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# Insulin mediated activation of PI3K/Akt signalling pathway modifies the proteomic cargo of extracellular vesicles

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## Abbreviations:

CRC : Colorectal cancer

EVs : Extracellular vesicles

II : Insulin induced

NI : Non-induced

Rsc : Relative spectral count

WCL : Whole cell lysate

**Keyword:** extracellular vesicles, PI3K/Akt signalling, colorectal cancer, intercellular communication

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## Statement of significance

Increasing evidences suggest that high levels of insulin in blood is correlated with colorectal cancer (CRC) progression and poor survival rates in CRC patients. It is well established that insulin induces the PI3K/Akt signalling pathway, in turn increasing cellular proliferation and inhibiting apoptosis. In this study, activation of PI3K/Akt by insulin modified the proteomic cargo of extracellular vesicles (EVs) secreted by CRC cells. These EVs has the potential to stimulate proliferation in the recipient cells and hence can amplify the proliferative signal in the tumor microenvironment.

## ABSTRACT

Epidemiological studies suggest that diabetes and obesity increases the risk of colorectal cancer (CRC) and lowers the patient survival rate. An important attribute in diabetes and obesity is the presence of high levels of growth factors including insulin in blood which can activate the PI3K/Akt signalling pathway. Dysregulation of PI3K/Akt signalling pathway leads to sustained proliferative signals thereby allowing the cells susceptible to cancer. Extracellular vesicles (EVs), secreted nanovesicles of endocytic origin, are implicated in mediating the transfer of oncogenic cargo in the tumour microenvironment. In this study, CRC cells were treated with insulin to activate PI3K/Akt signaling pathway. Insulin treatment significantly increased the number of EVs secreted by CRC cells. Furthermore, pAkt was exclusively packaged in EVs secreted by PI3K/Akt activated cells. Quantitative proteomics analysis confirmed that the protein cargo of EVs are modified upon activation of PI3K/Akt signaling pathway. Bioinformatics analysis highlighted the enrichment of proteins implicated in cell proliferation in EVs secreted by PI3K/Akt activated cells. Furthermore, incubation of EVs secreted by PI3K/Akt activated cells induced proliferation in recipient CRC cells. These findings suggest that EVs can amplify the signal provided by the growth factors in the tumor microenvironment and hence aid in cancer progression.

## 1. Introduction

Cancer is a leading cause of death worldwide [1]. In 2012 alone, an estimate of 14.1 million people were diagnosed with cancer and around 8.2 million cancer deaths were reported worldwide. Among these, a total of 1.4 million people were estimated to be diagnosed with

colorectal cancer (CRC) [1, 2]. Alarming, the CRC incidence rates increased to 1.27 million in 2008 [3]. Estimated mortality rate of CRC also increased more than 10% from 2008. Large epidemiological studies have established positive correlation of environmental factors such as diet and lifestyle with CRC risk [4, 5]. Physical inactivity and excess body weight is two of the most correlated lifestyle-factors associated with obesity, diabetes and CRC [6-10]. People with body mass index over than 31 have double the risk of CRC occurrence in their life [9]. Similarly, risk of CRC occurrence was determined to be 1.7-fold higher in diabetic patients compared to healthy individuals [11]. The correlation of diabetes and obesity in CRC occurrence rate is evident and one common factor in these conditions is the high levels of insulin in the blood [12]. Hyperinsulinemia is widely documented to link obesity and diabetes to cancer progression and to the increased amount of insulin circulating in the blood [13]. Consistently, high insulin levels in blood is documented to increase the risk of CRC by two fold [14]. In addition, recent studies have established that CRC survivors with excess amounts of insulin in the blood have a higher risk of recurrence [15]. In accordance with other studies, diabetes patients undertaking insulin therapy for 3-5 years were reported to have a 3-fold increase in CRC risk compared with those who did not take insulin therapy [16].

Insulin is one of the growth factors known to induce PI3K/Akt signalling [17-19] which subsequently activates various signalling cascades to induce proliferation [20], neuronal survival [21] and inhibition of apoptosis by phosphorylation of BAD [22]. In cancer, dysregulation of PI3K/Akt signalling is a common occurrence [23, 24]. Constitutive activation of PI3K/Akt cascade could enhance cancer progression and aid in the evasion of immune surveillance by cancer cells. Hence, PI3K/Akt signaling axis have remained as a major therapeutic target by various drugs to treat cancer [25].

Extracellular vesicles (EVs) are found in the extracellular space of various cell types under normal and pathological conditions [26, 27]. EVs package various biologically active content such as protein, mRNA and miRNA which are transferable to the recipient cells and hence have been considered as mediators of signalling cascades [28]. EVs are known to mediate various biological processes such enhancement of cancer progression, activation of Wnt signalling, promoting neurodegeneration and activation of PI3K/Akt signalling [29]. However, the role of insulin in activating PI3K/Akt signaling pathway and its effect on

exosomal cargo and function is poorly studied. As EVs secreted by PI3K/Akt signalling pathway activated cells can be a critical part of understanding CRC progression, it is important to study the role of EVs in CRC progression.

