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5 Human mesenchymal stromal cells in adhesion to cell-derived extracellular matrix and titanium: a comparative kinome profile analysis

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ABSTRACT

The extracellular matrix (ECM) is an essential component of tissue architecture that physically supports cells and actively influences stem cell behaviour, by modulating kinase-mediated signaling cascades that are the key regulators of signal transduction. Cell-derived ECMs have recently emerged in the context of bone regeneration, as they reproduce physiological tissue-architecture and ameliorate the promising properties of mesenchymal stromal cells (MSCs). Titanium scaffolds show good mechanical properties and porosity to facilitate cell adhesion, and thus have been routinely used for bone tissue engineering applications. The aim of this study was to analyze the kinomic signature of human MSCs in adhesion to an osteoblast-derived ECM that we have previously shown to be osteopromotive, and to compare it to MSCs on titanium. PamChip kinase array analysis revealed 63 phosphorylated peptides on ECM and 59 on titanium, with MSCs on ECM exhibiting significant higher levels of kinase activity than those on titanium. MSCs on the 2 substrates showed a substantial overlap of kinome profiles, with the activation of similar signaling pathways, such as FAK, ERK and PI3K signaling. Inhibition of PI3K signaling in cells significantly reduced cell adhesion to the ECM and increased the number of non-adherent cells both on ECM and on titanium. In summary, this study comprehensively characterized the kinase activity in MSCs on cell-derived ECM and on titanium, highlighting the role of PI3K signaling in the kinomic changes regulating osteoblast viability and adhesion. Kinome profile analysis represents a powerful tool to select pathways for a better understanding of cell behaviour. Osteoblast-derived ECM could be further investigated as coating for titanium scaffolds, to improve bone tissue engineering applications.

Key words: kinome profiling; osteoblasts; extracellular matrix; titanium; cell adhesion; peptide array

INTRODUCTION

The extracellular matrix (ECM) is an essential structural component present in every tissue, which physically supports cells, but also actively modulates their behaviour, by regulating the availability of bioactive molecules and by transducing mechanical signaling [1-4]. Bone matrix is composed of collagen and non-collagenous proteins to maintain bone flexibility, whereas its stiffness is achieved by hydroxyapatite crystals, which makes bone a peculiar type of connective tissue [5, 6]. Because of its composition, bone ECM is essential for the structure and the strength of the bone and it also actively participates in bone formation and bone metabolism, by regulating mineralization and modulating growth factor availability [6, 7]. The physical cues of bone ECM proteins are mechanosensed by bone cells via integrin-mediated signaling, which converts the biomechanical properties of the ECM, eventually acting on cell adhesion, proliferation and differentiation [8-10]. The molecular details of the signaling pathways that mediate the relay of information from integrin engagement to altered cellular physiology remain, however, largely obscure.

In recent years, the interest in bone ECM for regenerative purposes has grown rapidly. Bone tissue engineering (BTE) applications have been proposed as bone graft substitutes in large bone defects when bone healing capacity is lost. BTE involves the combination of scaffolds (osteoconduction), osteogenic factors (osteoinduction) and autologous mesenchymal stromal cells (MSCs; osteogenesis) to mimic the native bone ECM structure and stimulate the osteogenic differentiation of local progenitors, driving new bone formation [11, 12]. Scaffolds serve as a structural template for osteogenesis, being biocompatible and osteoconductive [13]. Among the several scaffolds that can be used for BTE, titanium shows good mechanical properties and it can be tailored in porosity to suit cell adhesion. Although not bioresorbable, titanium scaffolds are already clinically used for orthopaedic and dental implants and in load-bearing areas for their good mechanical properties [14]. As scaffolds do not reproduce the native structure of bone ECM, decellularized ECMs represent an alternative cell-instructive microenvironment to guide endogenous repair [15, 16]. In this context, cell-secreted ECMs have also been proposed, as they are readily available and can be customized for the use as scaffold-coating [17-19]. We and others demonstrated that osteoblast-derived ECM stimulates MSC osteogenesis and promotes bone formation (Baroncelli M., unpublished results) [20, 21]. Moreover, cell-secreted ECMs have already been used to coat and modify titanium surfaces, showing that ECM influences gene expression and enhances osteogenic differentiation of MSCs [20, 22, 23]. In this context, the aim of this study was to compare the naturally secreted devitalized ECM to titanium, and investigate how they differentially regulate cell adhesion.

Kinase activity lies at the core of cell signal transduction, as activation of specific kinases mediates the induction of signaling cascades resulting into cellular processes such as cell metabolism, differentiation and cytoskeletal rearrangements during cell adhesion [24-27]. Integrin-mediated activation of kinase signaling cascades such as FAK and Src family kinase converts mechanical forces into biochemical signals and results in the efficient adhesion of the cell to the surface. At the same time, deregulation of kinase-mediated signaling pathways leads to pathological states, emphasizing that studying kinase activity is crucial to understand biological functions.

The aim of our study was to assess specific kinomic changes upon MSC adhesion to cell-derived ECM and titanium surfaces, by using tyrosine kinase PamChip® array which to the best of our knowledge has not been used before to investigate cell adhesion of human MSCs.

MATERIALS AND METHODS

Cell culture and ECM preparations

Human bone marrow-derived MSCs were used to prepare the osteopromotive devitalized ECM as previously described [28]. Briefly, MSCs (5128 viable cells/cm^2; PT-2501, Lonza, Walkersville, MD, USA) from a single donor at passage 7 were cultured in growth medium for 2 days (alpha-Mem phenol-red free (GIBCO, Paisley, UK), 10% foetal bovine serum), and osteogenically differentiated for 11 days (culture medium supplemented with 100 nM dexamethasone and 10 mM β glycerophosphate (Sigma, St. Louis, MO, USA) to induce the deposition of the ECM. MSCs were devitalized by freeze/thaw cycles, DNAse treatment (10 U/ml; Sigma-Aldrich, St Louis MO, USA), extensive washings with Phosphate Buffer Saline (PBS) (GIBCO, Paisley, UK), and sterile air drying. Devitalized ECMs were stored at -20 °C until further use.

Tyrosine kinase activity profiling using PamChip peptide microarray

To check the effect of the devitalized ECM and titanium on MSC behavior, MSCs (28300 viable cells/cm^2) were cultured on these surfaces in growth medium. After 4 hours, cells were scraped in M-PER Mammalian protein extraction buffer (Thermo Scientific, Rockford, IL, USA) containing Halt phosphatase and protease inhibitors (Thermo Scientific), allowed to lyse at 4°C for 10 minutes and lysates were cleared by centrifugation at 14,000 g for 10 minutes. Supernatants were stored at -80°C until use. Cell lysates (5µg protein for all samples) were loaded on a PamChip tyrosine kinase microarray (PamGene International B.V., 's-Hertogenbosch, the Netherlands). PamChip® is a high-throughput and cost-effective peptide array that

allows the study of kinome profile changes without *a priori* assumptions [29]. In the PamChip platform, cell lysates are continuously pumped past 144 consensus peptide-sequences spotted on a 3D porous microarray, and the phosphorylation of their specific target substrates by kinases present in the whole cell lysate is fluorescently detected, describing the entire tyrosine-kinase activity profile within a single experiment [30-32]. Phosphorylation of the 144 kinase substrates on the array was detected by using FITC-labelled secondary antibody. After array washing, images were taken every 5 minutes to create real-time kinetics data. Signal intensities of the three technical replicates for each substrate were quantified using Bionavigator software (version 6.1.42.1, PamGene International BV). A complete list of phosphopeptides on PamChip is depicted in Supplementary Table 1. The internal positive control peptide ART_003_EAI(pY)AAPFAKKKXC was not considered for further analysis. Kinase reactions start at *t*=640 s. Subsequently, kinase reactions for different peptides show markedly different kinetics. Most peptides act according to classical biochemical theory, with the derivative of the initial reaction speed approximating maximal velocity (Vmax) for phosphorylation of this peptides. For data analysis of these peptides Vmax was established by calculating the tangent of apparent peptide phosphorylation between 640-1040 s and were classified as early Vmax peptides (Supplementary Figure 1A). Phosphorylation of other peptides showed considerable lag time, followed by a quick rise in speed of phosphorylation and subsequent decay according to conventional biochemical theory, yielding sigmoid curves of substrate phosphorylation when plotted against the time domain. For peptides which upon visual inspection displayed such a Maxwell-Boltzmann-like activation kinetics, Vmax was calculated by determining the tangent of substrate phosphorylation between 1040-1440 s and were classified as mid Vmax (Supplementary Figure 1B). Finally, a group of peptides displayed very slow initial activation followed by a rapid increase in reaction velocity towards the end of the experiment. These peptides were identified by inspection of the visual aspect of the curve and classified as late Vmax, whereas Vmax was calculated by using the tangent of apparent substrate phosphorylation between 1440-1840 s (Supplementary Figure 1C). A detailed flowchart of kinome profile analysis is presented in Supplementary Figure 1D. Vmax values below zero were artificially set to zero. Only Vmax values with average above zero were considered for further analysis. Markov state analysis was performed to determine 'on' and 'off' calls of peptide phosphorylation on ECM and titanium [33]. In detail, for each substrate the 143 peptides were ranked for Vmax intensity, and a linear trend line was set for the lowest 60 peptides considered as background. Peptides whose average phosphorylation minus 1.95 times the standard deviation being higher than the background signal were considered as Markov-positive 'on' calls and further analyzed (Supplementary Figure 2A and 2B).

Kinome array analysis

Protein and gene annotations of the kinase substrates on PamChip were searched through Uniprot Knowledgebase (www.uniprot.org). Markov-positive peptides were analyzed through QIAGEN's Ingenuity® Pathway Analysis (IPA®, QIAGEN Redwood City www.qiagen.com/ingenuity) against human genome provided by Ingenuity Knowledge Base as background. Gene IDs of the parent proteins of Markov-positive peptides on ECM and titanium were used in IPA. As the peptide ART_004_EAIYAAP-FAKKKXC is phosphorylated by ABL1 kinase if artificial [34] as in our case, ABL1 was also included in the IPA analysis. Consensus phosphopeptides representing different phosphorylation sites of the same protein were considered together. The Canonical Pathway analysis tool was used for IPA analysis. Intracellular signaling pathways not restricted to a specific cellular type were selected and used to generate the heat-map using R, together with the relative Gene IDs of the phosphorylated kinase substrates.

Since specific kinases activate signaling cascades, the kinase substrates that were phosphorylated in cells on ECM and on titanium were further fitted into signaling pathways and cell-related functions as previously described [32] to confirm IPA analysis.

Immunoblot analysis

Some of the activated kinases revealed by PamChip array were validated by Western blot as described (with modifications) [35, 36]. Briefly, cell lysates (40 µg) were prepared as for kinome profile analysis, mixed with 2x Laemmli buffer, separated by SDS-PAGE, transferred onto nitrocellulose membrane (Immobilon FL membrane, Merck KGaA, Darmstadt, Germany) and non-specifically blocked with Odyssey buffer (LI-COR Biosciences, Lincoln, NE). Membranes were incubated overnight with primary antibodies against pFAK (Y925) (rabbit polyclonal; Signalway Antibody, MD, USA), pERK, pPKB, pEGFR and pSMAD1/5/8 (Cell Signaling Technology, Beverly, MA) and β-actin loading control (mouse monoclonal; Clone sc-47778; Santa Cruz Biotechnology, Dallas, TX, USA). All antibodies were used 1:1,000. Membranes were probed with secondary antibody conjugated with goat-anti-mouse-Alexa Fluor 680 and goat-anti-rabbit IRDye 800CW at 1:5,000 (LI-COR Biosciences, Lincoln, NE). Odyssey infrared imaging (LI-COR Biosciences, Lincoln, NE) was used to detect proteins. Quantification was performed using Odyssey 3.0 software.

Cell viability analysis

To assess the effect of PI3K signaling inhibition on cell viability, PI3K signaling was inhibited by Wortmannin or LY294002 [37]. MSCs were cultured on ECM for 4 hours in growth culture medium in the presence of 10 µM Wortmannin (in dimethyl

sulfoxide [DMSO]; Sigma, St Louis, MO, USA), 10 μM LY294002 (in DMSO; Cayman Chemical, MI, USA) or vehicle control. After 4 hours, MSCs were gently rinsed with PBS and both floating and adherent cells were collected in Laemmli buffer.

To confirm that Wortmannin and LY294002 effectively inhibited PI3K signaling, cell lysates treated with and without PI3K inhibitors were probed for the presence of pPKB and pERK (p42/44) by immunoblot analysis.

To investigate if the PI3K inhibitors affected cell viability, the presence of poly ADP-ribose polymerase (PARP) and Caspase 3 and its relative cleaved forms were detected by Western blot analysis (antibodies from Cell Signaling Technology, Beverly, MA) [38].

Cell adhesion analysis

To check the effect of PI3K signaling inhibition on cell adhesion, MSCs were cultured in osteogenic conditions on cell-derived ECM, in the presence of increasing concentrations of Wortmannin and LY294002 (0.1 μM, 1 μM and 10 μM in DMSO for both). MSCs in adhesion to titanium with and without the highest concentration of Wortmannin and LY294002 (10 μM in DMSO) were used for the same purpose. MSCs with vehicle were considered as control (1/200 vol/vol). After 4 hours, both floating cells and cells adhering to the substrates were collected and the fraction of adhering cells was quantified by Flow Cytometry (Accuri C6 Flow Cytometer, BD Biosciences, San Jose, CA, USA), using counting beads (Liquid Counting Beads, BD Biosciences, San Jose, CA, USA).

Statistical analysis

Area under curve (AUC) of the kinetic reaction was calculated for each Markov-positive peptide, and nonparametric Wilcoxon matched-pairs signed rank test used to calculate significance.

Functional attachment data were representative of three independent experiments, with one or two technical replicates per each experiment, and all values were displayed as average ± Standard Deviation (SD) of biological replicates otherwise indicated elsewhere. One-way analysis of variance (ANOVA), followed by Bonferroni Post Hoc test was used to calculate significance, unless otherwise indicated.

RESULTS

PamChip array showed similar tyrosine kinase activity profiles of MSCs on ECM and on titanium

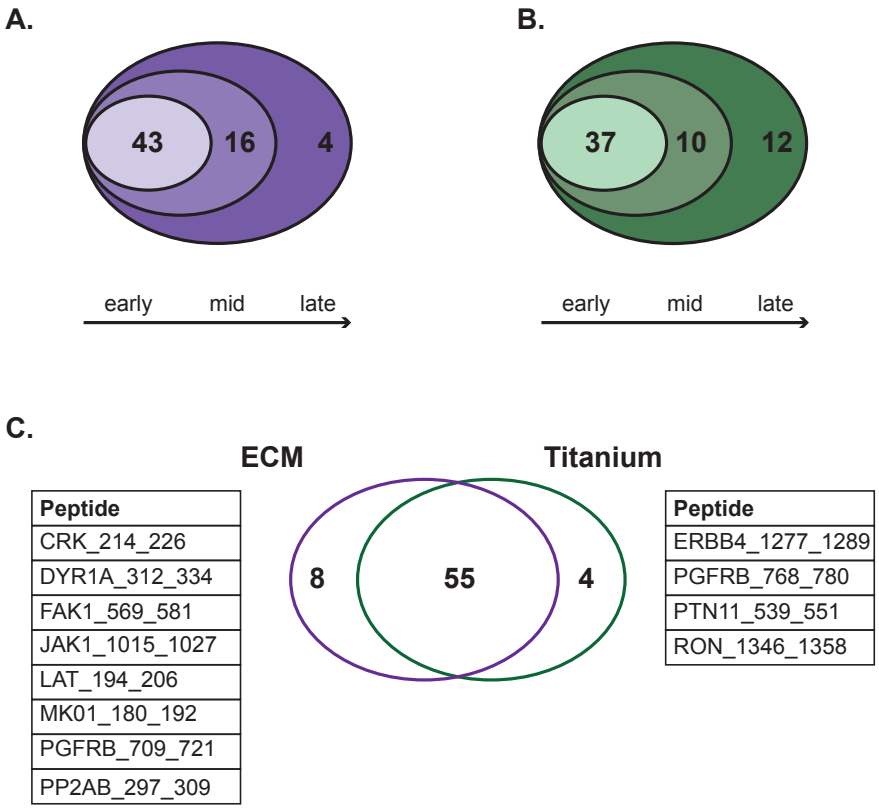
In order to investigate how surfaces influence cell behaviour, we cultured MSCs for 4 hours on either an osteopromotive osteoblast-derived ECM or on titanium and analyzed the kinome profiles by a high-throughput Tyrosine Kinase PamChip microarray system. Maximal velocity (Vmax) as the slope of phosphorylation kinetics was used as measure of peptide phosphorylation and depending on the kinetic behaviour observed, peptides were categorized as either early Vmax, mid Vmax or late Vmax (see Supplementary Figure 1A-C for examples). Following Markov state analysis, lysates obtained from MSCs cultured on ECM yielded significant phosphorylation of 43 early Vmax peptides, 16 mid Vmax peptides and 4 late Vmax peptides (Figure 1A and Supplementary Figure 2A). Thus, a total of 63 'on calls' were detected over time on ECM and considered for further analysis (Figure 1A) (complete list in Table 1).

On titanium, 37 peptides were phosphorylated with maximum reaction speed early in the analysis, with an additional 10 at mid stage and 12 at later stages (Supplementary Figure 2B), yielding a total of 59 Markov-positive peptides phosphorylated on titanium over time (Figure 1B) (complete list in Table 1). Scatter plots show the correlation of peptides at early, mid and late stage in MSCs on ECM and on titanium are shown in Supplementary Figure 2C and 2D. On both surfaces the correlation is stronger between mid and late peptides.

Figure 1C shows the number of phosphorylated peptides on ECM and on titanium. Despite a similar number of peptides being phosphorylated on the 2 substrates, cultures on ECM induced a significantly higher overall phosphorylation compared to titanium (AUC of 34.1 ± 7.6 vs 27.6 ± 6.5, $P<0.0001$, data not shown). Several of the peptides that were significantly phosphorylated early in MSCs cultured on ECM, only achieved significant phosphorylation at later time points when cells were cultured on titanium, suggesting lower levels of active kinase present in these latter lysates. Comparing the kinase activity profiles, most of the phosphorylated peptides (55) were shared between the 2 substrates, highlighting a substantial overlap between the kinome profiles of MSCs in ECM and titanium, whereas 8 kinase substrates were exclusively phosphorylated on the cell-derived ECM and 4 on titanium (Figure 1C).

PamChip array revealed activation of PI3K/AKT signaling pathway

We analyzed the identified activated kinase-mediated signaling cascades by IPA, to unravel meaningful signaling changes upon cell adhesion. The 63 kinase substrates phosphorylated on ECM and the 59 on titanium were involved in many signaling pathways (complete list in Supplementary Table 2 for ECM and Supplementary



Peptide
CRK_214_226
DYR1A_312_334
FAK1_569_581
JAK1_1015_1027
LAT_194_206
MK01_180_192
PGFRB_709_721
PP2AB_297_309

Peptide
ERBB4_1277_1289
PGFRB_768_780
PTN11_539_551
RON_1346_1358

Figure 1. Kinome profiling of MSCs cultured on cell-derived ECM and on titanium. A) 63 peptides on PamChip were phosphorylated by kinases in MSCs on ECM over time: 43 as early peptides, 16 as mid peptides and 4 as late peptides. Only Markov-positive peptides are considered. B) 59 peptides were phosphorylated on titanium over time: 37 peptides were detected since early stages, 10 were phosphorylated at mid stages and 12 at late stages. Only Markov-positive peptides are considered. C) Venn diagram showing the number of peptides phosphorylated on both ECM and titanium (55), uniquely phosphorylated on ECM (8) and uniquely phosphorylated on titanium (4). Unique peptides are indicated in the tables.

