Highly specific role of the insulin receptor in breast cancer progression

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Abstract

Accumulating evidence from clinical trials indicates that specific targeting of the IGF1 receptor (IGF1R) is not efficient as an anti-breast cancer treatment. One possible reason is that the mitogenic signals from the insulin receptor (IR) can be processed independently or as compensation to inhibition of the IGF1R. In this study, we highlight the role of the IR in mediating breast tumor progression in both WT mice and a hyperinsulinemic MKR mouse model by induction of Ir (Insr) or Igf1r knockdown (KD) in the mammary carcinoma Mvt-1 cell line. By using the specific IR antagonist-S961, we demonstrated that Igf1r-KD induces elevated responses by the IR to IGF1. On the other hand, Ir-KD cells generated significantly smaller tumors in the mammary fat pads of both WT and MKR mice, as opposed to control cells, whereas the Igf1r-KD cells did not. The tumorigenic effects of insulin on the Mvt-1 cells were also demonstrated using microarray analysis, which indicates alteration of genes and signaling pathways involved in proliferation, the cell cycle, and apoptosis following insulin stimulation. In addition, the correlation between IR and the potential prognostic marker for aggressive breast cancer, CD24, was examined in the Ir-KD cells. Fluorescence-activated cell sorting (FACS) analysis revealed more than 60% reduction in CD24 expression in the Ir-KD cells when compared with the control cells. Our results also indicate that CD24-expressing cells can restore, at least in part, the tumorigenic capacity of Ir-KD cells. Taken together, our results highlight the mitogenic role of the IR in mammary tumor progression with a direct link to CD24 expression.

Key Words
- hyperinsulinemia
- insulin receptor
- insulin-like growth factor 1 receptor
- breast cancer and CD24

Introduction

High levels of circulating insulin were demonstrated to be an important marker and/or mediator of greater breast cancer risk in type 2 diabetes mellitus (T2DM) patients (Peairs et al. 2011). Emerging evidence from follow-up and meta-analysis studies indicates that hyperinsulinemia is associated with a worse prognosis in women with breast cancer even in the absence of diabetes (Goodwin et al. 2002, Belardi et al. 2013). The action of insulin is normally processed through the insulin receptor (IR), but at supraphysiological concentrations, insulin can also activate the insulin-like growth factor 1 receptor (IGF1R). Given the fact that both receptors belong to the same tyrosine kinase receptor subfamily, IR shares great structural homology with the IGF1R especially in the tyrosine kinase domain. In addition, both receptors activate similar signaling pathways, the PI3K/Akt and the
MAPK pathways (Avruch 1998, Gallagher & LeRoith 2010), which regulate processes such as cell proliferation, cell division, migration, and apoptosis (de la Monte & Wands 2005). Both the IGF1 and IRs are frequently overexpressed in breast cancer. Importantly, high levels of expression of the IR (INSR) was found to be associated with decreased disease-free survival in node-negative breast cancer patients (Belfiore et al. 1996, Mathieu et al. 1997). Furthermore, results have indicated that the ratio of IR subtypes (IR-A being more mitogenic than IR-B) may be associated with a worse prognosis; at present, the subtypes are measurable by mRNA levels only (Belfiore et al. 2009, Rowzee et al. 2009, Huang et al. 2011). Investigators have also proposed that IGF1R:IR hybrid receptors may be important in cancer growth; however, their levels are usually much lower than those of holo-IGF1R and IRs and they primarily respond to IGF1 (Gallagher & LeRoith 2010). Thus, their significance is as yet undefined.

Over the past two decades, most efforts were invested in developing strategies for targeting the IGF1R in cancer therapy, sparing the IR, due to concern about metabolic abnormalities secondary to IR inhibition (Judson et al. 1985, Buttrum et al. 2003). However, results from phase 2 and 3 clinical trials indicate that IGF1R inhibition with specific antibodies may result in unacceptable side effects and limited efficacy (Yang & Yee 2012, Guha 2013). One possible reason for the limited efficacy is that the IR may deliver mitogenic signals independently or as compensation to the IGF1R inhibition. Crosstalk between tyrosine kinase receptors has been shown to confer therapy resistance upon specific targeting of only a single receptor (Engelman et al. 2007). Similarly, enhancement in IR signaling was exhibited following IGF1R downregulation in several breast cancer cell lines (Zhang et al. 2007). Furthermore, IGF2 can drive IR signaling when IGF1R is selectively targeted, which might present another drawback for specific anti-IGF1R targeting (Buck et al. 2010). Thus, determining the role of the IR in breast cancer progression has become highly relevant in the field of oncology.