Here, we isolated EVs secreted from PI3K/Akt activated CRC cells using differential centrifugation coupled with ultracentrifugation. Subsequently, quantitative proteomics analysis was used to characterize the change in the protein cargo of EVs. Using integrated bioinformatics and experimental approach, we uncover the potential of EVs in mediating signal cascades in neighbouring cells.

## **2. Material and methods**

### **2.1. Cell culture**

The human CRC LIM1215 cell line was cultured in RPMI 1640 medium (supplemented with 10% FCS (SAFC<sup>®</sup> Bioscience) and 100 Units/mL of Penicillin-Streptomycin in 150 cm<sup>2</sup> tissue culture flasks (BD Falcon<sup>™</sup>) at 37°C in 5% CO<sub>2</sub>.

### **2.2. Activation of PI3K/Akt signaling using human recombinant insulin**

LIM1215 CRC cells were grown up to 80% confluency in RPMI 1640 media supplemented with 10% FCS and 100 Unit/mL of Penicillin-Streptomycin. The cells were serum-starved for 10 h in RPMI 1640 media without FCS. The LIM1215 cells were later incubated with RPMI 1640 media containing 1.4 µg/mL of human recombinant insulin (Invitrogen) and 20 Unit/mL of Penicillin-Streptomycin. As negative control, LIM1215 cells were lysed prior to insulin induction. Meanwhile the positive control was taken 5 min after induction. Activation of PI3K/Akt signaling pathway was assessed for every hour after insulin induction and analysed by Western blotting.

### **2.3. Isolation of EVs from insulin-induced and non-induced LIM1215 CRC cells by differential centrifugation and ultracentrifugation**

LIM1215 cells were grown to 70-80% confluence in Integrid<sup>™</sup> 150 mm cell culture dishes. Cells were serum-starved in FCS depleted media for 10 h. Starved LIM1215 cells were cultured for another 10 h in RPMI 1640 medium with 1.4 µg/mL of human recombinant insulin and 20 Unit/mL of Penicillin-Streptomycin at 37°C in 5% CO<sub>2</sub>. As control, LIM1215 cells were cultured without insulin at 37°C in 5% CO<sub>2</sub>. Prior to EVs isolation from the culture media, Tryphan Blue assays were performed to assess the viability of the cells in culture.

Control and insulin-induced cell culture media from 480 plates were collected and centrifuged at  $500 \times g$  for 10 min. A subsequent spin at  $2000 \times g$  for 20 min was performed to remove floating cells and cell debris. In order to pellet down the EVs, the conditioned media was subjected to ultracentrifugation at  $100,000 \times g$  (SW-28 rotor, Beckman) for 1 h at  $4^{\circ}\text{C}$ . Pellets were washed with PBS to remove residual traces of media. The EVs preparation was stored in PBS at  $-80^{\circ}\text{C}$ .

#### **2.4. Characterization of EVs for insulin-induced and non-induced LIM1215 CRC cells using Nanoparticle Tracking Analysis**

EVs derived from  $2 \times 10^7$  LIM1215 cells, grown in FBS (Control), serum starved (NI), or insulin induced (II) were resuspended in  $500 \mu\text{L}$  of PBS. Number and size distribution of the EVs in the sample were analysed using NanoSight NS300. The samples were injected using syringe pump with constant speed of 60. The video was taken for duration of 1 min and analysis was performed with NTA 3.2 Dev Build 3.2.16 with the auto analysis settings. All samples were analysed in triplicate. The experiment details has been submitted to EV-TRACK [30].

#### **2.5. In-gel digestion**

EVs samples ( $30 \mu\text{g}$ ) were loaded onto precast NuPAGE<sup>®</sup> 4-12% Bis-Tris gels. EVs protein separation in SDS-PAGE was carried out at constant voltage of 150 Volt for 1 h. The gel was stained with coomassie for 1 h and destained (7.5% acetic acid, 20% ethanoic acid in milliQ) to obtain a clear background. Each protein lane was cut into 20 gel bands and subjected to a series of reduction, alkylation and trypsinization as previously described [31, 32]. Briefly, gel fragments were destained further using acetonitrile solution (50% (v/v) acetonitrile: 50 mM Amonium Bicarbonate (1:1)) overnight with constant shaking. Gel pieces were treated with 10 mM DTT for 30 min at  $55^{\circ}\text{C}$ , alkylated for 30 min with 25 mM iodoacetamide, and digested with 750 ng sequencing grade trypsin (Promega). Tryptic digestion was conducted overnight at  $37^{\circ}\text{C}$ , subsequently peptides were extracted from the gel using 50% acetonitrile with 0.1% trifluoroacetic acid. Prior to analysis with LC-MS/MS, samples were concentrated to  $10 \mu\text{L}$  by centrifugal lyophilisation. Extracted peptide samples were concentrated and subsequently loaded onto a micro fluidic trap column using an Eksigent NanoUltra cHiPLC system. Peptides were separated by using the following gradient: 2% to 20% B for 0.5 min,

20% to 40% B in 22.5 min, 40% to 80% B in 1 min and maintained at 80% B for 2 min followed by equilibration at 2% B for 3 min before the next sample injection where an additional 7 min of equilibration was carried out. The peptides were analysed with an AB SCIEX 5600 TripleTOF MS that was equipped with a Nanospray III ion source. The MS was scanning at 200-1250 Da and selecting 2+ to 5+ charged peptides for data dependent MS/MS. Repeated peptide exclusion was enabled after 3 occurrences for 90 s.

