Leptin stimulates the migration of colon carcinoma cells by multiple signaling pathways

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Abstract

Active migration of tumor cells is a prerequisite for the development of metastasis and tumor progression, and is regulated by a variety of extracellular ligands. Epidemiological studies have shown that obesity increases the risk of colon cancer by 1.5- to 2-fold with obesity-associated colon cancer accounting for 14–35% of total incidence. In obese individuals, serum levels of leptin are markedly increased, and therefore, we have investigated the impact of this adipocytokine on the migration of various human colon carcinoma cell lines such as SW480, SW620, and HCT116. Leptin significantly enhanced the migratory activity of all three cell lines, and the strongest effect was observed in SW480 cells, which increased their locomotor activity from 28% spontaneously locomoting cells to 50%. The intracellular signal transduction regulating this pro-migratory effect involves the activation of the transcription factor signal transducer and activator of transcription-3 via Janus kinases, but also the activity of src tyrosine kinases, focal adhesion kinase, exclusively protein kinase Cα, and the phosphatidylinositol-3-kinase, as proven by the use of particular inhibitors and target-specific small interfering RNAs. Herein, we deliver new evidence for a modulatory role of leptin in the regulation of colon cancer progression by stimulating tumor cell migration. Thus, our findings have potential clinical implications, because understanding the impact of leptin on tumor cell migration and the underlying signal transduction mechanisms is mandatory for future development of novel therapeutics to treat obesity-associated colorectal cancer.

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Introduction

Obesity is a dramatically increasing public health problem worldwide, because of its known consequences to be responsible for a plurality of disease such as diabetes, hypertension, or cardiovascular disease. Obesity is also correlated with an increased risk for certain types of cancer, including breast, colon, prostate, and other (Housa et al. 2006). Obese individuals have ~1.5- to 3.5-fold increased risk of developing these cancers compared with normal-weight individuals, and it is estimated that 15–45% of all deaths from cancer in Europe are attributable to obesity (Pischon et al. 2008). Furthermore, several studies have demonstrated that colon cancers occur more frequently in people who are obese than in those of a healthy weight (Shike 1996, Caan et al. 1998).

A key molecule in obesity is leptin, a 16-kDa peptide hormone primarily secreted by adipocytes. The main function of leptin is the regulation of energy expenditure and control of appetite. Indeed, lack of leptin in mice with a mutation in the gene encoding leptin, or absence of functional leptin receptor (db/db mice) results in obesity and many associated metabolic complications such as insulin resistance (Ceddia et al. 2002). Serum levels of leptin reflect the amount of energy stored in the adipose tissue and are in proportion to body fat mass (Fruhbeck 2006), i.e. increased in obese and decreased with weight reduction. Leptin acts via transmembrane receptors (Ob-R), which belong to the class I cytokine receptor family (Tartaglia et al. 1995), such as the receptors of interleukin-6 (IL-6), IL-11, IL-12, granulocyte colony-stimulating factor, or...
leukemia inhibitory factor. The Ob-R has at least six isoforms, whereas only one of them (Ob-Rb) has full signaling capabilities and is able to activate the Janus kinase (JAK)/signal transducer and activator of transcription (Stat) pathway, the major pathway used by leptin to exert its effects (Hegyi et al., 2004). High levels of this isoform exist in the hypothalamus, the main site of leptin action, but it has also direct effects on many cell types on the periphery. Accordingly, the receptor is also found to be expressed on a variety of immune cells (Guzik et al., 2006), endothelial cells, adipocytes, and diverse cancer cells derived from different tissues such as breast, colon, or prostate (Frankenberry et al., 2004, 2006, Jaffe & Schwartz, 2008). In breast, colon, esophageal, and prostate cancer cells, leptin increases proliferation (Frankenberry et al., 2004, 2006, Housa et al., 2006). Leptin not only promotes the proliferation, but also the invasion and migration of hepatocellular and endometrial carcinoma cells (Sharma et al., 2006, Saxena et al., 2007). Thus, leptin may affect processes related to cancer initiation or progression, resulting in metastatic development.