Table 3 for titanium). We further focused on intracellular signalings, not specific for a selected cell type, resulting in a total of 30 parent gene IDs of the Markov-positive peptides on ECM and on titanium, involved in 35 selected intracellular signaling pathways, as shown in the heat map in Figure 2. For ECM, the most induced kinases (11) were involved in phosphatase and tensin homolog (PTEN) signaling (P=5.01*10^{-15}), but also Tec kinase signaling (P=3.16*10^{-13}). Signaling cascades activated upon integrin activation such as FAK, PAK, Paxillin and ILK signaling pathways were also activated (P=3.98*10^{-14}, P=2.51*10^{-12}, P=3.09*10^{-10}, P=2.39*10^{-8} respectively). Of the activated kinases, 10 are identified in IPA as regulating ERK/

Table 1. List of 63 peptides phosphorylated on ECM and 59 phospho-peptides on titanium at early, mid and late stage. Peptides are in alphabetical order; numbers indicate the position of the first and last amino acid of the peptide in the complete human protein.

Peptide #	ECM			Titanium		
	early	mid	late	early	mid	late
1	ART_004_EAI-YAAPFAKKKXC			ART_004_EAIYA APFAKKKXC		
2	CD79A_181_193			CD79A_181_193		
3	CDK2_8_20			CDK2_8_20		
4	CDK7_157_169			CDK7_157_169		
5	DCX_109_121			DCX_109_121		
6	EFS_246_258			EFS_246_258		
7	ENOG_37_49			ENOG_37_49		
8	EPHA1_774_786			EPHA1_774_786		
9	EPHA2_765_777			EPHA2_765_777		
10	EPHB1_921_933			EPHB1_921_933		
11	FER_707_719			FER_707_719		
12	FES_706_718			FES_706_718		
13	FRK_380_392			FRK_380_392		
14	K2C6B_53_65			K2C6B_53_65		
15	MBP_198_210			MBP_198_210		
16	MK10_216_228			MK10_216_228		
17	NCF1_313_325			NCF1_313_325		
18	NTRK2_696_708			NTRK2_696_708		
19	P85A_600_612			P85A_600_612		
20	PAXI_111_123			PAXI_111_123		
21	PAXI_24_36			PAXI_24_36		
22	PDPK1_2_14			PDPK1_2_14		
23	PECA1_706_718			PECA1_706_718		
24	PGFRB_572_584			PGFRB_572_584		
25	PLCG1_764_776			PLCG1_764_776		
26	RAF1_332_344			RAF1_332_344		
27	RB_804_816			RB_804_816		
28	RET_1022_1034			RET_1022_1034		
29	SRC8_ CHICK_476_488			SRC8_ CHICK_476_488		
30	SRC8_ CHICK_492_504			SRC8_ CHICK_492_504		
31	TYRO3_679_691			TYRO3_679_691		
32	VGFR2_944_956			VGFR2_944_956		
33	VGFR2_989_1001			VGFR2_989_1001		
34	41_654_666				41_654_666	
35	EPHA7_607_619				EPHA7_607_619	

Peptide #	ECM			Titanium		
	early	mid	late	early	mid	late
36	JAK2_563_577				JAK2_563_577	
37	LAT_249_261				LAT_249_261	
38	PDPK1_369_381				PDPK1_369_381	
39	VGFR1_1326_1338					VGFR1_1326_1338
40	DYR1A_312_324					
41	MK01_180_192					
42	PGFRB_709_721					
43	PP2AB_297_309					
44		FAK2_572_584		FAK2_572_584		
45		PRRX2_202_214		PRRX2_202_214		
46		RASA1_453_465		RASA1_453_465		
47		EPHB1_771_783			EPHB1_771_783	
48		LCK_387_399			LCK_387_399	
49		MET_1227_1239			MET_1227_1239	
50		ANXA1_14_26				ANXA1_14_26
51		EGFR_1165_1177				EGFR_1165_1177
52		EPOR_361_373				EPOR_361_373
53		EPOR_419_431				EPOR_419_431
54		PGFRB_1002_1014				PGFRB_1002_1014
55		PGFRB_1014_1028				PGFRB_1014_1028
56		PGFRB_771_783				PGFRB_771_783
57		ZAP70_485_497				ZAP70_485_497
58		FAK1_569_581				
59		LAT_194_206				
60			TEC_512_524		TEC_512_524	
61			FGFR3_753_765			FGFR3_753_765
62			CRK_214_226			
63			JAK1_1015_1027			
64				PTN11_539_551		
65					RON_1346_1358	
66						ERBB4_1277_1289
67						PGFRB_768_780

MAPK signaling, and 7 modulate PI3K/AKT signaling (P=5.01*10^{-11} and P=2.08*10^{-08} respectively). Moreover, Mitogen-activated protein kinase 1 (MAPK1) (peptide MK01_180_192), phosphorylated only on ECM, was involved in most of the signaling pathways, together with Phosphatidylinositol 3-kinase regulatory subunit alpha (PI3KR1; peptide P85A_600_612) and Fibroblast growth factor receptor 3 (FGFR3; peptide FGFR3_753_765). These last kinase substrates were phosphorylated also on titanium, together with Tyrosine-protein phosphatase non-receptor type 11 (PTPN11; peptide PTN11_539_551), which was uniquely phosphorylated in MSCs on titanium and involved in most of the activated signaling pathways (Figure 2). Most of the kinases activated on titanium were involved in nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) signaling (P=2.51*10^{-13}), Tec kinase signaling (P=5.01*10^{-12}) and PTEN signaling (P=6.30*10^{-12}). PAK, FAK, Paxillin and ILK signaling pathways were activated on titanium as on the ECM (P=7.94*10^{-11}, P=6.30*10^{-11},

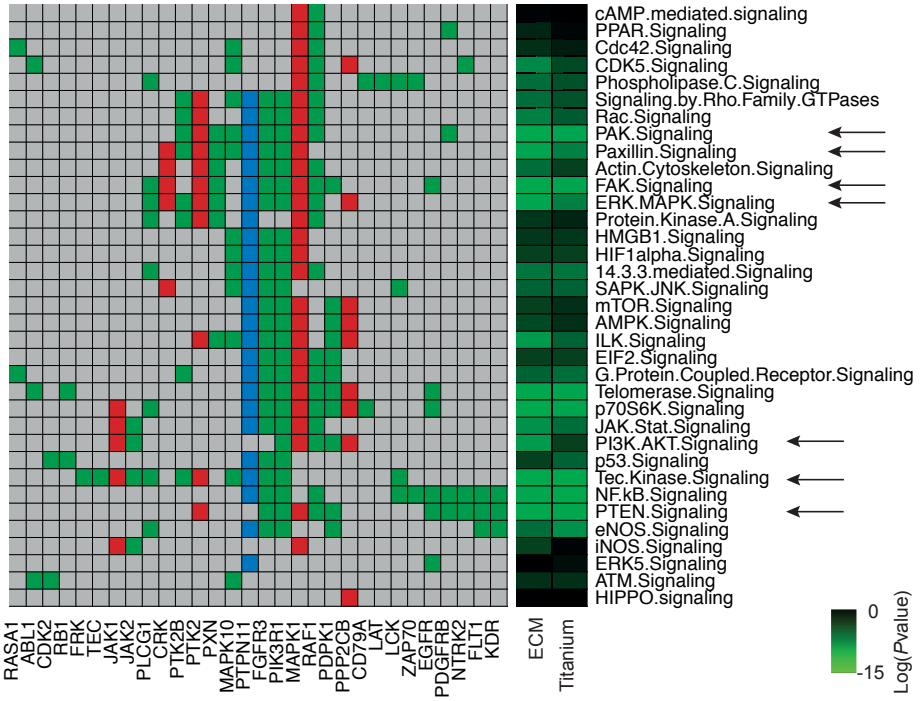


Figure 2. Comparative kinomes of MSCs on ECM and titanium. Heat-map of the 30 gene IDs of the parent proteins of Markov-positive phospho-peptides on ECM and titanium (bottom) involved in 35 selected intracellular signaling pathways (right) in IPA. Parent proteins of the Markov-positive peptides are indicated as gene IDs (bottom). Green: activated in both substrates, red: uniquely activated in ECM; blue: uniquely activated on titanium; grey: none. Color scale bar represents log (P value) of enrichment. Black arrows indicate signaling pathways highlighted in the text.

P=2.29*10^{-17}, P=5.75*10^{-6} respectively), as well as ERK/MAPK (P=3.46*10^{-7}) and PI3K/AKT (P=1.91*10^{-4}), confirming the overlap between these substrates.

IPA analysis revealed that the activated kinases were involved in multiple signaling cascades. In addition, we used the results of the peptide array to fit each kinase that phosphorylates a selected peptide into one specific signaling pathway, as previously done [32], in a more biased approach but more osteoblast-oriented (complete list in Supplementary Table 4). This approach confirmed the IPA analysis, as of the 63 kinase substrates phosphorylated on ECM, 4 induced the activation of FAK signaling and a total of 11 phospho-peptides were involved in cytoskeletal functions (Supplementary Figure 3A and 3B; Supplementary Table 5). Three peptides were clustered in MAPK signaling and 3 in PI3K signaling, illustrating that different approaches in kinase clustering lead to similar conclusions. Similar findings were found by clus-

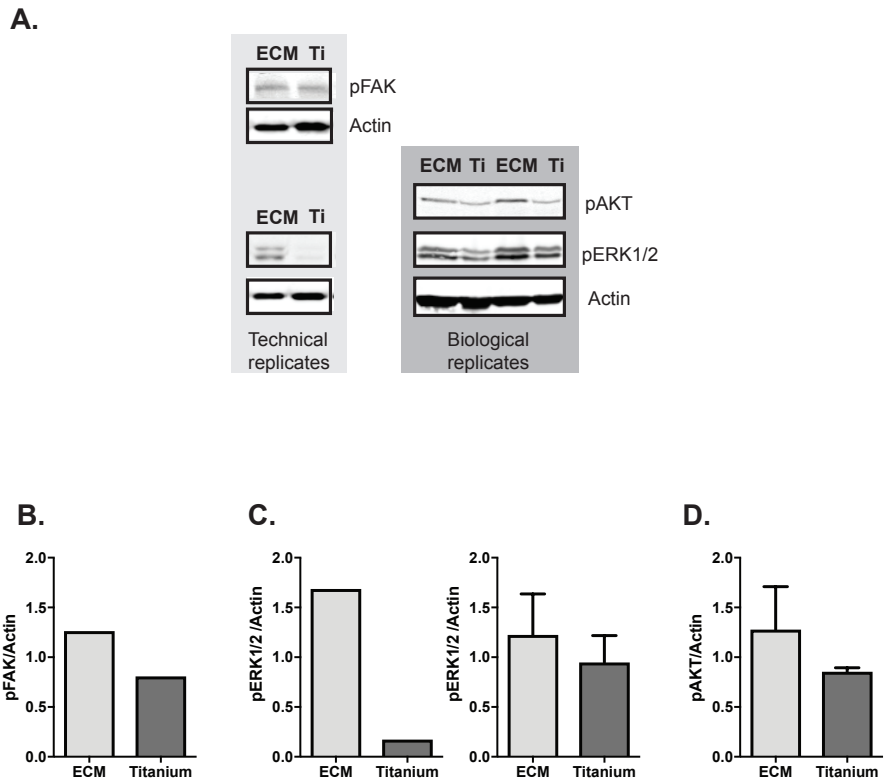


Figure 3. Immunoblot analysis of phosphoproteins confirmed the activation of specific intracellular signaling pathways revealed by PamChip. ECM: extracellular matrix, Ti: titanium. A) Western blot analysis of pFAK, pERK and pAKT in technical and biological replicates. β-actin was used as loading control. B-D) Quantification of immunoblot band intensities of the selected phosphorylated kinases over β-actin of technical and biological replicates. Bars represent Average ± SD.

tering the 59 kinase substrates phosphorylated on titanium (Supplementary Table 6). Phosphorylation of 4 peptides induce the activation of FAK signaling. Moreover, MAPK (2 peptides) and PI3K (3 peptides) signaling were activated also in cells in adhesion to titanium (Supplementary Figure 3C and 3D).

The activation of some signaling pathways on ECM and on titanium revealed by PamChip array was validated by immunoblot analysis, both in technical and biological replicates of cell lysates (Figure 3A). Overall and in line with the PamChip analyses, ECM tend to induce a higher kinase activity compared to titanium, as signaling pathways such as FAK, ERK/MAPK and PI3K/AKT pathways were more active in cells on ECM than on titanium, as shown in Figure 3B-D by the quantification of the induced kinase relative to the loading control in technical and biological replicates. This highlights the importance of the peptide array as high throughput screening technique to select candidate pathways.

Quantification of kinase substrate phosphorylation in the peptide array (Supplementary Figure 4A-C) followed the same trend as the quantification of the putative kinases of each signaling pathway by Western blot (Figure 3B-D). The activation of

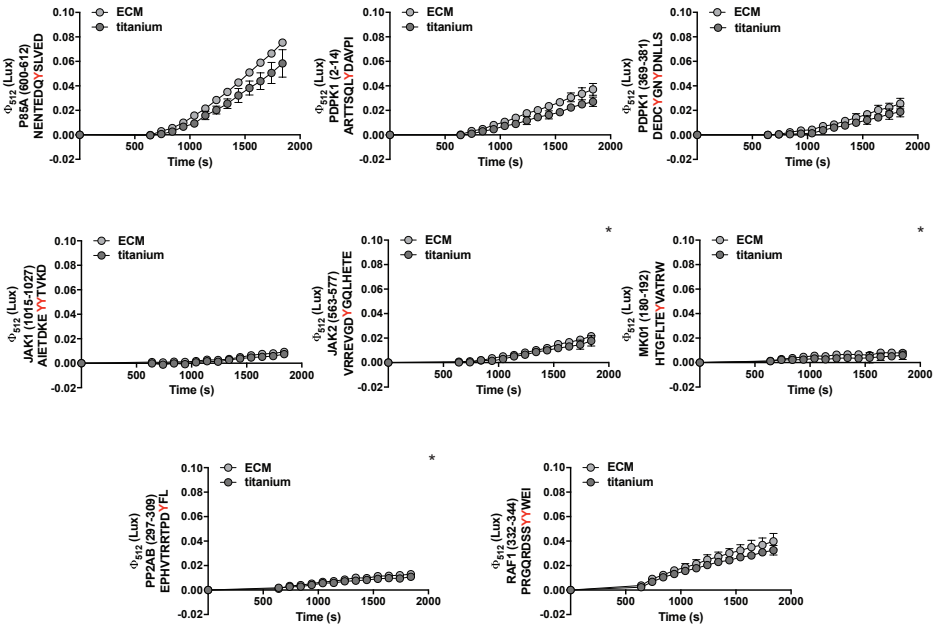


Figure 4. Temporal phosphorylation kinetics of selected peptides involved in PI3K/AKT signaling pathways as annotated by IPA. Peptide IDs and peptide sequences are displayed on Y axis; numbers indicate first and last amino acid of the complete human protein or the peptide sequence based on Uniprot annotation. Phospho-tyrosines are indicated in red. Peptides that were Markov-positive uniquely for cell lysates on ECM are indicated with *. Data represents Average ± SD of technical replicates.

signaling pathways revealed by IPA was the result of the phosphorylation of multiple kinase substrates (Supplementary Table 7). For instance, the phosphorylation of 8 peptides in PamChip revealed the activation of PI3K/AKT signaling in cells on ECM (Figure 4) (Supplementary Figure 4C), which was higher than on titanium (4 Markov-positive peptides), confirming the higher phosphorylation of pPKB on ECM compared to titanium (Figure 3A and 3D).

Functional consequences of reduced PI3K activation

PamChip microarray analysis revealed that the PI3K/AKT signaling pathway among others was activated in cells adhering to both substrates (Figure 2), but with a higher activity on ECM than on titanium (Figure 3A and 3D). The temporal kinetics of the peptides clustered in PI3K/AKT signaling are displayed in Figure 4. PI3K signaling has been shown to be important for several cellular functions, including cell adhesion. We validated this by allowing MSCs to adhere to ECM for 4 hours in the absence or presence of the PI3K kinase inhibitors Wortmannin or LY290042. Figure 5A showed that Wormannin significantly reduced cell attachment to ECM in a dose-dependent manner (P<0.001 for 10μM Wortmannin), by decreasing the number of cells in adhesion to the ECM (Figure 5A, left) and increasing the number of floating cells in culture medium (Figure 5A, right). Similarly, LY294002 increased the number of non-adherent cells (Figure 5B), albeit less efficiently. This is most likely due to residual PI3K activity, as shown in Figure 5C: both Wortmannin and LY294002 selectively inhibit PI3K signaling (as indicated by decreased phosphorylation of its downstream target, PKB) at a concentration of 10 μM, but Wortmannin showed a more prominent inhibitory effect. To confirm that the increase in non-adherent cells in response to PI3K inhibition was not due to induction of apoptosis by these compounds, MSCs on ECM were treated for 4 hours with and without PI3K inhibitors (Wortmannin 10μM and LY294002 10μM), and cleaving of PARP and Caspase 3 was measured by immunoblot analysis as a hallmark of apoptosis. The presence of cleaved caspase 3 was not detected in samples treated with and without PI3K inhibitors, nor were cleaved PARP levels increased, confirming that the PI3K inhibitors were not toxic (Figure 5D).

The phosphorylation of selected kinases such as PKB (as well as ERK) was also confirmed to be reduced in cells cultured on titanium compared to cultures on ECM, also when assessing non-adherent cells (Supplementary Figure 5A and 5B). Thus, we next investigated adhesion of MSCs to titanium and showed that while the highest concentration of PI3K inhibitors (10μM) reduced adhesion, this effect did not reach statistical significance, in accordance with the lesser PI3K activation in response to titanium engagement of MSCs (Figure 5E). Overall, our data confirm the importance of PI3K signaling in cell adhesion, and suggest that kinome activity differences observed are reflected by functional consequences in MSCs.