## **2.6. Database searching**

Total MS/MS spectra were merged and searched using Mascot (v2.4, Matrix Science, U.K.) against human RefSeq protein database (38,000 sequences). Search parameters used in Mascot were: Carboamidomethylation of cysteine residue (+57 Da) for fixed modification, oxidation of methionine (+16 Da) for variable modification, missed tryptic cleavage were set to 2 and for the tolerance of peptide and fragment ion were set to 20 ppm and 0.6 Da, respectively. Label-free spectral counting was performed as described previously [32]. Briefly, ratio of normalised spectral count (Rsc) was calculated to obtain the relative abundance between proteins identified in insulin-induced and non-induced EVs samples.

## **2.7. Bioinformatics analysis of proteomic data**

Protein list of insulin-induced and non-induced LIM1215 EVs generated by Mascot search engine were compared based on Rsc values. Highly abundant proteins found in insulin-induced sample (more than 2-fold) were analysed using Cytoscape [33] and FunRich [34, 35].

# **3. Result**

## **3.1. Activation of PI3K/Akt signalling pathway with insulin in CRC cells**

In order to study the effect of PI3K/Akt signaling pathway activation on CRC EVs, LIM1215 CRC cells were grown to 70% confluency and starved for 20 h in serum free media. After starvation, LIM1215 cells were treated with 0.14  $\mu\text{g}/\text{mL}$  human recombinant insulin in a time dependent manner. Activation of PI3K/Akt signalling pathway was assessed by Western blotting for the presence of the phosphorylated form of the serine/threonine kinase Akt (pAkt – S473) in the whole cell lysates (WCL). As shown in Figure 1A, upon insulin induction, the PI3K/Akt signalling was activated as confirmed by the presence of pAKT. However, the

pAkt signal diminished significantly after 60 min suggesting that the amount of insulin needs to be optimized to sustain the signal for longer time periods. In order to obtain adequate amount of EVs, LIM1215 cells were starved for shorter period of time (10 h) and amount of insulin was increased to 1.4  $\mu\text{g}/\text{mL}$ . Western blot analysis of pAkt confirmed the activation of PI3K/Akt signaling pathway up to 10 h when the LIM1215 CRC cells were treated with 1.4  $\mu\text{g}/\text{mL}$  of insulin (Figure 1B). Total Akt levels confirmed the reduction of pAkt signal was not due to the degradation of Akt.

### **3.2. Isolation and characterization of EVs from PI3K/Akt signalling activated LIM1215 CRC cells**

To isolate EVs from PI3K/Akt signalling activated cells, LIM1215 CRC cells were grown to 70-80% confluence and starved in serum free RPMI 1640 media for 10 h. After starvation, the cells were induced with 1.4  $\mu\text{g}/\text{mL}$  of insulin until 10 h. Conditioned media from insulin-induced (II) and non-induced (NI) cells were collected and subjected to differential centrifugation coupled with ultracentrifugation to isolate EVs. To ensure the viability of the cells after the serum starvation and insulin treatment, cell viability assay using Trypan blue exclusion was performed. As shown on Figure 1C, there are no significant difference in the viability among the cells grown under various conditions including FCS (Control), non-induced (NI) or insulin-induced (II). To quantify and characterize the EVs, nanoparticle tracking analysis was performed. Normalized to number of particle released by  $2 \times 10^7$  cells, II cells released higher number of EVs in comparison with NI cells,  $1.23 \times 10^9$  particle/mL and  $7.39 \times 10^8$  particle/mL, respectively (Figure 2A). Using size distribution analysis, it was further revealed that the population of EVs in the range of 50-150 nm were highly affected by the activation of PI3K/Akt signalling in the cells (Figure 2B). However, compared to control cells, II cells secreted less EVs. This could be attributed to the starvation of the cells prior to insulin treatment or lack of other growth factors which are normally present in the FBS. Further characterization using Western blot with equal protein amounts confirmed the presence of common EV enriched proteins such as Alix and TSG101 (Figure 2C) [36]. As Western blotting was performed with equal EV protein amount, there was no marked difference in the abundance of Alix and TSG101 in both II and NI cell derived EVs. Interestingly, pAKT was specifically packaged into II EVs during the activation of PI3K/Akt signaling pathway (Figure 2C).

