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POTENTIAL ROLE OF THE ISOFORM A OF THE INSULIN RECEPTOR AND ITS MOLECULAR PARTNER DISCOIDIN DOMAIN RECEPTORS-1 IN THE BIOLOGY OF BREAST CANCER CELLS

PhD Thesis

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1. INTRODUCTION

1.1. BREAST CANCER

Despite dramatic advances in the setting of cancer research, breast cancer (BC) remains a major health problem and a top biomedical research priority. Worldwide, BC is the most common cancer affecting women, and its incidence and mortality rates are expected to increase significantly in the next years [1]. Several genetic and environmental factors, especially when concomitant, increase the risk of development and progression of BC. Several lines of evidence show the influence of lifestyle and environmental factors on BC development (high-fat diet, alcohol consumption, lack of physical exercise, ionizing radiation, hormonal therapy, reproductive history), the elimination of which may contribute to a decrease in morbidity and mortality. Early menarche, late age at first birth, delayed menopause, genetic mutations are among commonly recognized and documented risk factors [2, 3]. Breastfeeding and more physical activity, on the contrary, are protective factors against BC. Better prognosis is associated with cancer detection at early stages, resulting in increased emphasis on timely and improved screening strategies [4].

Current classification of sub-types of BC includes genetic and immunohistochemistry expression profiling. Specifically, according to the expression of estrogen receptors (ER), progesterone receptors (PR), and human epidermal growth factor receptor 2 (HER2/neu), BCs are then

classified as Luminal A (ER and/or PR positive, and HER2 negative); Luminal B (ER and/or PR positive, and HER2 positive); HER2-enriched (ER and PR negative, and HER2 positive) and Basal Like (Triple negative breast cancer- ER, PR and HER2 negative) [5]. Triple-negative BCs grow and spread faster than most other types of BC. Patients with luminal A and B, and HER2-enriched subtypes are sensitive to targeted treatments, while patients with triple negative characteristic show poor prognosis. The status of these markers helps to determine which patients are likely to respond to targeted therapies (i.e., tamoxifen or aromatase inhibitors for ER+/PR+ patients and trastuzumab or lapatinib for HER2/neu patients) while triple negative patients have only chemotherapy as an alternative [6].

BC encompasses a large group of tumors with different morphological, phenotypic, and molecular characteristics, and the current classification includes a spectrum of in situ (pre-invasive) to invasive disease. Histological analysis of BC include the following subtypes: invasive ductal carcinoma (50%-75% of patients), invasive lobular carcinoma (5%-15% of patients), mixed ductal/lobular carcinomas and other rarer histological sub-types [7]. However, cancers of the same histologic type may show vastly different biological behavior. Assessment of these parameters may not comprise the varied clinical courses of individual BCs. Thus, in the current era of personalized medicine, a better understanding of BC behavior is needed.

1.2. THE IGF SYSTEM

The insulin-like growth factor (IGF) system plays a major role in growth, development and maintenance of homeostasis in normal cells and also contributes to the proliferation of malignant cells. It is composed of ligands (IGF-I, IGF-II, and insulin), receptors (IGF-IR, M6P/IGF-IIR, and insulin receptor), and IGF-binding proteins (IGFBP-1-6) [8].

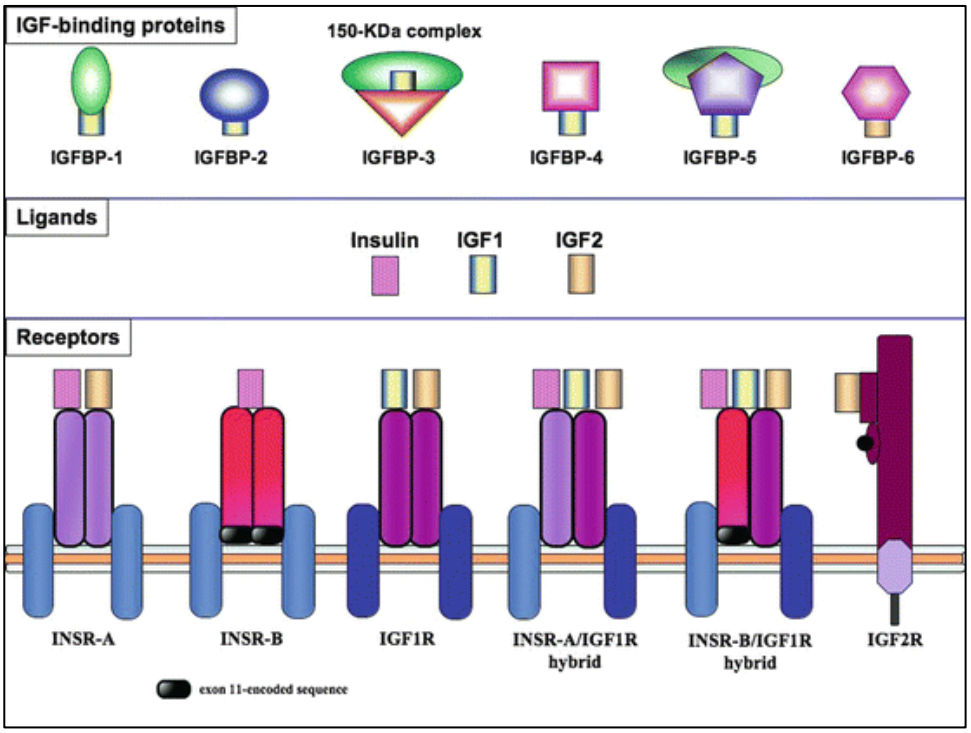

Figure 1: **The INSR/IGF1R family** (Sarfstein R., Werner H. The INSR/IGF1R Receptor Family. In: Wheeler D., Yarden Y. Receptor Tyrosine Kinases: Family and Subfamilies. Springer, Cham. 2015)

The ligands IGF-I, IGFII, and insulin have distinct tissues of expression and different physiological functions [9].