However, the development of metastases is a multistep process for which the metastasizing tumor cells need to acquire the ability of active migration. This migratory activity is not solely an intrinsic property of tumor cells, but a cell function which is regulated by signal substances similar to the regulation of migratory activity in leukocytes. We have shown previously that extracellular ligands such as neurotransmitters significantly induce the migratory activity of human SW480 colon carcinoma cells (Masur et al., 2001b). In this current work, we investigated the effect of leptin on the migration of distinct human colon carcinoma cells. Moreover, we addressed potential signal transduction pathways underlying the leptin-driven influence on the migration. Our experiments revealed that leptin has the strongest impact on the migration of SW480 colon carcinoma cells, and this effect is intracellularly integrated via multiple signaling pathways.

Materials and methods

Cell culture

All cell lines used in this study were derived from the American Type Culture Collection (Rockville, MA, USA). The human colon carcinoma cells lines SW480 (grade III–IV) and SW620 (metastatic) were cultured in Leibovitz’s L-15 culture medium (PAA, Linz, Austria) containing 10% FCS (PAA) at 37 °C humidified atmosphere. The human colon carcinoma cell line HCT 116 was grown in DMEM (PAA) supplemented with 10% FCS (PAA), penicillin (50 U/ml), and streptomycin (50 μg/ml; Gibco) at 37 °C humidified atmosphere with 5% CO₂.

Cell migration

We performed our conventional three-dimensional, collagen-based migration assay as described in detail previously (Lang et al., 2002). Mean locomotory activity and s.d. of 30 randomly selected cells were calculated for each single experiment at the steady state level.

Migratory activity was induced by 100 ng/ml recombinant human leptin (Biomol, Hamburg, Germany). Furthermore, we used LY294002 (5 μM, New England Biolabs, Frankfurt, Germany), Stat-3 inhibitor peptide (200 μg/ml, Merck), rottlerin (1 μM, Merck), JAK inhibitor I (20 nM, Merck), pp1 (100 nM), and pp2 (50 nM, both Merck). None of the substances affected the viability of the cells at the concentrations used, as was analyzed by propidium iodide staining and flow cytometry.

Immunoblotting

The detection of the leptin receptor and expression of the proteins focal adhesion kinase (FAK) and phospho-specific FAK were analyzed by immunoblotting. For phosphorylation studies, SW480 cells were incubated for 15 min in culture medium with or without 100 ng/ml leptin and then incubated for 5 min in PhosphoSafe Extraction Reagent (Novagen, Madison, WI, USA); whereas for the detection of FAK cells were incubated for 6 h. Cells were lysed in Laemmli sample buffer (10 min, 95 °C) (Laemmli, 1970), and lysates of 3×10⁵ cells and 5×10⁵ (for leptin receptor detection) of each sample were applied to gel-electrophoresis. The proteins were separated and transferred to an Immobilon-P membrane (Millipore, Bedford, MA, USA) as described previously (Lang et al., 2004), and subsequent immunoblotting was performed according to the method described therein. For the detection of FAK, an anti-FAK antibody (New England Biolabs) was used, and for visualization of the leptin receptor, an anti-Ob-R antibody (Abcam, Cambridge, UK) was used. For protein standardization in the case of FAK, the membrane was reprobed with a β-actin rabbit antibody (New England Biolabs). Anti-phospho-FAK (pY397) and anti-phospho-FAK (p576/p577) antibodies were obtained from Epitomics (Burlingame, CA, USA). Primary antibodies were detected using a HRP-conjugated anti-rabbit antibody (Southern Biotech, Birmingham, AL, USA). The luminescence signal was induced with chemiluminescence blotting.
Downregulation of the protein kinase Cα and protein kinase Cδ expression

For studying the role of protein kinase C (PKC)α and PKCδ in the leptin-induced migration of SW480 colon carcinoma cells, we have downregulated their expression in SW480 cells using small interfering RNA (siRNA) as described previously (Strell et al. 2007). The PKCα and PKCδ siRNA were derived as non-silencing control siRNA were derived from Dharmaco (ON-TARGET plus smart pool, Thermo Scientific, Bonn, Germany) and transfected using an Amaxa Nucleofector II (Cologne, Germany) with program L-024 and solution V. Silencing efficiency was controlled by immunoblotting using a mouse anti-PKCα or anti-PKCδ antibody (Becton Dickinson, Heidelberg, Germany) as described in the previous section. A mouse β-actin antibody (New England Biolabs) was used as a normalizing control. For migration experiments, cells were used 48 h post transfection.

