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APPLICATION NOTE 32

Analysis of Tyrosine Kinase Signaling in Human Cancer by Stable Isotope Labeling with Heavy Amino Acids in Mouse Xenografts Utilizing MouseExpress® Lysine $^{13}C_6$ Mouse Feed

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Tyrosine kinases (TK) play important roles in the induction of cell growth, survival and migration. They also have oncogenic activity when deregulated, a role originally described for the constitutively active v-SRC[1] and since then, observed with most TK in human cancer.[2] A large body of evidence indicates that aberrant TK activities contributes to cancer cell growth, survival, angiogenesis and cell dissemination leading to metastasis. This has been illustrated by the capacity of cancer cells transformed by oncogenic TK to induce tumor growth and metastasis formation when injected in nude mice. Since then, they have been considered as attractive therapeutic targets and several inhibitors are currently used in the clinic.[2] However, our knowledge of the TK-dependent oncogenic signaling in human tumors is largely incomplete, mostly because the majority of data has been obtained in two-dimensional cell culture models. Moreover, the standard culture conditions of transformed cells do not allow recapitulating all the kinase-dependent signaling cascades that are activated during tumorigenesis to promote tumor growth, angiogenesis and interactions with the microenvironment.

MS-based quantitative phosphoproteomic technology has been a valuable tool to decipher signaling pathways initiated by a given TK.[3] Particularly, the Stable Isotope Labeling with Amino acids in Cell culture (SILAC) method has been employed for the characterization of oncogenic TK signaling pathways in cell culture.[4, 5] We recently used this powerful approach to investigate oncogenic signaling dependent upon the activity of the TK SRC in colon cancer cells[6] and identified the first SRC-dependent tyrosine "phosphoproteome" in these cancer cells. Oncogenic signaling induced by TK could be investigated *in vivo* using similar MS-based quantitative phosphoproteomic approaches in mouse models or tumor biopsies. However, the application of the SILAC method *in vivo* has been challenging until recently because it requires efficient protein labeling in different tissues, which is conditioned by the rate of *de novo* protein synthesis. Recently, Mann *et al.* described the successful development of a SILAC approach for labeling mice that is based on the addition of L-Lysine•2HCl ($^{13}C_6$, 99%) (CLM-2247-H) into their food.[7] They reported complete labeling from the F2 generation.

In this note, we describe a novel proteomic approach to label tumors in *nude* mice xenografted with human cancer cells using MouseExpress® L-Lysine ($^{13}C_6$, 99%) Mouse Feed (MF-LYS-C).[8] We reasoned that the high rate of *de novo* protein synthesis occurring in tumors may induce an efficient labeling of xenografted tumors within a short period of time. We observed a consistent >88% labeling of the tumor proteome by feeding engrafted mice with the SILAC mouse diet for only 30 days. We then used this approach to compare the tyrosine phosphoproteome of SRC positive tumors (labeled with heavy amino acids) and of control tumors (labeled with light amino acids).

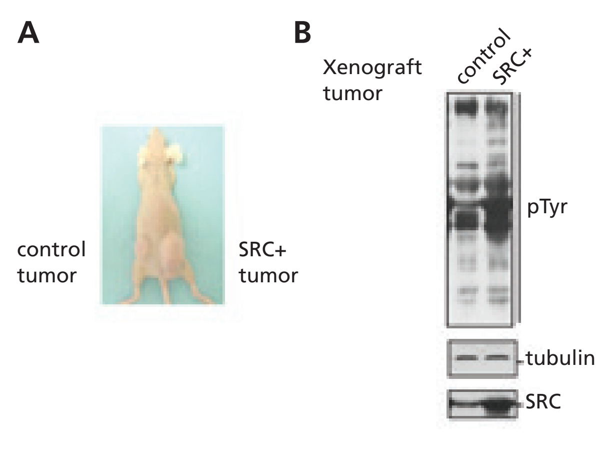

Figure 1. SRC increases tumor growth and pTyr content in CRC xenograft models. A) A representative example of xenograft tumors obtained by subcutaneous injection of controls SW620 CRC cells (left) and SRC-overexpressing SW620 CRC cells (right) in nude mice. B) A representative example of pTyr-level obtained from control and SRC-overexpressing tumor-lysates.

