Journal of Cell Science

Tyrosine phosphorylation of p145^{met} mediated by EGFR and Src is required for serum-independent survival of human bladder carcinoma cells

Natsumi Yamamoto[1], Gunay Mammadova[1], Robert X.-D. Song[2], Yasuo Fukami[1,3] and Ken-ichi Sato[3,*]

[1]Graduate School of Science and Technology, Kobe University, Nada, Kobe 657-8501, Japan
[2]Department of Internal Medicine, University of Virginia School of Medicine, Charlottesville, Virginia 22903, USA
[3]Laboratory of Molecular Biology, Research Center for Environmental Genomics, Kobe University, Nada, Kobe 657-8501, Japan
*Author for correspondence (e-mail: kksato@kobe-u.ac.jp)

Accepted 31 August 2006
Journal of Cell Science 119, 4623-4633 Published by The Company of Biologists 2006
doi:10.1242/jcs.03236

Summary

Here we address the molecular mechanism of serum-independent survival and growth of human bladder carcinoma cell line 5637. Serum starvation promoted tyrosine phosphorylation of a 145-kDa protein and activation of the tyrosine kinase Src and the receptor for epidermal growth factor (EGFR) over a slow time course (>8 hours). The phosphorylated 145-kDa protein was identified as the β-subunit of c-Met/hepatocyte growth factor (HGF) receptor, p145^{met}, in which tyrosine residues 1003, 1234, and 1235 were phosphorylated. Inhibitors of Src (PP2, SU6656) or EGFR (AG99), but not p145^{met} (K252a), effectively blocked tyrosine phosphorylation of p145^{met} and promoted cell death accompanied by activation of caspase-like proteases. Conditioned medium from the serum-starved 5637 cells or purified EGF readily promoted the activation of Src and EGFR, and tyrosine phosphorylation of p145^{met} in normally grown 5637 cells, suggesting that autocrine signaling of EGFR ligands is responsible for signal transduction events in serum-starved cells. Consistent with this idea, a monoclonal antibody against EGFR that would interfere with the ligand binding to EGFR blocked tyrosine phosphorylation events and promoted the caspase activation and cell death in serum-free conditions. Such apoptotic cell death was also induced by pretreatment of cells with a high concentration of HGF that downregulated endogenous p145^{met}. Nevertheless, Cu^{2+} ions, competitive inhibitors for HGF-binding to p145^{met}, did not show any effect on cellular functions in serum-free conditions. These results suggest that the serum-independent growth of 5637 cells involves the transmembrane signaling cascade via EGFR ligand(s) (but not HGF), EGFR, Src and p145^{met}.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/119/22/4623/DC1

Key words: EGFR, Src, Met, Cancer cells, Serum-independent growth, Signal transduction

Introduction

An ability to survive and proliferate under serum-free conditions is one of the well-known features of cancer cells in vitro (Deuel, 1987; Heldin and Westermark, 1989; Cross and Dexter, 1991). This abnormality might also be important for in vivo growth of cancer cells, especially at the early stage of carcinogenesis and invasion or metastasis, awaiting a regional angiogenesis responsible for the effective supply of energy resources for cancer cells. Thus, an understanding of the molecular nature of the serum-independent cancer cell growth is a prerequisite to improve the prognosis of cancer patients. Serum-independent function of cancer cells is often mediated by autocrine loop of survival and proliferative signals including growth factors and cytokines (Sporn and Roberts, 1985; Lang and Burgess, 1990). Because autocrine growth itself is not a cellular function peculiar to cancer cells, there should be a specific molecular mechanism explaining how cancer cells acquire the ability to drive the autocrine loop that allows cancer cells to survive under serum-depleted conditions. There are two major problems with this. First, how do cancer cells recognize or sense a 'serum-free' environment and translate that environmental signal into survival and a proliferative signal (e.g. autocrine of growth factors)? Second, how do survival and proliferative signals work for serum-independent function of cancer cells?

In this study, we address these problems by using the human bladder carcinoma cell line 5637 as a model system. It has been demonstrated that 5637 cells can undergo adaptation to serum-free conditions by autocrine growth stimulation (Ruck et al., 1994). It has also been shown that this autocrine growth involves epidermal growth factor (EGF) receptor/kinase (EGFR) and its ligands including EGF, transforming growth factor (TGF) α, amphiregulin and heparin-binding EGF (HB-EGF) (Ruck and Paulie, 1997; Ruck and Paulie, 1998). Function-blocking or neutralizing antibodies against EGFR or the EGFR ligands have been shown to inhibit growth of 5637 cells in serum-free conditions (Ruck and Paulie, 1997; Ruck and Paulie, 1998). As EGFR is a prototypical member of transmembrane receptor-type protein-tyrosine kinases (Carpenter, 1987), there is an assumption that protein tyrosine phosphorylation is involved in the autocrine-mediated survival and growth of

5637 cells under serum-free conditions. Therefore, we evaluated the occurrence as well as the importance of protein tyrosine phosphorylation in serum-starved 5637 cells, by performing phosphotyrosine-specific immunoblotting of cellular proteins and pharmacological application of tyrosine-kinase-specific inhibitors. We also evaluated the involvement of the Src family non-receptor protein tyrosine kinases (SFKs) (Brown and Cooper, 1996; Frame, 2002; Thomas and Brugge, 1997), because breakdown of normal cellular function in cancer cells often involves cooperated action of EGFR and SFKs (Maa et al., 1995; Biscardi et al., 2000; Bromann et al., 2004; Ishizawar and Parsons, 2004), both of whose deregulation and/or overexpression are often associated with poor clinical prognosis (Neal et al., 1990; Maa et al., 1995; Mellon et al., 1995; Biscardi et al., 2000; Irby and Yeatman, 2000; Summy and Gallick, 2006). We have identified a 145-kDa β-subunit of Met/hepatocyte growth factor (HGF) receptor ($p145^{met}$) (Bolanos-Garcia, 2005; Jiang et al., 2005) as a predominantly tyrosine-phosphorylated protein in serum-starved 5637 cells. Further analyses support the conclusion that the signaling cascade involving EGFR ligands (but not HGF, a ligand for $p145^{met}$), EGFR, SFKs and $p145^{met}$ is involved in suppression of pro-apoptotic action of caspase-like enzyme(s), and is thereby responsible for survival and growth of 5637 cells under serum-free conditions.

Results

We first examined whether adaptation of 5637 cells to the serum-free culture condition involved protein-tyrosine phosphorylation. To this end, 5637 cells were cultured in normal conditions (10% FCS) or serum-free conditions for several time points (1-48 hours). Growth rate of the serum-starved cells up to 48 hours of treatment was only slightly slower than that of normally cultured cells (see Fig. 1A, open circles versus closed circles), showing adaptation of this cell line to the serum-free conditions. Immunoblotting analysis of the whole cell extracts, as normalized by protein amounts (Fig. 1B, IB, tubulin), with anti-phosphotyrosine antibody demonstrated that remarkable tyrosine phosphorylation of a 145-kDa protein, designated pp145, became apparent at 8 hours and thereafter of serum starvation (Fig. 1B, IB: PY99), whereas the basal level of tyrosine phosphorylation of some proteins could be seen throughout the experimental conditions. Less evident but still significant elevation of tyrosine phosphorylation at 55-80 kDa was also seen in a similar time course of the pp145 appearance (Fig. 1B, IB: PY99), suggesting that Src family tyrosine kinases (SFKs), whose