### **3.3. Quantitative proteomic analysis of II and NI LIM1215 cell-derived EVs**

To profile the proteomic cargo of II and NI CRC cell-derived EVs, label-free quantitative MS-based proteomic analysis was performed. Equal amount of EVs proteins (30 µg) were separated by SDS-PAGE. The gel was stained with coomassie blue and each lane was excised to 20 bands. Excised bands were reduced, alkylated, and digested with trypsin and analysed by TripleTOF MS. The resulting MS/MS spectra were analysed using MASCOT (Version 2.4) against human RefSeq protein database (38,000 sequences). With a cut-off of at least 2 peptide identifications, a total of 2,135 proteins were identified in the II and NI LIM1215 CRC cell derived-EVs at a false discovery rate of < 1% (**Supplementary Table 2**). A total of 1834 and 1841 proteins were found in II and NI CRC cell-derived EVs, respectively (data deposited in ExoCarta and Vesiclepedia). A majority of the proteins 1540 (72%) were identified to be common between the EV samples while, 294 and 301 proteins were uniquely identified in II and NI cell-derived EVs, respectively (Figure 3A). Among the 1540 proteins that were common, EV markers such as Alix, TSG101, CD9, CD63, CD81, and HSC70 [28] were identified in abundance. In addition, equal abundance of small GTPase family members RAB4A, RAB6B, RAB14, RAB15 and RAB 21 which regulates MVB docking and membrane fusion were detected. These observations validate the presence of EVs in the isolated samples. In addition, several proteins implicated in CRC such as EGFR, CDH17, EPCAM, GPA33 and EPHB1 were also detected in both the EV samples [37]. In order to obtain quantitative data from the proteomic analysis, label-free spectral counting was performed using in-house software. Label-free quantitative proteomic analysis revealed that 712 proteins were differentially abundant in II and NI cell-derived EVs (arbitrary cut-off of 2-fold). Among these proteins, 364 were identified in more abundance in II EVs (Figure 3B).

### **3.4. Bioinformatics analysis reveals the enrichment of proteins implicated in cell proliferation**

In order to identify the pathways that are enriched in II EVs, network analysis was performed for 364 proteins that were highly abundant in II EVs. Analysis using Cytoscape protein-protein interaction visualization tool [38] highlighted four sub-networks in the II EVs (Figure 3C). The protein interaction networks identified in the analysis include “metabolism of proteins, translation and ribosome”, “formation and maturation of mRNA transcript and spliceosome” and “extracellular matrix (ECM) interaction”. In order to further identify the biological processes, molecular functions and subcellular compartments enriched in the

network, functional enrichment analysis were performed. As shown in Figure 4A, the analysis highlighted the biological processes enriched in II EVs. For instance, proteins implicated in DNA double strand break processing were found to be highly enriched (27-fold) in II EVs. Similarly, in subcellular compartment category, ribosomes (6.5-fold) and cytosolic ribosomal subunits were enriched in II EV sample. In molecular functions, structural constituent of ribosome and nucleoside diphosphate kinase activity were enriched (Figure 4B and 4C). Collectively, these data suggest that II EVs carry a rich cargo of proteins implicated in metabolism of proteins, translation and cell proliferation. In concordance with quantitative proteomics analysis, FAT1 and P-cadherin were enriched in the II EVs (Figure 4D).

FAT atypical cadherin 1 was originally identified in *Drosophila* and contains 34 tandem cadherin repeats with several epidermal growth factor domains in their extracellular region [39]. It has been implicated in maintaining cellular polarity and cell proliferation [40]. High expression of FAT1 is tightly correlated with early phase cell-cell contact, embryo development, and various types of cancer [39, 41, 42]. Somatic mutations of FAT1 gene leads to aberrant activation of Wnt signaling in several cancer such as head and neck cancer, glioblastoma and CRC [42]. In CRC specifically, FAT1 is mostly expressed in primary and metastatic stage and antibody treatment against FAT1 significantly impaired growth and invasiveness of CRC cells [43].

High-throughput phosphoproteomic studies also reveal that FAT1 can be phosphorylated upon insulin stimulation in mouse liver. It has been previously shown that phosphorylation of FasL, Annexin A2, tau protein and  $\gamma$ -synuclein can selectively package these cargo into EVs [44]. As FAT1 is enriched in the II EVs but not in the cells, it is tempting to speculate that the cargo sorting in EVs can be influenced by the phosphorylation state of a protein. Further studies are needed to validate this hypothesis.

Second cadherin family that was in high abundance in the II EVs is CDH3 or P-Cadherin. In comparison to FAT1, CDH3 contain 5 extracellular cadherin repeat and highly conserved cytoplasmic tail. In CRC, CDH3 is frequently demethylated and found in higher abundance in the liver metastatic CRC [45, 46]. In addition, P-Cadherin can enhance and stabilize IGF-1R and EGFR signaling hence leading to prolonged MAPK and Akt phosphorylation [47].

The presence of these proteins in II EVs may have a role in promoting and stabilizing insulin transduction signal cascades in recipient cells. Hence, further studies are needed to understand the role of FAT1 and CDH3 in II EVs-mediated functional regulation of the target cells.

### **3.5. II EVs mediate proliferation on target cells**

To establish a baseline for cellular uptake of EVs, LIM1215 EVs were labelled with PKH67 dye and incubated with two CRC cell lines, LIM1215 and HCT116. The underlying mutations and the plasma membrane protein expression for these two cell lines are different [31]. Interestingly, microscopic analysis suggested that LIM1215 cells took up relatively more number of LIM1215 cell-derived EVs compared to HCT116 cells (Figure 5A). Additional EV uptake assays were performed with LIM1215 cells as they were able to uptake more EVs as compared to HCT116 cells [48]. Furthermore, uptake efficiency of LIM1215 EVs were validated using FACS. The resulting FACS analysis suggested that EVs interact with the recipient cells in a time dependent manner. After 16 h of incubation with PKH67 labelled EVs, more than 70% of the cells efficiently interacted with the EVs (Figure 5B). To identify the function of pAkt containing II EVs in inducing proliferation on recipient cells, LIM1215 CRC cells were incubated with 20  $\mu$ g of II or NI EVs. Trypan blue assay highlighted the significant increase in the rate of cell proliferation after 48 h of treatment with II EVs (Figure 5C). From these results, it can be concluded that insulin can activate PI3K/Akt signaling pathway, modify the protein cargo of EVs and induce proliferation on the recipient cells.

