchymal stem cells undergo nemosis and have been shown to induce HGF-dependent keratinocyte wound repair *in vitro* [32]. However, MET-negative leukemia cells stimulated by cytokines produced by nemotic fibroblasts undergo growth arrest and differentiation to a dendritic phenotype [33]. The nemosis response displays differing characteristics between normal fibroblast and CAF strains [34], whereas nemotic fibroblasts exposed to malignant HaCaT keratinocytes or their conditioned medium showed myofibroblast-like differentiation [35].

In conclusion, nemotic fibroblasts display characteristics similar to fibroblasts in the inflammatory and tumor microenvironments. Since angiogenesis plays a crucial role in both processes, we followed the trail further. Our current results illustrate how nemosis induces angiogenic responses of endothelial cells.

Materials and methods

Antibodies and other reagents

The function-blocking antibodies goat-anti-human HGF (AF-294-NA) and goat-anti-human VEGF (AF-293-NA) were purchased from R&D Systems (Minneapolis, MN). Goat-anti-human COX-2, goat-anti-human PCNA (proliferating cell nuclear antigen) and mouse-pan-anti-human actin were from Neomarkers (LabVision, Fremont, CA). Anti-rabbit IgG-HRP was from Santa Cruz Biotechnologies (Santa Cruz, CA) and anti-mouse IgG+IgM-HRP from Jackson ImmunoResearch (West Grove, PA).

Cell lines and culture methods

Primary human dermal fibroblasts (CRL-2088, from American Type Culture Collection, Vanassas, VA) were cultured in a mixture of DMEM and F-12 (1:1) medium (Invitrogen, Carlsbad, CA), supplemented with 5% FBS (Invitrogen), 0.3 mg/ml glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin. The maximum passage number used was 20. Primary human dermal microvascular endothelial cells (HMEC-dNeo, Clonetics/Lonza, Basel, Switzerland) were cultured in Endothelial Cell Growth Medium MV (PromoCell, Heidelberg, Germany), supplemented with 100 U/ml penicillin and 0.1 mg/ml streptomycin. Experiments on HMEC-d cells were performed in Endothelial Cell Basal Medium MV (PromoCell), supplemented with 5% FBS, 100 U/ml penicillin and 0.1 mg/ml streptomycin. The maximum passage number used was 25. Primary human umbilical venous endothelial cells (HUVEC, in-house, a kind gift of Dr. Hannu Koistinen, University of Helsinki) were cultured in Endothelial Cell Growth Medium MV KIT (PromoCell), supplemented with 50 μg/ml gentamicin and 0.5 μg/ml amphotericin B. Experiments on HUVECs were performed in Endothelial Cell Basal Medium MV (PromoCell), supplemented with 5% FBS, 50 μg/ml gentamicin and 0.5 μg/ml amphotericin B. Before cell seeding, culture dishes were precoated with 0.2% gelatin in PBS for 2 h, then washed with PBS. Experiments on HUVEC cells were performed in Endothelial Cell Basal Medium MV (PromoCell), supplemented with 5% FBS, 50 μg/ml gentamicin and 0.5 μg/ml amphotericin B. The maximum passage number used was 8. Cells were cultured at +37 °C in a humidified atmosphere with 5% CO_2. Adherent cells were routinely detached from culture dishes by trypsinization.

For proliferation experiments, HUVECs were seeded onto 6-well plates at 50,000 cells/well and cultured in normal growth medium for 24 h. The medium was then replaced with conditioned media and incubated for a total of 48 h. Images were captured at time points 0, 24, and 48 h.

Spheroid formation and culture

Spheroids were prepared as described earlier [27], with some modification. Briefly, 96-well U-bottom plates (Nunc, Roskilde, Denmark) were coated with 0.8% low melting point agarose in sterile water, so that a thin agarose layer formed in the wells, making them nonadhesive. Cells were detached from the culture dishes by trypsinization and suspended in culture medium (experiments with endothelial cells only) or separate experimental medium (experiments involving fibroblast conditioned medium on endothelial monolayers) for HMEC-d or HUVEC cells (see above section). Cells were seeded in wells at a concentration of 10^4 cells/150 μl/well (=67,000/ml). Monolayer cultures of fibroblasts were used as a control, and were directly seeded in flat-bottom 96-well plates at a concentration of 10^4 cells/150 μl/well. Conditioned fibroblast monolayer or spheroid medium used in all experiments below was collected at 96 h after cell seeding.

Quantification of VEGF by fibroblast spheroids

Supernatants were collected from fibroblast spheroids at 0, 24, 48, 72 and 96 h after cell seeding. VEGF in conditioned medium was measured by ELISA according to instructions by the manufacturer (R&D Systems). All samples were assayed in duplicate and the experiments were repeated three times in total.

Scratch-wound repair assay

Endothelial cells were plated on 12-well or 24-well plates, 20,000 and 10,000 cells per well, respectively. HMEC-d cells were grown for 96 h on 12-well plates and HUVEC cells for 72 h on 24-well plates, after which the monolayers were scratch-wounded with a pipette tip and growth medium was substituted with experimental medium (conditioned fibroblast monolayer or spheroid medium). Optionally, a function-blocking antibody was added to the medium: anti-HGF IgG at 0.75 μg/ml and/or anti-VEGF IgG at 0.04 μg/ml. Samples were collected at time points 0, 24 and 48 h by fixing the monolayers with 4% paraformaldehyde and permeabilizing with 0.1% Triton X-100, and they were stored in PBS at +4 °C for further use. For image analysis, samples were stained with Hoechst dye for nuclear visualization and cell counting. Imaging was carried out with an Olympus CKX41 inverted microscope and a UPlan Fluorite phase-contrast objective with 4× magnification, connected to an Olympus DP12 CCD camera system.

Live-cell imaging of in vitro scratch-wound repair

The process of wound closure was followed with real-time microscopy, using a Cell-IQ cell culturing platform (Chip-Man Technologies, Tampere, Finland), equipped with a phase-contrast microscope (Nikon CFI Achromat phase contrast objective with 10× magnification) and a camera. The equipment was computer-controlled by Imagen software (Chip-Man Technologies). The experimental setup was identical to the scratch-wound repair assay. Images were captured at 30 min intervals for 24 h. Analysis was carried out with a freely distributed ImageJ software (McMaster Biophotonics Facility, Hamilton, ON, Canada, available for download at http://www.macbiophotonics.ca/downloads.htm), using the Manual Tracking plugin created by Fabrice Cordeliéres (Institut Curie, Orsay, France), available at http://rsb.info.nih.gov/ij/plugins/track/track.html. Images were stacked and the movement of eight cells per image, all located on the wound edge, was tracked. Means ± SEM of three identical experiments are displayed.