We have recently demonstrated that dual inhibition of both IR and IGF1R could reduce mammary tumor growth rate (Rostoker et al. 2013). In the current study, we employed short hairpin RNA (shRNA) technology to distinguish between the roles of the IR and IGF1R in mammary tumor growth. We demonstrate the therapeutic efficiency of targeting specific IR in a tumor-specific manner. Moreover, our current results with hyper-insulineemic MKR female mice indicate that, even at high levels, insulin mitogenic signals are mediated mainly through the IR and not through the IGF1R. Microarray analysis identified target genes and alterations in the cancer-associated pathway following IR activation. These results shed light on the effect of IR on cancer progression. Finally, we demonstrate for the first time, to our knowledge, a linkage between the IR and CD24 expression that supports the role of the IR in mammmary tumorigenesis.

Materials and methods

Cell culture

Mouse mammary cancer cell line, Mvt-1, has been described previously (Pei et al. 2004). Cells were grown in monolayer culture in DMEM (Biological Industries, Beit Haemek, Israel) supplemented with 10% fetal bovine serum (Biological Industries) and antibiotics (penicillin; streptomycin; Biological Industries) at 37 °C in a humidified atmosphere consisting of 5% CO₂ and 95% air.

Animals

FVB/N and MKR mice (FVB/N background) were used in this study. The generation of the MKR mice, transgenic mice with dominant negative IGF1R specifically targeted to the skeletal muscle, has been described previously (Fernández et al. 2001). Mice were kept on a 12 h light:12 h darkness cycle with access to standard mouse chow and fresh water ad libitum. Mouse studies were performed according to the protocol approved by the Technion Animal Inspection Committee. The Technion holds an NIH animal approval (license number A5026-01).

Knockdown of Igf1r and Ir by lentiviral-based delivery of shRNA

Vectors (GIPZ) encoding the following microRNA-adapted shRNA 5’-TGACTGTGAAATCTTCGGC-3’ (mouse/human IGF1R), 5’-AGACCAGACCCGAAGATTTCTC-3’ (mouse IR) were packed in high-titer lentiviral particles were purchased from Open Biosystems (Huntsville, AL, USA). These vectors or a vector containing a scrambled shRNA sequence (control shRNA; Open Biosystems) were used to infect Mvt-1 cells in the presence of 8 µg/ml polybrene (Sigma–Aldrich); all three vectors contained a GFP marker and puromycin resistance gene. Stable knockdown (KD) of Igf1r and Ir was achieved by selection with 2 µg/ml
puromycin (Sigma–Aldrich). It is important to note that the shRNA platform induces KD of both IR isoforms (IR-A and IR-B), as the shRNA targets the mRNA, from which both isoforms are derived.

**In vitro signaling assays**

Cells were washed twice with PBS from their serum-supplemented media, then grown for 16 h in serum-free media before a 10-min stimulation with insulin (10 nM) or IGF1 (10 nM). For IR blockade, cells were pretreated for 30 min with 100 nM of the high-affinity IR antagonist S961 (a gift from Novo Nordisk, Maaloev, Denmark).

**Syngeneic orthotopic tumor models**

Infected cells (scrambled/IR-KD/IGF1r-KD Mvt-1 cells) or sorted Mvt-1 cell populations (CD24− and CD24+ Mvt-1 cells) were suspended in 100 μl PBS and then injected (5×10^4 cells/mouse) into the left inguinal mammary fat pads (#4) of 8-week-old MKR female mice. Tumor volume was monitored once a week using calipers; the volume was calculated in mm³ by the following formula: (width²× length×0.5). After killing, tumors were removed and weighed, then flash frozen in liquid nitrogen, and kept at −80 °C for further analysis.

**Protein extraction and western blot analysis**

Western blot and densitometric analyses were carried out for protein detection in cells and tumor tissues as described previously (Rostoker et al. 2013). The following antibodies were used: phospho-IRβ(Y1150/51/IGFIR)[Y1135/36], IRβ, IGF1R, phospho-Akt(Thr308), total Akt, phospho-p44/42MAPK, total p44/42-MAPK (Erk1/2), and β-actin purchased from Cell Signaling Technology (Danvers, MA, USA), followed by a matched secondary antibody conjugated with HRP (Jackson Laboratories, Bar Harbor, ME, USA).

**Tail vein metastasis assay**

Ten thousand Mvt-1 cells were injected through the tail vein to assess lung metastatic activity.