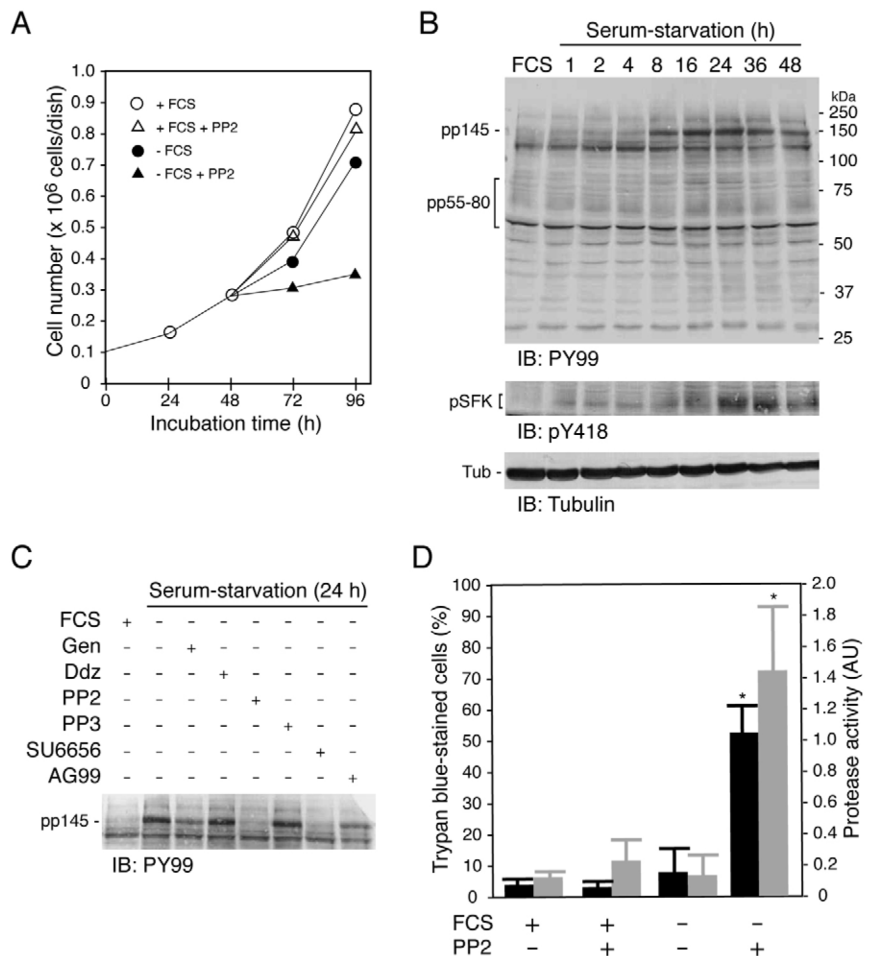


Fig. 1. Serum starvation promotes tyrosine phosphorylation of a 145 kDa protein in a Src- and EGFR-dependent manner in bladder carcinoma 5637 cells. (A) Human 5637 cells (1×10^6 cells/dish) were cultured in normal conditions (10% FCS) for 48 hours. After the treatment, cells were cultured for an additional 48 hours in normal conditions (○), 10% FCS plus 10 μM PP2 (△), without serum (●) or without serum plus 10 μM PP2 (▲). Cell number was determined at 24, 48, 72 and 96 hours post initial treatment. (B) Whole cell extracts (30 μg/lane) were prepared from carcinoma 5637 cells that had been cultured in normal (10% FCS, denoted FCS) or serum-free medium (for 1 to 48 hours), and analyzed by immunoblotting (IB) with an anti-phosphotyrosine antibody (PY99), an antibody against the activated and tyrosine-phosphorylated Src family protein-tyrosine kinases (pSFK) (pY418) or an anti-β tubulin antibody. In the top panel (IB: PY99), the positions of a 145 kDa tyrosine-phosphorylated protein (pp145) and molecular size markers (25-250 kDa) are indicated. (C) 5637 cells were cultured in normal (+ FCS) or serum-free (– FCS) medium for 24 hours. For the final 1 hour of the serum starvation treatment, cells were incubated in the absence or the presence of the following substances: 50 μM genistein (Gen), 50 μM daidzein (Ddz), 10 μM PP2, 10 μM PP3, 1 μM SU6656 or 5 μM AG99. Whole cell extracts (30 μg/lane) were analyzed by immunoblotting with anti-phosphotyrosine antibody. The position of pp145 is indicated. (D) Carcinoma 5637 cells were treated as in panel A. After the treatments (96 hours post treatment), cell death (black bars) and caspase 3/7 protease activity (grey bars) of the whole cell extracts (20 μg/assay) were determined by Trypan Blue exclusion and a synthetic substrate Ac-DEVD-AMC, respectively. Data shown are mean ± s.d. of three independent experiments. *$P<0.01$ compared with control.

molecular sizes are roughly in between 55 and 65 kDa, were activated and phosphorylated. In support of this idea, immunoblotting data with phospho-specific anti-SFK antibody, pY418, showed a time-dependent increase of the signal at 55-65 kDa (Fig. 1B, IB: pY418). Moreover, immunoprecipitation studies demonstrated that Src and Yes, but not Fyn, and EGFR were activated in response to serum starvation (Fig. 2).

We next examined the effect of some tyrosine kinase inhibitors. Human 5637 cells were serum-starved for 24 hours in the presence of inhibitors or their inactive analogs. Genistein, a global tyrosine kinase inhibitor and PP2 and SU6656, more potent SFK-specific inhibitors, were shown to inhibit the appearance of pp145 (Fig. 1C). However, daidzein and PP3, inactive analogs for genistein and PP2, respectively, had no effect (Fig. 1C). We confirmed that PP2 and SU6656 blocked the activation of Src in the serum-starved cells (supplementary material Fig. S1). AG99, a potent inhibitor of EGFR, but not its inactive analog AG9 (not shown), was also shown to inhibit the appearance of pp145 to some extent (Fig. 1C). Addition of 10% FCS to the 24 hour serum-starved cells resulted in a time-dependent disappearance of pp145, showing that the phosphorylation event is reversible (supplementary material Fig. S2).

We next evaluated the importance of SFKs and EGFR in the proliferation of 5637 cells. Although PP2 did not affect the time-dependent cell number in normal culture conditions, it reduced significantly the increase in cell number under the serum-free conditions (Fig. 1A), suggesting that SFK activity is required for serum-independent proliferation of 5637 cells. PP2 promoted a remarkable increase of cell death in serum-starved conditions (Fig. 1D, black bar). Protease activity toward Ac-DEVD-AMC (7-amino-4-methylcoumarin), a synthetic substrate of caspase 3/7, was also dramatically increased under the same condition (Fig. 1D, grey bar). PP2, when added from the beginning of serum starvation, induced caspase-like enzyme activity only after 8-12 hours of treatment (supplementary material Fig. S3A), but it could readily promote the caspase activation in the cells that had been serum-starved for 24 hours (within 30 minutes, supplementary material Fig. S3B). These results suggest that serum-starved cells will be primed to activate a caspase-like enzyme in the absence of SFK activity. Indirect fluorescent studies demonstrated that most of the serum-starved, PP2-treated cells were easily detached from the culture dishes and that most of the remaining, attached cells showed mitotic nuclear morphology (supplementary material Fig. S3C). AG99 showed an inhibitory effect on both normal as well as serum-starved culture conditions (data not shown). As EGFR activation was not seen in normal culture conditions (Fig. 2), we suggest that AG99 has a growth-inhibitory target(s) other than EGFR.

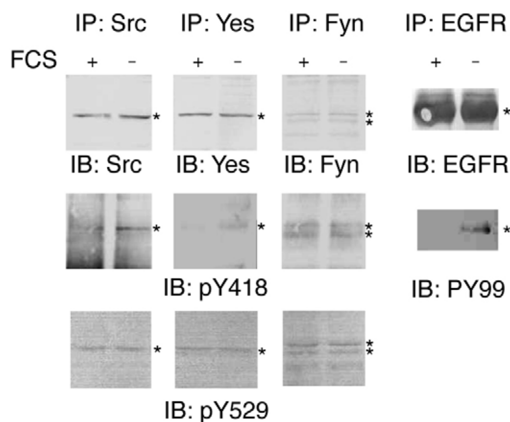


Fig. 2. Activation of SFKs and EGFR in serum-starved 5637 cells. Whole cell extracts (300 μg/lane) were prepared from carcinoma 5637 cells that had been cultured in normal (10% FCS, denoted '+') or serum-free conditions (for 24 hours, denoted '–') and subjected to immunoprecipitation (IP) with anti-Src antibody, anti-Yes antibody, anti-Fyn antibody or anti-EGFR antibody as described. The immunoprecipitates were analyzed by immunoblotting with the homologous antibody (Src, Yes, Fyn, or EGFR), anti-activated SFK antibody (pY418), an antibody that recognizes the inactive, carboxyl-terminal tyrosine-phosphorylated form of SFKs (pY529) and anti-phosphotyrosine antibody PY99. Asterisks indicate the positions of each protein.

Mass spectrometry analysis and database searches of pp145, which was immuno-affinity purified as a predominantly tyrosine-phosphorylated protein in serum-starved 5637 cells (Fig. 3A), demonstrated that it was the β-subunit of human c-Met/hepatocyte growth factor receptor ($p145^{met}$) (Fig. 3C). Of the four peptide fragments annotated to be parts of $p145^{met}$, one that corresponded to residues 988-1004 contained a phosphate group (Fig. 3B), suggesting that Y1003, a known phosphorylation site in $p145^{met}$, is phosphorylated (see Fig. 3C). To confirm that pp145 is identical to $p145^{met}$, and to examine whether Y1003 is actually phosphorylated in pp145, we performed immunoprecipitation studies with anti-Met antibody. As shown in Fig. 4A, 5637 cells express both precursor (180 kDa) and mature (145 kDa) forms of c-Met (left panels, see whole cell extract lanes). In addition, the anti-Met immunoprecipitates prepared from HGF-treated (50 ng/ml, 1 hour) or serum-starved (24 hours) 5637 cells, but not control cells, contained pp145, whose migration on SDS gels matched exactly with that of the band obtained with anti-Met antibody (Fig. 4A, left panels). Reciprocally, immunoprecipitation studies with PY99 demonstrated that pp145, which could be efficiently recovered from HGF-treated or serum-starved 5637 cells, but not control cells, contained a 145-kDa anti-Met immunoreactive protein (Fig. 4A, right panels). Therefore, we concluded that pp145 was tyrosine-phosphorylated $p145^{met}$. Immunoblotting analyses with phospho-specific anti-Met antibodies of the anti-Met immunoprecipitates confirmed that serum-starved 5637 cells involved the phosphorylation of Y1003 of $p145^{met}$ (Fig. 4B, –FCS). $pp145^{met}$ in serum-starved cells was also shown to contain tyrosine-phosphorylated Y1234/1235, which are located in the kinase domain (Fig. 4B, –FCS). However, $pp145^{met}$ in HGF-treated cells (50 ng/ml, 1 hour) was shown to be phosphorylated on not only Y1003 and Y1234/1235, but also the C-terminal Y1349 and Y1365 (Fig. 4B, +HGF).

The fact that pp145 is c-Met prompted us to examine whether the appearance of pp145 in serum-starved 5637 cells is due to HGF-dependent autophosphorylation of $p145^{met}$. As shown in Fig. 4C, exogenously added HGF promoted phosphorylation of $p145^{met}$ and p42/p44 mitogen-activated protein kinase (MAPK) in 5637 cells, and these events were effectively blocked by K252a, a potent c-Met kinase inhibitor. Under the same conditions, U0126, a MAPK kinase inhibitor,