Sprout formation assay

Ninety-six-well plates were coated with 50 μl Growth Factor Reduced Matrigel® basement membrane matrix (BD Biosciences, Franklin Lake, NJ) per well, and the matrix was allowed to polymerize for 1 h in cell culture conditions. Endothelial cells were seeded in a concentration of 6000 cells/well and covered with conditioned medium, 200 μl/well, with or without anti-HGF IgG at 0.75 μg/ml and anti-VEGF IgG at 0.04 μg/ml. Plates were then incubated in cell culture conditions, and wells were photographed 24 h after seeding, two randomly selected phase-contrast images per well. Images were analyzed and quantified as described below.

Image analysis

Analysis of the scratch-wound repair assay was carried out using a custom-made software (Molecular Imaging Unit Core Facility at Biomedicum, Faculty of Medicine, University of Helsinki, Finland) on Matlab platform (The MathWorks, Inc., Natick, MA). The software uses Otsu's tresholding function to recognize cells, which are then K-means clustered to two spatial groups: left and right. A boundary is drawn between monolayer and cell-free cut areas. Any necessary boundary corrections were carried out by hand and the final cut area was calculated in pixels. For further information, see Supplementary Method.

Cell proliferation was analyzed by counting the cells on images captured at desired time points. Two random images were analyzed per sample, and each sample was analyzed in duplicate. Counting was carried out with ImageJ software, using the Particle Analysis/Cell Counter plugin supplied with the software.

Analysis of endothelial cell sprout formation was carried out with ImageJ software. To assess average sprout length, a line was drawn on the image along the sprout, and its length was measured in pixels and scaled back to micrometers. Each sprout was measured as the distance between two branching points. Simultaneously, the number of sprouts and branching points per image was measured.

Statistical analysis

Results are presented as the mean ± SEM of at least three identical, independent experiments. The results were analyzed with a two-tailed paired or unpaired Student's *t*-test. $P \leq 0.05$ was considered significant.

Results

VEGF production by nemotic fibroblast spheroids

Supported by recent results showing elevated mRNA production and protein secretion of VEGF in nemotic fibroblasts at a late time point [35], we measured the secretion of VEGF in conditioned spheroid medium at time points 0–96 h after cell seeding. Compared to monolayers, quantification by ELISA showed a modest increase in VEGF secretion at time points 72 h and 96 h after cell seeding: 1254 pg/ml in monolayer medium vs. 2388 pg/ml in spheroid medium at time point 96 h, translating to a 1.9-fold increase (Fig. 1). However, the differences were not statistically significant due to high variation in triplicate samples.

Nemotic fibroblasts promote scratch-wound repair in endothelial cell monolayer cultures

Nemosis has been shown to promote invasiveness in MET-harboring tumor cells through paracrine signaling due to secretion of substantial amounts of HGF [31]. Judging by the secretion of angiogenic growth factors and cytokines (HGF, VEGF, CXCL8) we hypothesized that nemotic fibroblasts and their conditioned medium would also have an effect on endothelial cells. Using the scratch-wound repair assay, we studied migration and proliferation of endothelial cells exposed to conditioned fibroblast monolayer or spheroid medium. Endothelial monolayers were scratch-wounded, conditioned medium applied and wound closure analyzed on fixed and stained samples with microscope and a quantitative software. Experiments were performed with two endothelial cell types: umbilical venous HUVEC and microvascular dermal HMEC-d.

We observed that in the presence of conditioned fibroblast spheroid medium the wound area decreased more rapidly compared to monolayer medium (Fig. 2A). The differences were significant in HUVEC monolayers after 48 h: spheroid medium decreased the HUVEC wound area to 12% of zero-time point, whereas monolayer medium only narrowed the wound to 31% of the original size (Fig. 2B). The experiment was repeated with HMEC-d cells, with similar results and a significant difference in wound area (reduction to 26% by spheroid medium and 53% by monolayer medium) at time point 48 h (Fig. 2C). Wound closure induced by spheroid medium was partly inhibited in HUVEC cells by co-incubation with a function-blocking antibody against HGF (36% increase in wound area with anti-HGF at 24 h; 56% at 48 h), whereas the antibody had no effect in the presence of monolayer medium. A function-blocking antibody against VEGF had a modest effect without statistical significance, as did the combination anti-VEGF and anti-HGF (see Supplementary Figure 1).

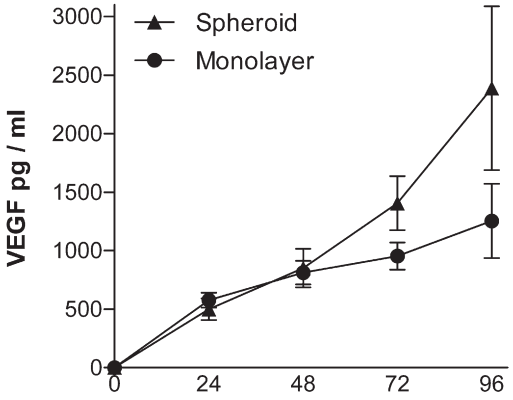

Fig. 1 – VEGF secretion by fibroblast spheroids and monolayers. VEGF was detected in the conditioned medium of spheroids 24 h after initiation of spheroid formation, increasing in concentration until the last time point (96 h). VEGF was also found in monolayer medium, however the concentration was lower than in spheroids at time points 72 and 96 h. Fold change = 1.9 at time point 96 h. Means ± SEM of three identical experiments are displayed.

Nemotic fibroblasts promote motility of endothelial cells

The wound repair effect of nemotic fibroblasts on endothelial cells could be due to proliferation and/or migration of the cells. To elucidate the response, we incubated conditioned spheroid and monolayer media with subconfluent HUVEC monolayers and compared the changes in population growth. The amount of HUVECs doubled after 24 h in presence of both spheroid and monolayer medium, and there was no significant difference in cell numbers between the samples (Fig. 3A). We also compared the levels of the proliferation marker PCNA in the cells. Whole-cell lysates were processed at 24 h intervals. Incubation with nemotic fibroblast medium increased the amount of PCNA at 24 h, but the increase was not significant compared to incubation with monolayer, and the PCNA levels returned to baseline at 48 h (Supplementary Figure 2).

To examine the possible motogenic activity of conditioned nemotic fibroblast spheroid medium, we performed real-time imaging of a scratch-wound repair experiment. Wound closure was followed for 24 h, and captured images were analyzed with a cell tracking software. The results showed a modest increase in the average velocity and total distance traveled by the cells (13% and 14%, respectively; Figs. 3B and C). The variation between samples was small; however, the change in means was not statistically significant.