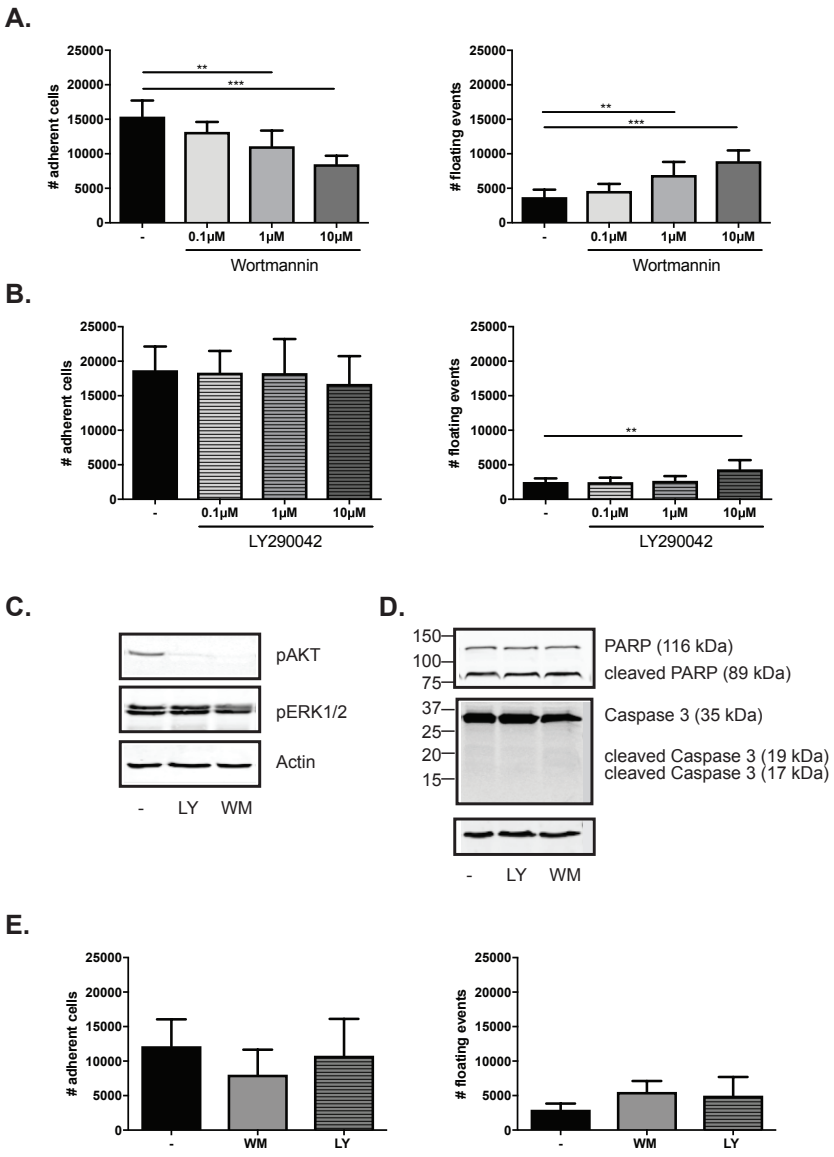


Figure 5. Functional effects of PI3K signaling inhibition. A) Number of cells in adhesion to ECM (left) for 4 hours with Wortmannin 0.1 μM, 1 μM and 10 μM and number of events floating in culture medium (right) in same culture conditions. B) Quantification of MSCs in adhesion to ECM with increasing concentrations of LY294002 (left) and floating in culture medium (right). C) Immunoblot of pAKT and pERK1/2 to confirm PI3K signaling inhibition by Wortmannin 10 μM and LY294002 10 μM in MSCs on ECM. D) Immunoblot validation of PARP, Caspase3 and the relative cleaved forms in extracts of MSCs on ECM without and with PI3K inhibitors Wortmannin 10 μM and LY294002 10 μM. E) Number of cells adherent to titanium (left) and non-adherent (right) after 4 hours of culture with Wortmannin 10 μM and LY294002 10 μM. Bars indicate Average ± SD of multiple independent experiments (N=3) (** P<0.01; *** P<0.001) (-: no inhibitors; WM: Wortmannin; LY: LY294002).

DISCUSSION

In this study, we comprehensively described the kinome profiles of human MSCs during adherence to a cell-derived ECM and to titanium, by successfully using PamChip array technology. MSCs on the two substrates showed a substantial overlap of kinase signatures. Cells on ECM typically activate kinase reactions that conform classical kinetics, *i.e.* that maximum reaction speeds are seen early in the experiment, and have higher level of active kinases. Importantly, without *a priori* assumptions, we used the PamChip kinase array to identify PI3K signaling and further functional experiments showed its importance in mesenchymal stromal cell viability and adhesion. This observation may guide rational design of novel scaffolds for tissue engineering.

Cell-surface interplay has been studied to develop biomaterials to improve bone tissue engineering [12, 24]. Upon cell adhesion to a surface, mechanical forces are converted into biochemical signals by integrins, that induce the activation of FAK, Src family kinases and an intricate network of signaling pathways, such as PI3K, MAPK ERK1/2, PKC and Rho-family GTPase, that eventually modulate cell behaviour [10]. Osteoblast adhesion is controlled mainly by PKA, PKC, RhoA proteins that promote cell cycle arrest and mediate cytoskeletal rearrangements [39]. In line with this, we showed that pathways such as FAK, PAK, Paxillin, ILK and Rho GTPase family signaling were activated upon MSC-adhesion to ECM and titanium. Moreover, PamChip kinase array revealed the activation of ERK/MAPK signaling. MAPKs are a central hub in controlling bone homeostasis, as they are activated by extracellular stimuli and ECM-mediated integrin activation via Src/FAK signaling network, but also promote osteoblast survival and differentiation by controlling osteogenic transcription factors [10, 40]. Cell adhesion to an ECM has been studied through mass spectrometry showing the high level of tyrosine phosphorylation in adhesion complexes, thus revealing the importance of kinases in cell adhesion [26, 27] The behaviour of calvarial osteoblasts has been analysed through PepChip kinase array screening technology. Milani *et al.* studied calvarial osteoblasts in adhesion to polystyrene and reported not only the induction of FAK, Src, PKA and PKC, but also kinases not directly related to cell adhesion such as GSK3β and Rap1A [41]. The activation of PKA, PKC, VEGF and Adducin-1 (ADD1) was reported in calvarial osteoblast adhesion to hydroxyapatite [42]. Recently, Marumoto *et al.* used the PepChip platform to study the interplay between ECM and osteoblasts, showing that Hedgehog signaling regulates morphological changes in calvarial osteoblasts during a 10-day culture on Matrigel™ [43], confirming some of the findings by Chaves Neto and co-workers who investigated the osteogenic differentiation on polystyrene [44]. In our study, PamChip kinase array was used to investigate how changes in the kinomic signature

regulate human MSC adhesion to diverse substrates, such as an osteoblast-derived ECM and titanium. PamChip contains a lower number of kinase substrates than PepChip, but allows kinetic measurements with strong reproducibility and giving quantification of end-point signals as well as temporal kinetics of the reaction [45]. Our PamChip results showed a big overlap between the distributions of phosphorylation in cells on the two surfaces, but also a delay in phosphorylation kinetics in cells cultured on titanium compared to MSCs on ECM, highlighting the importance of analyzing temporal kinetics.

PamChip is a cost-effective high-throughput array that can simultaneously identify rapid changes in kinome profiles [29]. PamChip and other kinase array platforms drive hypothesis formation, due to the variable number of putative upstream kinases that could phosphorylate the peptides. They represent powerful tools to select pathways that might be crucial in physiological functions, but the kinase activation needs to be validated by immunoblot analysis [32, 46]. We used IPA for functional clustering of the peptides into signaling pathways, as previously done [47], and we confirmed it by fitting each peptide in one specific signaling pathway. However, software to fit phospho-peptides into cascade signaling networks needs to be implemented with kinomic-oriented tools.

In this study, PamChip revealed the activation of PI3K/AKT in MSCs adhering to ECM and titanium, which corroborates with previous findings on polystyrene and on hydroxyapatite [41, 42]. Conversely, PI3K/AKT signaling was found to be down-regulated during osteogenic differentiation in standard culture conditions and on Matrigel [43, 44]. The PI3K/AKT signaling pathway regulates many cellular functions such as proliferation, adhesion and migration, and its activation promotes cell survival [48]. In osteoblasts, PI3K was shown to mediate BMP2 induction of osteogenic differentiation [49-52] and to interact with RUNX2 in controlling osteoblast and chondrocyte differentiation and migration [53], though findings are still controversial regarding the influence of PI3K signaling in osteogenic differentiation [54, 55]. PI3K signaling is involved in integrin-mediated signal transduction during cell adhesion [56, 57]. In this study, we showed that PI3K is involved in osteoblast adhesion to ECM and titanium, in agreement to previous studies where PI3K-mediated AKT activity was shown to be reduced when PI3K inhibitors were present in COS7 cells and mesenchymal stromal cells in adhesion to fibronectin [58, 59]. When PI3K inhibitors were used, the number of non-adherent cells increased, with a stronger effect on ECM than titanium. This is probably due to the fact that PI3K signaling was more active on ECM than on titanium, as shown by the temporal kinetics of the PI3K kinase substrates, thus making it easier to visualize the inhibitory effect and highlighting the importance of performing kinetic analyses.

The mechanisms of cell adhesion to titanium has been previously investigated by using FAK and Src phosphorylation as biomarkers to monitor cell/biomaterials interplay, as FAK and Src have been proven to be phosphorylated upon integrin activation in cells when adhering to different substrates [39, 60, 61]. Our study using the PamChip kinase array showed FAK signaling activation upon adhesion to titanium, confirming these previous findings. Further studies are needed to investigate how the cell-derived ECM as coating for titanium scaffolds would influence the kinome profile and osteoblast adhesion, to implement the use of ECM and titanium in bone tissue engineering applications.

In summary, with this study we employed a multiplex peptide array technology to assess global tyrosine kinase changes upon cell adhesion, showing that osteoblasts adhering to ECM exhibited a similar kinase signature compared to titanium, but with higher levels of active kinases present in MSCs on ECM. We successfully used PamChip kinase substrate platform to comprehensively study rapid changes in the phosphoproteomes of MSCs, and to investigate specific pathways, highlighting the importance of PI3K signaling in osteoblast viability and adhesion. We thus contributed to disentangle kinomic changes upon cell adhesion to different substrates, to develop biomaterials to improve bone tissue engineering applications.

ACKNOWLEDGEMENTS

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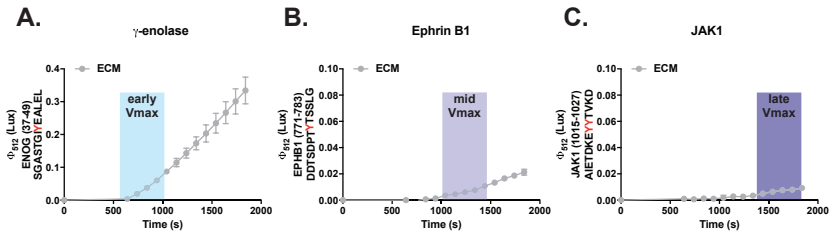
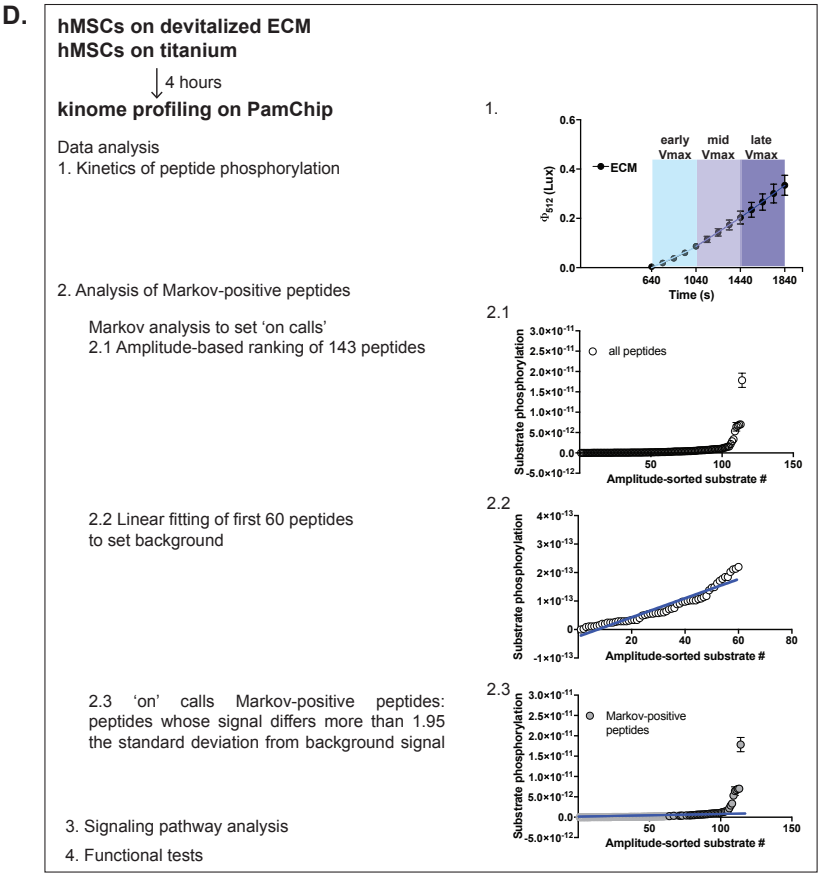
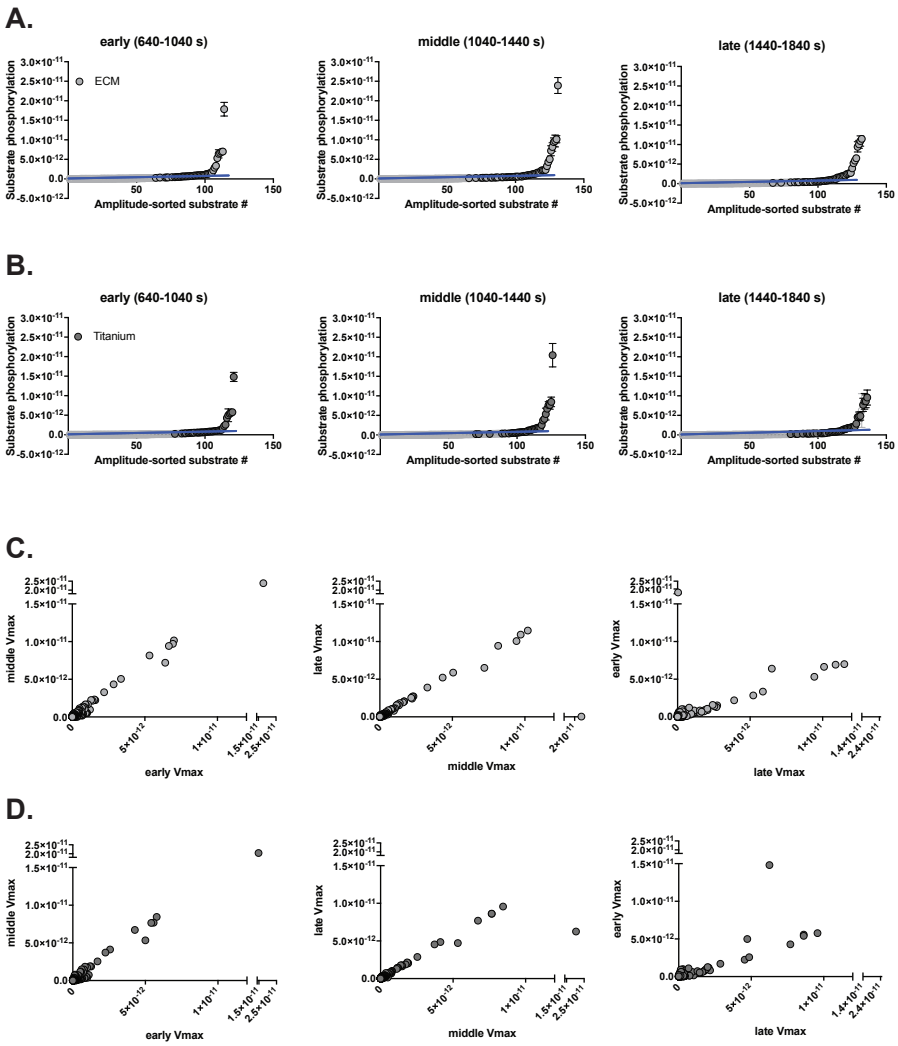
Author Contributions: All authors designed research. MB, GF, WZ performed research. MB, GF, JP, JL, MP and BE analyzed data and drafted manuscript. All authors revised the final version of manuscript and have approved the final article.

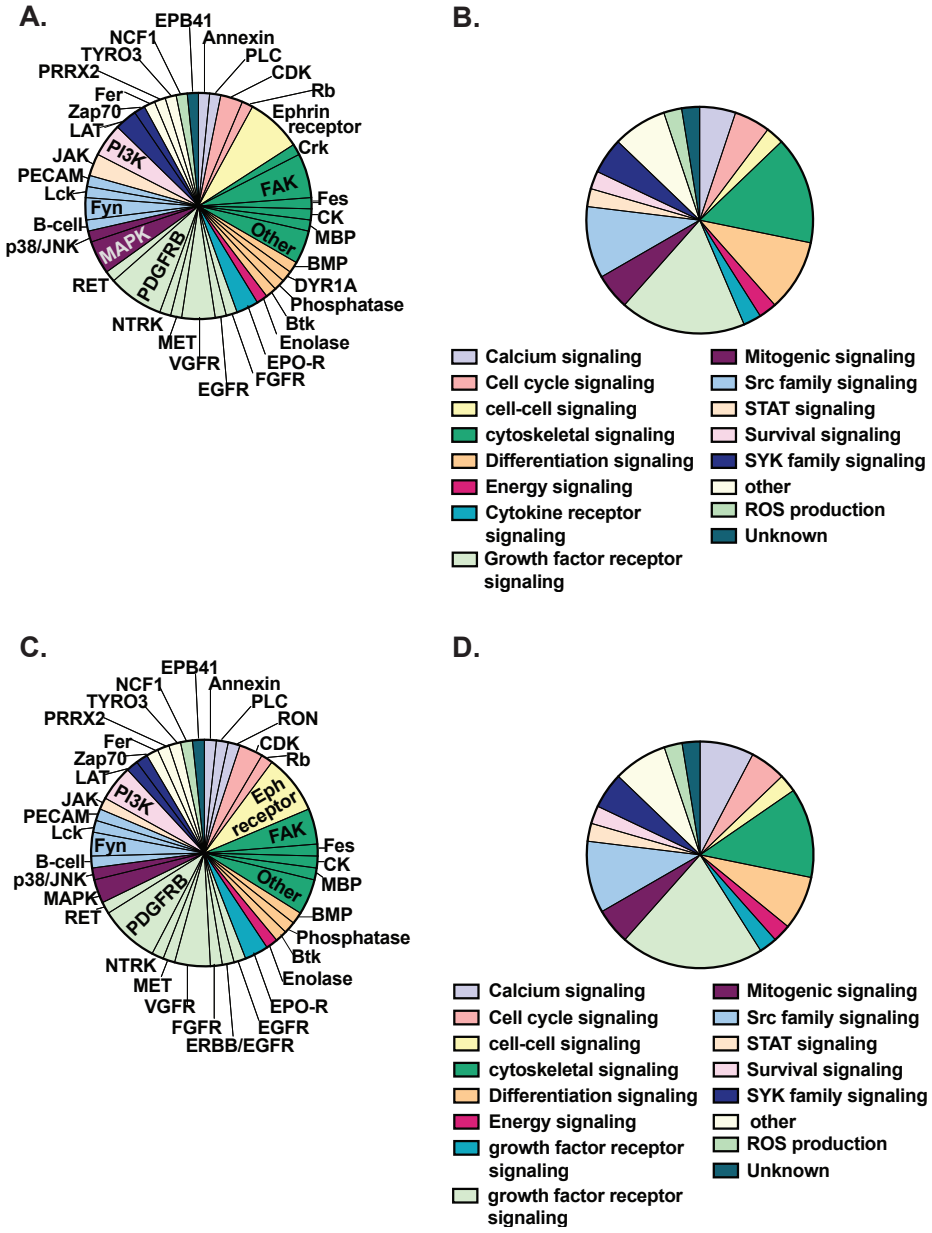




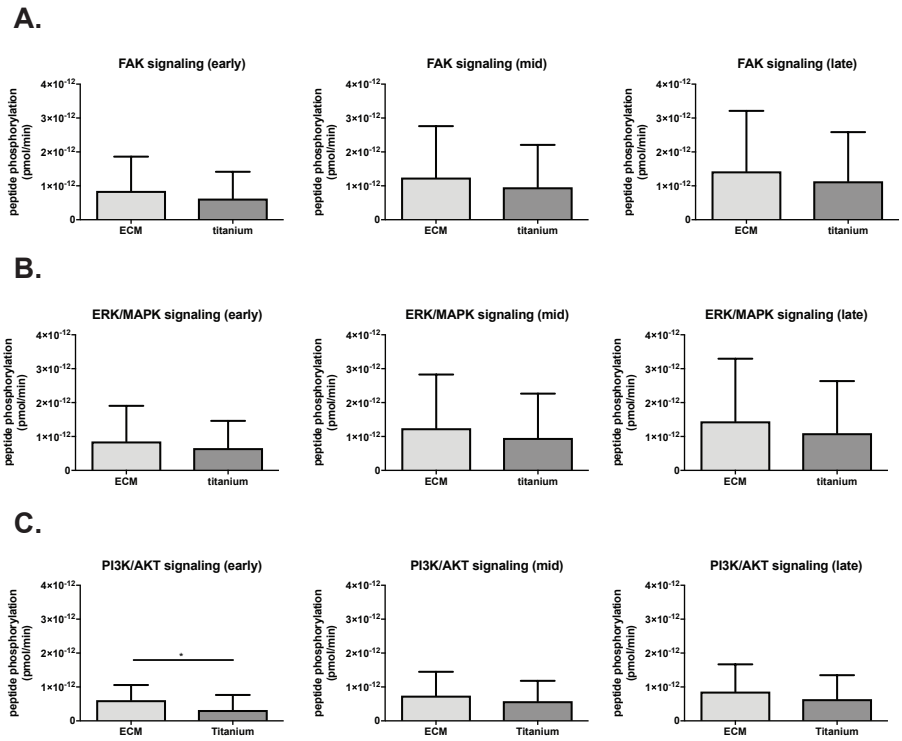
Supplementary Figure 1. A) Example of phosphorylation kinetic over time of ENOG_37_49, classified as early Vmax peptide. Vmax was calculated as tangent of apparent phosphorylation between 640-1040 s. B) Phosphorylation kinetic of EPHB1_771_783 as example of mid Vmax (1040-1440 s), and C) of JAK1_1015_1027 as late Vmax (1440-1840 s). Data: Average ± SD. D) Schematic overview of kinome profiling analysis. 1) Cell lysates of MSCs on ECM and titanium were loaded on PamChip, phosphorylation kinetics were analyzed, 2) Markov-positive phospho-peptides on ECM and titanium were selected by amplitude-ranking all the 143 peptides (2.1), linear-fitting the first 60 peptides to set the background (blue line) (2.2), and selecting as Markov-positive the peptides whose intensity differed more than 1.95 the standard deviation from the background (2.3). 3) Markov-positive peptides on ECM and titanium were further analyzed using IPA for signaling pathway analysis and 4) selected pathways were further used for functional testing.