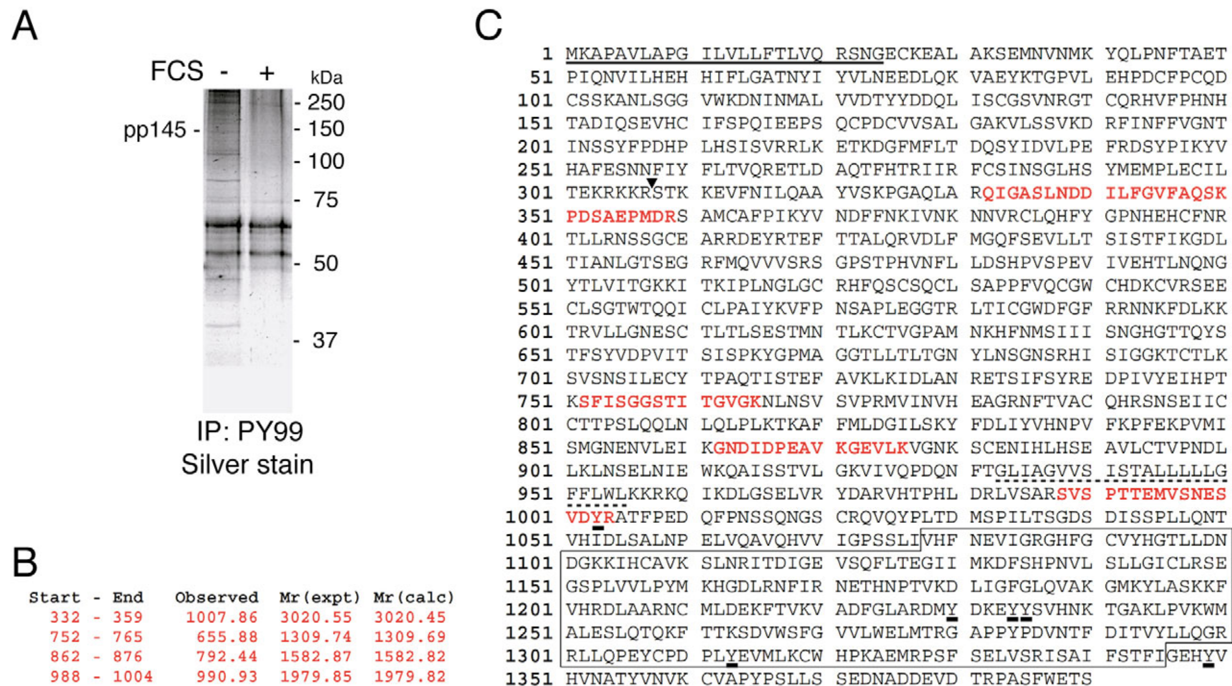


Fig. 3. Molecular identification of pp145 as β-subunit of c-Met protein. (A) Immunopurification of pp145. Whole cell extracts were prepared from carcinoma 5637 cells cultured in serum-starved conditions for 24 hours. The extracts (–FCS, 300 μg/lane) were subjected to immunoprecipitation with anti-phosphotyrosine antibody and the immunoprecipitates were analyzed by silver staining. A control immunoprecipitate prepared from the normally grown cell extracts (+ FCS, 300 μg/lane) was also analyzed. The position of a 145 kDa protein (pp145) is indicated. (B) MS identification of four peptide fragments annotated to be part of c-Met. Mass values of four peptide fragments were obtained from the 145 kDa tyrosine-phosphorylated protein digested with trypsin (see Materials and Methods for detail) and that were annotated to be part of the human $p145^{met}$ using the MASCOT database search algorithm. Also shown are amino acid numbers (start-end) and expected (expt) as well as calculated mass values (calc) for each peptide fragment. Note that a peptide fragment annotated to be the amino acid residues 988-1004 contained mass value equivalent to one phosphate. (C) Schematic structure of $p145^{met}$. The whole amino acid sequence (1390 amino acids) of the human $p145^{met}$ is shown. The amino acid sequences matching those of the known partial sequences by mass spectrometry analysis (see panel B) are shown in red. Also shown are the N-terminal signal sequence (residues 1-24, underlined), a C-terminal end of potential proteolytic cleavage site (residues 303-307, arrowhead), transmembrane region (residues 933-955, dashed line), catalytic domain (residues 1078-1345, squared), and tyrosine phosphorylation sites (residues 1003, 1230, 1234, 1235, 1313, 1349 and 1365, underlined in bold).

could block MAPK phosphorylation only (Fig. 4C, IB: pMAPK). On the other hand, K252a did not block phosphorylation of $p145^{met}$ in serum-free conditions (Fig. 4C). Serum starvation did not promote phosphorylation of p42/p44 MAPK (Fig. 4C, IB: pMAPK). As previously reported, prolonged treatment of cells with HGF promoted downregulation of $p145^{met}$ in a time-dependent manner (Fig. 4D, HGF). On the other hand, serum starvation did not show such an effect after up to 24 hours of treatment (Fig. 4D, –FCS). These results suggest that tyrosine phosphorylation of $p145^{met}$ in serum-starved cells is not due to HGF-dependent autophosphorylation of $p145^{met}$.

The aforementioned idea led us to analyze the biochemical properties of conditioned medium prepared from serum-starved 5637 cells. Conditioned media from the serum-starved 5637 cells readily promoted tyrosine phosphorylation of $p145^{met}$ in normally grown 5637 cells, as did exogenously added HGF (Fig. 5A, IB: PY99). Neither conditioned medium containing FCS nor fresh medium with or without FCS showed such an effect (Fig. 5A). The serum-free conditioned medium did not promote phosphorylation of p42/p44 MAPK in contrast with the case of HGF stimulation (Fig. 5A, IB: pMAPK), suggesting again a lack of the contribution of HGF in signaling events of serum-starved cells.

As noted in the Introduction, EGFR ligands are reported to be important for autocrine growth of serum-starved 5637 cells. So, we next examined whether exogenously added EGF could reconstitute signaling events seen in serum-starved cells. Purified EGF, when added to normally grown 5637 cells, promoted tyrosine phosphorylation of p170/EGFR and $p145^{met}$ (Fig. 5B,C). EGF also promoted activation of Src (Fig. 5B). A monoclonal anti-EGFR antibody mAb528, which would interfere with the binding of EGFR ligands to EGFR, inhibited all these events (Fig. 5B). We then used mAb528 to validate the importance of EGFR ligands in signaling events as well as growth in serum-starved 5637 cells. As shown in Fig. 5D, mAb528 effectively blocked tyrosine phosphorylation of $p145^{met}$, the molecular identity of which was determined in Fig. 4A, in serum-starved cells (– FCS data). mAb528 was also shown to decrease the growth rate (Fig. 5E) and promote cell death accompanied by caspase activation in serum-free conditions (Fig. 5F). The inhibitory effect of mAb528 was not seen in the case of the HGF (50 ng/ml, 1 hour)-induced tyrosine phosphorylation of $p145^{met}$ (Fig. 5D, +FCS/HGF). As

Journal of Cell Science

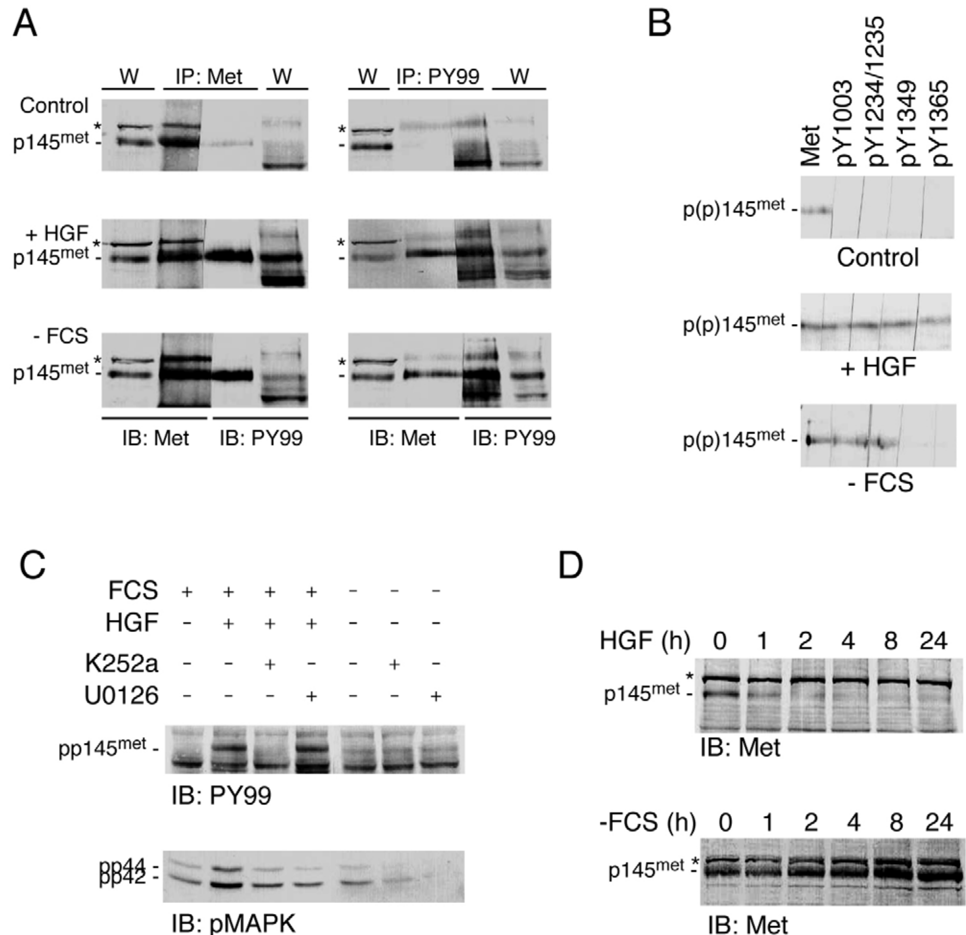


Fig. 4. Identification and characterization of p145^{met}, which is predominantly tyrosine phosphorylated in serum-starved 5637 cells. (A) Whole cell extracts were prepared from normally grown (control), HGF-treated (+ HGF, 125 ng/ml, 30 minutes), or serum-starved (– FCS, 24 hours) 5637 cells and immunoprecipitated (300 μg/lane) with either anti-Met antibody (IP: Met) or anti-phosphotyrosine antibody PY99 (IP: PY99). The immunoprecipitates were analyzed by immunoblotting with either anti-Met antibody (IB: Met) or anti-phosphotyrosine antibody PY99. The whole cell extracts (W, 30 μg/lane) were also analyzed by direct immunoblotting to serve as positive controls. Note that the position of p145^{met} matches exactly the position of the tyrosine-phosphorylated p145. Asterisks indicate the positions of p180^{met}, the precursor form of p145^{met}. (B) Whole cell extracts prepared as in A (control, + HGF, and – FCS: each 600 μg/analysis) were immunoprecipitated with anti-Met antibody and analyzed by immunoblotting with anti-Met antibody or phospho-specific anti-Met antibodies: pY1003, pY1234/1235, pY1349 or pY1365. The positions of the unphosphorylated or phosphorylated p145 [p(p)145^{met}] are indicated. (C) Carcinoma 5637 cells were cultured in normal (10% FCS, 1 hour) or serum-free condition (24 hours) in the absence or the presence of HGF (50 μg/ml), K252a (10 μM) and U0126 (10 μM). After treatment, cells were extracted and examined for phosphorylation of p145^{met} (IB: PY99) and p42/p44 MAPK (IB: pMAPK) (30 μg/lane). (D) Carcinoma 5637 cells were treated with either HGF (250 ng/ml) or serum-free medium (–FCS) for the indicated times. Whole cell extracts (30 μg/lane) were prepared and analyzed by immunoblotting with anti-Met antibody. The positions of p180^{met} (asterisk) and p145^{met} are indicated.

previously reported (Wright et al., 2004), Cu^{2+} ions could block the HGF-induced tyrosine phosphorylation of p145^{met} (Fig. 5D). However, they had no effect on the phosphorylation of p145^{met} (Fig. 5D, –FCS), caspase activation or cell viability in serum-free conditions (supplementary material Fig. S4). These results support the idea that EGFR ligands, but not HGF, are responsible for tyrosine phosphorylation of p145^{met} and survival/growth of 5637 cells in serum-free conditions.