Nemotic fibroblasts promote endothelial cell sprouting and networking

To further characterize the effect of nemotic fibroblasts on endothelial cells, we investigated the sprouting response of endothelial cells grown on basement membrane matrix, in our experiments Matrigel®. Endothelial cells were seeded on top of the matrix layer together with conditioned nemotic fibroblast spheroid or monolayer medium. In the presence of spheroid medium, HUVEC cells showed an increased sprouting response compared to monolayer medium, characterized as stouter sprouts and a denser network, measured as the number of sprouts and branching points per image field. No difference in sprout length could be observed (Fig. 4). Inhibition of HGF, VEGF or both in conditioned fibroblast spheroid medium by antibodies decreased the sprout number to 69%, 64%, and 63%, respectively. The blocking effect was not apparent in samples incubated with monolayer medium. Also, the amount of

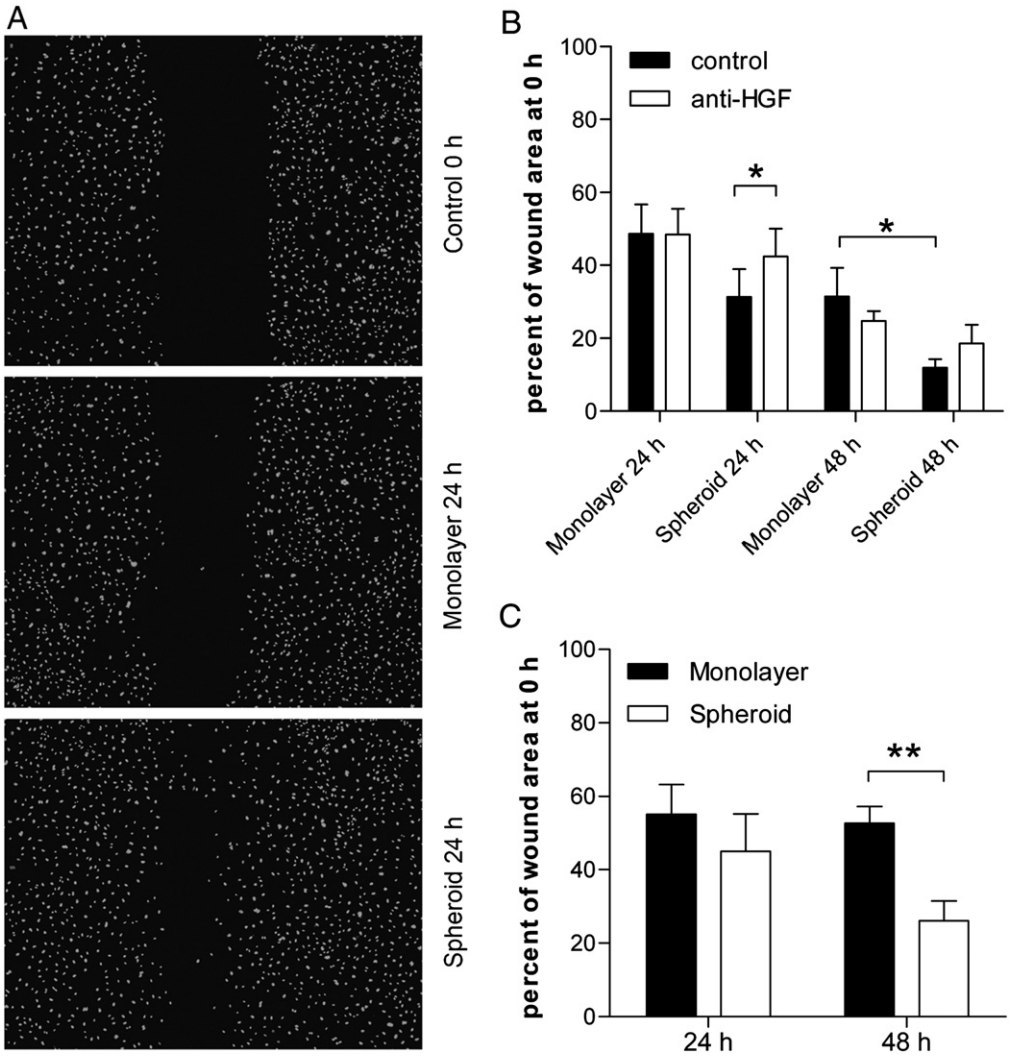

Fig. 2 – Nemotic fibroblasts promote wound repair in endothelial cell monolayer cultures. A. Fluorescence images of scratch wounds in HUVEC cells. HUVEC cells were scratch-wounded, conditioned medium was added, and samples were fixed at time points 0, 24, and 48 h after wounding and nuclear-stained. A more effective closure of the wound can be observed in the samples incubated with conditioned fibroblast spheroid medium. Imaged at 4× magnification with DAPI filter. Image background decreased and contrast enhanced by applying the Curves-function of Adobe Photoshop CS3. B. Quantitative analysis of wound repair by HUVEC cells. Wound areas were measured at time points 24 and 48 h after addition of conditioned media with or without antibodies. Results are shown as percentages of the wound area at zero time point. Means ± SEM of five identical experiments are displayed. *$P \leq 0.05$. C. Quantitative analysis of wound repair by HMEC-d cells. Wound areas were measured at time points 24 and 48 h after addition of conditioned media. Results are shown as percentages of the wound area at zero time point. Means ± SEM of six identical experiments are displayed. **$P \leq 0.01$.

branching points decreased after addition of inhibitory antibodies against HGF, VEGF, and both: 61%, 52%, and 49% of the control, respectively. Surprisingly, no additive effect of both anti-HGF and anti-VEGF could be observed in sprout or branch numbers.

In addition, we wanted to study the response of HUVEC and HMEC-d cells when grown in agarose-coated wells, similarly to nemotic fibroblasts. Both types of endothelial cells formed a loose spheroid by 24 h, which immediately began to disintegrate, forming a loose cloud by 96 h (see Supplementary Figures 3 and 4). In both cell types the proliferation marker PCNA was negative after 48 h, actin levels decreased markedly, and COX-2 remained negative, in contrast to fibroblast spheroids.

Discussion

Wound repair requires a complex scaffold or microenvironment, consisting of several cell types, ECM structures, and signaling processes that aid the reconstruction of the tissue. The activation process of stromal fibroblasts is of crucial importance in elucidating the role of the microenvironment in wound healing, of which angiogenesis is an important phenomenon. The vascular-forming response of endothelial cells is regulated by the tissue microenvironment [8].

We have characterized an activation process of fibroblasts, known as nemosis. Clustering of fibroblasts results in an

upregulation of COX-2 and increased secretion of prostaglandins. Nemosis induces leukocyte chemotaxis via the cytokines CXCL8, CCL3, and CCL5, and promotes cancer cell invasiveness through abundant secretion of HGF.

We have now followed VEGF secretion as a function of time and noticed that nemotic fibroblast spheroids produce elevated levels of VEGF, supported by previous results by our group [35]. The fold change compared to a fibroblast monolayer at 96 h is 1.9. However, from further experiments presented here it appears that the VEGF secreted by nemotic fibroblasts does play a functional role. Furthermore, our group has previously observed that malignant keratinocytes further augment VEGF and HGF produced in nemosis [35].