Flow cytometry

The leptin receptor expression was determined using a FacsCalibur flow cytometer (Becton Dickinson). Subsequently, 2.5×10⁵ cells were fixed with 1% formaldehyde, and then incubated for 10 min at room temperature (RT) with 10 μg/ml of the primary antibody monoclonal anti-human Leptin receptor (Ob-R) antibody (R&D Systems, Wiesbaden, Germany). After washing, we incubated the cells with 10 μg/ml FITC-conjugated anti-mouse antibody (Dianova, Hamburg, Germany). Non-specific binding was determined by isotypic control mouse antibody (Coulter-Immunotech, Hamburg, Germany).

Confocal laser scanning microscopy

To analyze the intracellular distribution of FAK and filamentous actin (F-actin) in the cells, collagen lattices containing tumor cells were generated as described in Cell Migration Assay. After incubation of the cells with 100 ng/ml leptin for 2 h in the gel, samples were treated with 4% paraformaldehyde overnight at 4°C for fixation, washed, and subsequently permeabilized with 0.5% Triton X-100 (10 min, RT). After washing, samples were incubated for 2 h at RT with a polyclonal IgG rabbit-anti-FAK antibody derived from Santa Cruz Biotechnology (Heidelberg, Germany). After washing, samples were incubated for 2 h at RT with a FITC-conjugated secondary goat anti-rabbit F(ab) fragment (Invitrogen) and AlexaFluor-phalloidin (Invitrogen). Confocal laser scanning microscopy was done using a TCS SP5 confocal laser scanning microscope (Leica, Bensheim, Germany) as described previously (Bastian et al. 2006).

Transcription factor measurement

The activation of transcription factors (Stat family: Stat-3, Stat-5A, Stat-5B and MAPK family: ATF-2, c-Jun, c-Myc, and MEF-2) was analyzed after leptin treatment using TransAM assay kits (Active Motif Europe, Rixensart, Belgium). After 30 min incubation with leptin alone or in combination with various inhibitors, 1×10⁵ SW480 cells per sample were lysed in 20 μl lysis buffer according to the manufacturer’s protocol (equivalent to 10 μg/ml protein as was measured by a standard Bradford assay), and the lysates were subsequently subjected to TransAM assays. Control experiments were conducted for leptin treatment for 15, 30, 60, and 120 min. Because Stat-3 activation had reached its maximum level in SW480 cells at 30 min, this time was chosen for transcription factor measurements throughout. The assays were performed as described previously (Lang et al. 2004).

Statistical analysis

Significant changes were calculated using the Student’s t-test (two-tailed, unpaired). A probability value of \( P < 0.05 \) was accepted as statistically significant throughout the experiments.

Results

Leptin stimulates the migration of colon carcinoma cells

Using our three-dimensional cell migration assay, we investigated the effects of leptin on the locomotory activity of various colon carcinoma cells, including SW480, its metastasis SW620, and HCT116. After incorporation into a collagen matrix, all carcinoma cells exhibited a significant increase of their migratory activity in response to leptin (100 ng/ml; Fig. 1A). The strongest pro-migratory effects were observed with SW480 and HCT116 cells, which demonstrated an enhanced locomotion from an average of 28.3 ± 5.1% spontaneously migrating cells to 43.8 ± 3.7% for SW480 cells (\( P = 0.013 \)) and from 21.5 ± 5.4% to...
35.3 ± 3.8% for HCT116 cells ( \( P = 0.02; \) Fig. 1A) respectively. The low spontaneous migration activity of the metastatic cell line SW620 in comparison to its parental tumor cell line (SW480) can be explained by previous findings, where we could demonstrate a strong correlation between the locomotory activity and the expression level of E-cadherin and PKC\( \alpha \). A high level of PKC\( \alpha \) expression simultaneously with a low E-cadherin level as we found in SW480 cells resulted in an elevated migratory activity in comparison to SW620 cells, which displayed a lower level of PKC\( \alpha \) expression in combination with a higher expression of E-cadherin (Masur et al. 2001a). Next, we examined whether the divergent colon carcinoma cells express a functional leptin receptor Ob-R1 to be able to respond to leptin. Using polyclonal antibody against Ob-R’s long and short isoforms, we showed that all three colon carcinoma cell lines express both isoforms (Fig. 1B). Two bands were evident: one band running at 100 kDa that corresponded to the short (Ob-Ra) and long (Ob-Rb) human isoforms. (C) Flow cytometry expression of the leptin receptor Ob-R on the surface of SW480 and HCT116 colon carcinoma cells as was assessed by flow cytometry. The FITC fluorescence of specific antibodies (gray area) was compared to an unspecific isotypic control (black line). MFI, mean fluorescence intensity of specific and isotypic (Iso) binding. The shown histogram holds true for five measurements that have been performed independently. In (D), the migration of SW480 carcinoma cells was induced by various concentrations of leptin. The graph shows mean values of three independent experiments (90 cells were analyzed per sample).