Mice were killed 28 days following injection, lungs were removed and fixed in 4% paraformaldehyde, embedded in paraffin, sectioned, and stained using hematoxylin and eosin (H&E).

**Gene expression array studies**

Mvt-1 cells were starved with a serum-free medium for 16 h and then treated for 90 min with 10 nM insulin. Total RNA was isolated using the total RNA purification kit (NORGEN Biotek Corp., Thorold, Canada) according to the manufacturer’s instructions. RNA was amplified into cRNA and labeled by in vitro transcription using the Illumina TotalPrep RNA Amplification Kit (Ambion, Applied Biosystems, Foster City, CA, USA). Samples were then hybridized to the Mouse WG-6 v2.0 Expression Beadchip according to manufacturer’s instructions for the direct hybridization assay (Illumina, Inc., San Diego, CA, USA). Arrays were scanned using the Illumina Bead Array Reader Confocal Scanner. Array data export processing and analyses were performed using the Illumina BeadStudio V.2009.1 Software for extraction and quality control. The raw data obtained were imported into the Eureka-DMA Software and were analyzed as described previously (Abelson 2014). Briefly, filtration of transcripts that were not detected in any sample and expressed at levels below the background intensities was omitted, and differentially expressed genes following insulin treatment were selected using P value <0.05 and fold change of at least 1.5. Pathway enrichment analysis was performed with differentially expressed genes (P value <0.05 and fold change of at least 1.3) to interrogate molecular pathways enriched upon insulin treatment. The list of genes for the pathway enrichment analysis is provided as Supplementary Table 1, see section on supplementary data given at the end of this article along with the enriched pathway gene sets (Supplementary Table 2). The data have been deposited in the National Center for Biotechnology Information, and are accessible through GEO Series accession number GSE57139.

In order to also identify molecular and functional interactions between the differentially expressed genes found following insulin treatment, the list of genes was imported into ingenuity pathway analysis (IPA; ingenuity.com). Genetic networks were ranked by score. Scores of 3 or higher have a 99.9% confidence of not being generated by a random chance alone.

**Quantitative PCR for cDNA products**

Quantitative PCR was performed using the Absolute Blue SYBR-Green ROX Mix (Thermo scientific, ABgene, Epsom, UK). RNA was extracted from treated Mvt-1 cells using the Total RNA Purification Kit (NORGEN Biotek Corp.) according to the manufacturer's instructions, followed by single-stranded cDNA synthesis using the Verso reverse transcriptase (Thermo scientific, ABgene). The expression measurement of
the designated genes was performed using the Rotor-Gene™ 6000 system (Corbett Research, Sydney, NSW, Australia) and its software, version 1.7. The relative gene copy number was normalized using b2 microglobulin (B2m) as an independent internal control gene, and calculated from the threshold cycle (Ct) by the $2^{-\Delta\Delta Ct}$ method. The primers used were as follows: B2m, forward: TTCTGGTGCTTCTCACTGA and reverse: CAGTATGTGCTTCCACCTC; Ets2, forward: CCTGCAATCTTTCATCAAGC and reverse: AGTTCTTGATCTTACGACAGT; Hebp1, forward: AGTGGATGATGACAGGTG and reverse: GCTGTGACCTGTGCTTGT; cyclin D1 (Ccnd1), forward: GCCCGGCTTTGATCTCTGCT and reverse: TGGAGGCTGCAGGACTTGT.

### Flow cytometry

The following antibodies were used for cell-surface staining of the Mvt-1 cell line, Pacific-Blue-conjugated anti-CD24, and Alexa Fluor 647 (AF647)-conjugated anti-CD49F (Biolegend, San Diego, CA, USA). 7-Amino-actinomycin D (7-AAD, Biolegend) was used to gate live cells. Cells were stained at a concentration of 5×10^6 cells/ml of FACS buffer (PBS containing 0.1% BSA) for 20 min on ice in the dark, after which, the cells were washed twice and resuspended in FACS buffer containing 7-AAD. Stained cells were analyzed using the CyAn ADP Instrument (Dako-Cytomation, Glostrup, Denmark) and the FlowJo 7.25 Analysis Software. Flow cytometry-based cell sorting for CD24^- and CD24^+ cells was performed using FACSARia (BD Biosciences, San Jose, CA, USA).