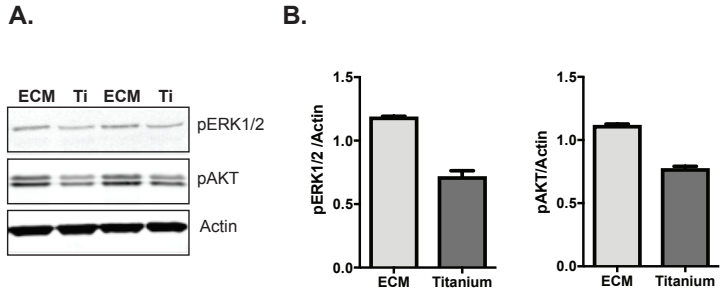
Supplementary Figure 2. Markov-positive peptides on ECM and titanium. A) Peptides whose intensity of phosphorylation differed more than 1.95 from background signal (blue line) were considered as Markov-positive, on ECM for early Vmax (left), mid (centre) and late (right). B) Early (left), mid (centre) and late (right) Markov-positive peptides on titanium. Data: Average ± SD. C) Correlation plots of Markov-positive peptides on ECM and D) on titanium, comparing early and mid peptides (left), mid and late (centre) and late versus early peptides (right).



Supplementary Figure 3. Clustering of phosphorylated peptides into signaling pathways. A) Clustering of the 63 peptides phosphorylated on ECM into signaling pathways and B) into cell function. C) Signaling pathway clustering of the 59 Markov-positive peptides phosphorylated on titanium and D) cell-related functions.



Supplementary Figure 4. Quantification of phosphorylation of the indicated peptides in the PamChip array representative of the activated signaling pathways by IPA analysis. A) FAK signaling, B) ERK/MAPK signaling, C) PI3K/AKT signaling. Bars represent Average ± SD of Vmax of phosphorylation (early, mid and late on left, central and right side, respectively) (*, $P<0.05$).



Supplementary Figure 5. Immunoblot analysis confirmed the activation of signaling pathways revealed by PamChip. A) Western blot analysis of pERK1/2 and pAKT in merged lysates of cells adherent and non-adherent to ECM and titanium (β-actin: loading control). B) Quantification of phosphoproteins over actin of technical replicates. Bars represent Average ± SD of intensity.

Supplementary Table 1. List of the 144 peptides on Pamchip array. Peptides are alphabetically ordered. Protein and Gene identifiers are based on Uniprot Knowledgebase. Numbers in the Peptide IDs indicate where the first and the last amino acid of the peptide are located in the complete human protein. Dark grey: artificial peptide ART_003, internal positive control, not considered for further analysis. Light grey: artificial peptide ART_004 considered for further analysis.

Peptide #	Peptide ID	Phosphorylation site	Peptide sequence	Uniprot ID	Gene ID	Encoding protein
1	41_654_666	Y660	LDGENIYIRHSNL	P11171	EPB41	Protein 4.1
2	ACHD_383_395	Y390	YISKAEEYFLLKS	Q07001	CHRND	Acetylcholine receptor subunit delta
3	AMPE_5_17	Y12	EREGSKRYCIQTK	Q07075	ENPEP	Glutamyl aminopeptidase
4	ANXA1_14_26	Y21	IENEEQEYVQTVK	P04083	ANXA1	Annexin A1
5	ANXA2_17_29	Y24	HSTPPSAYGSVKA	P07355	ANXA2	Annexin A2
6	ART_003_EAI(pY)AAPFAKKKXC		EAI(pY)AAPFAKKKXC			Artificial peptide, positive control
7	ART_004_EAIYAAPFAKKKXC		EAIYAAPFAKKKXC	P00519	ABL1	Tyrosine-protein kinase ABL1
8	B3AT_39_51	Y46	TEATATDYHTTSH	P02730	SLC4A1	Band 3 anion transport protein
9	C1R_199_211	Y204/S210	TEASGYISSLEYP	P00736	C1R	Complement C1r subcomponent
10	CALM_93_105	Y100	FDKDGNGYISAAE	P62158	CALM1	Calmodulin
11	CALM_95_107	Y100	KDGNGYISAAELR			
12	CBL_693_705	Y700	EGEEDTEYMTPSS	P22681	CBL	E3 ubiquitin-protein ligase CBL
13	CD3Z_116_128	Y123	KDKMAEAYSEIGM	P20963	CD247	T-cell surface glycoprotein CD3 zeta chain
14	CD3Z_146_158	Y153	STATKDTYDALHM			
15	CD79A_181_193	Y182/Y188	EYEDENLYEGLNL	P11912	CD79A	B-cell antigen receptor complex-associated protein alpha chain
16	CDK2_8_20	Y15/Y19	EKIGEGTYGVVYK	P24941	CDK2	Cyclin-dependent kinase 2
17	CDK7_157_169	Y169	GLAKSFGSPNRAY	P50613	CDK7	Cyclin-dependent kinase 7
18	CRK_214_226	Y221	GPPEPGPYAQPSV	P46108	CRK	Adapter molecule crk
19	CTNB1_79_91	Y86	VADIDGQYAMTRA	P35222	CTNNB1	Catenin beta-1
20	DCX_109_121	Y112	GIVYAVSSDRFRS	O43602	DCX	Neuronal migration protein doublecortin
21	DDR1_506_518	Y513	LLLSNPAYRLLLA	Q08345	DDR1	Epithelial discoidin domain-containing receptor 1
22	DYR1A_212_224	Y219/Y220	KHDTEMKYYIVHL	Q13627	DYRK1A	Dual specificity tyrosine-phosphorylation-regulated kinase 1A
23	DYR1A_312_324	Y319/Y321	CQLGQRIYQYIQS			
24	EFS_246_258	Y253	GGTDEGIYDVPLL	O43281	EFS	Embryonal Fyn-associated substrate
25	EFS_246_258_Y253F	Y253F	GGTDEGIFDVPLL			

Peptide #	Peptide ID	Phosphory-lation site	Peptide sequence	Uniprot ID	Gene ID	Encoding protein
26	EGFR_1062_1074	Y1069	EDSFLQRYSSDPT	P00533	EGFR	Epidermal growth factor receptor
27	EGFR_1103_1115	Y1110	GSVQNPVYHNQPL			
28	EGFR_1118_1130	Y1125	APSRDPHYQDPHS			
29	EGFR_1165_1177	Y1172	ISLDNPDYQQDFF			
30	EGFR_1190_1202	Y1197	STAENAEYLRVAP			
31	EGFR_862_874	Y869	LGAEEKEYHAEGG			
32	EGFR_908_920	Y915	MTFGSKPYDGIPA			
33	ENOG_37_49	Y44	SGASTGIYEALEL	P09104	ENO2	Gamma-enolase
34	EPHA1_774_786	Y781	LDDFDGTYETQGG	P21709	EPHA1	Ephrin type-A receptor 1
35	EPHA2_581_593	Y588	QLKPLKTYVDPHT	P29317	EPHA2	Ephrin type-A receptor 2
36	EPHA2_765_777	Y772	EDDPEATYTTSGG			
37	EPHA4_589_601	Y596	LNQGVRTYVDPFT	P54764	EPHA4	Ephrin type-A receptor 4
38	EPHA4_921_933	Y928	QAIKMDRYKDNFT			
39	EPHA7_607_619	Y608/Y614	TYIDPETYEDPNR	Q15375	EPHA7	Ephrin type-A receptor 7
40	EPHB1_771_783	Y778	DDTSDPTYTSSLG	P54762	EPHB1	Ephrin type-B receptor 1
41	EPHB1_921_933	Y928	SAIKMVQYRDSFL			
42	EPHB4_583_595	Y590	IGHGTKVYIDPFT	P54760	EPHB4	Ephrin type-B receptor 4
43	EPOR_361_373	Y368	SEHAQDTYLVLDK	P19235	EPOR	Erythropoietin receptor
44	EPOR_419_431	Y426	ASAASFEYTILDP			
45	ERBB2_1241_1253	Y1248	PTAENPEYLGLDV	P04626	ERBB2	Receptor tyrosine-protein kinase erbB-2
46	ERBB2_870_882	Y877	LDIDETEYHADGG			
47	ERBB4_1181_1193	Y1188	QALDNPEYHNASN	Q15303	ERBB4	Receptor tyrosine-protein kinase erbB-4
48	ERBB4_1277_1289	Y1284	IVAENPEYLSEFS			
49	FABPH_13_25	Y20	DSKNFDDYMKSLG	P05413	FABP3	Fatty acid-binding protein, heart
50	FAK1_569_581	Y570/Y576/ Y577	RYMEDSTYYKASK	Q05397	PTK2	Focal adhesion kinase
51	FAK2_572_584	Y573/Y579/ Y580	RYIEDEDYYKASV	Q14289	PTK2B	Protein-tyrosine kinase 2-beta
52	FER_707_719	Y714	RQEDGGVYSSSGL	P16591	FER	Tyrosine-protein kinase Fer
53	FES_706_718	Y713	REEADGVYAASGG	P07332	FES	Tyrosine-protein kinase Fes/Fps
54	FGFR1_761_773	Y766	TSNQEYLDLSMPL	P11362	FGFR1	Fibroblast growth factor receptor 1
55	FGFR2_762_774	Y769	TLTTNEEYLDLSQ	P21802	FGFR2	Fibroblast growth factor receptor 2
56	FGFR3_641_653	Y647/Y648	DVHNLDYYKKTTN	P22607	FGFR3	Fibroblast growth factor receptor 3
57	FGFR3_753_765	Y760	TVTSTDEYLDLSA			
58	FRK_380_392	Y387	KVDNEDIYESRHE	P42685	FRK	Tyrosine-protein kinase FRK
59	INSR_1348_1360	Y1355	SLGFKRSYEEHIP	P06213	INSR	Insulin receptor
60	INSR_992_1004	Y992/Y999	YASSNPEYLSASD			

Peptide #	Peptide ID	Phosphory-lation site	Peptide sequence	Uniprot ID	Gene ID	Encoding protein
61	JAK1_1015_1027	Y1022/ Y1023	AIETDKEYYTVKD	P23458	JAK1	Tyrosine-protein kinase JAK1
62	JAK2_563_577	Y570	VRREVGDYGQLHETE	O60674	JAK2	Tyrosine-protein kinase JAK2
63	K2C6B_53_65	Y62	GAGFGSRSLYGLG	P04259	KRT6B	Keratin, type II cytoskeletal 6B
64	K2C8_425_437	Y427/S437	SAYGGLTSPGLSY	P05787	KRT8	Keratin, type II cytoskeletal 8
65	KSYK_518_530	Y525/Y526	ALRADENYYKAQT	P43405	SYK	Tyrosine-protein kinase SYK
66	LAT_194_206	Y200	MESIDDYVNVPES	O43561	LAT	Linker for activation of T-cells family member 1
67	LAT_249_261	Y255	EEGAPDYENLQEL			
68	LCK_387_399	Y394	RLIEDNEYTAREG	P06239	LCK	Tyrosine-protein kinase Lck
69	MBP_198_210	Y203	ARTAHYGSLPQKS	P02686	MBP	Myelin basic protein
70	MBP_259_271	Y261/Y268	FGYGGRASDYKSA			
71	MBP_263_275	Y268	GRASDYKSAHKGF			
72	MET_1227_1239	Y1230/ Y1234/ Y1235	RDMYDKEYYSVHN	P08581	MET	Hepatocyte growth factor receptor
73	MK01_180_192	Y187	HTGFLTEYVATRW	P28482	MAPK1	Mitogen-activated protein kinase 1
74	MK01_198_210	Y205	IMLNSKGYTKSID			
75	MK07_211_223	Y215/Y220	AEHQYFMTEYVAT	Q13164	MAPK7	Mitogen-activated protein kinase 7
76	MK10_216_228	Y223/Y228	TSFMMTPYVVTRY	P53779	MAPK10	Mitogen-activated protein kinase 10
77	MK12_178_190	Y185	ADSEMTGYVVTRW	P53778	MAPK12	Mitogen-activated protein kinase 12
78	MK14_173_185	Y182	RHTDDEMTGYVAT	Q16539	MAPK14	Mitogen-activated protein kinase 14
79	NCF1_313_325	Y324	QRSRKRLSQDAYR	P14598	NCF1	Neutrophil cytosol factor 1
80	NPT2A_501_513	Y511	AKALGKRTAKYRW	Q06495	SLC34A1	Sodium-dependent phosphate transport protein 2A
81	NTRK1_489_501	Y496	HIIENPQYFSDAC	P04629	NTRK1	High affinity nerve growth factor receptor
82	NTRK2_509_521	Y516	PVIENPQYFGITN	Q16620	NTRK2	BDNF/NT-3 growth factors receptor
83	NTRK2_696_708	Y702/Y706/ Y707	GMSRDVYSTDYYR			
84	ODBA_340_352	Y345	DDSSAYRSVDEVN	P12694	BCKDHA	2-oxoisovalerate dehydrogenase subunit alpha, mitochondrial
85	ODPAT_291_303	Y299	SMSDPGVSYRTRE	P29803	PDHA2	Pyruvate dehydrogenase E1 component subunit alpha, testis-specific form, mitochondrial
86	P85A_600_612	Y607	NENTEDQYSLVED	P27986	PIK3R1	Phosphatidylinositol 3-kinase regulatory subunit alpha
87	PAXI_111_123	Y118	VGEEEHVYSFPNK	P49023	PXN	Paxillin
88	PAXI_24_36	Y31/Y33	FLSEETPYSYPTG			

Peptide #	Peptide ID	Phosphory-lation site	Peptide sequence	Uniprot ID	Gene ID	Encoding protein
89	PDPK1_2_14	Y9	ARTTSQLYDAVPI	O15530	PDPK1	3-phosphoinositide-dependent protein kinase 1
90	PDPK1_369_381	Y373/Y376	DEDCYGNYDNLLS			
91	PECA1_706_718	Y713	KKDTETVYSEVRK	P16284	PECAM1	Platelet endothelial cell adhesion molecule
92	PERI_458_470	Y470	QRSELDKSSAHSY	P41219	PRPH	Peripherin
93	PGFRB_1002_1014	Y1009	LDTSSVLYTAVQP	P09619	PDGFRB	Platelet-derived growth factor receptor beta
94	PGFRB_1014_1028	Y1021	PNEGDNDYIIPLPDP			
95	PGFRB_572_584	Y579/Y581	VSSDGHEYIYVDP			
96	PGFRB_709_721	Y716	RPPSAELYSNALP			
97	PGFRB_768_780	Y771/Y775/ Y778	SSNYMAPYDNYVP			
98	PGFRB_771_783	Y771/Y775/ Y778	YMAPYDNYVPSAP			
99	PLCG1_1246_1258	Y1253	EGSFESRYQQPFE	P19174	PLCG1	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase gamma-1
100	PLCG1_764_776	Y771/Y775	IGTAEPDYGALYE			
101	PLCG1_776_788	Y783	EGRNPGFYVEANP			
102	PP2AB_297_309	Y307	EPHVTRRTPDYFL	P62714	PPP2CB	Serine/threonine-protein phosphatase 2A catalytic subunit beta isoform
103	PRGR_545_557	Y557	LRPDSEASQSPQY	P06401	PGR	Progesterone receptor
104	PRGR_786_798	Y795	EQRMKESSFYSLC			
105	PRRX2_202_214	Y208/Y214	WTASSPYSTVPPY	Q99811	PRRX2	Paired mesoderm homeobox protein 2
106	PTN11_539_551	Y546/Y551	SKRKGHEYTNIKY	Q06124	PTPN11	Tyrosine-protein phosphatase non-receptor type 11
107	RAF1_332_344	Y340/Y341	PRGQRDSSYYWEI	P04049	RAF1	RAF proto-oncogene serine/ threonine-protein kinase
108	RASA1_453_465	Y460	TVDGKEIYNTIRR	P20936	RASA1	Ras GTPase-activating protein 1
109	RB_804_816	Y805/S813	IYISPLKSPYKIS	P06400	RB1	Retinoblastoma-associated protein
110	RBL2_99_111	Y111	VPTVSKGTVEGNY	Q08999	RBL2	Retinoblastoma-like protein 2
111	RET_1022_1034	Y1029	TPSDSLIYDDGLS	P07949	RET	Proto-oncogene tyrosine-protein kinase receptor Ret
112	RET_680_692	Y687	AQAFPVSYSSSGA			
113	RON_1346_1358	Y1353	SALLGDHYVQLPA	Q04912	MST1R	Macrophage-stimulating protein receptor
114	RON_1353_1365	Y1353/ Y1360	YVQLPATYMNLGP			
115	SRC8_CHICK_470_482	Y477	VSQREAEYEPETV	Q01406	CTTN1	Src substrate protein p85
116	SRC8_CHICK_476_488	Y477/Y483	EYEPETVYEVAGA			
117	SRC8_CHICK_492_504	Y492/Y499/ Y502	YQAEENTYDEYEN			

Peptide #	Peptide ID	Phosphory-lation site	Peptide sequence	Uniprot ID	Gene ID	Encoding protein
118	STA5A_687_699	Y694	LAKAVDGYVKPQI	P42229	STAT5A	Signal transducer and activator of transcription 5A
119	STAT1_694_706	Y701	DGPKGTGYIKTEL	P42224	STAT1	Signal transducer and activator of transcription 1-alpha/beta
120	STAT3_698_710	Y705	DPGSAAPYLKTKF	P40763	STAT3	Signal transducer and activator of transcription 3
121	STAT4_686_698	Y693	TERGDKGYVPSVF	Q14765	STAT4	Signal transducer and activator of transcription 4
122	STAT4_714_726	Y725	PSDLLPMSPSVYA			
123	STAT6_634_646	Y641	MGKDGRGYVPATI	P42226	STAT6	Signal transducer and activator of transcription 6
124	TEC_512_524	Y513/Y519	RYFLDDQYTSSSG	P42680	TEC	Tyrosine-protein kinase Tec
125	TNNT1_2_14	Y9	SDTEEQEYEEEQP	P13805	TNNT1	Troponin T, slow skeletal muscle
126	TYRO3_679_691	Y681/Y685/ Y686	KIYSGDYYRQGCA	Q06418	TYRO3	Tyrosine-protein kinase receptor TYRO3
127	VGFR1_1040_1052	Y1048	DFGLARDIYKNPD	P17948	FLT1	Vascular endothelial growth factor receptor 1
128	VGFR1_1046_1058_ Y1048F	Y1053	DIFKNPDYVRKGD			
129	VGFR1_1049_1061	Y1053	KNPDYVRKGDTRL			
130	VGFR1_1162_1174	Y1169	VQQDGKDYIPINA			
131	VGFR1_1206_1218	Y1213	GSSDDVRYVNAFK			
132	VGFR1_1235_1247	Y1242	ATSMFDDYQGDSS			
133	VGFR1_1320_1332_ C1320S/C1321S	Y1327	SSSPPPDYNSVVL			
134	VGFR1_1326_1338	Y1327/ Y1333	DYNSVVLYSTPPI			
135	VGFR2_1046_1058	Y1054	DFGLARDIYKDPD	P35968	KDR	Vascular endothelial growth factor receptor 2
136	VGFR2_1052_1064	Y1054/ Y1059	DIYKDPDYVRKGD			
137	VGFR2_1168_1180	Y1175	AQQDGKDYIVLPI			
138	VGFR2_1207_1219_ C1208S	Y1214	VSDPKFHYDNTAG			
139	VGFR2_944_956	Y951	RFRQGKDYVGAIP			
140	VGFR2_989_1001	Y996	EEAPEDLYKDFLT			
141	VGFR3_1061_1073	Y1063/ Y1068	DIYKDPDYVRKGS	P35916	FLT4	Vascular endothelial growth factor receptor 3
142	VINC_815_827	Y822	KSFLDSGYRILGA	P18206	VCL	Vinculin
143	ZAP70_485_497	Y492/Y493	ALGADDSYYTARS	P43403	ZAP70	Tyrosine-protein kinase ZAP-70
144	ZBT16_621_633	Y630	LRTHNGASPYQCT	Q05516	ZBTB16	Zinc finger and BTB domain-containing protein 16

Supplementary Table 2. List of Ingenuity Canonical Pathways in which the 63 Markov-positive peptides on ECM were involved. Markov-positive phosphopeptides are indicated as Gene IDs. Bold: selected intracellular pathways considered for further analysis.