Figure 1 Leptin stimulates the migration of human colon carcinoma cells. (A) Migration of colon carcinoma cell lines SW480, SW620, and HCT116 after stimulation with leptin. The graph shows mean values of three independent experiments (90 cells were analyzed per sample), and significant changes with a \( P \) value of \( P \leq 0.05 \) are marked by an asterisk. (B) Immunoblot analysis for detection of a functional leptin receptor. Two bands were evident, of 100 and 125 kDa, corresponding to the short (Ob-Ra) and long (Ob-Rb) human isoforms. (C) Flow cytometry expression of the leptin receptor Ob-R on the surface of SW480 and HCT116 colon carcinoma cells as was assessed by flow cytometry. The FITC fluorescence of specific antibodies (gray area) was compared to an unspecific isotypic control (black line). MFI, mean fluorescence intensity of specific and isotypic (Iso) binding. The shown histogram holds true for five measurements that have been performed independently. In (D), the migration of SW480 carcinoma cells was induced by various concentrations of leptin. The graph shows mean values of three independent experiments (90 cells were analyzed per sample).

Regulation of transcription factors by leptin
Leptin receptor Ob-R is a member of the class I cytokine receptor family, which is known to intracellularly activate the JAK/Stat and the MAPK pathway after leptin binding (Sweeney 2002, Fruhbeck 2006). Accordingly, incubation of SW480 cells with leptin led to an activation of Stat family members such as Stat-3, Stat-5a, and Stat-5b, but only the transcription factor Stat-3 was significantly activated (\( P < 0.05; \) Fig. 2). Furthermore, this activation of Stat-3 was accompanied by an increase of suppressor of cytokine signaling-3, its negative feedback regulator (data not shown). With regard to the activation state of members
of the MAPK family, leptin treatment of the cells resulted in an activation of c-Jun by its phosphorylation, whereas this change in regulation did not reach statistical significance (Fig. 2).

In addition to prove that the observed leptin effect on the migration is central and not mediated by other cytokines that overlap in this function, we measured the release of various cytokines such as IL-6 and IL-8 using a fluorescent bead immunoassay. We could not detect any IL-6 in the supernatant of the cells, and IL-8 was only present in very low concentrations (data not shown).

The intracellular signal transduction of leptin-induced cell migration

Since treatment of SW480 cells with leptin resulted in an activation of various transcription factors such as Stat-3, we analyzed whether this pathway is functionally involved in the leptin-induced migration, and if Stat-3 activation is mediated amongst others via JAKs. Using JAK-inhibitor I (20 nM), we significantly reduced the leptin-stimulated migration of SW480 cells from an average of 41.0 ± 5.2 to 18.0 ± 5.2% locomoting cells (P ≤ 0.05; Fig. 3A). Next, we utilized a Stat-3-specific inhibitory peptide, leading to the reduction of active Stat3:Stat3 dimers levels that can bind DNA, in our cell migration assay. Treatment of the cells with this specific peptide did not affect the spontaneous migration of SW480 cells, but completely abrogated the leptin-mediated increase of locomotion from 50.1 ± 7.0% migrating cells to 25.1 ± 6.2% (P = 0.0022; Fig. 3B).