### Statistical analysis

All data are expressed as mean ± S.E.M. The Mann–Whitney U test was used for statistical analysis of unmatched groups; the Wilcoxon’s signed-rank test was used for

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**Figure 1**

IR-KD cells were poorly responsive to insulin, whereas IGF1R-KD cells responded well to IGF1. (A) IR and IGF1R were downregulated in the Mvt-1 cells using shRNA constructs and a non-silencing (scrambled) shRNA construct was used as a control. To confirm KD efficiency, cellular lysates of Mvt-1 cells infected with lentiviral particles containing shRNA sequence against IR, IGF1R, and non-silencing sequence were separated by SDS–PAGE, and both IR and IGF1R protein levels were assessed using specific antibodies by western blotting. (B) Protein expression was quantified relative to β-actin expression by densitometric analysis. (C) Cells were starved with a serum-free medium for 16 h and then treated for 10 min with 10 nM insulin or IGF1. Cellular lysates were separated by SDS–PAGE, and expression of pAkt, Akt, and β-actin was assessed using specific antibodies by western blotting. (D) Protein expression was quantified by densitometric analysis; pAkt levels were normalized to Akt expression and phosphorylation for insulin and IGF1-treated cells was determined. Equal loading of proteins was demonstrated by immunoblotting with an antibody directed against β-actin. The Mann–Whitney U test was performed to compare the difference from the control cells treated with vehicle, *P<0.05, **P<0.005, and ***P<0.001.
matched group comparisons, with $P$ values $<0.05$ considered statistically significant.

**Results**

**KD of the $Ir$ and $Igf1r$ has different effects on their responses to their cognate ligand**

In order to distinguish between the roles of IR and IGF1R in breast cancer initiation and progression, we used shRNA lentiviral technology to knock down each receptor in the murine mammary Mvt-1 cell line. KD specificity and efficiency for each receptor were confirmed by immunoblotting of whole-cell lysates. Densitometric analysis indicates an approximately 70% reduction in IR expression in the $Ir$-KD cells with no effect on IGF1R expression when compared with control cells that were targeted with a non-targeting scrambled construct. In the $Igf1r$-KD cells, $Igf1r$ expression was reduced by 90% following shRNA lentiviral delivery when compared with the control cells (Fig. 1A and B).

Then, we examined the effect of each receptor KD on the functionality of cells. Cells were treated with 10 nM insulin or 10 nM IGF1 for 10 min. The cells were then lysed and phosphorylation of the downstream Akt kinase was measured by western blot analysis. $Ir$-KD cells displayed a poor response to insulin stimulation when compared with the control cells treated with scrambled siRNA however, they responded well to IGF1, as indicated by Akt phosphorylation levels. The $Igf1r$-KD cells responded to insulin stimulation in a manner similar to the control cells; however, Akt phosphorylation was also observed in response to IGF1 stimulation (Fig. 1C and D).

**$Igf1r$-KD induces an increased responsiveness of the IR to IGF1 stimulation**

As Akt is activated by IGF1 stimulation in the $Igf1r$-KD cells, we postulated that IGF1R downregulation in the cells give rise to highly responsive IR following IGF1 stimulation. To verify this assumption, cells were stimulated with insulin or IGF1, in the presence of the specific IR inhibitor, S961. A significant decrease in Akt phosphorylation level was observed in all three groups of cells when incubated with S961 before insulin stimulation. In the $Igf1r$-KD cells, S961 pretreatment significantly decreased Akt phosphorylation (47%) following IGF1 stimulation, an effect that was not observed in both control and $Ir$-KD cells (Fig. 2A and B). To further demonstrate the sensitivity acquired by IR to IGF1 following $Igf1r$ knockdown, we stimulated control and $Igf1r$-KD cells with a vehicle or IGF1, IR was immunoprecipitated, and phosphotyrosine was examined by western blot analysis. We found that IR was activated following IGF1 stimulation only in the $Igf1r$-KD cells (data not shown).

**Figure 2**

Severe IGF1R downregulation as accomplished by the shRNA construct results in increased IGF1 response via the IR. Cells were starved with a serum-free medium for 16 h and then treated for 30 min with 100 nM of the IR inhibitor S961 before treatment with 10 nM insulin or IGF1. (A) Cellular lysates were separated by SDS–PAGE, and expression of pAkt, Akt, and β-actin was assessed using specific antibodies by western blotting. (B) Protein expression was quantified by densitometric analysis; pAkt levels were normalized to Akt expression and relative phosphorylation for insulin and IGF1-treated cells was determined. Equal loading of proteins was demonstrated by immunoblotting with an antibody directed against β-actin. The Mann–Whitney $U$ test was performed to compare all groups with the control cells treated with a vehicle, *$P<0.05$, **$P<0.005$, ***$P<0.001$, and to compare IGF1 treatment with the IGF1 + S961 treatment, ##$P<0.005$. 