Our results here demonstrate that conditioned medium from nemotic fibroblasts promotes scratch-wound closure of endothelial monolayers, and the closure process is partly dependent on HGF but not VEGF. We also describe a positive effect of conditioned nemotic fibroblast medium on endothelial cell sprouting and networking, and its partial dependence on HGF and VEGF. Our previous results exhibit that at least 50% of the HGF produced is in its active form [33]. As nemotic fibroblasts secrete a variety of signaling molecules known to induce angiogenesis, blocking one or two such molecules does not result in completely effective inhibition of wound repair or sprouting responses to the level of that of monolayer medium. However, the significant decrease in the responses after blocking HGF and/or VEGF demonstrates the importance of these factors in the angiogenesis-promoting role of nemosis. Curiously, no additive effect on sprouting was perceived due to the combination of anti-HGF and anti-VEGF. A potential explanation is a possible convergence of downstream signaling routes resulting in a maximum inhibition of sprouting when blocking either HGF or VEGF separately.

We have previously characterized the chemokine secretion profile of nemotic fibroblasts and demonstrated their capability to attract leukocytes [29]. CXCL8 promotes angiogenesis and invasiveness [36,37], and has been detected in high levels in nemotic fibroblasts, suggesting an additional influence on the proangiogenic response of nemosis.

One hallmark of nemosis is strong induction of COX-2 [27] and an induced secretion of prostaglandins. COX-2 expression, chronic inflammation, and increased angiogenesis have been linked [38,39]. COX-2 products have also been shown to stimulate expression of VEGF and HGF [40,41], and its upregulation is known to be associated with HGF-promoted angiogenesis [42]. Both COX-2 and VEGF are known to be hypoxia-induced [43,44]. However, we have been unable to find hypoxic conditions in nemotic fibroblasts. Moreover, the clusters do not appear to have a necrotic center characteristic of a hypoxic environment, and COX-2 is shown to be induced uniformly, throughout the spheroid [27]. Therefore we assume that the induction of VEGF, together with other growth factors and cytokines produced, is an intrinsic property of the nemosis process.

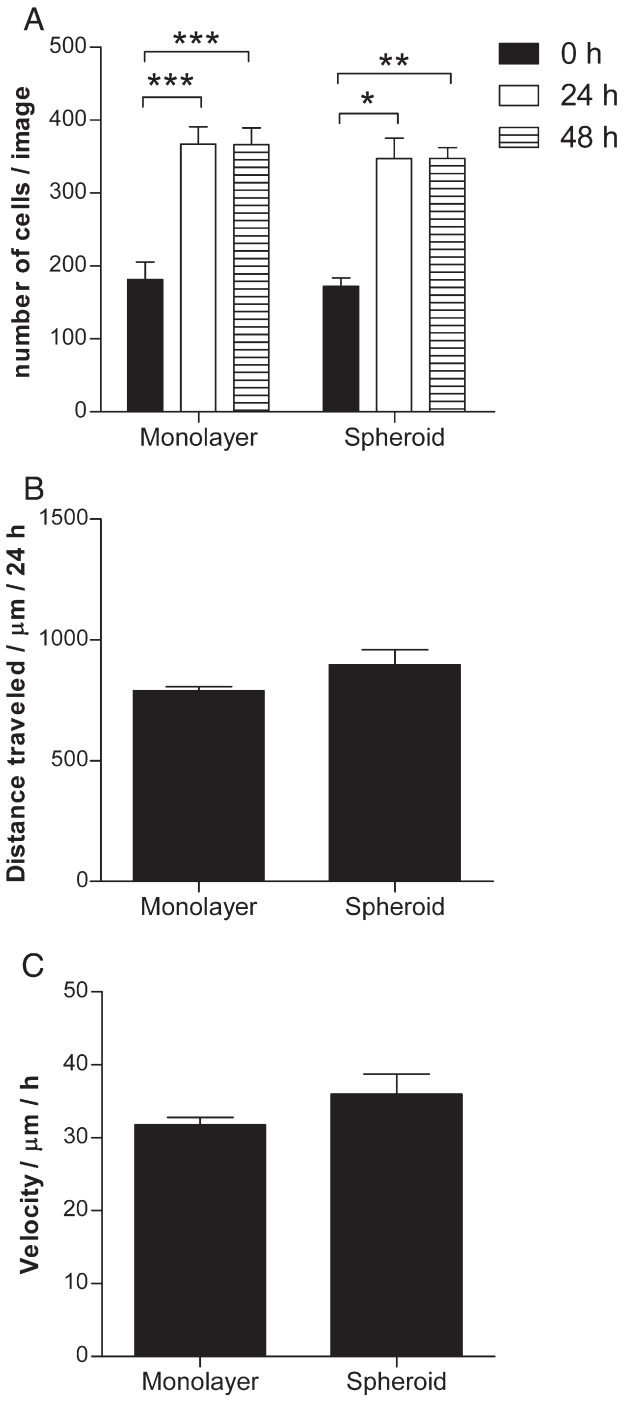

Fig. 3 – Nemotic fibroblasts promote motility of endothelial cells. A. Cell count of HUVEC cells exposed to conditioned fibroblast spheroid or monolayer medium. Cells were counted from phase-contrast images at several time points. Data are displayed as number of cells per image. Means ± SEM of three identical experiments are displayed. *$P \leq 0.005$; **$P \leq 0.01$; ***$P \leq 0.001$. B. Live-cell imaging of *in vitro* scratch-wound repair; average distance traveled by single cell in 24 h. Images were captured for 24 h at 30 min intervals, after which they were stacked and the movement of eight cells per image, all located on the wound edge, was tracked. Means ± SEM of three identical experiments are displayed. C. Live-cell imaging of *in vitro* scratch-wound repair; average velocity of single cell. Images were captured for 24 h at 30 min intervals, after which they were stacked and the movement of eight cells per image, all located on the wound edge, was tracked. Means ± SEM of three identical experiments are displayed.