In colon carcinoma cells, we distinguished a nonreceptor protein tyrosine kinase (PTK)-independent, spontaneous migration from a catecholamine-induced, PTK-dependent migration (Masur et al. 2001a). Treatment of the SW480 colon carcinoma cells with the selective inhibitors of the src family tyrosine kinases pp2 and pp1 did both not have any effect on the spontaneous, matrix-induced locomotory activity, but resulted in a significant reduction of the leptin-induced migratory activity (P ≤ 0.03; Fig. 3C and D).

Phosphatidylinositol-3-kinase (PI3K) is a known mediator in the signaling pathway of the leptin receptor (Sweeney 2002), and known to play an essential role for cell migration (Bastian et al. 2006). Here, inhibition of the PI3K with 5 μM LY294002 abolished the significant (P = 0.043) induction caused by leptin (Fig. 3E): as compared to the control (30.9 ± 4.0% locomoting cells), 44.8 ± 3.4% of the cells migrated after addition of leptin, but 26.8 ± 3.6% of the cells migrated after treatment with leptin and LY294002 in combination (P = 0.029). LY294002 alone had no effect (26.9 ± 3.6% locomoting cells). In conclusion, the PI3K is activated by leptin signaling and involved in this induced migration of SW480 cells.

To close the circle, we have further elucidated the role of Stat-3 in the leptin-mediated migration and investigated whether the inhibition of leptin-induced migration mediated by the use of several inhibitors is accompanied by an inactivation of Stat-3 (Fig. 3F). Treatment of the cells with leptin in combination with inhibitors for JAKs and src kinase did not only decrease the migratory activity of the cells, but resulted in a significant inactivation of Stat-3, too (Fig. 3F).

Which isotype of PKC in the cells is responsible for the regulation of migratory activity? From our own previous investigations and inquests on this topic, we know that both the PKCα and the PKCδ are expressed in SW480 cells and described to be involved in tumor cell migration (Masur et al. 2001a). However, we tested...
the involvement of these proteins in the leptin-induced migration of these cells by knocking out their expression via siRNA. After 48 h treatment with the PKCα or PKCδ downregulating siRNA, we observed a strong decrease of the gene products within the cells (Fig. 4A). At this timepoint, we subjected the cells to our migration assay. Knocking-down of the PKCα revealed no effect neither on the spontaneous, matrix-induced migration nor on the locomotion initiated by leptin treatment (Fig. 4B). In contrast, downregulation of the PKCδ completely abolished the leptin-induced migration of SW480 cells from 47.6 ± 6.3% migrating cells to 29.8 ± 1.9% (P = 0.016), but did not have any effect on the spontaneous locomotor activity (Fig. 4C). These results are further supported by migration experiments using the PKCδ-specific inhibitor rottlerin (1 μM), which led to a reduction of the leptin-induced migration, but did not have any effect on the spontaneous migratory activity of SW480 cells (Fig. 4D).

These results suggest a differential involvement of these two PKC isotypes in the migration of SW480 colon carcinoma cells, whereas PKCδ activity is exclusively involved in the leptin-induced migration, PKCα activity is not necessary for the regulation of SW480 migration. There are several lines of evidence that PKCs regulate the integrin-mediated adhesion, and the PKCα and δ are associated with focal adhesions (Miranti et al. 1999). The essential role of FAK for migration has been shown with fibroblasts from FAK-null mice, which exhibit a decreased rate of cell motility (Sieg et al. 1999). FAK, one of src’s major binding partners, is activated by phosphorylation at Tyr397 in response to integrin clustering, which can be induced by cell adhesion. Phosphorylation of FAK Tyr397 creates a binding site for src family kinases, and phosphorylation of FAK Tyr576/ Tyr577 in the kinase domain activation loop enhances catalytic activity, which is a binding site for src family