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IR plays a significant role in mammary tumor development and metastasis by reducing the activities of both the PI3K/Akt and MAPK pathways

To evaluate the role of IR and IGF1R in mammary tumor initiation and progression, control cells, Ir-KD cells, and Igf1r-KD cells were injected into the fourth mammary fat pad of WT and MKR female mice and the tumor volume was measured over the following 5 weeks. The tumor growth rate was significantly reduced in the Ir-KD groups, and at the end of the study, tumor size was reduced in these groups by approximately threefold in both WT female mice and the MKR female mice when compared with the mice that received injections of control cells (Fig. 3A and C). Similar results were obtained for tumor weight, as indicated by the significantly smaller tumors generated following injection of Ir-KD cells (Fig. 3B and D). In contrast, the Igf1r-KD in the MVT-1 cells did not affect tumor growth in either WT or MKR mice (Fig. 3A, B, C and D). IR and IGF1R intracellular signals are primarily mediated via the PI3K/Akt and the MAPK pathways. In order to determine the activity of both pathways in the mammary tumors generated by the control, Ir-KD, and the Igf1r-KD cells in both WT and MKR mice, tumor tissues from the three groups of WT and MKR mice were examined. Western blot analysis revealed a significant decrease in both PI3K/Akt and MAPK pathways in the Ir-KD-derived tumors compared with the control tumors as shown by a 63% reduction in Akt phosphorylation level in the WT mice (Fig. 3E and G) and an 85% reduction in the MKR mice (Fig. 3H and I). Erk1/2 phosphorylation levels were reduced by 47% in the WT mice (Fig. 3E and G) and by 67% in the MKR mice (Fig. 3H and I). These changes were not observed in the Igf1r-KD-derived tumors. Then, we compared the levels of the metastatic potential (independent of tumor size) of the control, Ir-KD, and Igf1r-KD cells in both WT and MKR mice. Cells were injected into the tail vein and 28 days following injection mice were killed and lung metastasis was determined by histological analysis. Control cells formed lesions in each of the inoculated WT mice, whereas only 33% and 50% of the lungs were found to contain metastases following injection of Ir-KD and Igf1r-KD cells respectively (not statistically significant). Similar results were obtained in the MKR mice (Table 1). These results indicate that IR plays a significant role in mammary tumor growth and that both IR and IGF1R mediate metastatic activity.

Gene array analysis reveal alterations in genes and cancer-associated pathways following insulin stimulation

To evaluate the effect of insulin on the cancer cell transcriptome, we examined gene expression variation along with enriched signaling pathways and gene networks in the Mvt-1 cells following insulin stimulation. In order to identify direct alterations in transcription factors and other primary gene alterations, cells were stimulated for 90 min with 10 nM insulin. RNA was extracted and subjected to gene expression array studies. A total of 15 genes were found to be differentially expressed (fold change > 1.5 and P value < 0.05) following insulin stimulation as shown in Fig. 4A. Several of the highlighted transcripts have been associated with various processes such as cell growth, migration, and regulation of immune cells. For example, upregulation of the Ets2 transcription factor has been found to be involved in the regulation of the cell cycle and cell transformation (Sementchenko et al. 1998). On the other hand, Hebp1, a transcriptional repressor of several oncogenes, has been demonstrated to be a regulator of cell differentiation that negatively regulates cell proliferation (Sampson et al. 2001). The microarray analysis results indicate a reduction in Hebp1 expression in Mvt-1 cells following insulin stimulation. In order to reveal the regulation of aberrant pathways, which include genes with a smaller change in expression, appropriate for the 90-min insulin signal induction in this experiment, a cutoff of fold change > 1.3 and P < 0.05 were chosen. Pathway enrichment analysis of the differently expressed genes revealed the affiliation of insulin and cancer-associated pathways (Supplementary Table 2) as anticipated. In
addition to the mTOR and PI3K–Akt signaling pathways, we found that insulin alters several other pathways that are associated with the cell cycle, proliferation, and apoptosis. To validate the microarray results, changes in mRNA levels of two genes, *Ets2* and *Hebp1*, that regulate the cell cycle and apoptosis were quantified using qRT-PCR in new independent RNA samples. The qRT-PCR results confirmed the microarray data for these three genes, with similar fold changes. Furthermore, we demonstrate that the alterations in expression levels were stable for at least 4 h for two of these genes (Fig. 4B). Unsurprisingly, we found that IGF1 similarly regulates the same genes (data not shown). To identify gene networks that are active following insulin stimulation, the differentially expressed genes were analyzed using the IPA Software (Ingenuity Systems, Redwood City, CA, USA). Genes were mapped to genetic networks that describe functional relationships, and then the IPA Software was used to associate these networks with known biological pathways. Five networks were found to be significantly altered in the insulin-treated cells, and these networks were associated with cellular growth and development, proliferation, the cell cycle, and immune cell trafficking (Supplementary Table 3, see section on supplementary data given at the end of this article). The top scored network contains genes associated with cellular development, cellular growth and proliferation, and cellular assembly and organization, and this is illustrated in Supplementary Figure 1. This analysis further highlights the mitogenic effects of insulin on cancer cells.