Ingenuity Canonical Pathways	Gene IDs of phosphopeptides
Leukocyte Extravasation Signaling	PXN,PTK2B,MAPK1,PIK3R1,ABL1,PLCG1,CRK,TEC,FGFR3,PTK2,NCF1,FER,MAPK10,PECAM1,CTTN
PDGF Signaling	FGFR3,RAF1,JAK1,MAPK1,PIK3R1,ABL1,PLCG1,CRK,JAK2,RASA1,PDGFRB
Axonal Guidance Signaling	EPHA7,RAF1,PXN,FES,MAPK1,PIK3R1,EPHA1,ABL1,PLCG1,CRK,FGFR3,PTK2,MET,EPHB1,NTRK2,RASA1,EPHA2
STAT3 Pathway	FGFR3,RAF1,NTRK2,MAPK1,FLT1,MAPK10,JAK2,KDR,EGFR,PDGFRB
Pancreatic Adenocarcinoma Signaling	FGFR3,RB1,RAF1,JAK1,MAPK1,PIK3R1,MAPK10,ABL1,JAK2,CDK2,EGFR
PTEN Signaling	PTK2,FGFR3,RAF1,NTRK2,MAPK1,FLT1,PIK3R1,PDPK1,KDR,EGFR,PDGFRB
Ephrin Receptor Signaling	PTK2,EPHA7,RAF1,EPHB1,PXN,MAPK1,EPHA1,ABL1,CRK,JAK2,EPHA2,RASA1
FAK Signaling	PTK2,FGFR3,RAF1,PXN,MAPK1,PIK3R1,PLCG1,PDPK1,CRK,EGFR
VEGF Signaling	PTK2,FGFR3,RAF1,PXN,MAPK1,FLT1,PTK2B,PIK3R1,PLCG1,KDR
IGF-1 Signaling	PTK2,FGFR3,RAF1,PXN,JAK1,MAPK1,PIK3R1,PDPK1,JAK2,RASA1
T Cell Receptor Signaling	TEC,FGFR3,RAF1,LCK,MAPK1,PIK3R1,ZAP70,LAT,PLCG1,RASA1
HGF Signaling	PTK2,MET,FGFR3,RAF1,PXN,MAPK1,PIK3R1,MAPK10,PLCG1,CDK2
IL-15 Signaling	PTK2,FGFR3,RAF1,LCK,JAK1,MAPK1,PIK3R1,PLCG1,JAK2
Molecular Mechanisms of Cancer	RAF1,JAK1,MAPK1,CDK7,PIK3R1,ABL1,CRK,JAK2,FGFR3,PTK2,RB1,MAPK10,RASA1,CDK2
Non-Small Cell Lung Cancer Signaling	FGFR3,RB1,RAF1,MAPK1,PIK3R1,ABL1,PLCG1,PDPK1,EGFR
Tec Kinase Signaling	PTK2,TEC,FGFR3,LCK,JAK1,PTK2B,PIK3R1,MAPK10,PLCG1,JAK2,FRK
G alpha 12/13 Signaling	PTK2,TEC,FGFR3,RAF1,PXN,MAPK1,PTK2B,PIK3R1,MAPK10,RASA1
p70S6K Signaling	FGFR3,PPP2CB,RAF1,JAK1,MAPK1,PIK3R1,PLCG1,PDPK1,CD79A,EGFR
PAK Signaling	PTK2,FGFR3,RAF1,PXN,MAPK1,PTK2B,PIK3R1,MAPK10,PDGFRB
EGF Signaling	FGFR3,RAF1,JAK1,MAPK1,PIK3R1,PLCG1,RASA1,EGFR
Glioma Signaling	FGFR3,RB1,RAF1,MAPK1,PIK3R1,ABL1,PLCG1,EGFR,PDGFRB
Telomerase Signaling	FGFR3,RB1,PPP2CB,RAF1,MAPK1,PIK3R1,ABL1,PDPK1,EGFR
GDNF Family Ligand-Receptor Interactions	FGFR3,RAF1,MAPK1,PIK3R1,MAPK10,PLCG1,RET,RASA1
Renin-Angiotensin Signaling	PTK2,FGFR3,RAF1,MAPK1,PTK2B,PIK3R1,MAPK10,PLCG1,JAK2
Erythropoietin Signaling	FGFR3,RAF1,MAPK1,EPOR,PIK3R1,PLCG1,PDPK1,JAK2
NF-kB Signaling	FGFR3,RAF1,LCK,NTRK2,FLT1,PIK3R1,ZAP70,KDR,EGFR,PDGFRB
IL-8 Signaling	PTK2,FGFR3,RAF1,MAPK1,FLT1,PTK2B,PIK3R1,MAPK10,KDR,EGFR
ERK/MAPK Signaling	PTK2,FGFR3,PPP2CB,RAF1,PXN,MAPK1,PTK2B,PIK3R1,PLCG1,CRK
Prostate Cancer Signaling	FGFR3,RB1,RAF1,MAPK1,PIK3R1,ABL1,PDPK1,CDK2
ErbB Signaling	FGFR3,RAF1,MAPK1,PIK3R1,MAPK10,PLCG1,PDPK1,EGFR
CTLA4 Signaling in Cytotoxic T Lymphocytes	FGFR3,PPP2CB,LCK,PIK3R1,ZAP70,LAT,PLCG1,JAK2
Integrin Signaling	PTK2,FGFR3,RAF1,PXN,MAPK1,PIK3R1,ABL1,PLCG1,CRK,CTTN
Glioblastoma Multiforme Signaling	FGFR3,RB1,RAF1,MAPK1,PIK3R1,PLCG1,CDK2,EGFR,PDGFRB
IL-2 Signaling	FGFR3,RAF1,LCK,JAK1,MAPK1,PTK2B,PIK3R1
Paxillin Signaling	PTK2,FGFR3,PXN,MAPK1,PTK2B,PIK3R1,MAPK10,CRK

Ingenuity Canonical Pathways	Gene IDs of phosphopeptides
Role of JAK1 and JAK3 in Œ≥c Cytokine Signaling	FGFR3,JAK1,FES,MAPK1,PTK2B,PIK3R1,JAK2
NGF Signaling	FGFR3,RAF1,MAPK1,PIK3R1,MAPK10,PLCG1,PDPK1,CRK
Fc Epsilon RI Signaling	FGFR3,RAF1,MAPK1,PIK3R1,LAT,MAPK10,PLCG1,PDPK1
Natural Killer Cell Signaling	FGFR3,RAF1,LCK,MAPK1,PIK3R1,ZAP70,LAT,PLCG1
Role of Tissue Factor in Cancer	FGFR3,LCK,MAPK1,PTK2B,PIK3R1,JAK2,FRK,EGFR
Role of NFAT in Regulation of the Immune Response	FGFR3,RAF1,LCK,MAPK1,PIK3R1,ZAP70,LAT,PLCG1,CD79A
B Cell Receptor Signaling	PTK2,FGFR3,RAF1,MAPK1,PTK2B,PIK3R1,ABL1,PDPK1,CD79A
Neurotrophin/TRK Signaling	FGFR3,RAF1,NTRK2,MAPK1,PIK3R1,PLCG1,PDPK1
Regulation of the Epithelial-Mesenchymal Transition Pathway	MET,FGFR3,RAF1,JAK1,MAPK1,PIK3R1,JAK2,EGFR,PDGFRB
Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	FGFR3,PPP2CB,NCF1,JAK1,MAPK1,PIK3R1,MAPK10,PLCG1,JAK2
CD28 Signaling in T Helper Cells	FGFR3,LCK,PIK3R1,ZAP70,LAT,MAPK10,PLCG1,PDPK1
Prolactin Signaling	FGFR3,RAF1,MAPK1,PIK3R1,PLCG1,PDPK1,JAK2
Insulin Receptor Signaling	FGFR3,RAF1,JAK1,MAPK1,PIK3R1,PDPK1,CRK,JAK2
Neuregulin Signaling	RAF1,MAPK1,PIK3R1,PLCG1,PDPK1,CRK,EGFR
VEGF Family Ligand-Receptor Interactions	FGFR3,RAF1,MAPK1,FLT1,PIK3R1,PLCG1,KDR
FGF Signaling	MET,FGFR3,RAF1,MAPK1,PIK3R1,PLCG1,CRK
FcŒ≥ Receptor-mediated Phagocytosis in Macrophages and Monocytes	PXN,NCF1,MAPK1,PTK2B,PIK3R1,PLCG1,CRK
Cholecystokinin/Gastrin-mediated Signaling	PTK2,RAF1,PXN,MAPK1,PTK2B,MAPK10,EGFR
Regulation of Cellular Mechanics by Calpain Protease	PTK2,RB1,PXN,MAPK1,CDK2,EGFR
IL-15 Production	PTK2,JAK1,PTK2B,JAK2,FRK
Chronic Myeloid Leukemia Signaling	FGFR3,RB1,RAF1,MAPK1,PIK3R1,ABL1,CRK
CXCR4 Signaling	PTK2,FGFR3,RAF1,PXN,MAPK1,PIK3R1,MAPK10,CRK
Ephrin A Signaling	PTK2,EPHA7,FGFR3,PIK3R1,EPHA1,EPHA2
Germ Cell-Sertoli Cell Junction Signaling	PTK2,FGFR3,PXN,MAPK1,PIK3R1,FER,MAPK10,PDPK1
CNTF Signaling	FGFR3,RAF1,JAK1,MAPK1,PIK3R1,JAK2
Thrombopoietin Signaling	FGFR3,RAF1,MAPK1,PIK3R1,PLCG1,JAK2
Agrin Interactions at Neuromuscular Junction	PTK2,PXN,MAPK1,MAPK10,CTTN,EGFR
iCOS-iCOSL Signaling in T Helper Cells	FGFR3,LCK,PIK3R1,ZAP70,LAT,PLCG1,PDPK1
Sphingosine-1-phosphate Signaling	PTK2,FGFR3,MAPK1,PTK2B,PIK3R1,PLCG1,PDGFRB
PI3K/AKT Signaling	PPP2CB,RAF1,JAK1,MAPK1,PIK3R1,PDPK1,JAK2
ErbB4 Signaling	FGFR3,RAF1,MAPK1,PIK3R1,PLCG1,PDPK1
ILK Signaling	PTK2,FGFR3,PPP2CB,PXN,MAPK1,PIK3R1,MAPK10,PDPK1
PI3K Signaling in B Lymphocytes	RAF1,MAPK1,PIK3R1,ABL1,PLCG1,PDPK1,CD79A
Glucocorticoid Receptor Signaling	FGFR3,RAF1,JAK1,MAPK1,ANXA1,PIK3R1,CDK7,MAPK10,JAK2
Thrombin Signaling	PTK2,FGFR3,RAF1,MAPK1,PIK3R1,PLCG1,PDPK1,EGFR
PKCŒ∏ Signaling in T Lymphocytes	FGFR3,LCK,MAPK1,PIK3R1,ZAP70,LAT,PLCG1
IL-17A Signaling in Airway Cells	FGFR3,JAK1,MAPK1,PIK3R1,MAPK10,JAK2
Cyclins and Cell Cycle Regulation	RB1,PPP2CB,RAF1,CDK7,ABL1,CDK2

Ingenuity Canonical Pathways	Gene IDs of phosphopeptides
Regulation of IL-2 Expression in Activated and Anergic T Lymphocytes	RAF1,MAPK1,ZAP70,LAT,MAPK10,PLCG1
Growth Hormone Signaling	FGFR3,MAPK1,PIK3R1,PLCG1,PDPK1,JAK2
Renal Cell Carcinoma Signaling	MET,FGFR3,RAF1,MAPK1,PIK3R1,CRK
IL-3 Signaling	FGFR3,RAF1,JAK1,MAPK1,PIK3R1,JAK2
JAK/Stat Signaling	FGFR3,RAF1,JAK1,MAPK1,PIK3R1,JAK2
Small Cell Lung Cancer Signaling	PTK2,FGFR3,RB1,PIK3R1,ABL1,CDK2
Ovarian Cancer Signaling	FGFR3,RB1,RAF1,MAPK1,PIK3R1,ABL1,EGFR
IL-17 Signaling	FGFR3,JAK1,MAPK1,PIK3R1,MAPK10,JAK2
Bladder Cancer Signaling	FGFR3,RB1,RAF1,MAPK1,ABL1,EGFR
Superpathway of Inositol Phosphate Compounds	MET,FGFR3,LCK,PIK3R1,PLCG1,RASA1,EGFR,PDGFRB
Reelin Signaling in Neurons	FGFR3,LCK,PIK3R1,MAPK10,FRK,DCX
Melanocyte Development and Pigmentation Signaling	FGFR3,RAF1,MAPK1,PIK3R1,PLCG1,CRK
CDK5 Signaling	PPP2CB,RAF1,NTRK2,MAPK1,MAPK10,ABL1
RANK Signaling in Osteoclasts	FGFR3,RAF1,MAPK1,PTK2B,PIK3R1,MAPK10
UVA-Induced MAPK Signaling	FGFR3,MAPK1,PIK3R1,MAPK10,PLCG1,EGFR
FcŒ≥RIIB Signaling in B Lymphocytes	FGFR3,PIK3R1,MAPK10,PDPK1,CD79A
Mouse Embryonic Stem Cell Pluripotency	FGFR3,RAF1,JAK1,MAPK1,PIK3R1,JAK2
Melanoma Signaling	FGFR3,RB1,RAF1,MAPK1,PIK3R1
Role of JAK family kinases in IL-6-type Cytokine Signaling	JAK1,MAPK1,MAPK10,JAK2
Rac Signaling	PTK2,FGFR3,RAF1,MAPK1,PTK2B,PIK3R1
Endometrial Cancer Signaling	FGFR3,RAF1,MAPK1,PIK3R1,PDPK1
Role of NANOG in Mammalian Embryonic Stem Cell Pluripotency	FGFR3,RAF1,JAK1,MAPK1,PIK3R1,JAK2
3-phosphoinositide Biosynthesis	MET,FGFR3,LCK,PIK3R1,RASA1,EGFR,PDGFRB
UVB-Induced MAPK Signaling	FGFR3,MAPK1,PIK3R1,MAPK10,EGFR
IL-6 Signaling	FGFR3,RAF1,MAPK1,PIK3R1,MAPK10,JAK2
ErbB2-ErbB3 Signaling	FGFR3,RAF1,MAPK1,PIK3R1,PDPK1
14-3-3-mediated Signaling	FGFR3,RAF1,MAPK1,PIK3R1,MAPK10,PLCG1
Chemokine Signaling	PTK2,RAF1,MAPK1,PTK2B,PLCG1
GM-CSF Signaling	FGFR3,RAF1,MAPK1,PIK3R1,JAK2
G Protein Signaling Mediated by Tubby	LCK,ABL1,PLCG1,JAK2
Systemic Lupus Erythematosus Signaling	FGFR3,LCK,MAPK1,PIK3R1,LAT,PLCG1,CD79A
Angiopoietin Signaling	PTK2,FGFR3,PIK3R1,CRK,RASA1
Actin Cytoskeleton Signaling	PTK2,FGFR3,RAF1,PXN,MAPK1,PIK3R1,CRK
Oncostatin M Signaling	RAF1,JAK1,MAPK1,JAK2
Phospholipase C Signaling	RAF1,LCK,MAPK1,ZAP70,LAT,PLCG1,CD79A
FLT3 Signaling in Hematopoietic Progenitor Cells	FGFR3,RAF1,MAPK1,PIK3R1,PDPK1
Leptin Signaling in Obesity	FGFR3,MAPK1,PIK3R1,PLCG1,JAK2
eNOS Signaling	FGFR3,FLT1,PIK3R1,PLCG1,PDPK1,KDR
LPS-stimulated MAPK Signaling	FGFR3,RAF1,MAPK1,PIK3R1,MAPK10