We next examined the effect of removal of p145^{met} on serum-starved 5637 cells. Pretreatment of 5637 cells with a high concentration of HGF (250 ng/ml) for 24 hours resulted in a complete loss of endogenous p145^{met} (Fig. 6A, IB: Met), whereas the expression level of the precursor Met (p180) remained a little higher than the control condition (Fig. 6A, IB: Met, shown by an asterisk). Under these conditions, serum starvation did not promote tyrosine phosphorylation of p145^{met} (Fig. 6A, IB: PY99). However, the same treatment did induce Src activation (Fig. 6A, IB: pY418). The results indicate that Src activation can occur independently of p145^{met}. Neither tyrosine phosphorylation of p145^{met} nor Src activation occurred in FCS-treated cells (Fig. 6A). Cell proliferation assays demonstrated that p145^{met}-depleted, serum-starved cells became inactive as in PP2-treated condition (Fig. 6B, +FCS/HGF, –FCS). A similar growth rate to control cells was observed in p145^{met}-depleted, FCS-treated cells (data not shown). Also, inclusion of $CuCl_2$ during HGF treatment, which cancels p145^{met} depletion (supplementary material Fig. S4A), supported active proliferation of serum-starved cells (Fig. 6B, +FCS/HGF/$CuCl_2$, –FCS). Data obtained by Trypan Blue staining and caspase assays were consistent with the idea that p145^{met}-depleted, serum-staved cells underwent apoptotic cell death (Fig. 6C).

Discussion

Malignant cell transformation is often associated with a cooperative action of receptor and non-receptor protein tyrosine kinases in the vicinity of plasma membranes. Therefore, a number of studies have been devoted to corroborate the functional relationship between transmembrane signal transduction pathways and cellular functions in cancer cells. Previously, human bladder carcinoma 5637 cells have been shown to undergo adaptation to serum-free culture conditions

(Ruck et al., 1994). It has also been demonstrated that the adaptation is mediated by autocrine mechanism involving all four known EGFR ligands (EGF, TGFα, amphiregulin, and HB-EGF) and EGFR (Ruck and Paulie, 1997; Ruck and Paulie, 1998). However, further detail of the signal transduction pathways has not yet been reported. Our present study extended those studies and shed light for the first time on the crucial role of tyrosine kinase signaling in serum-independent growth of 5637 cells (Fig. 7). Our results demonstrated that: (1) EGFR and SFKs (Src and Yes) are activated in response to serum-starvation; (2) the β-subunit of c-Met/HGF receptor p145^{met} is predominantly tyrosine phosphorylated in the serum-starved cells; (3) abrogation of EGFR/SFK activation or the tyrosine phosphorylation of p145^{met} promotes cell death accompanied

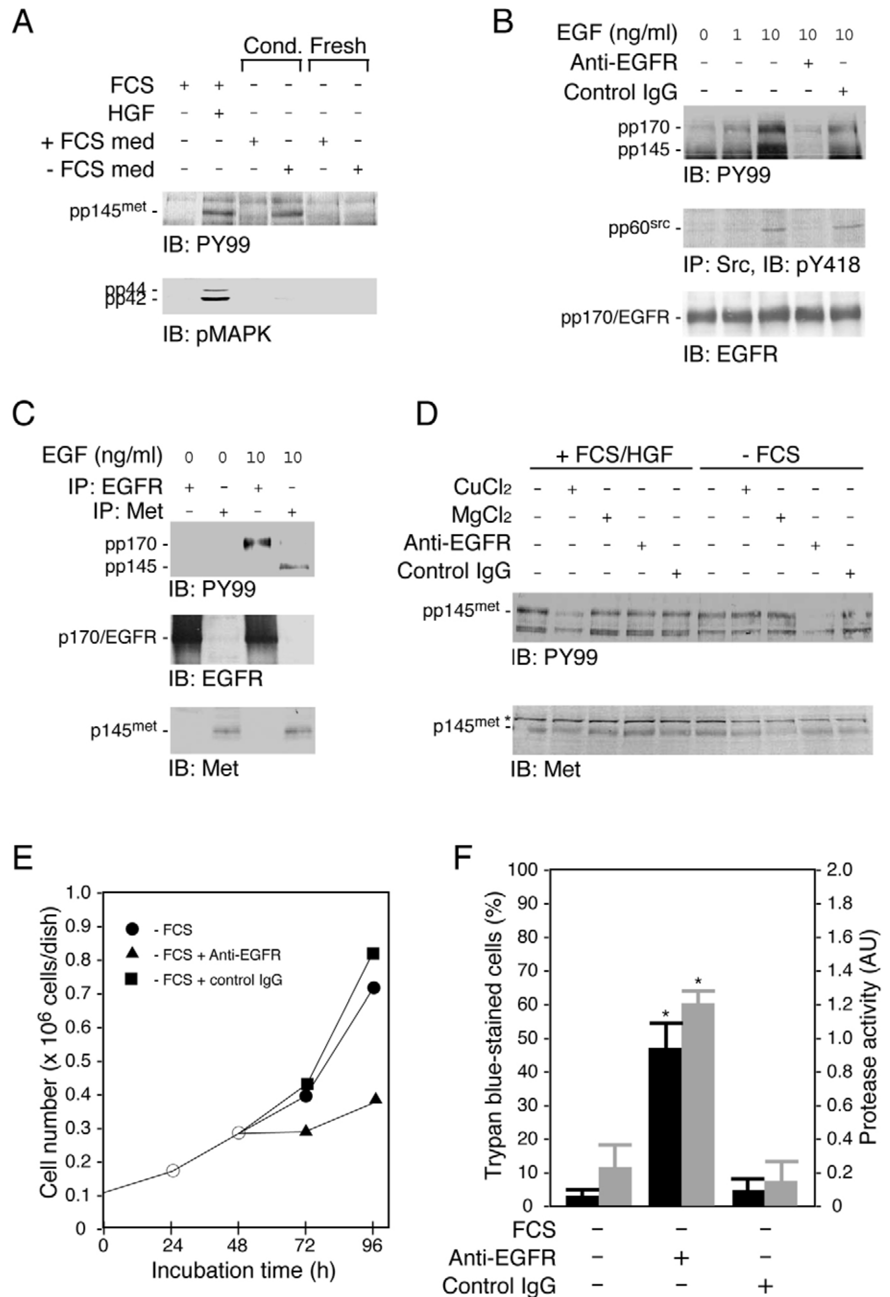


Fig. 5. EGFR ligands and EGFR act as an upstream regulator of tyrosine phosphorylation of p145^{met}, activation of Src and survival in serum-starved 5637 cells. (A) Normally grown 5637 cells (+ FCS) were treated with 125 ng/ml HGF (+ FCS/HGF), conditioned RPMI1640 media from 24-hour normally grown 5637 cells (Cond./+ FCS med), conditioned RPMI1640 media from 24-hour serum-starved 5637 cells (Cond./– FCS med), or fresh RPMI1640 medium containing either 10% FCS (Fresh/+ FCS med) or no FCS (Fresh/– FCS med) for 1 hour. After treatment, whole cell extracts (20 μg/lane) were prepared and analyzed by immunoblotting with either anti-phosphotyrosine antibody PY99 or anti-phosphoMAPK antibody. The positions of pp145^{met}, pp44, and pp42 are indicated. (B) Normally grown 5637 cells were treated with the indicated concentrations (0, 1 or 10 ng/ml) of EGF for 10 minutes in the absence or presence of 1 μg/ml anti-EGFR monoclonal antibody mAb528 or 1 μg/ml normal mouse IgG. After treatment, whole cell extracts were prepared and analyzed by immunoprecipitation (200 μg/lane) and/or immunoblotting (20 μg/lane for direct analysis) with the indicated antibodies. The positions of pp170, pp145, pp60^{src} and p170/EGFR are indicated. (C) Whole cell extracts were prepared from 5637 cells that had been treated with or without 10 ng/ml EGF for 10 minutes as in panel B, and subjected to immunoprecipitation (200 μg/lane) with either anti-EGFR or anti-Met antibodies followed by immunoblotting with either anti-phosphotyrosine antibody PY99, anti-EGFR or anti-Met antibodies. The positions of pp170, pp145, p170/EGFR and p145^{met} are indicated. (D) Carcinoma 5637 cells were treated with either normal medium (10% FCS) containing 125 ng/ml HGF for 1 hour or serum-free medium for 24 hours in the absence or the presence of 1.25 mM $CuCl_2$, 1.25 mM $MgCl_2$, 1 μg/ml anti-EGFR monoclonal antibody mAb528 or 1 μg/ml normal mouse IgG. After the treatments, whole cell extracts (20 μg/lane) were prepared and analyzed by immunoblotting with either anti-phosphotyrosine antibody PY99 or anti-Met antibody. The positions of pp145^{met}, p145^{met} and p180^{met} (asterisk) are indicated. (E) Carcinoma 5637 cells (1×10^6 cells/dish) were cultured in normal conditions (10% FCS) for 48 hours, and then further cultured for an additional 48 hours in serum-free medium containing none (closed circles), 1 μg/ml anti-EGFR monoclonal antibody mAb528 (closed triangles) or 1 μg/ml normal mouse IgG (closed squares). Cell number was determined at 24, 48, 72 and 96 hour post initial treatment as in Fig. 1A. (F) Carcinoma 5637 cells were treated as in E. After treatment (96 hours post treatment), cell death (black bar) and caspase 3/7 protease activity (grey bar) of the whole cell extracts (20 μg/assay) were determined as in Fig. 1D. Data shown are mean ± s.d. of three independent experiments. *P<0.01 compared with levels in the control.