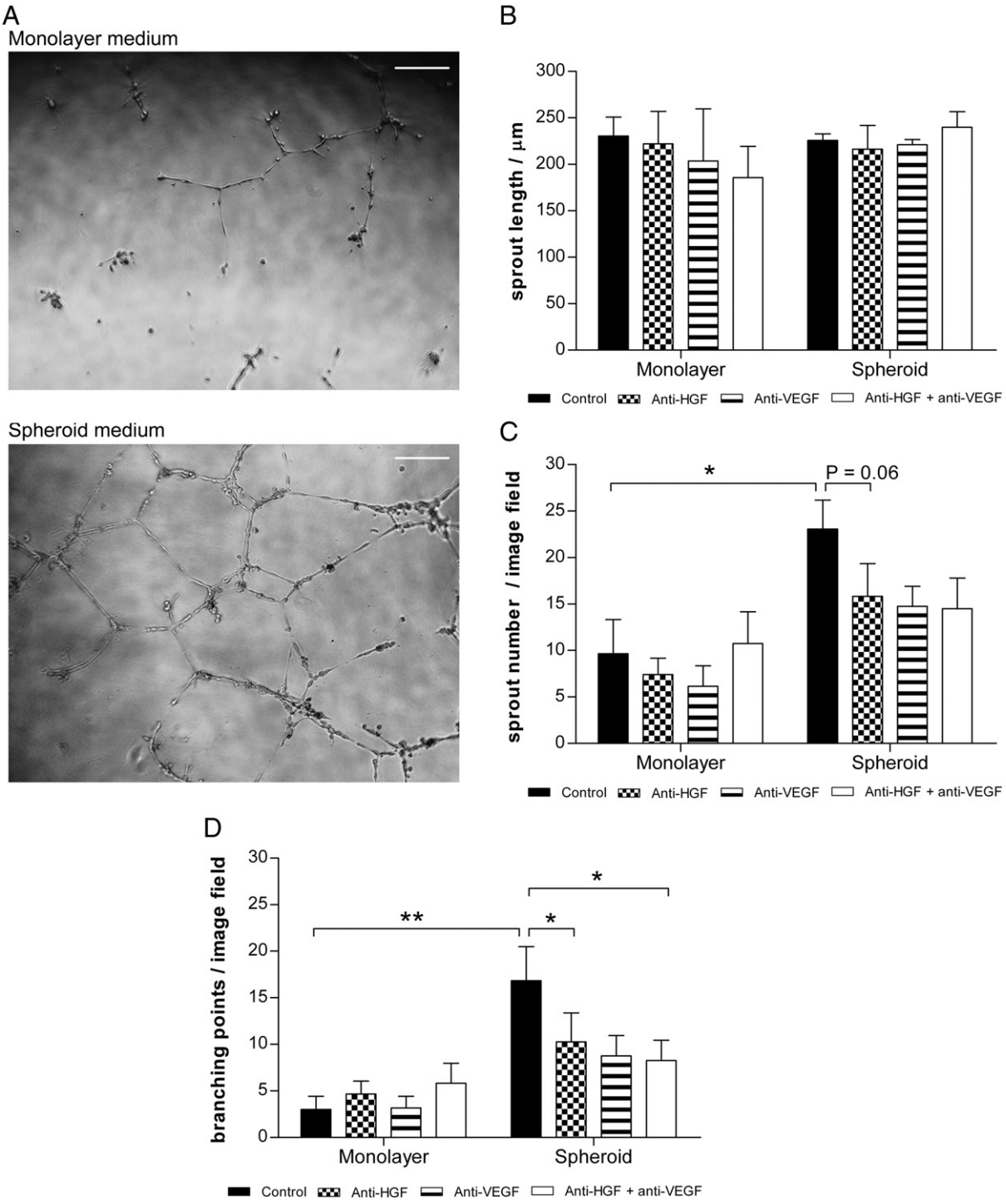

Fig. 4 – Nemotic fibroblasts promote endothelial cell sprouting and networking. A. Phase-contrast image of sprout formation by HUVEC cells on Matrigel® after exposure to conditioned fibroblast spheroid or monolayer medium. Imaged at 4× magnification, time point 24 h after cell seeding. Bar length = 200 µm. Image background decreased and contrast enhanced by applying the Curves-function of Adobe Photoshop CS3. B. Sprout length. Average sprout length from two random images per sample, measured at time point 24 h. Means ± SEM of six identical experiments are displayed. C. Sprout number. Average sprout number from two random images per sample, measured at time point 24 h. Means ± SEM of six identical experiments are displayed. *$P \leq 0.05$. D. Number of branching points. Average sprout branching point number from two random images per sample, measured at time point 24 h. Means ± SEM of six identical experiments are displayed.*$P \leq 0.05$; **$P \leq 0.01$.

The transcription factor NF-κB is implicated in many aspects of inflammation and inflammation-related malignancies [45]. Recent work has pointed out the importance of NF-κB in angiogenesis, such as via control of VEGF transcription [46,47]. We have previously demonstrated that NF-κB is active in nemosis [29], whereby a role for it in the regulation of angiogenesis seems plausible. Nemotic fibroblasts produce and secrete abundant amounts of HGF [31], and HGF has been shown to be capable of activating NF-κB [48]. The HGF receptor MET, mostly present on epithelium and endothelium, can be activated by IL-1 and IL-6 [49], cytokines which also are produced by nemotic fibroblasts [33]. Such transcriptional feedback loops would pose interesting implications on the angiogenesis-promoting qualities of nemosis.

The ECM protein fibronectin has been shown to promote endothelial cell adhesion, growth, survival, and HGF and VEGF activity [50–52], and it also plays a role in nemosis, initiating cluster formation [30]. Fibroblasts are an important source of fibronectin and may in such a way play an indirect role in angiogenesis [53]. Nemotic fibroblasts also produce matrix metalloproteinases [28], which are known to break down the ECM and aid the formation of vasculature [54].

We show that endothelial cells do not respond to conditioned nemotic fibroblast medium by proliferation, but display a modest increase in motility, which alone may be sufficient for enhanced scratch-wound closure. Nemotic fibroblast medium did not induce a significant increase in endothelial cell numbers, nor did the proliferation marker PCNA differ markedly between the two conditions, and the endothelial cells presented increased motility when tracked live with light microscopy. Growth factors such as VEGF and HGF are known to be mitogenic for endothelial cells but especially HGF induces a motogenic response in many cell types, including endothelial cells [9,13].

Finally, we investigated the response of endothelial cells encouraged to grow as multicellular spheroids. Based on two cell types, we conclude that in our model of experimentation, endothelial cells rather face an anoikis-like fate [55].

Inflammation is clearly implicated in the development and progress of many types of cancer, and malignancies are known to arise at sites of chronic inflammation [56]. Solid tumors are usually surrounded by stroma, a structure resembling the inflammatory microenvironment and composed of a mixture of components such as ECM, various inflammatory cell types, and often also vasculature. Activated, stromal fibroblasts produce cytokines and growth factors required for tissue repair as well as for tumor progression, whereas normal, non-activated fibroblasts have been observed to act as tumor suppressors [24,57,58]. Fibroblasts found in tumor stroma have been noted to remain constantly activated, and are often called cancer-associated fibroblasts (CAF). They frequently display a myofibroblastic phenotype [5,59,60].