Ingenuity Canonical Pathways	Gene IDs of phosphopeptides
NF-ŒʃB Activation by Viruses	FGFR3,RAF1,LCK,MAPK1,PIK3R1
Colorectal Cancer Metastasis Signaling	FGFR3,JAK1,MAPK1,PIK3R1,MAPK10,JAK2,EGFR
Signaling by Rho Family GTPases	PTK2,FGFR3,RAF1,MAPK1,PTK2B,PIK3R1,MAPK10
Regulation of eIF4 and p70S6K Signaling	FGFR3,PPP2CB,RAF1,MAPK1,PIK3R1,PDPK1
G Beta Gamma Signaling	RAF1,MAPK1,PLCG1,PDPK1,EGFR
G alpha q Signaling	FGFR3,RAF1,MAPK1,PTK2B,PIK3R1,PLCG1
Aldosterone Signaling in Epithelial Cells	FGFR3,RAF1,MAPK1,PIK3R1,PLCG1,PDPK1
UVC-Induced MAPK Signaling	RAF1,MAPK1,MAPK10,EGFR
Gap Junction Signaling	FGFR3,RAF1,MAPK1,PIK3R1,PLCG1,EGFR
G-Protein Coupled Receptor Signaling	FGFR3,RAF1,MAPK1,PTK2B,PIK3R1,PDPK1,RASA1
SAPK/JNK Signaling	FGFR3,LCK,PIK3R1,MAPK10,CRK
Endothelin-1 Signaling	FGFR3,RAF1,MAPK1,PIK3R1,MAPK10,PLCG1
RAR Activation	MAPK1,PIK3R1,CDK7,MAPK10,PDPK1,JAK2
Dendritic Cell Maturation	FGFR3,MAPK1,PIK3R1,MAPK10,PLCG1,JAK2
Role of NFAT in Cardiac Hypertrophy	FGFR3,RAF1,MAPK1,PIK3R1,MAPK10,PLCG1
Nitric Oxide Signaling in the Cardiovascular System	FGFR3,MAPK1,FLT1,PIK3R1,KDR
Semaphorin Signaling in Neurons	PTK2,MET,FES,MAPK1
Neuropathic Pain Signaling In Dorsal Horn Neurons	FGFR3,NTRK2,MAPK1,PIK3R1,PLCG1
Breast Cancer Regulation by Stathmin1	FGFR3,PPP2CB,RAF1,MAPK1,PIK3R1,CDK2
fMLP Signaling in Neutrophils	FGFR3,RAF1,NCF1,MAPK1,PIK3R1
Type II Diabetes Mellitus Signaling	FGFR3,MAPK1,PIK3R1,MAPK10,PDPK1
GNRH Signaling	PTK2,RAF1,MAPK1,MAPK10,EGFR
P2Y Purigenic Receptor Signaling Pathway	FGFR3,RAF1,MAPK1,PIK3R1,PLCG1
Pyridoxal 5′-phosphate Salvage Pathway	MAPK1,CDK7,CDK2,DYRK1A
Lymphotoxin Œ≤ Receptor Signaling	FGFR3,MAPK1,PIK3R1,PDPK1
Role of IL-17A in Arthritis	FGFR3,MAPK1,PIK3R1,MAPK10
Cardiac Hypertrophy Signaling	FGFR3,RAF1,MAPK1,PIK3R1,MAPK10,PLCG1
CCR5 Signaling in Macrophages	MAPK1,PTK2B,MAPK10,PLCG1
Human Embryonic Stem Cell Pluripotency	FGFR3,NTRK2,PIK3R1,PDPK1,PDGFRB
Glioma Invasiveness Signaling	PTK2,FGFR3,MAPK1,PIK3R1
Huntington's Disease Signaling	FGFR3,MAPK1,PIK3R1,PDPK1,RASA1,EGFR
IL-22 Signaling	JAK1,MAPK1,MAPK10
Role of JAK1, JAK2 and TYK2 in Interferon Signaling	RAF1,JAK1,JAK2
IL-17A Signaling in Gastric Cells	MAPK1,MAPK10,EGFR
Ephrin B Signaling	PTK2,EPHB1,PXN,MAPK1
Antiproliferative Role of TOB in T Cell Signaling	RB1,MAPK1,CDK2
Role of PI3K/AKT Signaling in the Pathogenesis of Influenza	FGFR3,MAPK1,PIK3R1,CRK
Estrogen-Dependent Breast Cancer Signaling	FGFR3,MAPK1,PIK3R1,EGFR
CD40 Signaling	FGFR3,MAPK1,PIK3R1,MAPK10
Macropinocytosis Signaling	MET,FGFR3,PIK3R1,PLCG1

Ingenuity Canonical Pathways	Gene IDs of phosphopeptides
PEDF Signaling	FGFR3,RAF1,MAPK1,PIK3R1
Acute Phase Response Signaling	RAF1,MAPK1,PIK3R1,PDPK1,JAK2
HER-2 Signaling in Breast Cancer	FGFR3,PIK3R1,PLCG1,EGFR
IL-4 Signaling	FGFR3,JAK1,PIK3R1,JAK2
Acute Myeloid Leukemia Signaling	FGFR3,RAF1,MAPK1,PIK3R1
Inhibition of Angiogenesis by TSP1	MAPK1,MAPK10,KDR
Role of JAK2 in Hormone-like Cytokine Signaling	JAK1,EPOR,JAK2
Ceramide Signaling	FGFR3,PPP2CB,RAF1,PIK3R1
Hepatic Fibrosis / Hepatic Stellate Cell Activation	MET,FLT1,KDR,EGFR,PDGFRB
Cell Cycle Regulation by BTG Family Proteins	RB1,PPP2CB,CDK2
Salvage Pathways of Pyrimidine Ribonucleotides	MAPK1,CDK7,CDK2,DYRK1A
CREB Signaling in Neurons	FGFR3,RAF1,MAPK1,PIK3R1,PLCG1
AMPK Signaling	FGFR3,PPP2CB,MAPK1,PIK3R1,PDPK1
Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	FGFR3,RAF1,MAPK1,PIK3R1,PLCG1,JAK2
Virus Entry via Endocytic Pathways	FGFR3,PIK3R1,ABL1,PLCG1
Antioxidant Action of Vitamin C	MAPK1,MAPK10,PLCG1,JAK2
mTOR Signaling	FGFR3,PPP2CB,MAPK1,PIK3R1,PDPK1
Thyroid Cancer Signaling	NTRK2,MAPK1,RET
Type I Diabetes Mellitus Signaling	JAK1,MAPK1,MAPK10,JAK2
p53 Signaling	FGFR3,RB1,PIK3R1,CDK2
Role of p14/p19ARF in Tumor Suppression	FGFR3,RB1,PIK3R1
iNOS Signaling	JAK1,MAPK1,JAK2
HIF1alpha Signaling	FGFR3,MAPK1,PIK3R1,MAPK10
IL-9 Signaling	FGFR3,JAK1,PIK3R1
EIF2 Signaling	FGFR3,RAF1,MAPK1,PIK3R1,PDPK1
Primary Immunodeficiency Signaling	LCK,ZAP70,CD79A
Cancer Drug Resistance By Drug Efflux	RAF1,MAPK1,PIK3R1
Sperm Motility	PTK2,PTK2B,PLCG1,FRK
Role of Osteoblasts, Osteoclasts and Chondrocytes in Rheumatoid Arthritis	FGFR3,MAPK1,PTK2B,PIK3R1,MAPK10
Docosahexaenoic Acid (DHA) Signaling	FGFR3,PIK3R1,PDPK1
CCR3 Signaling in Eosinophils	FGFR3,RAF1,MAPK1,PIK3R1
HMGB1 Signaling	FGFR3,MAPK1,PIK3R1,MAPK10
Th1 Pathway	FGFR3,JAK1,PIK3R1,JAK2
Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	FGFR3,MAPK1,PIK3R1,MAPK10
Protein Kinase A Signaling	PTK2,RAF1,PXN,MAPK1,PTK2B,PLCG1
MSP-RON Signaling Pathway	FGFR3,PIK3R1,JAK2
Synaptic Long Term Depression	PPP2CB,RAF1,MAPK1,PLCG1
IL-12 Signaling and Production in Macrophages	FGFR3,MAPK1,PIK3R1,MAPK10
Epithelial Adherens Junction Signaling	MET,FER,CRK,EGFR

Ingenuity Canonical Pathways	Gene IDs of phosphopeptides
Th2 Pathway	FGFR3,JAK1,PIK3R1,JAK2
Cell Cycle: G1/S Checkpoint Regulation	RB1,ABL1,CDK2
Calcium-induced T Lymphocyte Apoptosis	LCK,ZAP70,PLCG1
Xenobiotic Metabolism Signaling	FGFR3,PPP2CB,RAF1,MAPK1,PIK3R1
Myc Mediated Apoptosis Signaling	FGFR3,PIK3R1,MAPK10
Melatonin Signaling	RAF1,MAPK1,PLCG1
Role of MAPK Signaling in the Pathogenesis of Influenza	RAF1,MAPK1,MAPK10
Cdc42 Signaling	RAF1,MAPK1,MAPK10,RASA1
TREM1 Signaling	MAPK1,PLCG1,JAK2
Antiproliferative Role of Somatostatin Receptor 2	FGFR3,MAPK1,PIK3R1
BMP signaling pathway	RAF1,MAPK1,MAPK10
PPARŒ±/RXRŒ± Activation	RAF1,MAPK1,PLCG1,JAK2
Sertoli Cell-Sertoli Cell Junction Signaling	RAF1,EPB41,MAPK1,MAPK10
ATM Signaling	MAPK10,ABL1,CDK2
Th1 and Th2 Activation Pathway	FGFR3,JAK1,PIK3R1,JAK2
alpha Adrenergic Signaling	RAF1,MAPK1,PLCG1
NRF2-mediated Oxidative Stress Response	FGFR3,RAF1,MAPK1,PIK3R1
Apoptosis Signaling	RAF1,MAPK1,PLCG1
Clathrin-mediated Endocytosis Signaling	MET,FGFR3,PIK3R1,CTTN
PPAR Signaling	RAF1,MAPK1,PDGFRB
Estrogen-mediated S-phase Entry	RB1,CDK2
Corticotropin Releasing Hormone Signaling	RAF1,MAPK1,PLCG1
4-1BB Signaling in T Lymphocytes	MAPK1,MAPK10
Synaptic Long Term Potentiation	RAF1,MAPK1,PLCG1
RhoA Signaling	PTK2,PTK2B,EPHA1
Phagosome Formation	FGFR3,PIK3R1,PLCG1
Interferon Signaling	JAK1,JAK2
Estrogen Receptor Signaling	RAF1,MAPK1,CDK7
April Mediated Signaling	MAPK1,MAPK10
Cell Cycle Control of Chromosomal Replication	CDK7,CDK2
B Cell Activating Factor Signaling	MAPK1,MAPK10
MIF Regulation of Innate Immunity	MAPK1,MAPK10
Aryl Hydrocarbon Receptor Signaling	RB1,MAPK1,CDK2
Hereditary Breast Cancer Signaling	FGFR3,RB1,PIK3R1
Role of IL-17F in Allergic Inflammatory Airway Diseases	RAF1,MAPK1
Relaxin Signaling	FGFR3,MAPK1,PIK3R1
Cell Cycle: G2/M DNA Damage Checkpoint Regulation	CDK7,ABL1
Role of CHK Proteins in Cell Cycle Checkpoint Control	PPP2CB,CDK2
IL-10 Signaling	JAK1,MAPK1

Ingenuity Canonical Pathways	Gene IDs of phosphopeptides
Caveolar-mediated Endocytosis Signaling	ABL1,EGFR
NAD Biosynthesis from 2-amino-3-carboxymuconate Semialdehyde	ABL1
TGF-beta Signaling	RAF1,MAPK1
IL-1 Signaling	MAPK1,MAPK10
TR/RXR Activation	FGFR3,PIK3R1
Amyotrophic Lateral Sclerosis Signaling	FGFR3,PIK3R1
Androgen Signaling	MAPK1,CDK7
DNA Double-Strand Break Repair by Homologous Recombination	ABL1
G alpha I Signaling	RAF1,MAPK1
NAD biosynthesis II (from tryptophan)	ABL1
Parkinson's Signaling	MAPK1
Adipogenesis pathway	FGFR3,CDK7
GADD45 Signaling	CDK2
DNA damage-induced 14-3-3œÉ Signaling	CDK2
D-myo-inositol-5-phosphate Metabolism	PLCG1,RASA1
Dopamine-DARPP32 Feedback in cAMP Signaling	PPP2CB,PLCG1
Glycolysis I	ENO2
Gluconeogenesis I	ENO2
Tight Junction Signaling	PPP2CB,EPB41
D-myo-inositol (1,4,5)-Trisphosphate Biosynthesis	PLCG1
Sonic Hedgehog Signaling	DYRK1A
MIF-mediated Glucocorticoid Regulation	MAPK1
B Cell Development	CD79A
IL-17A Signaling in Fibroblasts	MAPK1
Nucleotide Excision Repair Pathway	CDK7
Pyrimidine Ribonucleotides Interconversion	ANXA1
cAMP-mediated signaling	RAF1,MAPK1
Pyrimidine Ribonucleotides De Novo Biosynthesis	ANXA1
Role of Oct4 in Mammalian Embryonic Stem Cell Pluripotency	RB1
Assembly of RNA Polymerase II Complex	CDK7
Amyloid Processing	MAPK1
CD27 Signaling in Lymphocytes	MAPK10
Wnt/Ca+ pathway	PLCG1
Induction of Apoptosis by HIV1	MAPK10
Activation of IRF by Cytosolic Pattern Recognition Receptors	MAPK10
Phospholipases	PLCG1
ERK5 Signaling	EGFR
PCP pathway	MAPK10

Ingenuity Canonical Pathways	Gene IDs of phosphopeptides
Mitotic Roles of Polo-Like Kinase	PPP2CB
Remodeling of Epithelial Adherens Junctions	MET
GPCR-Mediated Integration of Enteroendocrine Signaling Exemplified by an L Cell	PLCG1
Toll-like Receptor Signaling	MAPK1
Dopamine Receptor Signaling	PPP2CB
Role of BRCA1 in DNA Damage Response	RB1
GPCR-Mediated Nutrient Sensing in Enteroendocrine Cells	PLCG1
HIPPO signaling	PPP2CB
OX40 Signaling Pathway	MAPK10
Altered T Cell and B Cell Signaling in Rheumatoid Arthritis	CD79A
Factors Promoting Cardiogenesis in Vertebrates	CDK2
Sumoylation Pathway	MAPK10
GŒ±s Signaling	MAPK1
FXR/RXR Activation	MAPK10
Cellular Effects of Sildenafil (Viagra)	PLCG1
Cardiac Œ≤-adrenergic Signaling	PPP2CB
D-myo-inositol (1,4,5,6)-Tetrakisphosphate Biosynthesis	RASA1
D-myo-inositol (3,4,5,6)-tetrakisphosphate Biosynthesis	RASA1
3-phosphoinositide Degradation	RASA1
Hepatic Cholestasis	MAPK10
Wnt/beta-catenin Signaling	PPP2CB
Mitochondrial Dysfunction	MAPK10
Granulocyte Adhesion and Diapedesis	PECAM1
Calcium Signaling	MAPK1
Agranulocyte Adhesion and Diapedesis	PECAM1

Supplementary Table 3. List of Ingenuity Canonical Pathways of the 59 Markov-positive peptides on titanium. Phosphopeptides are indicated as Gene IDs. Selected intracellular pathways considered for further analysis are indicated in bold.

Ingenuity Canonical Pathways	Gene IDs of phosphopeptides
Leukocyte Extravasation Signaling	PXN,PTK2B,PIK3R1,ABL1,PLCG1,FGFR3,TEC,NCF1,PTPN11,FER,MAPK10,PECAM1,CTTN
Axonal Guidance Signaling	EPHA7,RAF1,PXN,FES,PIK3R1,EPHA1,ABL1,PLCG1,FGFR3,MET,EPHB1,NTRK2,PTPN11,RASA1,EPHA2
T Cell Receptor Signaling	TEC,FGFR3,RAF1,LCK,PTPN11,PIK3R1,ZAP70,LAT,PLCG1,RASA1
STAT3 Pathway	FGFR3,RAF1,NTRK2,FLT1,MAPK10,JAK2,KDR,EGFR,PDGFRB
Non-Small Cell Lung Cancer Signaling	FGFR3,RB1,RAF1,PTPN11,PIK3R1,ABL1,PLCG1,PDPK1,EGFR
Pancreatic Adenocarcinoma Signaling	FGFR3,RB1,RAF1,PTPN11,PIK3R1,MAPK10,ABL1,JAK2,CDK2,EGFR
NF-kB Signaling	FGFR3,RAF1,LCK,NTRK2,FLT1,PTPN11,PIK3R1,ZAP70,KDR,EGFR,PDGFRB
PDGF Signaling	FGFR3,RAF1,PTPN11,PIK3R1,ABL1,PLCG1,JAK2,RASA1,PDGFRB
ErbB Signaling	FGFR3,RAF1,PTPN11,PIK3R1,ERBB4,MAPK10,PLCG1,PDPK1,EGFR
VEGF Signaling	FGFR3,RAF1,PXN,FLT1,PTK2B,PTPN11,PIK3R1,PLCG1,KDR
Glioma Signaling	FGFR3,RB1,RAF1,PTPN11,PIK3R1,ABL1,PLCG1,EGFR,PDGFRB
HGF Signaling	MET,FGFR3,RAF1,PXN,PTPN11,PIK3R1,MAPK10,PLCG1,CDK2
Tec Kinase Signaling	TEC,FGFR3,LCK,PTK2B,PTPN11,PIK3R1,MAPK10,PLCG1,JAK2,FRK
Ephrin Receptor Signaling	EPHA7,RAF1,EPHB1,PXN,PTPN11,EPHA1,ABL1,JAK2,EPHA2,RASA1
PTEN Signaling	FGFR3,RAF1,NTRK2,FLT1,PIK3R1,PDPK1,KDR,EGFR,PDGFRB
GDNF Family Ligand-Receptor Interactions	FGFR3,RAF1,PTPN11,PIK3R1,MAPK10,PLCG1,RET,RASA1
Erythropoietin Signaling	FGFR3,RAF1,PTPN11,EPOR,PIK3R1,PLCG1,PDPK1,JAK2
G alpha 12/13 Signaling	TEC,FGFR3,RAF1,PXN,PTK2B,PTPN11,PIK3R1,MAPK10,RASA1
CD28 Signaling in T Helper Cells	FGFR3,LCK,PTPN11,PIK3R1,ZAP70,LAT,MAPK10,PLCG1,PDPK1
Prostate Cancer Signaling	FGFR3,RB1,RAF1,PTPN11,PIK3R1,ABL1,PDPK1,CDK2
CTLA4 Signaling in Cytotoxic T Lymphocytes	FGFR3,LCK,PTPN11,PIK3R1,ZAP70,LAT,PLCG1,JAK2
FAK Signaling	FGFR3,RAF1,PXN,PTPN11,PIK3R1,PLCG1,PDPK1,EGFR
PAK Signaling	FGFR3,RAF1,PXN,PTK2B,PTPN11,PIK3R1,MAPK10,PDGFRB
Glioblastoma Multiforme Signaling	FGFR3,RB1,RAF1,PTPN11,PIK3R1,PLCG1,CDK2,EGFR,PDGFRB
IGF-1 Signaling	FGFR3,RAF1,PXN,PTPN11,PIK3R1,PDPK1,JAK2,RASA1
Superpathway of Inositol Phosphate Compounds	MET,FGFR3,LCK,PTPN11,PIK3R1,ERBB4,PLCG1,RASA1,EGFR,PDGFRB
Telomerase Signaling	FGFR3,RB1,RAF1,PTPN11,PIK3R1,ABL1,PDPK1,EGFR
EGF Signaling	FGFR3,RAF1,PTPN11,PIK3R1,PLCG1,RASA1,EGFR
Fc Epsilon RI Signaling	FGFR3,RAF1,PTPN11,PIK3R1,LAT,MAPK10,PLCG1,PDPK1
ErbB4 Signaling	FGFR3,RAF1,PTPN11,PIK3R1,ERBB4,PLCG1,PDPK1
Renin-Angiotensin Signaling	FGFR3,RAF1,PTK2B,PTPN11,PIK3R1,MAPK10,PLCG1,JAK2
Natural Killer Cell Signaling	FGFR3,RAF1,LCK,PTPN11,PIK3R1,ZAP70,LAT,PLCG1