**Ir-KD is associated with downregulation of CD24<sup>+</sup>-expressing cells**

Recently, a subpopulation of breast tumor cells with high tumorigenic capacity have been identified in the MMTV-Wnt-1 transgenic mice which express CD24 and CD49f, two common cell surface markers for breast cancer stem cells (Vaillant et al. 2008). It was also demonstrated that CD24 downregulation alters the gene expression pattern and pathways associated with insulin (Sagiv et al. 2008). For this reason, we characterized the cell-surface expression of CD24 and CD49f in the Mvt-1 cells, using flow cytometry analysis. While CD49f expression remained uniform in Mvt-1 cells, two distinct populations were observed: CD24<sup>-</sup> (62.4%) and CD24<sup>+</sup> (37.6%) cells (Fig. 5A) that were separated into pure (>98%, as determined by FACS analysis) CD24<sup>-</sup> and CD24<sup>+</sup> cell populations (data not shown). In *vitro*, both cell populations display a distinct appearance with the CD24<sup>+</sup> cells having a slightly more mesenchymal morphology (Fig. 5B). To examine whether IR might be involved in the regulation of CD24 expression, CD24 and CD49f expression was evaluated in the Ir-KD and Igfr1-KD cells compared with the control cells. FACS analysis revealed a similar percentage of CD24<sup>-</sup> and CD24<sup>+</sup> subpopulations in both the Igfr1-KD cells and the control cells; however, a significant reduction (approximately 66%) in the CD24<sup>+</sup> subpopulation was observed in the Ir-KD cells when compared with the control cells (Fig. 5C).

**Table 1** The number of lungs with lesions out of the total number in the group, following injections of cells into the tail vein

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Metastatic lungs/total lungs</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT mice</td>
<td>5/5 (100%)</td>
</tr>
<tr>
<td>Control</td>
<td>2/6 (33%)</td>
</tr>
<tr>
<td>IR-KD</td>
<td>3/6 (50%)</td>
</tr>
<tr>
<td>IGF1R-KD</td>
<td>4/6 (66%)</td>
</tr>
<tr>
<td>MKR mice</td>
<td>5/6 (83%)</td>
</tr>
<tr>
<td>Control</td>
<td>3/6 (50%)</td>
</tr>
<tr>
<td>IR-KD</td>
<td>5/6 (83%)</td>
</tr>
<tr>
<td>IGF1R-KD</td>
<td>4/6 (66%)</td>
</tr>
</tbody>
</table>

**Figure 4**

Volcano plot and networks of differently expressed genes following insulin stimulation. Mvt-1 cells were stimulated with insulin (10 nM) for 90 min, RNA was extracted from the vehicle-treated and insulin-treated cells and tested on the Illumina gene-array platform. Volcano plot (A) of log<sub>2</sub> fold-change shows 15 genes that were differentially expressed by at least 1.5-fold at P value < 0.05. (B) RNA was extracted from new independent samples of cells stimulated with insulin (10 nM and 90 min), and these samples were analyzed using qPCR. The Mann-Whitney test was performed to compare insulin and vehicle treatments, *P < 0.05. A full colour version of this figure is available at [http://dx.doi.org/10.1530/ERC-14-0490](http://dx.doi.org/10.1530/ERC-14-0490).
CD24 expression is associated with partial restoration of tumorigenic capacity of the Ir-KD cells

CD24 expression has been recently proposed to be a marker for aggressive breast tumors. Based on the observation of reduced tumor size following injection of the Ir-KD cells, combined with the significant reduction in the CD24+ cell subpopulation following Ir-KD (in vitro), we examined whether CD24 expression is associated with restoration of the tumorigenic capacity of Ir-KD cells in vivo. For this purpose, control cells and Ir-KD cells were double sorted into pure (>98%) CD24− and CD24+ cell subpopulations. As shown in Fig. 6, control/CD24− cells generated significantly larger tumors by approximately 2.5-fold when compared with the control/CD24+ cells.