Journal of Cell Science

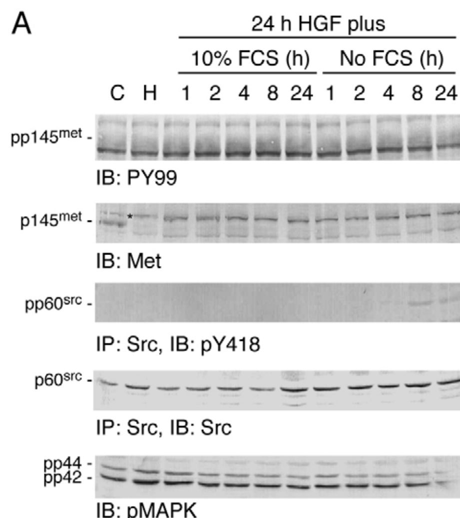
Journal of Cell Science

by activation of caspase-like proteases; (4) conditioned medium from the serum-starved 5637 cells or purified EGF, when applied to normally grown 5637 cells, readily promotes EGFR/SFK activation and tyrosine phosphorylation of p145^{met}; (5) abrogation of EGFR ligands, but not HGF, also promote cell death.

A relatively slow time course of tyrosine phosphorylation induced by serum starvation (>8 hours, see Fig. 1A) suggests that production of a trigger for tyrosine kinase signaling, i.e. autocrine of EGFR ligands (see below), requires some time to be stimulated. Results obtained with use of conditioned media (Fig. 5A), purified EGF (Fig. 5B,C), and a neutralizing anti-EGFR antibody (Fig. 5B,D-F) support the idea that EGFR ligands are primal mediators for tyrosine kinase signaling and survival in 5637 cells (Fig. 7). There are two major mechanisms for the upregulation of EGFR ligands: one is a genomic, transcriptional upregulation of the ligand expression (Carpenter, 1987; Deuel, 1987; Heldin and Westermark, 1989); the other is a non-genomic, cell surface protease-mediated release of transmembrane EGFR ligands (Suzuki et al., 1997; Prenzel et al., 1999; Fischer et al., 2003; Miyamoto et al., 2006). This second mechanism is particularly intriguing, because application of several protease inhibitors effectively blocked the signal transduction events as well as growth in serum-starved 5637 cells (our unpublished observations). Among known transmembrane EGFR ligands, HB-EGF is overexpressed in 5637 cells (Thogersen et al., 2001). Also, amphiregulin is released upon application of lysophosphatidic acid to 5637 cells (Schafer et al., 2004). Some reports have shown that SFKs can activate metalloproteinase, which can be responsible for ectodomain shedding of the transmembrane EGFR ligands, through its enhanced gene expression or tyrosine-phosphorylation-mediated enzymatic upregulation (Kadono et al., 1998; Guerrero et al., 2004; Zhang et al., 2004; Neel et al., 2005; Luo et al., 2006).

Immunoprecipitation studies demonstrated that EGFR and two SFKs – Src and Yes – were activated in serum-starved cells (Fig. 2). Importantly, specific inhibitors of EGFR or SFK were shown to block tyrosine phosphorylation of p145^{met} (Fig. 1C), which was shown to be a crucial event for serum-independent growth of 5637 cells in this study (see below). Accordingly, PP2-treated 5637 cells did not proliferate and underwent cell death accompanied by activation of caspase-like proteases (Fig. 1A,D). Such an effect of PP2 was not seen in normally grown 5637 cells. These results clearly demonstrate that SFK activity is specifically required for serum-independent growth of 5637 cells. On the other hand, the EGFR inhibitor AG99 showed a growth-inhibitory effect on not only serum-starved cells but also normally grown cells. It is suggested, therefore, that the importance of EGFR is not limited to serum-independent growth and/or the EGFR inhibitor has an alternative target to exert its growth-inhibitory effect in normally grown cells.

One question arises as to how SFKs are activated in serum-starved 5637 cells. The fact that exogenously added EGF could promote Src activation (Fig. 5B) supports the idea that EGFR is directly involved in the SFK activation. In any case, the SFK activation seems to be independent of dephosphorylation of the



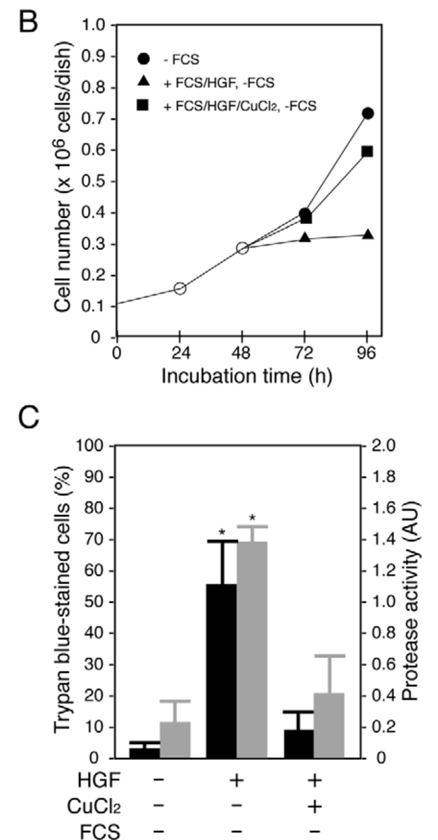


Fig. 6. p145^{met} is required for survival of serum-starved 5637 cells: effect of HGF-induced down-regulation of p145^{met}. (A) Normally grown 5637 cells (C, control) were treated with 10% FCS plus 125 ng/ml HGF for 24 hours (H, HGF-treated). After treatment, the cells were further treated for the indicated times (1-24 hours) in new medium containing 10% FCS plus 125 ng/ml HGF (10% FCS) or 125 ng/ml HGF alone (no FCS). After treatment, whole cell extracts were prepared and analyzed by immunoprecipitation and/or immunoblotting as in Fig. 1B, Fig. 4C and Fig. 5B. The positions of pp145^{met}, p145^{met}, pp60^{src}, p60^{src}, pp44 and pp42 are indicated. (B) Carcinoma 5637 cells (1×10^6 cells/dish) were cultured in normal conditions (10% FCS) for 24 hours, and then cultured for 24 hours in 10% FCS plus 125 ng/ml HGF (▲) or 10% FCS plus 125 ng/ml HGF plus 1.25 mM $CuCl_2$ (■). After treatment, cells were treated for an additional 48 hours in serum-free media. Cell number was determined at 24, 48, 72 and 96 hours post initial treatment as in Fig. 1A. As a control, data obtained with normally grown cells (10% FCS, 48 hours) and serum-starved cells (– FCS, additional 48 hours) are shown (●). (C) Carcinoma 5637 cells were treated as in panel B. After treatment (96 hours post treatment), cell death (black bars) and caspase 3/7 protease activity (grey bars) of the whole cell extracts (20 μg/assay) were determined as in Fig. 1D. Data shown are representative of three independent experiments. Results are the mean ± s.d. of three independent experiments. *P<0.01 compared with levels in the control.

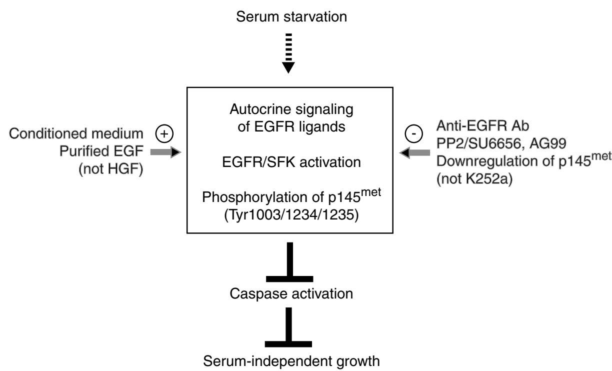


Fig. 7. Schematic model for signal transduction pathways in serum-starved 5637 cells. In carcinoma 5637 cells, serum starvation promotes signaling events involving EGFR ligands, EGFR, SFKs (e.g. Src) and p145^{met} to suppress activation of caspase-like proteases. Serum-starved cells can therefore survive and proliferate. In this study, several materials are used to validate this scheme: positive regulators such as conditioned medium and purified EGF, but not HGF; negative regulators such as a neutralizing anti-EGFR antibody (mAb528), PP2, SU6656, AG99 and downregulation of p145^{met}, but not K252a. See text for details.

C-terminal tyrosine residue (Y529) (Fig. 2). Such a dephosphorylation-independent activation of SFK may involve protein-protein interaction mediated by functional domains of SFKs, e.g. Src homology 3 and 2 domains, and a part of the kinase domain (for a review, see Sato et al., 2004). In particular, EGF-dependent Src activation has been shown to involve Shc, a Src-homology-2-containing adaptor protein (Sato et al., 2000; Sato et al., 2002; Sato et al., 2003). Shc can also be important for anti-apoptotic events under the control of p145^{met} (Saucier et al., 2004). Activated SFKs may, in turn, activate EGFR in an EGF-independent manner through the phosphorylation of Y845 in the EGFR (Sato et al., 1995; Biscardi et al., 1999; Luo et al., 2006). Further study will address this bidirectional signal transduction between SFKs and EGFR in serum-starved 5637 cells.

In this study, we identified p145^{met} as a major tyrosine-phosphorylated protein in serum-starved 5637 cells. Several reports have demonstrated that p145^{met} and its ligand HGF can act as anti-apoptotic proteins and as enhancers for the malignancy of cancer cells (Ponzetto et al., 1994; Rahimi et al., 1996, Rahimi et al., 1998; Qiao et al., 2000; Hung and Elliott, 2001; Xiao et al., 2001; Han et al., 2006; Wojcik et al., 2006). As the tyrosine phosphorylation of p145^{met} required EGFR ligands (Fig. 5D), SFK and EGFR (Fig. 1C), but not HGF (Fig. 5D), we suggest that the phosphorylation is due to trans-phosphorylation by SFKs/EGFR, but not to the autophosphorylation of p145^{met}. This is also supported by the fact that, K252a, an inhibitor for HGF-dependent kinase activity of p145^{met} (Morotti et al., 2002), did not block serum-starvation-induced phosphorylation of p145^{met} (Fig. 4C). Src-dependent phosphorylation and/or modulation of p145^{met} has been documented (Rahimi et al., 1998; Cutrupi et al., 2000; Chan et al., 2003; Maejima et al., 2003; Popsueva et al., 2003; Sridhar et al., 2005), although there was no clear demonstration of the phosphorylation site(s).