Ingenuity Canonical Pathways	Gene IDs of phosphopeptides
iCOS-iCOSL Signaling in T Helper Cells	FGFR3,LCK,PTPN11,PIK3R1,ZAP70,LAT,PLCG1,PDPK1
Role of Tissue Factor in Cancer	FGFR3,LCK,PTK2B,PTPN11,PIK3R1,JAK2,FRK,EGFR
Role of NFAT in Regulation of the Immune Response	FGFR3,RAF1,LCK,PTPN11,PIK3R1,ZAP70,LAT,PLCG1,CD79A
IL-15 Signaling	FGFR3,RAF1,LCK,PTPN11,PIK3R1,PLCG1,JAK2
Neurotrophin/TRK Signaling	FGFR3,RAF1,NTRK2,PTPN11,PIK3R1,PLCG1,PDPK1
p70S6K Signaling	FGFR3,RAF1,PTPN11,PIK3R1,PLCG1,PDPK1,CD79A,EGFR
IL-8 Signaling	FGFR3,RAF1,FLT1,PTK2B,PTPN11,PIK3R1,MAPK10,KDR,EGFR
3-phosphoinositide Biosynthesis	MET,FGFR3,LCK,PTPN11,PIK3R1,ERBB4,RASA1,EGFR,PDGFRB
Molecular Mechanisms of Cancer	FGFR3,RB1,RAF1,PTPN11,PIK3R1,CDK7,MAPK10,ABL1,JAK2,RASA1, CDK2
Prolactin Signaling	FGFR3,RAF1,PTPN11,PIK3R1,PLCG1,PDPK1,JAK2
Neuregulin Signaling	RAF1,PTPN11,PIK3R1,ERBB4,PLCG1,PDPK1,EGFR
VEGF Family Ligand-Receptor Interactions	FGFR3,RAF1,FLT1,PTPN11,PIK3R1,PLCG1,KDR
Reelin Signaling in Neurons	FGFR3,LCK,PTPN11,PIK3R1,MAPK10,FRK,DCX
FcŒ≥RIIB Signaling in B Lymphocytes	FGFR3,PTPN11,PIK3R1,MAPK10,PDPK1,CD79A
Ephrin A Signaling	EPHA7,FGFR3,PTPN11,PIK3R1,EPHA1,EPHA2
IL-2 Signaling	FGFR3,RAF1,LCK,PTK2B,PTPN11,PIK3R1
Thrombopoietin Signaling	FGFR3,RAF1,PTPN11,PIK3R1,PLCG1,JAK2
NGF Signaling	FGFR3,RAF1,PTPN11,PIK3R1,MAPK10,PLCG1,PDPK1
B Cell Receptor Signaling	FGFR3,RAF1,PTK2B,PTPN11,PIK3R1,ABL1,PDPK1,CD79A
Regulation of the Epithelial-Mesenchymal Transition Pathway	MET,FGFR3,RAF1,PTPN11,PIK3R1,JAK2,EGFR,PDGFRB
Role of JAK1 and JAK3 in Œ≥c Cytokine Signaling	FGFR3,FES,PTK2B,PTPN11,PIK3R1,JAK2
PKCŒ∏ Signaling in T Lymphocytes	FGFR3,LCK,PTPN11,PIK3R1,ZAP70,LAT,PLCG1
Growth Hormone Signaling	FGFR3,PTPN11,PIK3R1,PLCG1,PDPK1,JAK2
Integrin Signaling	FGFR3,RAF1,PXN,PTPN11,PIK3R1,ABL1,PLCG1,CTTN
Ovarian Cancer Signaling	FGFR3,RB1,RAF1,PTPN11,PIK3R1,ABL1,EGFR
Small Cell Lung Cancer Signaling	FGFR3,RB1,PTPN11,PIK3R1,ABL1,CDK2
FGF Signaling	MET,FGFR3,RAF1,PTPN11,PIK3R1,PLCG1
eNOS Signaling	FGFR3,FLT1,PTPN11,PIK3R1,PLCG1,PDPK1,KDR
RANK Signaling in Osteoclasts	FGFR3,RAF1,PTK2B,PTPN11,PIK3R1,MAPK10
UVA-Induced MAPK Signaling	FGFR3,PTPN11,PIK3R1,MAPK10,PLCG1,EGFR
Germ Cell-Sertoli Cell Junction Signaling	FGFR3,PXN,PTPN11,PIK3R1,FER,MAPK10,PDPK1
Chronic Myeloid Leukemia Signaling	FGFR3,RB1,RAF1,PTPN11,PIK3R1,ABL1
Melanoma Signaling	FGFR3,RB1,RAF1,PTPN11,PIK3R1
Paxillin Signaling	FGFR3,PXN,PTK2B,PTPN11,PIK3R1,MAPK10
MSP-RON Signaling Pathway	FGFR3,PTPN11,PIK3R1,MST1R,JAK2
Glucocorticoid Receptor Signaling	FGFR3,RAF1,PTPN11,ANXA1,PIK3R1,CDK7,MAPK10,JAK2

Ingenuity Canonical Pathways	Gene IDs of phosphopeptides
Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	FGFR3,NCF1,PTPN11,PIK3R1,MAPK10,PLCG1,JAK2
CNTF Signaling	FGFR3,RAF1,PTPN11,PIK3R1,JAK2
ERK/MAPK Signaling	FGFR3,RAF1,PXN,PTK2B,PTPN11,PIK3R1,PLCG1
Endometrial Cancer Signaling	FGFR3,RAF1,PTPN11,PIK3R1,PDPK1
Sphingosine-1-phosphate Signaling	FGFR3,PTK2B,PTPN11,PIK3R1,PLCG1,PDGFRB
Thrombin Signaling	FGFR3,RAF1,PTPN11,PIK3R1,PLCG1,PDPK1,EGFR
IL-15 Production	PTK2B,MST1R,JAK2,FRK
UVB-Induced MAPK Signaling	FGFR3,PTPN11,PIK3R1,MAPK10,EGFR
IL-6 Signaling	FGFR3,RAF1,PTPN11,PIK3R1,MAPK10,JAK2
PI3K Signaling in B Lymphocytes	RAF1,PIK3R1,ABL1,PLCG1,PDPK1,CD79A
14-3-3-mediated Signaling	FGFR3,RAF1,PTPN11,PIK3R1,MAPK10,PLCG1
Agrin Interactions at Neuromuscular Junction	PXN,ERBB4,MAPK10,CTTN,EGFR
ErbB2-ErbB3 Signaling	FGFR3,RAF1,PTPN11,PIK3R1,PDPK1
GM-CSF Signaling	FGFR3,RAF1,PTPN11,PIK3R1,JAK2
Systemic Lupus Erythematosus Signaling	FGFR3,LCK,PTPN11,PIK3R1,LAT,PLCG1,CD79A
Insulin Receptor Signaling	FGFR3,RAF1,PTPN11,PIK3R1,PDPK1,JAK2
G Protein Signaling Mediated by Tubby	LCK,ABL1,PLCG1,JAK2
Human Embryonic Stem Cell Pluripotency	FGFR3,NTRK2,PTPN11,PIK3R1,PDPK1,PDGFRB
IL-17A Signaling in Airway Cells	FGFR3,PTPN11,PIK3R1,MAPK10,JAK2
Cyclins and Cell Cycle Regulation	RB1,RAF1,CDK7,ABL1,CDK2
Regulation of IL-2 Expression in Activated and Anergic T Lymphocytes	RAF1,ZAP70,LAT,MAPK10,PLCG1
Renal Cell Carcinoma Signaling	MET,FGFR3,RAF1,PTPN11,PIK3R1
Macropinocytosis Signaling	MET,FGFR3,PTPN11,PIK3R1,PLCG1
IL-3 Signaling	FGFR3,RAF1,PTPN11,PIK3R1,JAK2
JAK/Stat Signaling	FGFR3,RAF1,PTPN11,PIK3R1,JAK2
IL-17 Signaling	FGFR3,PTPN11,PIK3R1,MAPK10,JAK2
FLT3 Signaling in Hematopoietic Progenitor Cells	FGFR3,RAF1,PTPN11,PIK3R1,PDPK1
Leptin Signaling in Obesity	FGFR3,PTPN11,PIK3R1,PLCG1,JAK2
LPS-stimulated MAPK Signaling	FGFR3,RAF1,PTPN11,PIK3R1,MAPK10
NF-ŒʃB Activation by Viruses	FGFR3,RAF1,LCK,PTPN11,PIK3R1
Bladder Cancer Signaling	FGFR3,RB1,RAF1,ABL1,EGFR
G alpha q Signaling	FGFR3,RAF1,PTK2B,PTPN11,PIK3R1,PLCG1
HER-2 Signaling in Breast Cancer	FGFR3,PTPN11,PIK3R1,PLCG1,EGFR
CXCR4 Signaling	FGFR3,RAF1,PXN,PTPN11,PIK3R1,MAPK10
Aldosterone Signaling in Epithelial Cells	FGFR3,RAF1,PTPN11,PIK3R1,PLCG1,PDPK1

Ingenuity Canonical Pathways	Gene IDs of phosphopeptides
FcŒ≥ Receptor-mediated Phagocytosis in Macrophages and Monocytes	PXN,NCF1,PTK2B,PIK3R1,PLCG1
Gap Junction Signaling	FGFR3,RAF1,PTPN11,PIK3R1,PLCG1,EGFR
Melanocyte Development and Pigmentation Signaling	FGFR3,RAF1,PTPN11,PIK3R1,PLCG1
G-Protein Coupled Receptor Signaling	FGFR3,RAF1,PTK2B,PTPN11,PIK3R1,PDPK1,RASA1
Role of p14/p19ARF in Tumor Suppression	FGFR3,RB1,PTPN11,PIK3R1
Cholecystokinin/Gastrin-mediated Signaling	RAF1,PXN,PTK2B,MAPK10,EGFR
Virus Entry via Endocytic Pathways	FGFR3,PTPN11,PIK3R1,ABL1,PLCG1
SAPK/JNK Signaling	FGFR3,LCK,PTPN11,PIK3R1,MAPK10
Endothelin-1 Signaling	FGFR3,RAF1,PTPN11,PIK3R1,MAPK10,PLCG1
Mouse Embryonic Stem Cell Pluripotency	FGFR3,RAF1,PTPN11,PIK3R1,JAK2
Dendritic Cell Maturation	FGFR3,PTPN11,PIK3R1,MAPK10,PLCG1,JAK2
Role of NFAT in Cardiac Hypertrophy	FGFR3,RAF1,PTPN11,PIK3R1,MAPK10,PLCG1
p53 Signaling	FGFR3,RB1,PTPN11,PIK3R1,CDK2
ILK Signaling	FGFR3,PXN,PTPN11,PIK3R1,MAPK10,PDPK1
Nitric Oxide Signaling in the Cardiovascular System	FGFR3,FLT1,PTPN11,PIK3R1,KDR
Docosahexaenoic Acid (DHA) Signaling	FGFR3,PTPN11,PIK3R1,PDPK1
Neuropathic Pain Signaling In Dorsal Horn Neurons	FGFR3,NTRK2,PTPN11,PIK3R1,PLCG1
Rac Signaling	FGFR3,RAF1,PTK2B,PTPN11,PIK3R1
fMLP Signaling in Neutrophils	FGFR3,RAF1,NCF1,PTPN11,PIK3R1
Role of NANOG in Mammalian Embryonic Stem Cell Pluripotency	FGFR3,RAF1,PTPN11,PIK3R1,JAK2
Regulation of Cellular Mechanics by Calpain Protease	RB1,PXN,CDK2,EGFR
Type II Diabetes Mellitus Signaling	FGFR3,PTPN11,PIK3R1,MAPK10,PDPK1
P2Y Purigenic Receptor Signaling Pathway	FGFR3,RAF1,PTPN11,PIK3R1,PLCG1
Cardiac Hypertrophy Signaling	FGFR3,RAF1,PTPN11,PIK3R1,MAPK10,PLCG1
Lymphotoxin Œ≤ Receptor Signaling	FGFR3,PTPN11,PIK3R1,PDPK1
Role of IL-17A in Arthritis	FGFR3,PTPN11,PIK3R1,MAPK10
Phospholipase C Signaling	RAF1,LCK,ZAP70,LAT,PLCG1,CD79A
Huntington's Disease Signaling	FGFR3,PTPN11,PIK3R1,PDPK1,RASA1,EGFR
Myc Mediated Apoptosis Signaling	FGFR3,PTPN11,PIK3R1,MAPK10
Colorectal Cancer Metastasis Signaling	FGFR3,PTPN11,PIK3R1,MAPK10,JAK2,EGFR
Signaling by Rho Family GTPases	FGFR3,RAF1,PTK2B,PTPN11,PIK3R1,MAPK10

Ingenuity Canonical Pathways	Gene IDs of phosphopeptides
IL-12 Signaling and Production in Macrophages	FGFR3,PTPN11,PIK3R1,MAPK10,MST1R
Role of JAK family kinases in IL-6-type Cytokine Signaling	PTPN11,MAPK10,JAK2
Angiopoietin Signaling	FGFR3,PTPN11,PIK3R1,RASA1
Estrogen-Dependent Breast Cancer Signaling	FGFR3,PTPN11,PIK3R1,EGFR
Regulation of eIF4 and p70S6K Signaling	FGFR3,RAF1,PTPN11,PIK3R1,PDPK1
CD40 Signaling	FGFR3,PTPN11,PIK3R1,MAPK10
PEDF Signaling	FGFR3,RAF1,PTPN11,PIK3R1
Acute Phase Response Signaling	RAF1,PTPN11,PIK3R1,PDPK1,JAK2
G Beta Gamma Signaling	RAF1,PLCG1,PDPK1,EGFR
IL-4 Signaling	FGFR3,PTPN11,PIK3R1,JAK2
Acute Myeloid Leukemia Signaling	FGFR3,RAF1,PTPN11,PIK3R1
Ceramide Signaling	FGFR3,RAF1,PTPN11,PIK3R1
Hepatic Fibrosis / Hepatic Stellate Cell Activation	MET,FLT1,KDR,EGFR,PDGFRB
CREB Signaling in Neurons	FGFR3,RAF1,PTPN11,PIK3R1,PLCG1
Role of JAK2 in Hormone-like Cytokine Signaling	PTPN11,EPOR,JAK2
Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	FGFR3,RAF1,PTPN11,PIK3R1,PLCG1,JAK2
RAR Activation	PIK3R1,CDK7,MAPK10,PDPK1,JAK2
CDK5 Signaling	RAF1,NTRK2,MAPK10,ABL1
Clathrin-mediated Endocytosis Signaling	MET,FGFR3,PTPN11,PIK3R1,CTTN
Breast Cancer Regulation by Stathmin1	FGFR3,RAF1,PTPN11,PIK3R1,CDK2
UVC-Induced MAPK Signaling	RAF1,MAPK10,EGFR
HIF1alpha Signaling	FGFR3,PTPN11,PIK3R1,MAPK10
IL-9 Signaling	FGFR3,PTPN11,PIK3R1
EIF2 Signaling	FGFR3,RAF1,PTPN11,PIK3R1,PDPK1
Actin Cytoskeleton Signaling	FGFR3,RAF1,PXN,PTPN11,PIK3R1
Phagosome Formation	FGFR3,PTPN11,PIK3R1,PLCG1
Primary Immunodeficiency Signaling	LCK,ZAP70,CD79A
PI3K/AKT Signaling	RAF1,PIK3R1,PDPK1,JAK2
Role of Osteoblasts, Osteoclasts and Chondrocytes in Rheumatoid Arthritis	FGFR3,PTK2B,PTPN11,PIK3R1,MAPK10
Sperm Motility	PTK2B,PLCG1,MST1R,FRK
CCR3 Signaling in Eosinophils	FGFR3,RAF1,PTPN11,PIK3R1
HMGB1 Signaling	FGFR3,PTPN11,PIK3R1,MAPK10

Ingenuity Canonical Pathways	Gene IDs of phosphopeptides
Th1 Pathway	FGFR3,PTPN11,PIK3R1,JAK2
Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	FGFR3,PTPN11,PIK3R1,MAPK10
Hereditary Breast Cancer Signaling	FGFR3,RB1,PTPN11,PIK3R1
Th2 Pathway	FGFR3,PTPN11,PIK3R1,JAK2
Cell Cycle: G1/S Checkpoint Regulation	RB1,ABL1,CDK2
Calcium-induced T Lymphocyte Apoptosis	LCK,ZAP70,PLCG1
CCR5 Signaling in Macrophages	PTK2B,MAPK10,PLCG1
Glioma Invasiveness Signaling	FGFR3,PTPN11,PIK3R1
Chemokine Signaling	RAF1,PTK2B,PLCG1
Antiproliferative Role of Somatostatin Receptor 2	FGFR3,PTPN11,PIK3R1
Role of PI3K/AKT Signaling in the Pathogenesis of Influenza	FGFR3,PTPN11,PIK3R1
ATM Signaling	MAPK10,ABL1,CDK2
Th1 and Th2 Activation Pathway	FGFR3,PTPN11,PIK3R1,JAK2
AMPK Signaling	FGFR3,PTPN11,PIK3R1,PDPK1
NRF2-mediated Oxidative Stress Response	FGFR3,RAF1,PTPN11,PIK3R1
mTOR Signaling	FGFR3,PTPN11,PIK3R1,PDPK1
Role of JAK1, JAK2 and TYK2 in Interferon Signaling	RAF1,JAK2
Estrogen-mediated S-phase Entry	RB1,CDK2
TR/RXR Activation	FGFR3,PTPN11,PIK3R1
IL-17A Signaling in Gastric Cells	MAPK10,EGFR
Antiproliferative Role of TOB in T Cell Signaling	RB1,CDK2
Antioxidant Action of Vitamin C	MAPK10,PLCG1,JAK2
Protein Kinase A Signaling	RAF1,PXN,PTK2B,PTPN11,PLCG1
Amyotrophic Lateral Sclerosis Signaling	FGFR3,PTPN11,PIK3R1
Oncostatin M Signaling	RAF1,JAK2
Inhibition of Angiogenesis by TSP1	MAPK10,KDR
Cell Cycle Regulation by BTG Family Proteins	RB1,CDK2
GNRH Signaling	RAF1,MAPK10,EGFR
Cell Cycle Control of Chromosomal Replication	CDK7,CDK2
Thyroid Cancer Signaling	NTRK2,RET
Xenobiotic Metabolism Signaling	FGFR3,RAF1,PTPN11,PIK3R1
Epithelial Adherens Junction Signaling	MET,FER,EGFR
Relaxin Signaling	FGFR3,PTPN11,PIK3R1

Ingenuity Canonical Pathways	Gene IDs of phosphopeptides
Cell Cycle: G2/M DNA Damage Checkpoint Regulation	CDK7,ABL1
Cancer Drug Resistance By Drug Efflux	RAF1,PIK3R1
D-myo-inositol-5-phosphate Metabolism	PTPN11,PLCG1,RASA1
Semaphorin Signaling in Neurons	MET,FES
Cdc42 Signaling	RAF1,MAPK10,RASA1
PPARŒ±/RXRŒ± Activation	RAF1,PLCG1,JAK2
Sertoli Cell-Sertoli Cell Junction Signaling	RAF1,EPB41,MAPK10
ERK5 Signaling	PTPN11,EGFR
Pyridoxal 5′-phosphate Salvage Pathway	CDK7,CDK2
Caveolar-mediated Endocytosis Signaling	ABL1,EGFR
Melatonin Signaling	RAF1,PLCG1
Role of MAPK Signaling in the Pathogenesis of Influenza	RAF1,MAPK10
Ephrin B Signaling	EPHB1,PXN
TREM1 Signaling	PLCG1,JAK2
BMP signaling pathway	RAF1,MAPK10
NAD Biosynthesis from 2-amino-3-carboxymuconate Semialdehyde	ABL1
Alpha Adrenergic Signaling	RAF1,PLCG1
Apoptosis Signaling	RAF1,PLCG1
PPAR Signaling	RAF1,PDGFRB
Salvage Pathways of Pyrimidine Ribonucleotides	CDK7,CDK2
Type I Diabetes Mellitus Signaling	MAPK10,JAK2
Corticotropin Releasing Hormone Signaling	RAF1,PLCG1
Synaptic Long Term Potentiation	RAF1,PLCG1
DNA Double-Strand Break Repair by Homologous Recombination	ABL1
RhoA Signaling	PTK2B,EPHA1
NAD biosynthesis II (from tryptophan)	ABL1
Estrogen Receptor Signaling	RAF1,CDK7
Adipogenesis pathway	FGFR3,CDK7
Aryl Hydrocarbon Receptor Signaling	RB1,CDK2
GADD45 Signaling	CDK2
DNA damage-induced 14-3-3œÉ Signaling	CDK2