## **4. Discussion**

Increasing evidences suggest that high levels of insulin in blood is correlated with CRC progression and poor survival rates in CRC patients [7, 8]. It is well established that insulin induces the PI3K/Akt signalling pathway, in turn increasing cellular proliferation and inhibiting apoptosis [18, 19]. Soluble secreted factors and EVs are thought to play a critical role in the intercellular communication within the tumour microenvironment [49-52]. Whilst the focus of this study was on EVs, the role of soluble secreted factors in cancer progression cannot be ignored. Currently, the role of PI3K/Akt signalling pathway in modulating the protein cargo in EVs is unknown. In addition, the role of EVs secreted by II CRC cells in the tumour microenvironment is not explored. In this study, useful insights into putative functions of EVs from PI3K/Akt activated cells were obtained by bioinformatics analysis of

the proteomics data and experimental approach. From the proteomics and Western blot analysis, it is evident that PI3K/Akt signalling regulates the protein cargo of EVs. LIM1215 cells-secreted EVs which were enriched with proteins implicated with mRNA formation and translation suggesting a possible role of EVs as mediators of cell proliferation in the tumor microenvironment. It is possible that EVs can shuttle the recombinant insulin substituted in the conditioned media of the cells. Hence, the proliferative effect of EVs exerted on the recipient cells could also be attributed to the insulin shuttled by the EVs. Perhaps, insulin receptor knockout cells could be used as controls to rule out the intracellular transfer of insulin. Regardless, it is physiologically possible for the EVs to shuttle insulin and regulate the recipient cell proliferation. As high insulin levels is correlated with high CRC risk, it can be speculated that high amounts of insulin in turn can regulate the secretion of more EVs and may result in the amplification of proliferative signals in the tumor microenvironment. As shown in Figure 6, a 3D tumor can receive nutrients/growth factors including insulin via the blood vessels. The PI3K/Akt activated CRC cells can then produce EVs that can amplify the proliferative signal in other CRC cells (that seldom receive sufficient growth factors) in the tumor proliferative core. As the deeper sections of the proliferative cores are depleted with nutrients/growth factors, EVs could be amplifying the proliferative signal and aid in cancer progression. Though this exact proposed model need additional concrete data, it is tempting to speculate that EVs may have a role in regulating the proliferation in inner cores of 3D tissues.

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### **Conflict of interest**

The authors have declared no conflict of interest

### **5. References**

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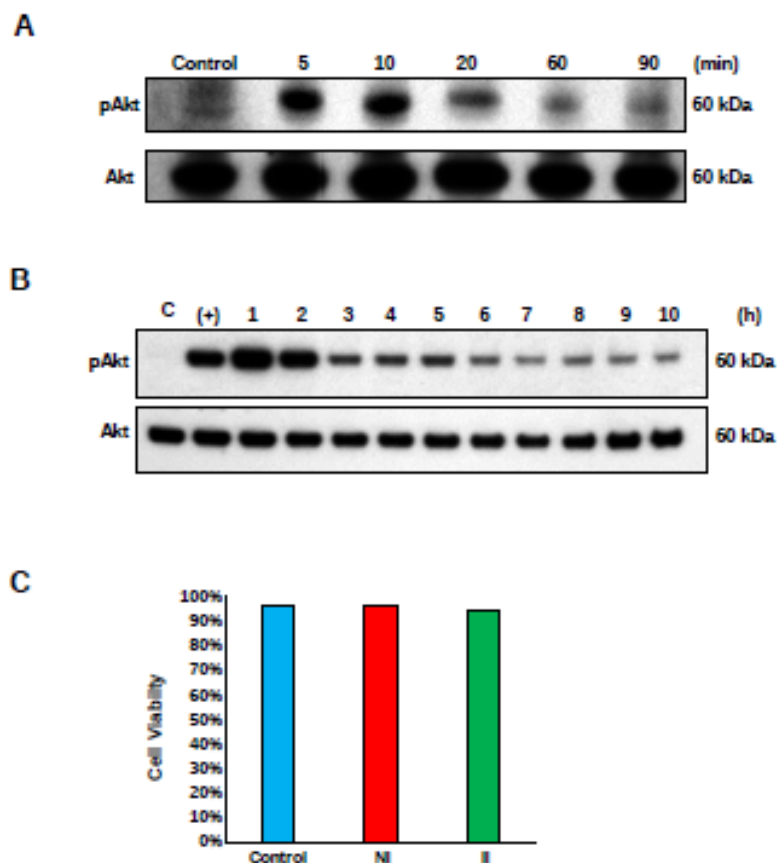
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## FIGURE LEGEND

### Figure 1. Stimulation of PI3K/Akt signalling pathway with insulin.

(A) LIM1215 cells were stimulated with 0.14  $\mu\text{g/mL}$  insulin and the WCL was prepared at various time points. The pAkt signal peaked at 5 min and was detectable in very low amounts at 90 min. Total Akt protein levels did not change irrespective of the pAkt levels and served as a loading control. (B) In order to retain the activation of PI3K/Akt signalling, LIM1215 cells were stimulated with 1.4  $\mu\text{g/mL}$  insulin. As a positive control (+), cells were induced with insulin for 5 min. The pAkt signal peaked at 1 h of stimulation and sustained up to 10 h. (C) Trypan blue exclusion assay depicts no significant difference in cell viability between treatment conditions (control, NI and II). Error bars represent standard error of the mean,  $n=3$ .