In their need of growth-allowing nutrition, tumors also depend on neovascularization for development beyond a critical volume [61]. The microenvironment plays a crucial role in supporting vascularization: CAFs isolated from invasive breast carcinoma have been shown to promote tumor vascularization when mixed with breast cancer cells, in contrast to fibroblasts from normal tissue [62]. In neuroblastoma tumors, CAFs have been associated with microvascular proliferation [63], and increased vascularity in a tumor often correlates with poor prognosis. Vascularization of the tumor also facilitates metastatic escape of malignant cells, providing a way into the circulation system.

VEGF is a powerful proangiogenic growth factor. Its principal source in a tumor lies within the inflammatory cells and fibroblasts of the stroma [64]. However, it would seem that instead of VEGF release dynamics, regulation of the receptor itself is more important in controlling tumor vascularization [65]. HGF is another stromal-derived growth factor more recently linked to angiogenesis. Moreover, many carcinomas have abundant expression of the HGF ligand MET, and high levels seem to be substantial for tumor growth and survival [66]. MET and HGF are implicated in a variety of cancers, and over-expression of either one results in tumorigenesis and aggressive metastasis [15,67].

Nemosis is currently an experimental model providing a possible explanatory mechanism for the activation of fibroblasts. A plausible *in vivo* relevance can be depicted in the stroma of inflammatory sites and inflammation-associated tumors, where fibroblasts are found in abundance. Interactions of fibroblasts in such conditions may result in the activation experimentally profiled as nemosis. The results displayed here expand the concept to include endothelial cells as targets of nemosis, describing events observed during angiogenesis. Taken together, the increasing evidence around nemosis points to a close resemblance to inflammation- and cancer-associated fibroblasts, and further experimentation will reveal the actual role of nemosis in such pathogenic conditions. Because the production of angiogenic growth factors is an intrinsic property of clustering fibroblasts, it can be considered a means of inducing angiogenesis, promoting a wound healing response and subsequent tissue regeneration, and possessing a therapeutic potential.

Acknowledgments

We are indebted to Irina Suomalainen for her excellent technical assistance and advice. We also thank the Molecular Imaging Unit Core Facility at Biomedicum Helsinki, Faculty of Medicine, University of Helsinki for the use of their instruments, as well as excellent support and software development services. The Biomedicum Stem Cell Center is acknowledged for the use of their live-cell imaging system, as well as the excellent assistance, provided by Milla Mikkola, MSc. Drs. Ismo Virtanen and Hannu Koistinen at the University of Helsinki, Faculty of Medicine, are thanked for the kind donation of primary HMEC-d and HUVEC cells.

This work is supported by Helsinki Graduate School for Biotechnology and Molecular Biology, Svenska Läkaresällskapet Foundation, Nylands Nation Foundation, Jenny and Antti Wihuri Foundation, Academy of Finland, Finnish Cancer Organizations, and Joint Authority for the Hospital District of Helsinki and Uusimaa, Finland. The authors declare that they have no competing financial interests. The funding sources have no active involvement in the contents of this work.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.yexcr.2009.11.012.