Interestingly, CD24 expression in Ir-KD cells was associated with the rescue, at least partially, of tumorigenic capacity; Ir-KD/CD24+ cells formed significantly (approximately 3.7-fold) larger tumors, when compared with the Ir-KD/CD24− cells (Fig. 6B and C). Moreover, while both tumor volume and tumor weight for the Ir-KD/CD24− cells were significantly reduced in comparison to the control/CD24− cells, tumors generated by Ir-KD/CD24+ cells demonstrated similar weight to tumors generated by the controls/CD24+ group. However, the tumor volume was significantly reduced compared with tumors from the control/CD24− group (Fig. 6C and D). These results further support our in vitro observations and indicate that cell-surface expression of CD24 might be related to IR expression and more aggressive tumor growth.

Figure 5
Ir-KD is associated with downregulation of CD24+—expressing cells.
(A) A FACS dot plot showing CD24 and CD49f expression in the Mvt-1 cell line. (B) Mvt-1 cells were double sorted into CD24− and CD24+ cells, and different phenotypes for each group are presented in phase-contrast bright field images of cells grown in adherent cultures. (C) Representative FACS dot plots of CD24 and CD49f cell surface expression in control, Ir-KD, and Igf1r-KD cells, and CD24− cells are gated. A full colour version of this figure is available at http://dx.doi.org/10.1530/ERC-14-0490.
In the current study, we employed shRNA technology in order to discriminate between the roles of IR and IGF1R in tumorigenesis. As expected, we found that Ir-KD cells are poorly responsive to insulin, but maintain responsiveness to IGF1. Surprisingly, our results indicate that the Igf1r-KD cells remain IGF1-responsive. It has previously been suggested that IR responsiveness may compensate for IGF1R inhibition. Zhang et al. (2007) demonstrated that IGF1R downregulation induces IR hypersensitivity to insulin as a result of an incremental increase in holo-IR formation. On the other hand, it was demonstrated that IGF1R blockade with the specific SCH717454 antibody can be overcome by IGF2-IR crosstalk with the IR (Bid et al. 2012). Using S961, which specifically blocks IR, we confirmed hypersensitivity of the IR not only to insulin but also to IGF1 as a result of Igf1r KD. These results, along with the finding that the IMC-A12 antibody (and IGF1R specific antibody) induces IR–IGF1 interaction (Weinstein et al. 2004), indicate that, in order to eliminate the mitogenic signals of IGFs, inhibition of both receptors should be considered.

Previously, it has been demonstrated using the transgenic PyVmT/MKR and rtTA-Neu mice models and the syngeneic Met-1 and MCNeuA orthotopic mice models that hyperinsulinemia promotes accelerated mammary tumor growth through IR/IGF1R activation. However, there was no discrimination between the two receptor tyrosine kinases (Fierz et al. 2010, Novosyadlyy et al. 2010). In this study, we found that Ir-KD cells form significantly smaller tumors in both WT and MKR mice compared with the control cells. On the other hand, Igf1r-KD had no inhibitory effect on tumor growth, which could be the result of mitogenic signals delivered by both insulin and IGF1 through the IR, as indicated by the in vitro results. Furthermore, the reduction in mammary tumor growth, following injection of Ir-KD cells into the hyperinsulinemic MKR mice, indicates that the mitogenic signals of insulin are mainly delivered via the IR and not via the IGF1R as proposed previously (Noto et al. 2013). The results of the current study indicate that mammary tumor-specific treatment effectively prevents tumor growth and avoids metabolic abnormalities. Alternatively, both receptors can be inhibited simultaneously to a lesser degree, as we have described previously, thereby reducing tumor burden with minimal metabolic side effects (Rostoker et al. 2013). The PI3K/Akt and the MAPK pathways are the primary signaling cascades for delivering insulin and IGF1 signals. In this study, we demonstrate a dramatic decrease in the activity of the PI3K/Akt pathway and a moderate but
significant decrease in the activity of the MAPK pathway in the Ir-KD-derived mammary tumors.

An additional finding in our study involves the metastatic potential of tumors imparted by the IR and IGF1R. IGF1R has been recently found to mediate the metastatic activity of the MDA-MB-231 human breast carcinoma cells but not their tumorigenicity (Zhang et al. 2013). In this study, we demonstrated that both IR and IGF1R mediate metastasis of Mvt-1 cancer cells to the lungs, while only IR affects primary tumor growth in this study.