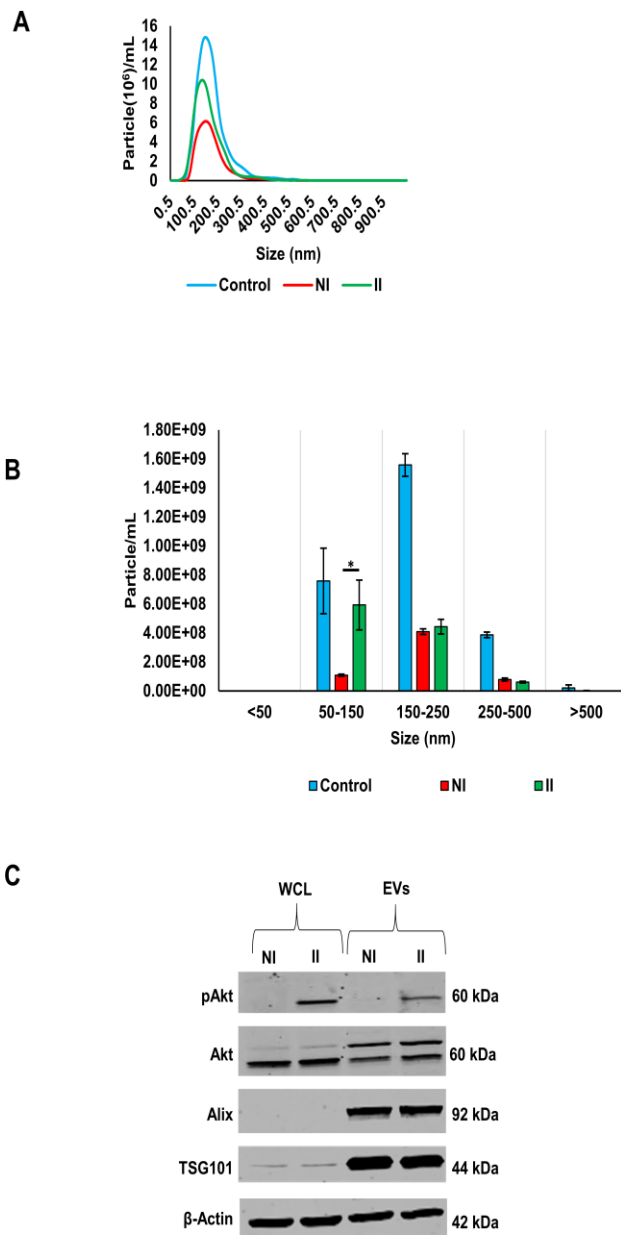
Figure 1



## Figure 2. Characterization of EV release upon PI3K/Akt activation from CRC cells.

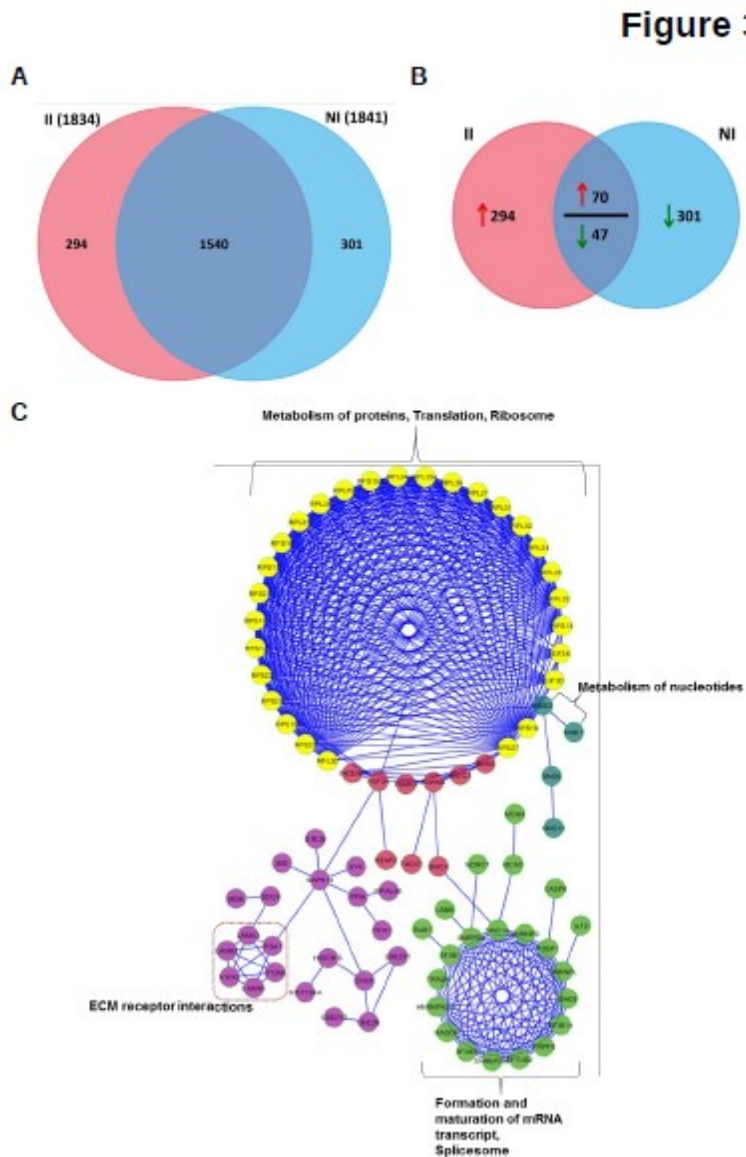
(A) NTA were performed to count number of particles released (normalized to  $2 \times 10^7$  cells) in different treatment conditions. (B) Size distribution analysis indicates significant increase of 50-150 nm EVs secreted by LIM1215 CRC cells upon induction with 1.4  $\mu\text{g/mL}$  Insulin. Error bars represent standard error of the mean,  $n=3$ , \* denotes significance ( $p<0.05$ ). (C) Western blot analysis identified enrichment of EV protein markers; Alix and TSG101 in the isolated NI and II EVs. Phosphorylated Akt was exclusively identified in II EVs.