The phosphorylation pattern of p145^{met} in serum-starved 5637 cells was different from that in HGF-treated cells (Fig. 4B). Namely, Tyr1003 and Tyr1234/1235, but not other autophosphorylation sites: Tyr1349 and Tyr1365, were found to be phosphorylated in serum-starved cells. It has been reported that Tyr1003, which is located in the juxtamembrane Asp1002-Tyr1003-Arg1004 motif of p145^{met}, when phosphorylated, forms an atypical binding site for c-Cbl that induces ubiquitylation and degradation of p145^{met} (Jeffers et al., 1997; Hammond et al., 2001; Peschard et al., 2001; Peschard and Park, 2003; Peschard et al., 2004; Abella et al., 2005). Such downregulation scheme of p145^{met} was actually working in HGF-treated 5637 cells (Fig. 4D). However, Tyr1003-phosphorylated p145^{met} did not show downregulation in serum-starved cells (Fig. 4D). The results suggest that phosphorylation of Tyr1003 is not sufficient for downregulation of p145^{met} in 5637 cells and that there is an unknown function of this phosphorylation event. Other phosphorylation sites, Tyr1234 and Tyr1235 reside in the activation segment of the kinase domain of p145^{met}. Phosphorylation in the activation segment is believed to be important for enzymatic activation of p145^{met} (Wang et al., 2006) and of other protein kinases in general (Fukami et al., 1999). As discussed above, however, it seems that serum-starvation-induced tyrosine phosphorylation of p145^{met} is not accompanied by enzymatic activation. Further study is necessary to understand the functional importance of these phosphorylation events.

Abrogation of EGFR ligands, EGFR, SFKs or p145^{met} in serum-starved 5637 cells always resulted in cell death accompanied by activation of caspase-like proteases. We suggest that these signaling molecules are involved in the prevention of caspase activation and cell death (Fig. 7). In this study, we evaluated the activation of proteolytic activity toward a synthetic DEVD substrate, which is preferentially targeted by ‘executioner caspases’ such as caspase 3 and 7 (Stennicke and Salvesen, 2000). To be activated, these ‘executioner caspases’ require upstream ‘initiator caspases’ such as caspase 8 and 9. Reportedly, caspase 9 is activated by the action of cytochrome c oxidase, which is released from mitochondria upon intrinsic pro-apoptotic signaling (Stennicke and Salvesen, 2000). Until now, however, no report is available for the involvement of tyrosine phosphorylation in the regulation of mitochondria-mediated pro-apoptotic event. Caspase 8 is regulated by an extrinsic pro-apoptotic signalling, such as that in Fas-ligand-mediated apoptosis triggered by the transmembrane receptor (Stennicke and Salvesen, 2000). It is interesting to note that caspase 8 is reported to be tyrosine phosphorylated by SFKs, and thereby maintained in the inactive state without proteolytic processing (Cursi et al., 2006).

The serum-independent ability to survive and proliferate has been recognized as one of the abnormal characteristics of cancer cells. To date, several biochemical and cell biological events have been documented to understand the molecular insight of such cancer-cell-specific biological function (Epstein et al., 1990; Ruck et al., 1994). Our present study demonstrates for the first time that serum-independent growth of bladder carcinoma cells is accompanied by upregulation of EGFR and SFKs. In particular, Src activity is specifically required for serum-independent cellular function. We identified p145^{met} as an EGFR and SFK substrate, which acts to prevent caspase

activation. Future studies will aim to determine the physiological relevance of the molecular circuit involving autocrine EGFR ligands, EGFR, SFKs and p145^{met} in several malignant stages of bladder carcinoma cells. In addition, comparative studies with cancer cells from different types of tissues and organs should be performed to know general importance of serum-starvation-induced signal transduction. For example, a recent report by Murillo et al. (Murillo et al., 2005) has shown that TGFβ1-induced survival of hepatocytes require EGFR and Src, and Griffiths et al. (Griffiths et al., 2004) have shown that kinase-inactive mutants of Src increase Fas-induced apoptosis in colon cancer cells. We should learn more from these experimental models about the physiological relevance of cancer cell growth, by which we might establish a more effective strategy to target cancer cells in vivo.

Materials and Methods

Reagents and antibodies

Human recombinant HGF and mouse submaxillary gland EGF were obtained from Calbiochem (San Diego, CA) and Wako Pure Chemicals (Osaka, Japan), respectively. An anti-phosphotyrosine mouse monoclonal antibody PY99 and a rabbit anti-Met/HGF receptor antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Phospho-specific polyclonal anti-Met antibodies: pY1003 and pY1365 were from Abcam (Cambridge, UK); pY1234/1235 and pY1349 were from Cell Signaling (Beverly, MA). Mouse anti-Src antibody mAb327 was obtained from Oncogene Research Products (San Diego, CA). Phospho-specific rabbit anti-active and inactive SFK antibodies, pY418 and pY529, were obtained from Biosource International (Camarillo, CA). A mouse monoclonal antibody against Yes and a rabbit polyclonal antibody against Fyn were purchased from Upstate Biotechnology (Lake Placid, NY) and Santa Cruz Biotechnology, respectively. A rabbit polyclonal anti-EGF receptor (EGFR) antibody, which was raised against a synthetic peptide that corresponds to residues 1155-1175 of the human EGFR, was prepared as previously described (Sato et al., 1995). An inhibitory monoclonal anti-EGFR antibody mAb528 and a rabbit polyclonal antibody against phosphorylated, active MAPK, were purchased from Cell Signaling. Mouse monoclonal antibody against β-tubulin was from BD Biosciences (Tokyo, Japan). Protein-tyrosine kinase inhibitors and inactive analogs: PP2 (4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine), PP3 (4-amino-7-phenylpyrazol[3,4-d]pyrimidine), genistein, daidzein, SU6656, AG99 (α-cyano-(3,4-dihydroxy)cinnamide) and AG9 [α-cyano-(4-methoxy)cinnamonnitrile] were obtained from Calbiochem. Protease inhibitors leupeptin and (*p*-amidino-phenyl)methanesulphonyl fluoride hydrochloride (APMSF) were purchased from the Peptide Institute (Osaka, Japan) and Wako Pure Chemicals, respectively. $CuCl_2 \cdot 2H_2O$ and $MgCl_2 \cdot H_2O$ were from Nacalai Tesque (Kyoto, Japan). A synthetic substrate for caspase 3/7, Ac-Asp-Glu-Val-Asp-AMC (Ac-DEVD-AMC), was obtained from Calbiochem. Propidium iodide was from Sigma-Aldrich (St Louis, MO). Protein-A-Sepharose was obtained from Amersham Biosciences (Uppsala, Sweden). Other chemicals were analytical grade and purchased from Sigma, Wako Pure Chemicals or Nacalai Tesque.

Cell maintenance, culture conditions and viability assays

Human bladder carcinoma cell line 5637 was obtained from American Type Culture Collection (Rockville, MD) and maintained in RPMI1640 medium supplemented with 10% fetal calf serum (FCS), 1 mM L-glutamine, and antibiotics. Cells were grown at 37°C in a humidified 5% CO_2 atmosphere. For all experiments, confluent cells were trypsinized, and aliquots of single-cell suspension were re-seeded at 0.5×10^6 cells/dish (100-mm diameter) or 0.1×10^6 cells/dish (60-mm diameter) and cultured in poly-L-lysine-coated new culture dishes containing normal culture media for 24-48 hours. After the normal growth treatment, cells were treated under several different conditions as specified in the text. When cell number and cell viability were determined, both attached cells and unattached cells were collected by trypsinization and sedimentation of the culture media, respectively, and combined in one tube. The cells were washed twice with phosphate-buffered saline (PBS) and aliquots of the cells were stained with Trypan Blue solution. After the staining, cells were mounted in a hemocytometer for microscopic observation. Total cell number was determined by counting more than 300 cells per condition. The percentage of Trypan-Blue-stained cells (versus total cell number) was determined by calculation of data obtained with three independent counting of more than 100 cells. When nuclear morphology was evaluated, cells were grown and treated in glass-bottom dishes (MatTek Corporation, Ashland, MA). After treatment, the cells were washed three times with PBS, fixed with 4% paraformaldehyde in PBS for 10 minutes, permeabilized with 0.2% Triton X-100 in PBS for 2 minutes and blocked with 3% BSA in PBS for 1 hour. Cells were then treated with propidium iodide at 2 μg/ml in PBS for 1 hour. After washing with PBS (three times 5 minutes), samples were mounted in PBS containing 50 mg/ml 1,4-diazabicyclo[2,2,2]octane and 90% glycerol and subjected to confocal microscopic observation (FMV-300 model, Olympus, Tokyo, Japan). The fluorescence was detected at 543 nm argon excitation using optics of a 590 nm long-pass barrier filter. The fluorescent images were recorded and reconstituted with the use of software Adobe Photoshop CS (Adobe Systems, Seattle, WA).

Cell extraction, immunoprecipitation and immunoblotting

Whole cell extracts were prepared by extracting cell samples with buffer containing 1% Triton X-100, 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM β-mercaptoethanol, 1 mM sodium orthovanadate, 10 μg/ml leupeptin, and 20 μM APMSF. Specified amounts of proteins, as determined by the dye-binding assay using a Bio-Rad Protein Assay Mixture (Bio-Rad Laboratories, Hercules, CA), were analyzed for the expression level of proteins of interest, protein phosphorylation and molecular interaction by immunoprecipitation and/or immunoblotting as essentially described previously (Sato et al., 2002). Specifically in this study, separation of the cellular proteins was done with use of 8% or 10% SDS-polyacrylamide gel electrophoresis according the method of Laemmli (Laemmli, 1970). Immunoprecipitation was done at 4°C overnight by using 1-5 μg mouse or rabbit IgG against 200-1000 μg protein per assay, followed by collection of the immune complexes with protein-A-Sepharose. In immunoblotting analysis, detection of the immune complex was done by enzyme-linked color development with biotin-conjugated secondary antibodies against mouse or rabbit IgG (Vecstatin) and an avidin-conjugated horseradish peroxidase (Vecstatin ABC kit, Vector Laboratories, Burlingame, CA). In all immunoblotting experiments, normalized application of cellular proteins was confirmed by anti-tubulin immunoblotting data.