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Experimental Design

Mouse xenografts, [$^{13}C_6$]-Lysine tumor labeling and protein extraction. Swiss *nu/nu (nude)* mice (Charles River, L'Arbresle, France) were injected s.c. with 2x10^6 cells (SRC-SW620 or control SW620 cells) in the flank and fed respectively with L-Lysine $^{13}C_6$ feed or unlabeled feed using MouseExpress® L-Lysine ($^{13}C_6$, 99%) Mouse Feed Labeling Kit (MLK-LYS-C). After 30 days, animals were then sacrificed, tumors dissected and protein extracted from frozen tumors using lysis buffer (20 mM Hepes, 150 mM NaCl, 0.5% Triton, 6 mM β-octylglucoside, 100 µM orthovanadate, 100 µM aprotinin, 100 mM DTT, 100 mM NAF) and a Duall® Glass Tissue Grinder size 21.

Mass spectrometry analysis. Phosphotyrosine immunoaffinity purification (using a mixture of 4G10 and pY100 antibodies), and tryptic digestion were essentially performed as described in ref. 9. Purified proteins were separated on 9% SDS-PAGE gels, digested with Lysine C endoproteinase (Thermo Scientific) and analyzed on-line using nanoflow HPLC-nano-electrospray ionization on a LTQ-Orbitrap XL mass spectrometer (ThermoScientific, Waltham, MA USA) coupled with an Ultimate 3000 HPLC apparatus (Dionex, Amsterdam, Netherland). Spectra were acquired with the instrument operating in the information-dependent acquisition mode throughout the HPLC gradient. Survey scans were acquired in the Orbitrap system with resolution set at a value of 60,000. Up to five of the most intense ions per cycle were fragmented and analyzed in the linear trap. Peptide fragmentation was performed using nitrogen gas on the most abundant and at least doubly charged ions detected in the initial MS scan and an active exclusion time of 1 min. Ion selection was set at 5.000 counts.

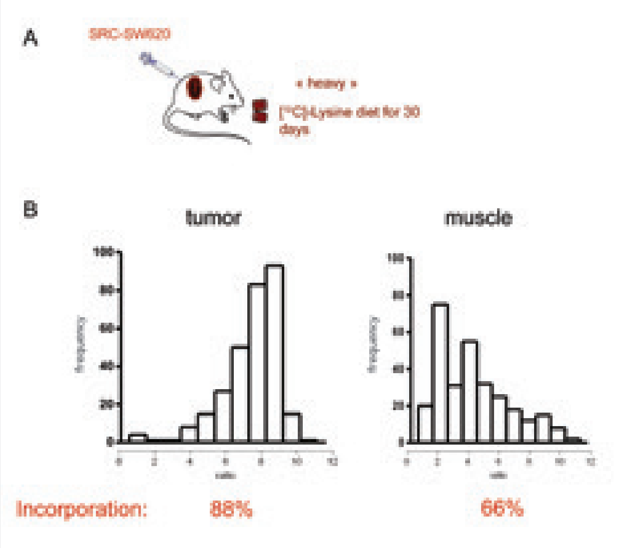

Figure 2. Time course of [$^{13}C_6$]-Lysine incorporation in xenograft tumors. A. Schematic of the analysis of heavy [$^{13}C_6$]-Lysine incorporation in mouse xenograft tumors. After subcutaneous injection of SRC-SW620 cells into the flank of nude mice, animals were subjected to heavy SILAC diet containing [$^{13}C_6$]-Lysine for 30 days. B. Histogram showing the distribution of the incorporation ratios in tumor proteins and in proteins of the muscle tissue surrounding the tumor. The mean ratio incorporation (%) is indicated.