### Figure 2



### Figure 3. High resolution MS-based proteomics analysis of NI and II EVs.

(A) Venn diagram depicting the number of proteins identified in II and NI CRC cell-derived EVs. A large majority of the proteins (72%) were identified in both the II and NI EVs. (B) In total, 712 proteins were found to be differentially abundant (at least 2-fold) in II and NI EVs. Upon insulin induction, 364 proteins were enriched in EVs (up to 40-fold). On the contrary, 349 proteins were found to be depleted in EVs upon insulin induction (up to 15-fold). (C) Sub-network of proteins that are enriched in II EVs as analysed in Cytoscape.

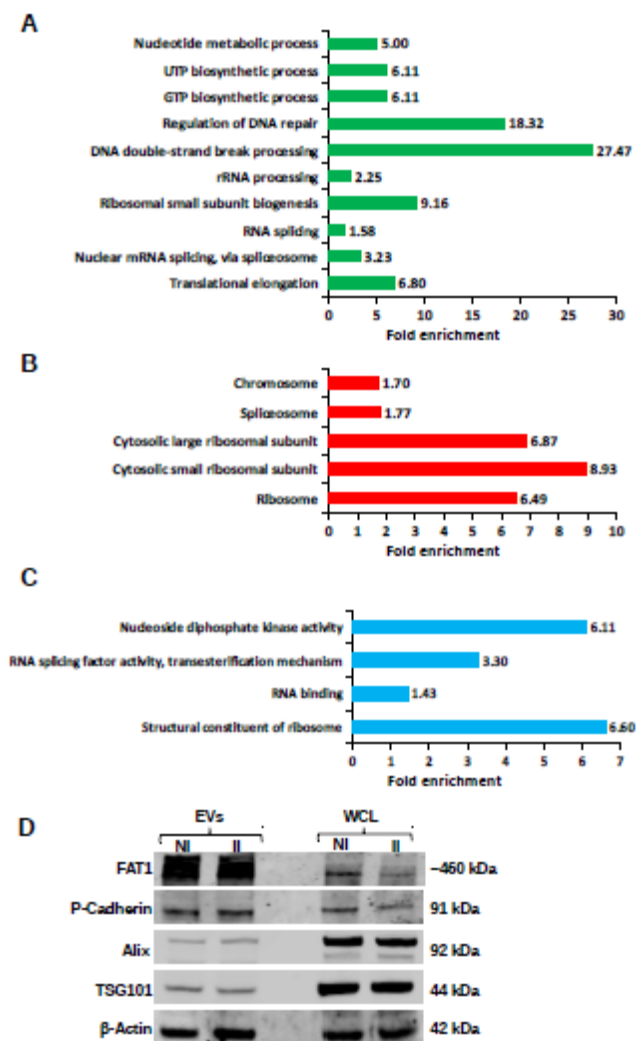


A

**Figure 4. Gene ontology and Western blot analysis of proteins enriched in II EVs.**

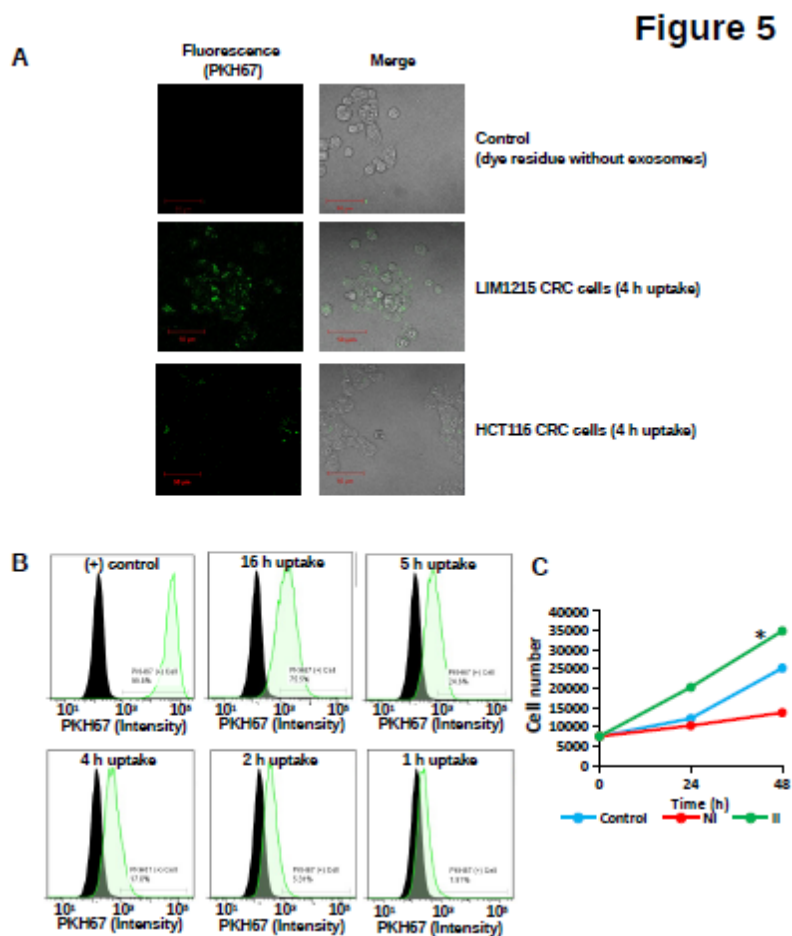
(A) Biological process that are enriched in proteins exclusively detected in II EVs. (B) Subcellular compartments that are enriched in proteins exclusively detected in II EVs. (C) Molecular function that are enriched in proteins exclusively detected in II EVs. (D) Western blot analysis validated MS proteomics data where several proteins including FAT1 and P-cadherin were enriched in II EVs. EV enriched proteins Alix and TSG101 were detected in similar levels in both the EVs.

**Figure 4**



**Figure 5. LIM1215 CRC cell-derived EVs induce cell proliferation.**

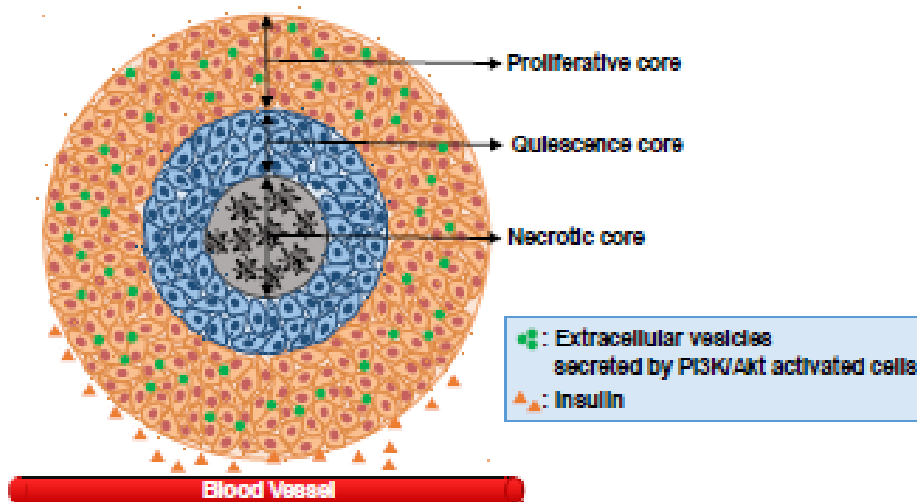