Caspase assay

A reaction mixture (200 μl) containing 10-20 μg cellular proteins and 20 μM Ac-DEVD-AMC in a buffer consisting of 20 mM HEPES, pH 7.5, 10% glycerol and 2 mM dithiothreitol, was incubated for 1 h in the dark at 37°C. To minimize non-specific effect of the extraction buffer, volume of the cellular proteins was set at 10% of the total volume of the reaction mixture. After the reaction, fluorescence was measured in a Micro-cuvette Fluorescence Reader (VersaFluor, Bio-Rad) with an excitation wavelength of 380 nm and an emission wavelength of 440 nm. Fluorescence units obtained were subtracted with that obtained with a control sample (extraction buffer containing 10-20 μg bovine serum albumin alone) and plotted as shown in figures.

Silver stain, in-gel protein digestion and mass spectrometry

Tyrosine-phosphorylated 145 kDa protein, designated pp145, was concentrated by immunoprecipitation of the whole cell extracts of serum-starved 5637 cells and separated by SDS-polyacrylamide gel electrophoresis. Proteins were visualized by using the Bio-Rad Silver Stain Plus kit. In-gel digestion of the pp145-containing SDS gels with trypsin, liquid chromatography mass spectrometry and database search were done by Apro Science (Tokushima, Japan).

Transfer of conditioned media

To transfer conditioned media to other cell dishes, 5637 cells were normally grown for 48 hours post re-seeding and then treated in serum-free medium for additional 24 hours. After the treatments, the culture media (i.e. conditioned media) were taken, centrifuged for 10 minutes to remove cells and debris, and immediately added to normally grown 5637 cells (48 hours post re-seeding). As a control transfer experiment, the culture medium taken from the normally grown 5637 cells (48 hours post re-seeding) were also used. Medium was removed after 1 hour for immunoblotting analysis or 48 hours for determination of cell number, as described above.

We are grateful to members of the Laboratory of Molecular Biology, Research Center for Environmental Genomics, Kobe University for critical comments and helpful discussion. Special thanks are also due to Misato Yoshikawa for secretarial assistance. This work was partly supported by grants (16022243, 16026244, 16570174) from the Ministry of Education, Culture, Sports, Science, and Technology (MEXT), Japan, and a grant from the Sumitomo Foundation to K.S.

References

Abella, J. V., Peschard, P., Naujokas, M. A., Lin, T., Saucier, C., Urbe, S. and Park, M. (2005). Met/Hepatocyte growth factor receptor ubiquitination suppresses transformation and is required for Hrs phosphorylation. *Mol. Cell. Biol.* **25**, 9632-9645.

Biscardi, J. S., Ishizawar, R. C., Silva, C. M. and Parsons, S. J. (2000). Tyrosine kinase signalling in breast cancer: epidermal growth factor receptor and c-Src interactions in breast cancer. *Breast Cancer Res.* **2**, 203-210.

Bolanos-Garcia, V. M. (2005). MET meet adaptors: functional and structural implications in downstream signalling mediated by the Met receptor. *Mol. Cell. Biochem.* **276**, 149-157.

Journal of Cell Science

Bromann, P. A., Korkaya, H. and Courtneidge, S. A. (2004). The interplay between Src family kinases and receptor tyrosine kinases. *Oncogene* **23**, 7957-7968.

Brown, M. T. and Cooper, J. A. (1996). Regulation, substrates and functions of src. *Biochim. Biophys. Acta* **1287**, 121-149.

Carpenter, G. (1987). Receptors for epidermal growth factor and other polypeptide mitogens. *Annu. Rev. Biochem*. **56**, 881-914.

Chan, P. C., Chen, Y. L., Cheng, C. H., Yu, K. C., Cary, L. A., Shu, K. H., Ho, W. L. and Chen, H. C. (2003). Src phosphorylates Grb2-associated binder 1 upon hepatocyte growth factor stimulation. *J. Biol. Chem*. **278**, 44075-44082.

Cross, M. and Dexter, T. M. (1991). Growth factors in development, transformation, and tumorigenesis. *Cell* **64**, 271-280.

Cursi, S., Rufini, A., Stagni, V., Condo, I., Matafora, V., Bachi, A., Bonifazi, A. P., Coppola, L., Superti-Furga, G., Testi, R. et al. (2006). Src kinase phosphorylates caspase-8 on Tyr380: a novel mechanism of apoptosis suppression. *EMBO J*. **25**, 1895-1905.

Cutrupi, S., Baldanzi, G., Gramaglia, D., Maffe, A., Schaap, D., Giraudo, E., van Blitterswijk, W., Bussolino, F., Comoglio, P. M. and Graziani, A. (2000). Src-mediated activation of alpha-diacylglycerol kinase is required for hepatocyte growth factor-induced cell motility. *EMBO J*. **19**, 4614-4622.

Deuel, T. F. (1987). Polypeptide growth factors: roles in normal and abnormal cell growth. *Annu. Rev. Cell Biol*. **3**, 443-492.

Epstein, R. J., Druker, B. J., Roberts, T. M. and Stiles, C. D. (1990). Modulation of a Mr 175,000 c-neu receptor isoform in G8/DHFR cells by serum starvation. *J. Biol. Chem*. **265**, 10746-10751.

Fischer, O. M., Hart, S., Gschwind, A. and Ullrich, A. (2003). EGFR signal transactivation in cancer cells. *Biochem. Soc. Trans*. **31**, 1203-1208.

Frame, M. C. (2002). Src in cancer: deregulation and consequences for cell behaviour. *Biochim. Biophys. Acta* **1602**, 114-130.

Fukami, Y., Tokmakov, A. A., Konaka, K. and Sato, K. (1999). Peptide inhibitors of the mitogen-activated protein kinase pathway: a structure-mimetic peptide corresponding to the conserved inter-DFG-APE region in the kinase domain. *Pharmacol. Ther*. **82**, 399-407.

Griffiths, G. J., Koh, M. Y., Brunton, V. G., Cawthorne, C., Reeves, N. A., Greaves, M., Tilby, M. J., Pearson, D. G., Ottley, C. J., Workman, P. et al. (2004). Expression of kinase-defective mutants of c-Src in human metastatic colon cancer cells decreases Bcl-xL and increases oxaliplatin- and Fas-induced apoptosis. *J. Biol. Chem*. **279**, 46113-46121.

Guerrero, J., Santibanez, J. F., Gonzalez, A. and Martinez, J. (2004). EGF receptor transactivation by urokinase receptor stimulus through a mechanism involving Src and matrix metalloproteinases. *Exp. Cell Res*. **292**, 201-208.

Hammond, D. E., Urbe, S., Vande Woude, G. F. and Clague, M. J. (2001). Down-regulation of MET, the receptor for hepatocyte growth factor. *Oncogene* **20**, 2761-2770.

Han, C., Michalopoulos, G. K. and Wu, T. (2006). Prostaglandin E_2 receptor EP_1 transactivates EGFR/MET receptor tyrosine kinases and enhances invasiveness in human hepatocellular carcinoma cells. *J. Cell. Physiol*. **207**, 261-270.

Heldin, C. H. and Westermark, B. (1989). Growth factors as transforming proteins. *Eur. J. Biochem*. **184**, 487-496.

Hung, W. and Elliott, B. (2001). Co-operative effect of c-Src tyrosine kinase and Stat3 in activation of hepatocyte growth factor expression in mammary carcinoma cells. *J. Biol. Chem*. **276**, 12395-12403.

Irby, R. B. and Yeatman, T. J. (2000). Role of Src expression and activation in human cancer. *Oncogene* **19**, 5636-5642.

Ishizawar, R. and Parsons, S. J. (2004). c-Src and cooperating partners in human cancer. *Cancer Cell* **6**, 209-214.

Jeffers, M., Taylor, G. A., Weidner, K. M., Omura, S. and Vande Woude, G. F. (1997). Degradation of the Met tyrosine kinase receptor by the ubiquitin-proteasome pathway. *Mol. Cell. Biol*. **17**, 799-808.

Jiang, W. G., Martin, T. A., Parr, C., Davies, G., Matsumoto, K. and Nakamura, T. (2005). Hepatocyte growth factor, its receptor, and their potential value in cancer therapies. *Crit. Rev. Oncol. Hematol*. **53**, 35-69.

Kadono, Y., Okada, Y., Namiki, M., Seiki, M. and Sato, H. (1998). Transformation of epithelial Madin-Darby canine kidney cells with p60(v-src) induces expression of membrane-type 1 matrix metalloproteinase and invasiveness. *Cancer Res*. **58**, 2240-2244.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Lang, R. A. and Burgess, A. W. (1990). Autocrine growth factors and tumourigenic transformation. *Immunol. Today* **11**, 244-249.

Luo, L., Yano, N. and Luo, J. Z. (2006). The molecular mechanism of EGF receptor activation in pancreatic β-cells by thyrotropin-releasing hormone. *Am. J. Physiol. Endocrinol. Metab*. **290**, E889-E899.

Maa, M. C., Leu, T. H., McCarley, D. J., Schatzman, R. C. and Parsons, S. J. (1995). Potentiation of epidermal growth factor receptor-mediated oncogenesis by c-Src: implications for the etiology of multiple human cancers. *Proc. Natl. Acad. Sci. USA* **92**, 6981-6985.

Maejima, Y., Ueba, H., Kuroki, M., Yasu, T., Hashimoto, S., Nabata, A., Kobayashi, N., Ikeda, N., Saito, M. and Kawakami, M. (2003). Src family kinases and nitric oxide production are required for hepatocyte growth factor-stimulated endothelial cell growth. *Atherosclerosis* **167**, 89-95.