Although the effect of insulin on gene expression has been studied on normal cells such as muscle cells and fibroblasts (Dupont et al. 2001, Rome et al. 2003), much remains to be learned about alteration of transcripts in cancer cells. We found that insulin alters the levels of transcripts associated with the cell cycle, proliferation, and apoptosis in Mvt-1 cells. Moreover, several cancer-associated pathways were enriched upon insulin treatment, in addition to altered genetic networks involved in cellular growth and development, proliferation, cell-to-cell signaling, and immune cell interaction.

CD24 is an anchored cell-surface glycoprotein, mainly associated with the progression of invasive tumors through P-selectin binding, which is expressed by activated endothelial cells and platelets (Akashi et al. 1994, Kristiansen et al. 2004, Lim & Oh 2005). CD24-positive cells were found to possess tumor-initiating cell properties in colon, pancreatic, and hepatocellular cancers (Thomas et al. 2012). It is important to note, however, that results from several studies have indicated that human breast cancer cells with stem-cell-like characteristics are CD24-negative (Abraham et al. 2005, Phillips et al. 2006, Wright et al. 2008).

Using FACS analysis, we demonstrated the existence of two distinct and stable CD24− and CD24+ subpopulations. Gene expression analysis of a human colorectal cancer cell line (HT29) revealed that transient CD24 downregulation induces a decrease in genes related to the Ras pathway, and other proteins in its downstream cascade, in addition to genes of the MAPK family and Akt1 (Sagiv et al. 2008). Our results indicating the generation of smaller mammary tumors by Ir-KD cells and CD24 cells (R Rostoker, S Abelson, K Bitton-Worms, I Genkin, S Ben-Shmuel, M Dakwar, Z S Orr, A Caspi, M Tzukerman and D LeRoith. unpublished observations) along with the similarity in the signaling pathways that both insulin and CD24 are found to be involved in prompted us to look for a correlation between IR and CD24 expression. FACS analysis revealed a decrease of more than 60% in CD24 expression in the Ir-KD cells compared with that in the control cells. These results indicate a possible mechanism for the lower tumorigenic capacity of Ir-KD cells; however, it is important to note that CD24 (in the Ir-KD/CD24− cells) did not rescue the cells’ response to insulin in vitro and, on the other hand, insulin treatment for up to 3 weeks had no effect on in vitro expression of CD24 (data not shown). Lipid rafts are plasma membrane domains that are known to contain many signaling proteins including IR (Vainio et al. 2002).

Results from many studies have indicated that lipid rafts are critical for proper insulin activation of both the PI3K/Akt signaling pathway and the MAPK pathway (Bickel 2002). It has been recently suggested that CD24 acts as a gatekeeper for lipid rafts, thereby regulating signaling processes (Pike 2003). It is plausible that CD24 associates with IR in lipid raft domains, which may explain the reduction in CD24 following Ir-KD.

Then, we examined the hypothesis that the reduction in the high tumorigenic CD24+ subpopulation may explain the low tumorigenic capacity of the Ir-KD cells. Indeed, using Ir-KD/CD24+ cells, we demonstrated that CD24-expressing cells can partially restore the tumorigenic capacity of Ir-KD cells.

Taken together, the results of this study indicate a significant role for IR in the progression of mammary tumors, not only through mediating the mitogenic effect of insulin but also by a crosstalk with IGF1. We suggest that both IR and IGF1R should be targeted to achieve the desired inhibitory effect on tumor growth; however, such a strategy should be carried out in parallel with constant monitoring of blood glucose levels. Furthermore, we demonstrate in this study for the first time, to our knowledge, that CD24 expression is linked to IR in mediating mammary tumor growth. This may reveal a novel mechanism for the mitogenic effects of IR; however, further studies are required to explore the relationship between the two. These results indicating an important role for the IR in cancer progression could lead to new targeted therapeutics in women with breast cancer and other cancers that have similarly been shown to be associated with hyperinsulinemia in obesity, prediabetes, and diabetes.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/ERC-14-0490.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.
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Author contribution statement
D LeRoith, R Rostoker, and M Tzukerman developed ideas, and edited the manuscript. R Rostoker, S Abelson, K Bitton-Worms, I Genkin, S Ben-Shmuel, M Dakwar, Z S Orr, and A Caspi conducted the experiments. R Rostoker wrote the manuscript. All authors contributed to the analysis of data.

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