(A) LIM1215 cell-derived EVs were labelled with PKH67 dye (green fluorescent) prior to incubation with LIM1215 and HCT116 cells. Uptake assay was performed at 37°C for 4 h. Fluorescence was captured using confocal microscope Zeiss LSM 510. (B) FACS analysis was performed to identify the interaction efficiency of LIM1215 EVs against the recipient cells during 1, 2, 4, 5 and 16 h. Cells interacted with PKH67 labelled EVs are represented by green peak. Black graph indicates background fluorescence from control population. (C) Trypan blue proliferation assay performed on LIM1215 cells after 24 and 48 h of treatment using 20 µg of NI or II EVs. Error bars represent standard error of the mean, n=3, \* denotes significance (p<0.05).



**Figure 6. EVs amplify the proliferative signal in the tumor microenvironment.**

A proposed speculative model of how EVs can induce proliferation in 3D tumor tissues. Tumor cells in 3D space can have an outer proliferative core that is accessible by the blood vessels. Nutrients and growth factors are enriched in this core and hence account for constitutive proliferation. The inner core can be divided into the quiescent and necrotic cores that are depleted with nutrients and growth factors. EVs secreted by cells in the outer layer of the proliferative core in response to growth factors can induce proliferation on the recipient cells inside the proliferative core.

**Figure 6**



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