Mellon, K., Wright, C., Kelly, P., Horne, C. H. and Neal, D. E. (1995). Long-term outcome related to epidermal growth factor receptor status in bladder cancer. *J. Urol*. **153**, 919-925.

Miyamoto, S., Yagi, H., Yotsumoto, F., Kawarabayashi, T. and Mekada, E. (2006). Heparin-binding epidermal growth factor-like growth factor as a novel targeting molecule for cancer therapy. *Cancer Sci*. **97**, 341-347.

Morotti, A., Mila, S., Accornero, P., Tagliabue, E. and Ponzetto, C. (2002). K252a inhibits the oncogenic properties of Met, the HGF receptor. *Oncogene* **21**, 4885-4893.

Murillo, M. M., del Casillo, G., Sanchez, A., Fernandez, M. and Fabregat, I. (2005). Involvement of EGF receptor and c-Src in the survival signals induced by TGF-β1 in hepatocytes. *Oncogene* **24**, 4580-4587.

Neal, D. E., Sharples, L., Smith, K., Fennelly, J., Hall, R. R. and Harris, A. L. (1990). The epidermal growth factor receptor and the prognosis of bladder cancer. *Cancer* **65**, 1619-1625.

Neel, B. D., Aouacheria, A., Nouvion, A. L., Ronot, X. and Gillet, G. (2005). Distinct protease pathways control cell shape and apoptosis in v-src-transformed quail neuroretina cells. *Exp. Cell Res*. **311**, 106-116.

Peschard, P. and Park, M. (2003). Escape from Cbl-mediated downregulation: a recurrent theme for oncogenic deregulation of receptor tyrosine kinases. *Cancer Cell* **3**, 519-523.

Peschard, P., Fournier, T. M., Lamorte, L., Naujokas, M. A., Band, H., Langdon, W. Y. and Park, M. (2001). Mutation of the c-Cbl TKB domain binding site on the Met receptor tyrosine kinase converts it into a transforming protein. *Mol. Cell* **8**, 995-1004.

Peschard, P., Ishiyama, N., Lin, T., Lipkowitz, S. and Park, M. (2004). A conserved DpYR motif in the juxtamembrane domain of the Met receptor family forms an atypical c-Cbl/Cbl-b tyrosine kinase binding domain binding site required for suppression of oncogenic activation. *J. Biol. Chem*. **279**, 29565-29571.

Ponzetto, C., Bardelli, A., Zhen, Z., Maina, F., dalla Zonca, P., Giordano, S., Graziani, A., Panayotou, G. and Comoglio, P. M. (1994). A multifunctional docking site mediates signaling and transformation by the hepatocyte growth factor/scatter factor receptor family. *Cell* **77**, 261-271.

Popsueva, A., Poteryaev, D., Arighi, E., Meng, X., Angers-Loustau, A., Kaplan, D., Saarma, M. and Sariola, H. (2003). GDNF promotes tubulogenesis of GFRα1-expressing MDCK cells by Src-mediated phosphorylation of Met receptor tyrosine kinase. *J. Cell Biol*. **161**, 119-129.

Prenzel, N., Zwick, E., Daub, H., Leserer, M., Abraham, R., Wallasch, C. and Ullrich, A. (1999). EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF. *Nature* **402**, 884-888.

Qiao, H., Saulnier, R., Patryzkat, A., Rahimi, N., Raptis, L., Rossiter, J., Tremblay, E. and Elliott, B. (2000). Cooperative effect of hepatocyte growth factor and fibronectin in anchorage-independent survival of mammary carcinoma cells: requirement for phosphatidylinositol 3-kinase activity. *Cell Growth Differ*. **11**, 123-133.

Rahimi, N., Tremblay, E., McAdam, L., Park, M., Schwall, R. and Elliott, B. (1996). Identification of a hepatocyte growth factor autocrine loop in a murine mammary carcinoma. *Cell Growth Differ*. **7**, 263-270.

Rahimi, N., Hung, W., Tremblay, E., Saulnier, R. and Elliott, B. (1998). c-Src kinase activity is required for hepatocyte growth factor-induced motility and anchorage-independent growth of mammary carcinoma cells. *J. Biol. Chem*. **273**, 33714-33721.

Ruck, A. and Paulie, S. (1997). The epidermal growth factor receptor is involved in autocrine growth of human bladder carcinoma cell lines. *Anticancer Res*. **17**, 1925-1931.

Ruck, A. and Paulie, S. (1998). EGF, TGF alpha, AR and HB-EGF are autocrine growth factors for human bladder carcinoma cell lines. *Anticancer Res*. **18**, 1447-1452.

Ruck, A., Jakobson, E., Bjorkman, S. and Paulie, S. (1994). Adaptation of human bladder carcinoma cell lines to serum-free growth. Evidence for autocrine growth stimulation. *Anticancer Res*. **14**, 55-60.

Sato, K., Sato, A., Aoto, M. and Fukami, Y. (1995). c-Src phosphorylates epidermal growth factor receptor on tyrosine 845. *Biochem. Biophys. Res. Commun*. **215**, 1078-1087.

Sato, K., Kimoto, M., Kakumoto, M., Horiuchi, D., Iwasaki, T., Tokmakov, A. A. and Fukami, Y. (2000). Adaptor protein Shc undergoes translocation and mediates up-regulation of the tyrosine kinase c-Src in EGF-stimulated A431 cells. *Genes Cells* **5**, 749-764.

Sato, K., Nagao, T., Kakumoto, M., Kimoto, M., Otsuki, T., Iwasaki, T., Tokmakov, A. A., Owada, K. and Fukami, Y. (2002). Adaptor protein Shc is an isoform-specific activator of the tyrosine kinase c-Src. *J. Biol. Chem*. **277**, 29568-29576.

Sato, K., Nagao, T., Iwasaki, T., Nishihira, Y. and Fukami, Y. (2003). Src-dependent phosphorylation of the EGF receptor Tyr-845 mediates Stat-p21^{waf1} pathway in A431 cells. *Genes Cells* **8**, 935-943.

Sato, K., Iwasaki, T., Hirahara, S., Nishihira, Y. and Fukami, Y. (2004). Molecular dissection of egg fertilization signaling with the aid of tyrosine kinase-specific inhibitor and activator strategies. *Biochim. Biophys. Acta* **1697**, 103-121.

Saucier, C., Khoury, H., Lai, K. M., Peschard, P., Dankort, D., Naujokas, M. A., Holash, J., Yancopoulos, G. D., Muller, W. J., Pawson, T. et al. (2004). The Shc adaptor protein is critical for VEGF induction by Met/HGF and ErbB2 receptors and for early onset of tumor angiogenesis. *Proc. Natl. Acad. Sci. USA* **101**, 2345-2350.

Schafer, B., Gschwind, A. and Ullrich, A. (2004). Multiple G-protein-coupled receptor signals converge on the epidermal growth factor receptor to promote migration and invasion. *Oncogene* **23**, 991-999.

Sporn, M. B. and Roberts, A. B. (1985). Autocrine growth factors and cancer. *Nature* **313**, 745-747.

Sridhar, S. C. and Miranti, C. K. (2005). Tetraspanin KAI1/CD82 suppresses invasion by inhibiting integrin-dependent crosstalk with c-Met receptor and Src kinases. *Oncogene* **25**, 2367-2378.

Stennicke, H. R. and Salvesen, G. S. (2000). Caspases – controlling intracellular signals by protease zymogen activation. *Biochim. Biophys. Acta* **1477**, 299-306.

Summy, J. M. and Gallick, G. E. (2006). Treatment for advanced tumors: SRC reclaims center stage. *Clin. Cancer Res*. **12**, 1398-1401.

Suzuki, M., Raab, G., Moses, M. A., Fernandez, C. A. and Klagsbrun, M. (1997). Matrix metalloproteinase-3 releases active heparin-binding EGF-like growth factor by cleavage at a specific juxtamembrane site. *J. Biol. Chem*. **272**, 31730-31737.

Thomas, S. M. and Brugge, J. S. (1997). Cellular functions regulated by Src family kinases. *Annu. Rev. Cell Dev. Biol*. **13**, 513-609.

Thogersen, V. B., Sorensen, B. S., Poulsen, S. S., Orntoft, T. F., Wolf, H. and Nexo, E. (2001). A subclass of HER1 ligands are prognostic markers for survival in bladder cancer patients. *Cancer Res*. **61**, 6227-6233.

Wang, W., Marimuthu, A., Tsai, J., Kumar, A., Krupka, H. I., Zhang, C., Powell, B., Suzuki, Y., Nguyen, H., Tabrizizad, M. et al. (2006). Structural characterization of autoinhibited c-Met kinase produced by coexpression in bacteria with phosphatase. *Proc. Natl. Acad. Sci. USA* **103**, 3563-3568.

Wojcik, E. J., Sharifpoor, S., Miller, N. A., Wright, T. G., Watering, R., Tremblay, E. A., Swan, K., Mueller, C. R. and Elliott, B. E. (2006). A novel activating function of c-Src and Stat3 on HGF transcription in mammary carcinoma cells. *Oncogene* **25**, 2773-2784.

Wright, T. G., Tsai, J., Jia, Z. and Elliott, B. E. (2004). Inhibition by Cu^{2+} binding of hepatocyte growth factor (HGF) interaction with its receptor Met and blockade of HGF/Met function. *J. Biol. Chem*. **279**, 32499-32506.

Xiao, G. H., Jeffers, M., Bellacosa, A., Mitsuuchi, Y., Vande Woude, G. F. and Testa, J. R. (2001). Anti-apoptotic signaling by hepatocyte growth factor/Met via the phosphatidylinositol 3-kinase/Akt and mitogen-activated protein kinase pathways. *Proc. Natl. Acad. Sci. USA* **98**, 247-252.

Zhang, Q., Thomas, S. M., Xi, S., Smithgall, T. E., Siegfried, J. M., Kamens, J., Gooding, W. E. and Grandis, J. R. (2004). SRC family kinases mediate epidermal growth factor receptor ligand cleavage, proliferation, and invasion of head and neck cancer cells. *Cancer Res.* **64**, 6166-6173.