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**Figure 3** Involvement of JAKs, Stat-3, src kinase, and PI3K in the leptin-induced migration of SW480 cells. Inhibition of JAKs was performed by treatment of the cells with 20 nM JAK inhibitor I (A), activity of Stat-3 was blocked using 200 μg/ml of specific Stat-3 inhibitor peptide (B), and the src kinase was inhibited using 50 nM of the specific inhibitor pp2 (C) or 100 nM of pp1 (D). The PI3K was inhibited by treatment of the cells with 5 μM of the specific inhibitor LY294002 (E). The graphs show mean values of three independent experiments (90 cells were analyzed per sample). None of the inhibitor concentrations reduced the viability of the cells as was assessed by propidium-iodide staining and flow cytometry. In (F), investigation of Stat-3 activation. The graph shows mean intensities and s.d. of the measured optical density (450 nm) of control cells (black), and cells treated with leptin in combination with various inhibitors for 30 min (white) of three independently performed experiments. We used Student’s t-test to calculate statistical significance; changes with a P value of P ≤ 0.05 are marked by an asterisk.
FAK regulates these contacts for the interaction of the cells with the surrounding development of focal adhesion contacts as a key structure in independent pathways are activated.

Experiments (120 cells were analyzed per sample). 577 (58%; Fig. 5 A) in comparison to untreated FAK at Tyr397 (33%) and even stronger at Tyr576/leptin treatment stimulated the phosphorylation of with leptin (Fig. 5 A). Furthermore, already 15 min expression of total FAK after stimulation of the cells Immunoblotting studies demonstrate an increased combination with JAK-inhibitor I (Fig. 5 A and B). We have shown before that inhibition of src kinase or JAKs resulted in an abrogation of the leptin-induced migration. Therefore, we have investigated, if the expression of FAK and its phosphorylation change by treatment of the cells with leptin alone or in combination with JAK-inhibitor I (Fig. 5A and B). Immunoblotting studies demonstrate an increased expression of total FAK after stimulation of the cells with leptin (Fig. 5A). Furthermore, already 15 min leptin treatment stimulated the phosphorylation of FAK at Tyr397 (33%) and even stronger at Tyr576/577 (58%; Fig. 5A) in comparison to untreated control cells. Treatment of the cells with the src kinase-specific inhibitor pp2 abrogated the enhanced phosphorylation of FAK, but not the protein expression (data not shown). To investigate whether a JAK-independent pathway in response to leptin exists, too, we monitored the leptin-induced phosphorylation of FAK Tyr397 in the presence or absence of JAK-inhibitor I. We could show that JAK inhibition did not significantly block the phosphorylation of FAK Tyr397 (Fig. 5B), thus demonstrating that two independent pathways are activated.

Established tumor cell migration models postulate the development of focal adhesion contacts as a key structure for the interaction of the cells with the surrounding matrix, in which FAK regulates these contacts dynamically. Therefore, we did an immunostaining of FAK in SW480 cells to determine its intracellular distribution together with filamentous actin (F-actin, Fig. 5C). After 2 h in 47% of the leptin-stimulated cells, demonstrating a migratory phenotype, we found FAK primarily in the periphery, and at certain matrix contact sites of the cells higher concentrations of FAK were co-localized with F-actin (white arrows; Fig. 5C). In contrast, untreated SW480 cells displayed a weak and diffuse distribution of FAK in the cytosol, and F-actin revealed a cortical localization (Fig. 5C, second row, third column).

Discussion

We have reported previously that SW480 colon carcinoma cells migrated spontaneously, immediately after incorporation of the cells within a three-dimensional collagen matrix (Masur et al. 2001a). Besides this migratory activity, another type of locomotion was inducible by physiological extracellular ligands such as the neurotransmitter norepinephrine. In obese individuals, serum levels of leptin are markedly increased, and a recent study indicates that leptin is overexpressed in human colorectal cancer, which suggests that the hormone might contribute to colorectal cancer development and progression (Koda et al. 2007). Therefore, we have
investigated herein the impact of leptin on the migration of SW480 colon carcinoma cells and the underlying regulatory machinery. Treatment of the cells with leptin resulted in a significant increase of the locomotor activity from 28% spontaneously migrating cells to 50%. This migration type is different from the spontaneous migration with regard to the src-PTK involvement (Fig. 3C and D). Whereas the spontaneous migration of SW480 colon carcinoma cells was independent of PTK activity, the leptin-induced type of migration involves PTKs, because using PTK-specific inhibitors completely abrogated leptin-mediated locomotion.