REFERENCES

[1] S.A. Eming, T. Krieg, J.M. Davidson, Inflammation in wound repair: molecular and cellular mechanisms, J. Invest. Dermatol. 127 (2007) 514–525.
[2] J. Li, J. Chen, R. Kirsner, Pathophysiology of acute wound healing, Clin. Dermatol. 25 (2007) 9–18.
[3] C.J. Baglole, D.M. Ray, S.H. Bernstein, S.E. Feldon, T.J. Smith, P.J. Sime, R.P. Phipps, More than structural cells, fibroblasts create and orchestrate the tumor microenvironment, Immunol. Invest. 35 (2006) 297–325.
[4] C.D. Buckley, D. Pilling, J.M. Lord, A.N. Akbar, D. Scheel-Toellner, M. Salmon, Fibroblasts regulate the switch from acute resolving to chronic persistent inflammation, Trends Immunol 22 (2001) 199–204.
[5] B. Hinz, Formation and function of the myofibroblast during tissue repair, J. Invest. Dermatol. 127 (2007) 526–537.
[6] S.A. Eming, B. Brachvogel, T. Odorisio, M. Koch, Regulation of angiogenesis: wound healing as a model, Prog. Histochem. Cytochem. 42 (2007) 115–170.
[7] R.H. Adams, K. Alitalo, Molecular regulation of angiogenesis and lymphangiogenesis, Nat. Rev., Mol. Cell Biol. 8 (2007) 464–478.
[8] E. Langenkamp, G. Molema, Microvascular endothelial cell heterogeneity: general concepts and pharmacological consequences for anti-angiogenic therapy of cancer, Cell Tissue Res. 335 (2009) 205–222.
[9] N. Ferrara, H.P. Gerber, J. LeCouter, The biology of VEGF and its receptors, Nat. Med. 9 (2003) 669–676.
[10] K.M. Weidner, N. Arakaki, G. Hartmann, J. Vandekerckhove, S. Weingart, H. Rieder, C. Fonatsch, H. Tsubouchi, T. Hishida, Y. Daikuhara, W. Birchmeier, Evidence for the identity of human scatter factor and human hepatocyte growth factor, Proc. Natl. Acad. Sci. U. S. A. 88 (1991) 7001–7005.
[11] K. Conway, P. Price, K.G. Harding, W.G. Jiang, The molecular and clinical impact of hepatocyte growth factor, its receptor, activators, and inhibitors in wound healing, Wound Repair Regen. 14 (2006) 2–10.
[12] T. Nakamura, H. Teramoto, A. Ichihara, Purification and characterization of a growth factor from rat platelets for mature parenchymal hepatocytes in primary cultures, Proc. Natl. Acad. Sci. U. S. A. 83 (1986) 6489–6493.
[13] F. Bussolino, M.F. Di Renzo, M. Ziche, E. Bocchietto, M. Olivero, L. Naldini, G. Gaudino, L. Tamagnone, A. Coffer, P.M. Comoglio, Hepatocyte growth factor is a potent angiogenic factor which stimulates endothelial cell motility and growth, J. Cell Biol. 119 (1992) 629–641.
[14] K. Kajiya, S. Hirakawa, B. Ma, I. Drinnenberg, M. Detmar, Hepatocyte growth factor promotes lymphatic vessel formation and function, EMBO J. 24 (2005) 2885–2895.
[15] C. Birchmeier, W. Birchmeier, E. Gherardi, G.F. Vande Woude, Met, metastasis, motility and more, Nat. Rev. 4 (2003) 915–925.
[16] M. Park, M. Dean, C.S. Cooper, M. Schmidt, S.J. O'Brien, D.G. Blair, G.F. Vande Woude, Mechanism of met oncogene activation, Cell 45 (1986) 895–904.
[17] Y. Nakamura, R. Morishita, J. Higaki, I. Kida, M. Aoki, A. Moriguchi, K. Yamada, S. Hayashi, Y. Yo, K. Matsumoto, T. Nakamura, T. Ogihara, Expression of local hepatocyte growth factor system in vascular tissues, Biochem. Biophys. Res. Commun. 215 (1995) 483–488.
[18] E. Sonnenberg, D. Meyer, K.M. Weidner, C. Birchmeier, Scatter factor/hepatocyte growth factor and its receptor, the c-met tyrosine kinase, can mediate a signal exchange between mesenchyme and epithelia during mouse development, J. Cell Biol. 123 (1993) 223–235.
[19] J. Chmielowiec, M. Borowiak, M. Morkel, T. Stradal, B. Munz, S. Werner, J. Wehland, C. Birchmeier, W. Birchmeier, c-Met is essential for wound healing in the skin, J. Cell Biol. 177 (2007) 151–162.
[20] A. Mantovani, P. Allavena, A. Sica, F. Balkwill, Cancer-related inflammation, Nature 454 (2008) 436–444.
[21] N.A. Bhowmick, E.G. Neilson, H.L. Moses, Stromal fibroblasts in cancer initiation and progression, Nature 432 (2004) 332–337.
[22] W. Schurch, T.A. Seemayer, R. Lagace, Stromal myofibroblasts in primary invasive and metastatic carcinomas. A combined immunological, light and electron microscopic study, Virchows Arch. A Pathol. Anat. Histol. 391 (1981) 125–139.
[23] M.T. Dimanche-Boitrel, L. Vakaet Jr., P. Pujuguet, B. Chauffert, M.S. Martin, A. Hammann, F. Van Roy, M. Mareel, F. Martin, In vivo and in vitro invasiveness of a rat colon-cancer cell line maintaining E-cadherin expression: an enhancing role of tumor-associated myofibroblasts, Int. J. Cancer 56 (1994) 512–521.
[24] A.F. Olumi, G.D. Grossfeld, S.W. Hayward, P.R. Carroll, T.D. Tlsty, G.R. Cunha, Carcinoma-associated fibroblasts direct tumor progression of initiated human prostatic epithelium, Cancer Res. 59 (1999) 5002–5011.
[25] P. Micke, A. Ostman, Tumour–Stroma interaction: cancer-associated fibroblasts as novel targets in anti-cancer therapy? Lung Cancer 45 (2004) S163–S175.
[26] A. Vaheri, A. Enzerink, K. Räsänen, P. Salmenperä, Nemosis, a novel way of fibroblast activation, in inflammation and cancer, Exp. Cell Res. 315 (2009) 1633–1638.
[27] J. Bizik, E. Kankuri, A. Ristimaki, A. Taieb, H. Vapaatalo, W. Lubitz, A. Vaheri, Cell–Cell contacts trigger programmed necrosis and induce cyclooxygenase-2 expression, Cell Death Differ. 11 (2004) 183–195.
[28] V. Sirén, P. Salmenperä, E. Kankuri, J. Bizik, T. Sorsa, T. Tervahartiala, A. Vaheri, Cell–Cell contact activation of fibroblasts increases the expression of matrix metalloproteinases, Ann. Med. 38 (2006) 212–220.
[29] A. Enzerink, P. Salmenperä, E. Kankuri, A. Vaheri, Clustering of fibroblasts induces proinflammatory chemokine secretion promoting leukocyte migration, Mol. Immunol. 46 (2009) 1787–1795.
[30] P. Salmenperä, E. Kankuri, J. Bizik, V. Sirén, I. Virtanen, S. Takahashi, M. Leiss, R. Fassler, A. Vaheri, Formation and activation of fibroblast spheroids depend on fibronectin–integrin interaction, Exp. Cell Res. 314 (2008) 3444–3452.
[31] E. Kankuri, D. Cholujova, M. Comajova, A. Vaheri, J. Bizik, Induction of HGF/SF by cell–cell contacts in fibroblasts directly promotes tumor invasiveness, Cancer Res. 65 (2005) 9914–9922.
[32] M. Peura, J. Bizik, P. Salmenpera, A. Noro, M. Korhonen, T. Patila, A. Vento, A. Vaheri, R. Alitalo, J. Vuola, A. Harjula, E. Kankuri, Bone marrow mesenchymal stem cells undergo nemosis and induce keratinocyte wound healing utilizing the HGF/c-Met/PI3K pathway, Wound Repair Regen. 17 (2009) 569–577.
[33] E. Kankuri, O. Babusikova, K. Hlubinova, P. Salmenpera, C. Boccaccio, W. Lubitz, A. Harjula, J. Bizik, Fibroblast nemosis arrests growth and induces differentiation of human leukemia cells, Int. J. Cancer 122 (2008) 1243–1252.
[34] K. Rasanen, I. Virtanen, P. Salmenpera, R. Grenman, A. Vaheri, Differences in the nemosis response of normal and cancer-associated fibroblasts from patients with oral squamous cell carcinoma, PLoS One 4 (2009) e6879.
[35] K. Räsänen, P. Salmenperä, M. Baumann, I. Virtanen, A. Vaheri, Nemosis of fibroblasts is inhibited by benign HaCaT keratinocytes but promoted by malignant HaCaT cells, Mol. Oncol. 2 (2008) 340–348.
[36] Y. Matsuo, N. Ochi, H. Sawai, A. Yasuda, H. Takahashi, H. Funahashi, H. Takeyama, Z. Tong, S. Guha, CXCL8/IL-8 and CXCL12/SDF-1alpha co-operatively promote invasiveness and angiogenesis in pancreatic cancer, Int. J. Cancer 124 (2009) 853–861.
[37] Y. Kitadai, K. Haruma, K. Sumii, S. Yamamoto, T. Ue, H. Yokozaki, W. Yasui, Y. Ohmoto, G. Kajiyama, I.J. Fidler, E. Tahara, Expression of interleukin-8 correlates with vascularity in human gastric carcinomas, Am. J. Pathol. 152 (1998) 93–100.