Ingenuity Canonical Pathways	Gene IDs of phosphopeptides
D-myo-inositol (1,4,5,6)-Tetrakisphosphate Biosynthesis	PTPN11,RASA1
D-myo-inositol (3,4,5,6)-tetrakisphosphate Biosynthesis	PTPN11,RASA1
Synaptic Long Term Depression	RAF1,PLCG1
3-phosphoinositide Degradation	PTPN11,RASA1
IL-22 Signaling	MAPK10
Glycolysis I	ENO2
Gluconeogenesis I	ENO2
D-myo-inositol (1,4,5)-Trisphosphate Biosynthesis	PLCG1
4-1BB Signaling in T Lymphocytes	MAPK10
B Cell Development	CD79A
Nucleotide Excision Repair Pathway	CDK7
Interferon Signaling	JAK2
April Mediated Signaling	MAPK10
B Cell Activating Factor Signaling	MAPK10
MIF Regulation of Innate Immunity	MAPK10
Pyrimidine Ribonucleotides Interconversion	ANXA1
Role of IL-17F in Allergic Inflammatory Airway Diseases	RAF1
iNOS Signaling	JAK2
Pyrimidine Ribonucleotides De Novo Biosynthesis	ANXA1
Role of Oct4 in Mammalian Embryonic Stem Cell Pluripotency	RB1
Assembly of RNA Polymerase II Complex	CDK7
CD27 Signaling in Lymphocytes	MAPK10
Role of CHK Proteins in Cell Cycle Checkpoint Control	CDK2
Wnt/Ca+ pathway	PLCG1
Induction of Apoptosis by HIV1	MAPK10
Activation of IRF by Cytosolic Pattern Recognition Receptors	MAPK10
Phospholipases	PLCG1
PCP pathway	MAPK10
Remodeling of Epithelial Adherens Junctions	MET
GPCR-Mediated Integration of Enteroendocrine Signaling Exemplified by an L Cell	PLCG1

Ingenuity Canonical Pathways	Gene IDs of phosphopeptides
Role of BRCA1 in DNA Damage Response	RB1
GPCR-Mediated Nutrient Sensing in Enteroendocrine Cells	PLCG1
TGF-beta Signaling	RAF1
OX40 Signaling Pathway	MAPK10
Altered T Cell and B Cell Signaling in Rheumatoid Arthritis	CD79A
IL-1 Signaling	MAPK10
Factors Promoting Cardiogenesis in Vertebrates	CDK2
Sumoylation Pathway	MAPK10
Androgen Signaling	CDK7
G alpha l Signaling	RAF1
FXR/RXR Activation	MAPK10
Cellular Effects of Sildenafil (Viagra)	PLCG1
Hepatic Cholestasis	MAPK10
Dopamine-DARPP32 Feedback in cAMP Signaling	PLCG1
Tight Junction Signaling	EPB41
Mitochondrial Dysfunction	MAPK10
Granulocyte Adhesion and Diapedesis	PECAM1
Agranulocyte Adhesion and Diapedesis	PECAM1
cAMP-mediated signaling	RAF1

Supplementary Table 4. Clustering of all 143 peptides into signaling pathways and cell-related functions.

Peptide #	Peptide ID	Signaling pathway	Cell-related function
1	ANXA1_14_26	Annexin	Calcium signaling
2	ANXA2_17_29		
3	CALM_93_105	Calmodulin	
4	CALM_95_107		
5	PLCG1_1246_1258	PLC	
6	PLCG1_764_776		
7	PLCG1_776_788		
8	RON_1346_1358	RON	
9	RON_1353_1365		
10	CDK2_8_20	CDK	Cell cycle signaling
11	CDK7_157_169		
12	RB_804_816	Rb	
13	RBL2_99_111		
14	ZBT16_621_633	Other 1	
15	C1R_199_211		
16	EPHA1_774_786	Eph Receptor	cell-cell signaling
17	EPHA2_581_593		
18	EPHA2_765_777		
19	EPHA4_589_601		
20	EPHA4_921_933		
21	EPHA7_607_619		
22	EPHB1_771_783		
23	EPHB1_921_933		
24	EPHB4_583_595		
25	CRK_214_226	Crk	cytoskeletal signaling
26	FAK1_569_581	FAK	
27	FAK2_572_584		
28	PAXI_24_36		
29	PAXI_111_123		
30	FES_706_718	Fes	
31	K2C6B_53_65	CK	
32	K2C8_425_437		
33	MBP_198_210	MBP	
34	MBP_259_271		
35	MBP_263_275		
36	PERI_458_470	Other 2	
37	SRC8_CHICK_470_482		
38	SRC8_CHICK_476_488		
39	SRC8_CHICK_492_504		
40	TNNT1_2_14		
41	DCX_109_121		

Peptide #	Peptide ID	Signaling pathway	Cell-related function
42	DDR1_506_518	DDR1	Extracellular matrix signaling
43	VINC_815_827	Other 3	
44	ART_004_EAIYAAPFAKKKXC	BMP	Differentiation signaling
45	DYR1A_212_224	DYR1A	
46	DYR1A_312_324		
47	PP2AB_297_309	Phosphatase	
48	PTN11_539_551		
49	AMPE_5_17	Other 4	
50	TEC_512_524	Btk	
51	B3AT_39_51	solute carrier family	Ion transport
52	NPT2A_501_513		
53	ENOG_37_49	Enolase	Energy signaling
54	ODBA_340_352	BCKDHA	
55	ODPAT_291_303	PDHA2	
56	EPOR_361_373	EPO-R	Cytokine receptor signaling
57	EPOR_419_431		
58	FGFR1_761_773	FGFR	growth factor receptor signaling/bone morphogenesis
59	FGFR2_762_774		
60	FGFR3_641_653		
61	FGFR3_753_765		
62	EGFR_1062_1074	EFGR	growth factor receptor signaling
63	EGFR_1103_1115		
64	EGFR_1118_1130		
65	EGFR_1165_1177		
66	EGFR_1190_1202		
67	EGFR_862_874		
68	EGFR_908_920		
69	ERBB2_1241_1253	ERBB/EGFR	
70	ERBB2_870_882		
71	ERBB4_1181_1193		
72	ERBB4_1277_1289		
73	INSR_1348_1360	INSR	
74	INSR_992_1004		

Peptide #	Peptide ID	Signaling pathway	Cell-related function
75	VGFR1_1040_1052	VGFR	growth factor receptor signaling
76	VGFR1_1046_1058_Y1048F		
77	VGFR1_1049_1061		
78	VGFR1_1162_1174		
79	VGFR1_1206_1218		
80	VGFR1_1235_1247		
81	VGFR1_1320_1332_C1320S-C1321S		
82	VGFR1_1326_1338		
83	VGFR2_1046_1058		
84	VGFR2_1052_1064		
85	VGFR2_1168_1180		
86	VGFR2_1207_1219_C1208S		
87	VGFR2_944_956		
88	VGFR2_989_1001		
89	VGFR3_1061_1073		
90	MET_1227_1239	MET	
91	NTRK1_489_501	NTRK1	
92	NTRK2_509_521	NTRK2	
93	NTRK2_696_708		
94	PGFRB_1014_1028	PDGFRB	
95	PGFRB_1002_1014		
96	PGFRB_572_584		
97	PGFRB_709_721		
98	PGFRB_768_780		
99	PGFRB_771_783		
100	RET_680_692	RET	
101	RET_1022_1034		
102	RAF1_332_344	MAPK	Mitogenic signaling
103	RASA1_453_465		
104	MK01_180_192		
105	MK01_198_210		
106	MK07_211_223		
107	MK10_216_228	p38/JNK	
108	MK12_178_190		
109	MK14_173_185		
110	PRGR_545_557	PRGR	nuclear receptor signaling
111	PRGR_786_798		

Peptide #	Peptide ID	Signaling pathway	Cell-related function
112	CBL_693_705	Cbl	Src family signaling
113	CD3Z_116_128	T-cell	
114	CD3Z_146_158		
115	CD79A_181_193	B-cell	
116	EFS_246_258	Fyn	
117	EFS_246_258_Y253F		
118	FRK_380_392		
119	LCK_387_399	Lck	
120	PECA1_706_718	PECAM	
121	JAK1_1015_1027	JAK	STAT signaling
122	JAK2_563_577		
123	STA5A_687_699	STAT	
124	STAT1_694_706		
125	STAT3_698_710		
126	STAT4_686_698		
127	STAT4_714_726		
128	STAT6_634_646		
129	P85A_600_612	PI3K	Survival signaling
130	PDPK1_2_14		
131	PDPK1_369_381		
132	KSYK_518_530	Syk	SYK family signaling
133	LAT_194_206	LAT	
134	LAT_249_261		
135	ZAP70_485_497	Zap70	
136	ACHD_383_395	CHRND	WNT signaling
137	CTNB1_79_91	Beta-catenin	
138	FABPH_13_25	FABP3	other
139	FER_707_719	Fer	
140	PRRX2_202_214	PRRX2	
141	TYRO3_679_691	TYRO3	
142	NCF1_313_325	NCF1	ROS production
143	41_654_666	EPB41	Unknown

Supplementary Table 5. Clustering of the 63 peptides phosphorylated on ECM into signaling pathways and cell-related functions.

Peptide #	Peptide ID	Signaling pathway	Cell-related function
1	ANXA1_14_26	Annexin	Calcium signaling
2	PLCG1_764_776	PLC	
3	CDK2_8_20	CDK	Cell cycle signaling
4	CDK7_157_169		
5	RB_804_816	Rb	
6	EPHA1_774_786	Eph Receptor	cell-cell signaling
7	EPHA2_765_777		
8	EPHA7_607_619		
9	EPHB1_771_783		
10	EPHB1_921_933		
11	CRK_214_226	Crk	cytoskeletal signaling
12	FAK1_569_581	FAK	
13	FAK2_572_584		
14	PAXI_24_36		
15	PAXI_111_123		
16	FES_706_718	Fes	
17	K2C6B_53_65	CK	
18	MBP_198_210	MBP	
19	SRC8_CHICK_476_488	Other 2	
20	SRC8_CHICK_492_504		
21	DCX_109_121		
22	ART_004_EAIYAAPFAKKKXC	BMP	Differentiation signaling
23	DYR1A_312_324	DYR1A	
24	PP2AB_297_309	Phosphatase	
25	TEC_512_524	Btk	
26	ENOG_37_49	Enolase	Energy signaling
27	EPOR_361_373	EPO-R	Cytokine receptor signaling
28	EPOR_419_431		
29	FGFR3_753_765	FGFR	growth factor receptor signaling/ bone morphogenesis

Peptide #	Peptide ID	Signaling pathway	Cell-related function
30	EGFR_1165_1177	EFGR	growth factor receptor signaling
31	VGFR1_1326_1338	VGFR	
32	VGFR2_944_956		
33	VGFR2_989_1001		
34	MET_1227_1239	MET	
35	NTRK2_696_708	NTRK2	
36	PGFRB_1014_1028	PDGFRB	
37	PGFRB_1002_1014		
38	PGFRB_572_584		
39	PGFRB_709_721		
40	PGFRB_771_783		
41	RET_1022_1034	RET	
42	RAF1_332_344	MAPK	Mitogenic signaling
43	RASA1_453_465		
44	MK01_180_192		
45	MK10_216_228	p38/JNK	
46	CD79A_181_193	B-cell	Src family signaling
47	EFS_246_258	Fyn	
48	FRK_380_392		
49	LCK_387_399	Lck	
50	PECA1_706_718	PECAM	
51	JAK1_1015_1027	JAK	STAT signaling
52	JAK2_563_577		
53	P85A_600_612	PI3K	Survival signaling
54	PDPK1_2_14		
55	PDPK1_369_381		
56	LAT_194_206	LAT	SYK family signaling
57	LAT_249_261		
58	ZAP70_485_497	Zap70	
59	FER_707_719	Fer	Other
60	PRRX2_202_214	PRRX2	
61	TYRO3_679_691	TYRO3	
62	NCF1_313_325	NCF1	ROS production
63	41_654_666	EPB41	Unknown

Supplementary Table 6. Signaling pathways and cell-related functions of the 59 Markov-positive phospho-peptides on titanium.

Peptide #	Peptide ID	Signaling pathway	Cell-related function
1	ANXA1_14_26	Annexin	Calcium signaling
2	PLCG1_764_776	PLC	
3	RON_1346_1358	RON	
4	CDK2_8_20	CDK	Cell cycle signaling
5	CDK7_157_169		
6	RB_804_816	Rb	
7	EPHA1_774_786	Eph Receptor	cell-cell signaling
8	EPHA2_765_777		
9	EPHA7_607_619		
10	EPHB1_771_783		
11	EPHB1_921_933		
12	FAK2_572_584	FAK	cytoskeletal signaling
13	PAXI_24_36		
14	PAXI_111_123		
15	FES_706_718	Fes	
16	K2C6B_53_65	CK	
17	MBP_198_210	MBP	
18	SRC8_CHICK_476_488	Other 2	
19	SRC8_CHICK_492_504		
20	DCX_109_121		
21	ART_004_EAIYAAPFAKKKXC	BMP	Differentiation signaling
22	PTN11_539_551	Phosphatase	
23	TEC_512_524	Btk	
24	ENOG_37_49	Enolase	Energy signaling
25	EPOR_361_373	EPO-R	Cytokine receptor signaling
26	EPOR_419_431		
27	FGFR3_753_765	FGFR	growth factor receptor signaling/ bone morphogenesis

Peptide #	Peptide ID	Signaling pathway	Cell-related function
28	EGFR_1165_1177	EFGR	growth factor receptor signaling
29	ERBB4_1277_1289	ERBB/EGFR	
30	VGFR1_1326_1338	VGFR	
31	VGFR2_944_956		
32	VGFR2_989_1001		
33	MET_1227_1239	MET	
34	NTRK2_696_708	NTRK2	
35	PGFRB_1014_1028	PDGFRB	
36	PGFRB_1002_1014		
37	PGFRB_572_584		
38	PGFRB_768_780		
39	PGFRB_771_783		
40	RET_1022_1034	RET	
41	RAF1_332_344	MAPK	Mitogenic signaling
42	RASA1_453_465		
43	MK10_216_228	p38/JNK	
44	CD79A_181_193	B-cell	Src family signaling
45	EFS_246_258	Fyn	
46	FRK_380_392		
47	LCK_387_399	Lck	
48	PECA1_706_718	PECAM	
49	JAK2_563_577	JAK	STAT signaling
50	P85A_600_612	PI3K	Survival signaling
51	PDPK1_2_14		
52	PDPK1_369_381		
53	LAT_249_261	LAT	SYK family signaling
54	ZAP70_485_497	Zap70	
55	FER_707_719	Fer	Other
56	PRRX2_202_214	PRRX2	
57	TYRO3_679_691	TYRO3	
58	NCF1_313_325	NCF1	ROS production
59	41_654_666	EPB41	Unknown

Supplementary Table 7. List of peptides belonging to the selected signaling pathways (IPA) and the relative kinase detected by Immunoblot analysis. A) FAK signaling, putative kinase confirmed by Western blot: pFAK (Y925), B) ERK/MAPK signaling, putative kinase confirmed by Western blot: pERK, C) PI3K/AKT signaling, putative kinase confirmed by Western blot: pPKB. Values indicate early, mid and late Vmax for each peptide.

A)

Gene ID	Peptide ID	ECM			titanium		
		early	mid	late	early	mid	late
FGFR3	FGFR3_753_765	0	0	3.51E-13	0	0	3.24E-13
RAF1	RAF1_332_344	1.23E-12	9.71E-13	7.98E-13	1.10E-12	7.47E-13	6.8E-13
PXN	PAXI_111_123	2.19E-12	3.28E-12	3.90E-12	1.70E-12	2.54E-12	2.87E-12
PXN	PAXI_24_36	1.39E-12	2.16E-12	2.54E-12	9.98E-13	1.73E-12	1.89E-12
PIK3R1	P85A_600_612	1.3E-12	2.25E-12	2.69E-12	8.38E-13	1.84E-12	2.16E-12
PLCG1	PLCG1_764_776	3.36E-12	5.06E-12	5.88E-12	2.57E-12	4.13E-12	4.86E-12
PDPK1	PDPK1_2_14	8.51E-13	1.06E-12	1.15E-12	6.22E-13	7.8E-13	9.29E-13
PDPK1	PDPK1_369_381	3.91E-13	7.93E-13	9.47E-13	0	6.98E-13	7.56E-13
EGFR	EGFR_1165_1177	0	3.22E-13	0	0	0	3.09E-13
PTK2	FAK1_569_581	0	3.02E-13	0	0	0	0
MAPK1	MK01_180_192	3.38E-13	0	0	0	0	0
CRK	CRK_214_226	0	0	3.22E-13	0	0	0
PTPN11	PTN11_539_551	0	0	0	3.11E-13	0	0

B)

Gene ID	Peptide ID	ECM			titanium		
		early	mid	late	early	mid	late
FGFR3	FGFR3_753_765	0	0	3.51E-13	0	0	3.24E-13
RAF1	RAF1_332_344	1.22E-12	9.71E-13	7.98E-13	1.10E-12	7.47E-13	6.8E-13
PXN	PAXI_111_123	2.19E-12	3.28E-12	3.90E-12	1.70E-12	2.54E-12	2.87E-12
PXN	PAXI_24_36	1.39E-12	2.16E-12	2.54E-12	9.98E-13	1.73E-12	1.89E-12
PTK2B	FAK2_572_584	0	6.16E-13	6.69E-13	3.64E-13	5.06E-13	4.02E-13
PIK3R1	P85A_600_612	1.3E-12	2.25E-12	2.69E-12	8.38E-13	1.84E-12	2.16E-12
PLCG1	PLCG1_764_776	3.36E-12	5.06E-12	5.88E-12	2.57E-12	4.13E-12	4.86E-12
PTK2	FAK1_569_581	0	3.02E-13	0	0	0	0
PPP2CB	PP2AB_297_309	4.33E-13	3E-13	2.4E-13	0	0	0
MAPK1	MK01_180_192	3.38E-13	0	0	0	0	0
CRK	CRK_214_226	0	0	3.22E-13	0	0	0
PTPN11	PTN11_539_551	0	0	0	3.11E-13	0	0

C)

Gene ID	Peptide ID	ECM			titanium		
		early	mid	late	early	mid	late
PIK3R1	P85A_600_612	1.3E-12	2.25E-12	2.69E-12	8.38E-13	1.84E-12	2.16E-12
PDPK1	PDPK1_2_14	8.51E-13	1.06E-12	1.15E-12	6.22E-13	7.8E-13	9.29E-13
PDPK1	PDPK1_369_381	3.91E-13	7.93E-13	9.47E-13	0	6.98E-13	7.56E-13
JAK2	JAK2_563_577	3.76E-13	5.87E-13	7.62E-13	0	5.91E-13	6.09E-13
RAF1	RAF1_332_344	1.22E-12	9.71E-13	7.98E-13	1.10E-12	7.47E-13	6.8E-13
PPP2CB	PP2AB_297_309	4.33E-13	3E-13	2.4E-13	0	0	0
JAK1	JAK1_1015_1027	0	0	3.38E-13	0	0	0
MAPK1	MK01_180_192	3.38E-13	0	0	0	0	0

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