The herein investigated adipocytokine leptin binds to transmembrane receptors, which belong to the class I cytokine receptor family. Leptin predominantly activates the JAK/Stat signal transduction pathway (Fig. 6). The engagement of Ob-Rb by leptin leads to the JAK-2 phosphorylation, which can then recruit Stat-3 tyrosine phosphorylation, finally leading to a nuclear translocation and stimulation of transcription (Fruhbeck 2006). In addition, the leptin receptor is also

Figure 5 Activation and distribution of FAK in leptin-induced locomotion of SW480 cells. (A) Immunoblot analysis of the expression of total FAK and phosphorylated FAK in SW480 cells in a time-dependent manner. The representative histograms are the densitometric analysis of bands showing fold increase in levels of phosphorylated FAK on p397 and p576/577 with respect to total FAK. Columns represent mean densitometric values of band intensities (n=3). Changes with a P value ≤ 0.05 are marked by an asterisk. (B) Immunoblot analysis of phosphorylated FAK after incubation of SW480 cells with leptin alone or in combination with 20 nM JAK inhibitor I. The histogram represents the densitometric analysis of bands showing fold increase in levels of phosphorylated FAK on p397 in comparison to total FAK. Columns represent mean densitometric values of band intensities (n=3). Changes with a P value ≤ 0.05 are marked by an asterisk. (C) Intracellular distribution of FAK and filamentous actin in migrating SW480 colon carcinoma cells. The first row display a cell stimulated with leptin for 2 h, and in the second row, a control cell is displayed. The immunostaining of FAK is displayed in green, and the Alexa Fluor-phalloidin staining for actin is shown in red. In the third column, we show an overlay of FAK (green) and filamentous actin staining (red). The fourth column depicts the transmission light images showing the morphologies of the cells. The white bar in the lower left-hand corner represents the scale bar of 10 µm.
known to intracellularly activate MAPK pathway after leptin binding (Sweeney 2002). These results on the involvement of leptin in the regulation of transcription are partially in concurrence with our findings on tumor cells. We observed a significant activation of Stat-3 after incubation with leptin, whereas phosphorylation of c-Jun was not significant. In further accordance, treatment of the leptin-stimulated cells with a Stat-3 inhibitor peptide, resulted in a reduction of the migratory activity of SW480 cells, with a residual locomotor activity of 26%; which is Stat-3 independent (Fig. 3B). In this context, we could significantly reduce the leptin-mediated migration by incubation of SW480 cells with an inhibitor of JAKs, too (Fig. 3A). Moreover, in studies with human breast carcinoma cells, it was shown that Stat-3 can not only be activated via the classical JAK/Stat-pathway, but also by src kinases, because an inhibition of these tyrosine kinases was accompanied by a dose-dependent blocking of Stat-3 signaling (Garcia et al. 2001). Src-specific inhibitors such as pp2 significantly attenuated leptin-stimulated tyrosine phosphorylation of Stat-3 in both fibroblast and neurons (Jiang et al. 2008). We can approve this correlation, because incubation of the cells with leptin in combination with pp2, resulted in a significant decrease of Stat-3 activation (Fig. 3F). Thus, src kinases might be able to activate Stat-3, too, and this explains why in our experiments addition of pp2 significantly attenuated leptin-stimulated migration to the same extent as in migration experiments using a Stat-3-specific inhibitor peptide. Moreover, treatment with JAK-inhibitor I decreased the leptin-induced Stat-3 activation, too. Thus, leptin seems to mediate its pro-migratory effect via two independent signaling pathways. One is activated via the classical JAK–Stat pathway, and the second is transduced via src kinases, independent of JAK activation. This JAK-independent signaling is also reflected in the activation of FAK (Fig. 5B). Moreover, a possible complex forming of Stat-3 with FAK and an increase of FAK activation, which includes influences on cell migration, cannot be completely excluded (Silver et al. 2004). But in our experiments, activation of FAK is not dependent on complex forming of activated Stat-3, because inhibition of Stat-3 by JAK-inhibitor I does not have any influence on FAK phosphorylation (Fig. 5B).