[38] S.H. Chang, C.H. Liu, R. Conway, D.K. Han, K. Nithipatikom, O.C. Trifan, T.F. Lane, T. Hla, Role of prostaglandin E2-dependent angiogenic switch in cyclooxygenase 2-induced breast cancer progression, Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 591–596.
[39] W. Wang, A. Bergh, J.E. Damber, Cyclooxygenase-2 expression correlates with local chronic inflammation and tumor neovascularization in human prostate cancer, Clin. Cancer Res. 11 (2005) 3250–3256.
[40] X.H. Liu, A. Kirschenbaum, S. Yao, M.E. Stearns, J.F. Holland, K. Claffey, A.C. Levine, Upregulation of vascular endothelial growth factor by cobalt chloride-simulated hypoxia is mediated by persistent induction of cyclooxygenase-2 in a metastatic human prostate cancer cell line, Clin. Exp. Metastasis 17 (1999) 687–694.
[41] T. Matsunaga, E. Gohda, T. Takebe, Y.L. Wu, M. Iwao, H. Kataoka, I. Yamamoto, Expression of hepatocyte growth factor is up-regulated through activation of a cAMP-mediated pathway, Exp. Cell Res. 210 (1994) 326–335.
[42] C. Boccaccio, G. Sabatino, E. Medico, F. Girolami, A. Follenzi, G. Reato, A. Sottile, L. Naldini, P.M. Comoglio, The MET oncogene drives a genetic programme linking cancer to haemostasis, Nature 434 (2005) 396–400.
[43] R.M. Tuder, B.E. Flook, N.F. Voelkel, Increased gene expression for VEGF and the VEGF receptors KDR/Flk and Flt in lungs exposed to acute or to chronic hypoxia. Modulation of gene expression by nitric oxide, J. Clin. Invest. 95 (1995) 1798–1807.
[44] M. Chida, N.F. Voelkel, Effects of acute and chronic hypoxia on rat lung cyclooxygenase, Am. J. Physiol. 270 (1996) L872–878.
[45] Q. Li, S. Withoff, I.M. Verma, Inflammation-associated cancer: NF-kappaB is the lynchpin, Trends Immunol. 26 (2005) 318–325.
[46] S.P. Tabruyn, A.W. Griffioen, NF-kappa B: a new player in angiostatic therapy, Angiogenesis 11 (2008) 101–106.
[47] S. Kiriakidis, E. Andreakos, C. Monaco, B. Foxwell, M. Feldmann, E. Paleolog, VEGF expression in human macrophages is NF-kappaB-dependent: studies using adenoviruses expressing the endogenous NF-kappaB inhibitor IkappaBalpha and a kinase-defective form of the IkappaB kinase 2, J. Cell Sci. 116 (2003) 665–674.
[48] S. Fan, M. Gao, Q. Meng, J.J. Laterra, M.H. Symons, S. Coniglio, R.G. Pestell, I.D. Goldberg, E.M. Rosen, Role of NF-kB signaling in hepatocyte growth factor/scatter factor-mediated cell protection, Oncogene 24 (2005) 1749–1766.
[49] A. Moghul, L. Lin, A. Beedle, A. Kanbour-Shakir, M.C. DeFrances, Y. Liu, R. Zarnegar, Modulation of c-MET proto-oncogene (HGF receptor) mRNA abundance by cytokines and hormones: evidence for rapid decay of the 8 kb c-MET transcript, Oncogene 9 (1994) 2045–2052.
[50] S. Rahman, Y. Patel, J. Murray, K.V. Patel, R. Sumathipala, M. Sobel, E.S. Wijelath, Novel hepatocyte growth factor (HGF) binding domains on fibronectin and vitronectin coordinate a distinct and amplified Met-integrin induced signalling pathway in endothelial cells, BMC Cell Biol. 6 (2005) 8.
[51] J. Sottile, Regulation of angiogenesis by extracellular matrix, Biochim. Biophys. Acta 1654 (2004) 13–22.
[52] E.S. Wijelath, J. Murray, S. Rahman, Y. Patel, A. Ishida, K. Strand, S. Aziz, C. Cardona, W.P. Hammond, G.F. Savidge, S. Rafii, M. Sobel, Novel vascular endothelial growth factor binding domains of fibronectin enhance vascular endothelial growth factor biological activity, Circ. Res. 91 (2002) 25–31.
[53] C.C. Hughes, Endothelial–Stromal interactions in angiogenesis, Curr. Opin. Hematol. 15 (2008) 204–209.
[54] V.W. van Hinsbergh, P. Koolwijk, Endothelial sprouting and angiogenesis: matrix metalloproteinases in the lead, Cardiovasc. Res. 78 (2008) 203–212.
[55] P. Chiarugi, E. Giannoni, Anoikis: a necessary death program for anchorage-dependent cells, Biochem. Pharmacol. 76 (2008) 1352–1364.
[56] M. Schafer, S. Werner, Cancer as an overhealing wound: an old hypothesis revisited, Nat. Rev., Mol. Cell Biol. 9 (2008) 628–638.
[57] A. Orimo, R.A. Weinberg, Stromal fibroblasts in cancer: a novel tumor-promoting cell type, Cell Cycle 5 (2006) 1597–1601.
[58] G.B. Pierce, C.G. Pantazis, J.E. Caldwell, R.S. Wells, Specificity of the control of tumor formation by the blastocyst, Cancer Res. 42 (1982) 1082–1087.
[59] R. Kalluri, M. Zeisberg, Fibroblasts in cancer, Nat. Rev., Cancer 6 (2006) 392–401.
[60] O. De Wever, P. Demetter, M. Mareel, M. Bracke, Stromal myofibroblasts are drivers of invasive cancer growth, Int. J. Cancer 123 (2008) 2229–2238.
[61] J. Folkman, E. Merler, C. Abernathy, G. Williams, Isolation of a tumor factor responsible for angiogenesis, J. Exp. Med. 133 (1971) 275–288.
[62] A. Orimo, P.B. Gupta, D.C. Sgroi, F. Arenzana-Seisdedos, T. Delaunay, R. Naeem, V.J. Carey, A.L. Richardson, R.A. Weinberg, Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion, Cell 121 (2005) 335–348.
[63] R. Zeine, H.R. Salwen, R. Peddinti, Y. Tian, L. Guerrero, Q. Yang, A. Chlenski, S.L. Cohn, Presence of cancer-associated fibroblasts inversely correlates with Schwannian stroma in neuroblastoma tumors, Mod. Pathol. 22 (2009) 950–958.
[64] D. Fukumura, R. Xavier, T. Sugiura, Y. Chen, E.C. Park, N. Lu, M. Selig, G. Nielsen, T. Taksir, R.K. Jain, B. Seed, Tumor induction of VEGF promoter activity in stromal cells, Cell 94 (1998) 715–725.
[65] M. Skobe, P. Rockwell, N. Goldstein, S. Vosseler, N.E. Fusenig, Halting angiogenesis suppresses carcinoma cell invasion, Nat. Med. 3 (1997) 1222–1227.
[66] N. Shinomiya, C.F. Gao, Q. Xie, M. Gustafson, D.J. Waters, Y.W. Zhang, G.F. Vande Woude, RNA interference reveals that ligand-independent met activity is required for tumor cell signaling and survival, Cancer Res. 64 (2004) 7962–7970.
[67] S. Rong, S. Segal, M. Anver, J.H. Resau, G.F. Vande Woude, Invasiveness and metastasis of NIH 3T3 cells induced by Met-hepatocyte growth factor/scatter factor autocrine stimulation, Proc. Natl. Acad. Sci. U. S. A. 91 (1994) 4731–4735.