Data Analysis

Analysis was performed using the MaxQuant software (version 1.1.1.36). All MS/MS spectra were searched using Andromeda against a decoy database consisting of a combination of *Homo sapiens* and *Mus musculus* CPS databases (97,681 entries, release Jun 2011 http://www.expasy.ch) and 250 classical contaminants, containing forward and reverse entities. The statistical validity of the results and the determination of over-represented proteins were assessed using significance B, as defined using Perseus (version 1.1.1.36, standard parameters) on the logarithmized normalized ratio (base 2).

Results

Stable isotope labeling with amino acids in mouse xenografts. Expression of SRC in SW620 cells, a human metastatic colorectal cell line that exhibits a low level of endogenous SRC, increased cell transforming properties as it significantly promoted tumor growth when subcutaneous injected in *nude* mice (Figure 1A). These SRC oncogenic effects were associated with a strong increase of the pTyr content in xenograft tumors in which SRC was overexpressed (Figure 1B). We then applied a MS-based quantitative phosphoproteomic method based on stable isotope labeling with amino acids in mouse xenografts, to thoroughly characterize the SRC-dependent oncogenic signaling pathway in xenograft tumors. Mice were subcutaneously injected with 2x10^6 SRC-SW620 cells and then fed with MouseExpress® L-Lysine ($^{13}C_6$, 99%) Mouse Feed, as done to obtain the SILAC mouse, but only during the time required for tumors to reach a volume of about 900 mm^3 (30 days). Tumor proteins were then solubilized from isolated tumors and separated on 1D SDS-PAGE gels, then in-gel digested with the endoproteinase Lys-C and analyzed by liquid chromatography-tandem MS. Digested peptides were then quantified based on the relative Lys intensities. We observed a median SILAC ratio of 1:7.4 at day 30, which corresponded to >88% of tumor protein labeling (Figure 2). These ratios were very consistent over time and in tumors from different animals, further validating our *in vivo* SILAC approach. In contrast, the median SILAC ratio of non-transformed surrounding tissue (*i.e.* muscle) reached 1.97, which corresponded to 66% of protein labeling (Figure 2B). Altogether these results indicate that, while insufficient for labeling non-transformed tissues of the host mice, a 30-day SILAC mouse diet is sufficient to label xenograft tumors to a level that is adequate for quantitative proteomic analysis.

Quantitative phosphoproteomics in xenograft tumors. We next applied this mouse SILAC approach to investigate the SRC-dependent oncogenic signaling pathway in xenografted tumors. SRC-SW620 cells were injected in animals that were fed a diet of MouseExpress® L-Lysine ($^{13}C_6$, 99%) Mouse Feed. As a control, parental SW620 cells were injected in mice that were fed with a "light" diet of MouseExpress® Unlabeled Mouse Feed (MF-UNLABELED). After 30 days of this regimen, xenograft tumors were isolated and lysed, and three pairs of lysates were prepared by mixing (1:1) one SRC-SW620 xenograft tumor lysate with one control tumor lysate. pTyr proteins were then purified using anti-