A prerequisite for cell migration is the flexible change of the cells between adhesive and nonadhesive states as well as the cytoskeletal rearrangements, which are regulated by enzymatically active proteins that are present in these focal adhesions. The catalytic activity of tyrosine kinases like src and the FAK are required for these events (Playford & Schaller 2004). Activation of FAK by integrin clustering leads to autophosphorylation at Tyr397, which is a binding site for src family kinases and PI3K (Chen et al. 1996). Binding of src kinase in turn potentiates the activation of FAK through further phosphorylation of additional tyrosine residues (Calalb et al. 1995). Accordingly, in our study leptin treatment of the cells was found to be accompanied by an increase of total FAK expression, and a significant upregulation of FAK phosphorylation at Tyr397 as well as Tyr576/577. The phosphorylation of FAK at Tyr397 is independent of JAK activation as proven by specific JAK-inhibitor I. Consequently, FAK activation seems to be primarily mediated by src kinases, thus facilitating a subsequent activation of...
downstream target molecules such as PI3K and rac-1 (Fig. 6; Kallergi et al. 2007). Viewed in a more general way, the phosphorylation of FAK seems to be the decisive step for the leptin-induced locomotion of the cells, because phosphorylation of FAK as well as the migration of cells already occurs 15 min after addition of leptin. In contrast, an increase of total FAK expression is no more than 4 h after leptin treatment detectable, further supporting the phosphorylation of FAK as key prerequisite for cell migration. However, it would be possible that the time-dependent increase of total FAK expression performs the task to continuously allocate new substrate for future phosphorylation steps, thus enabling the tumor cell to keep up its migratory activity over a long period of time.

The PI3K is known to be a key molecule regulating cell migration (Bastian et al. 2006). Herein, treatment of SW480 cells with leptin resulted in an activation of PI3K, because the leptin-mediated increase of SW480 cell migration was abrogated by using LY294002, a specific inhibitor of the PI3K. This is consistent with previous findings demonstrating that the migratory activity of MDA-MB-468 breast carcinoma cells was impaired by the inhibition of PI3K (Bastian et al. 2006). Furthermore, PI3K products typically stimulate protein kinases such as Akt and PKC isoforms (Sweeney 2002). PKCs are known to be crucially involved in the regulation of tumor cell migration, e.g. by regulating proteins that interact with the actin cytoskeleton such as myristoylated, alanine-rich C-kinase substrate and gelsolin, but also by participating in focal adhesion formation (Miranti et al. 1999). In our own previous investigations, we could demonstrate that both the PKCα and the PKCδ are expressed in SW480 cells (Masur et al. 2001a). Herein, we present strong evidence for a specific involvement of the calcium-independent PKCδ isotype, and not the calcium-requiring PKCα, in the leptin-induced migration of SW480 cells. Whereas downregulation of the PKCδ by target-specific siRNA did not have any effect on the spontaneous locomotion of SW480 cells, it completely abolished the leptin-mediated locomotion of these cells (Fig. 4D). In contrast, knocking-down the expression of the PKCα did neither affect the spontaneous nor affect the leptin-induced migration. The exclusive involvement of the calcium-independent PKCδ in the leptin-induced migration is further supported by calcium measurements, which demonstrated no cytosolic calcium changes after stimulation of the cells with leptin (data not shown).

Leptin is a key molecule in obesity, which is predominantly produced by white adipose tissue, for regulation of energy expenditure and control of appetite. Epidemiologic data collected clearly demonstrate that obesity in adults is associated with an increased risk of cardiovascular disease, diabetes, and numerous other health disorders (Klein et al. 2002). Thus, we herein deliver molecular evidence for a functional link between obesity namely the increasing release of the adipocytokine leptin in vivo, and its pro-migratory and proliferation-promoting influence on tumor cells in vitro. This pro-migratory effect was intracellularly mediated by activation of transcription factors such as Stat-3 via JAK, and participation of various PTKs, including FAK, src kinase, PI3K, and the PKCδ (Fig. 6). By illuminating the signaling molecules mediating this leptin effect, our study represents a big step forward understanding the molecular mechanism of leptin, which thus delivers a starting point for the design of novel therapeutics for the treatment but also prevention of obesity-associated colon cancer.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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