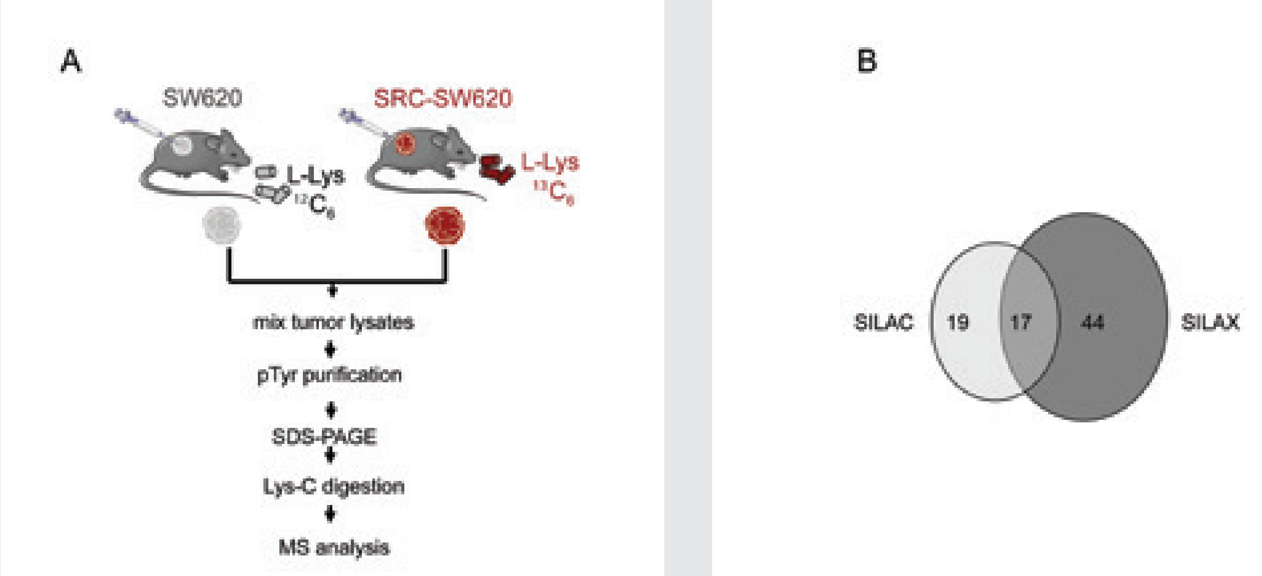

Figure 3. Analysis of SRC oncogenic signaling in xenograft tumors by SILAC mouse. A. Schematic overview of the SILAC experimental procedure applied to mouse xenografts. B. Comparison of *in vivo* and *in vitro* SRC signaling by MS analysis. Venn diagram showing the number of common and specific SRC targets identified by mouse SILAC *in vivo* and SILAC in cell culture.

pTyr antibodies and analyzed by MS.[9] A scheme of the procedure is illustrated in Figure 3A. Quantitative phosphoproteomic analysis led to the identification of 61 SRC targets *in vivo* that were obtained with a ratio significantly >1 in two out of three separate experiments (tyrosine phosphorylated proteins or proteins whose association with pTyr proteins is increased). A comparison of SRC targets obtained by our *in vivo* analysis with the one obtained by SILAC analysis of the same cancer cells in culture indicates that only 17/61 were also targets of SRC *in vitro* (Figure 3B). This data indicates that oncogenic signaling induced by SRC in tumors significantly differs from the one induced by SRC in cell culture.

Discussion

Here we describe a novel SILAC approach to investigate oncogenic TK signaling *in vivo* in mouse xenografts. This method is based on the efficient labeling of tumor proteins by feeding xenografted mice with the mouse SILAC diet for a limited period of time (30 days) thanks to the high rate of *de novo* protein synthesis in tumors. Indeed, we could successfully label xenograft tumors derived from human colon cells that are characterized by a much slower *in vitro* growth rate than human leukemic cells. Therefore, we think that this approach may be suitable for most human cancer cells that induce significant tumor growth in nude mice. We also predict that our mouse SILAC approach will have a large number of applications, including for the analysis of the dynamic signaling of oncogenic TK during tumor progression from early tumorigenesis to metastasis formation, and also for evaluating the activity of TK inhibitors on the tumor phosphoproteome over time. In this case, this methodology could be particularly useful for determining the molecular cause(s) of innate or acquired resistance to such inhibitors.

Related Products

Catalog No.	Description
MLK-LYS-C	MouseExpress® L-Lysine ($^{13}C_6$, 99%) Mouse Feed Labeling Kit
MF-LYS-C	MouseExpress® L-Lysine ($^{13}C_6$, 99%) Mouse Feed
CLM-2247-H	L-Lysine•2HCl ($^{13}C_6$, 99%)

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