The IGF-I gene maps on chromosome 12 and consists of six exons that encodes for a small peptide of 70 amino acids with molecular weight of 7,649 kDa [10,11]. The IGFII gene is located on chromosome 11 and encodes for a 7.5 kDa peptide of 67 amino acids. The IGF-II gene is parentally imprinted, with only the paternal allele being expressed [9] except for in the liver, choroid plexus, and leptomeninges, where there is biallelic expression [12,13].

IGF-I and IGF-II regulate several physiological processes in different stages of life. For instance, IGF-I operates in an endocrine manner promoting longitudinal growth, and in a paracrine and autocrine manner enhancing cell proliferation. Originally called “somatomedin C”, IGF-I is stimulated by growth hormone (GH) which is produced in the pituitary gland under the control of the hypothalamic factors. On the other side, IGF-II regulates fetal growth and development and it is abundant in fetal skeletal muscle [9]. Moreover, IGF-I and IGF-II have similar growth-promoting activities and they share a high homology with insulin in the amino acid sequence.

Insulin is a peptide of 51 amino acids, formed by two chains (A and B) cross-linked by disulfide bonds. Classically, insulin is an anabolic hormone produced after a meal by the islets of Langerhans and stimulated by increased blood glucose levels. Its actions are exerted mainly on glucose metabolism as it reduces hepatic glucose output and increases glucose uptake in striated muscles and adipose tissue through the translocation of the glucose transporter GLUT4 to the cell membrane. Together to its

metabolic functions, insulin plays also important roles in the regulation of several processes including cellular growth, apoptosis and differentiation [14].

IGFs ligands directly regulate cellular functions by interacting with specific cell surface receptors and activating various intracellular signaling cascades. Among them, the insulin receptor (IR) and insulin-like growth factor receptor (IGF-IR) belong to the family of tyrosine kinase receptor (RTKs) and have high homology ranging from 45-65% in the ligand binding domains to 60-85% in the tyrosine kinase and substrate recruitment domains [15, 16]. The disulfide-linked homodimer of both human IR and IGF-IR consist of two extracellular α subunits and two transmembrane β subunits. The binding of ligands to the extracellular domains induces the activation of intrinsic kinase activity in the intracellular domains of the receptor. Afterword, the receptor is able to autophosphorylate and phosphorylate intracellular molecular adaptors thus propagating the biological response upon ligand stimulation. These two receptors originate by a common ancestor gene and are involved in glucose metabolism and growth in response to nutrient availability. Although IR is believed to be involved in metabolic regulation, recent work has suggested that IR might have a role in human cancer, including BC. Otherwise, IGF-IIR has a monomeric structure able to bind IGF-II, proteins containing mannose-6-phosphate (M6P) and TGFβ [17], but it lacks the catalytic activity [18]. Thus IGF-IIR may behave as tumor suppressor acting as an IGF-II scavenger [19] [20].

Finally, to add more complexity to the system, IGFs actions can be modulated by a superfamily of six soluble IGFBPs which bind IGFs with high affinity and specificity but not insulin. IGFBPs can influence IGF signaling by regulating both the half-life and the clearance of IGF-I and IGF-II, thus interfering with receptor interaction [21]. IGF-IBPs role in human cancer it is not yet fully known, but they may act as oncoproteins or tumor suppressors depending of cell context [19].

1.2.1. THE INSULIN RECEPTOR ISOFORMS AND HYBRID RECEPTORS

The human IR gene is located on chromosome 19 and includes 22 exons. The IR primary transcript undergoes alternative splicing producing two isoforms: the isoform A (IR-A), lacking exon 11, and the isoform B (IR-B), which includes exon 11 encoding for a 12 amino acid fragment in the α subunit. IR alternative splicing is cell specific, and the relative proportions of the different isoforms vary during development, aging and different disease states. For instance, IR-A is ubiquitously expressed and up-regulated in fetal life and in cancer, while IR-B is expressed in liver, muscle and adipose tissue representing the peculiar target tissues of insulin actions [22]. Furthermore, the physiological roles of IR isoforms appear to be determined by their different binding affinities for IGFs [23] as they have different affinities for IGF-I and IGF-II, while having a similar affinity for native insulin. Indeed, IR-A has a greater affinity for IGF-II and lower for IGF-I than IR-B. By contrast IR-B has a lower affinity for IGF-II and a very low affinity for IGF-

I compared to IR-A [24]. On the contrary, IGF-II affinity for IR-A and IGF-IR is very similar [25].

Previous studies have shown that, by binding to IRA, IGF-II is a more potent mitogen stimulus than insulin. Moreover, insulin is more effective in promoting glucose uptake upon binding IR-B compared to IR-A [25]. These data suggest that $IR^{-}A$ is mainly involved in the regulation of cell growth while $IR^{-}B$ is the isoform modulating the classical metabolic effects of insulin [26].

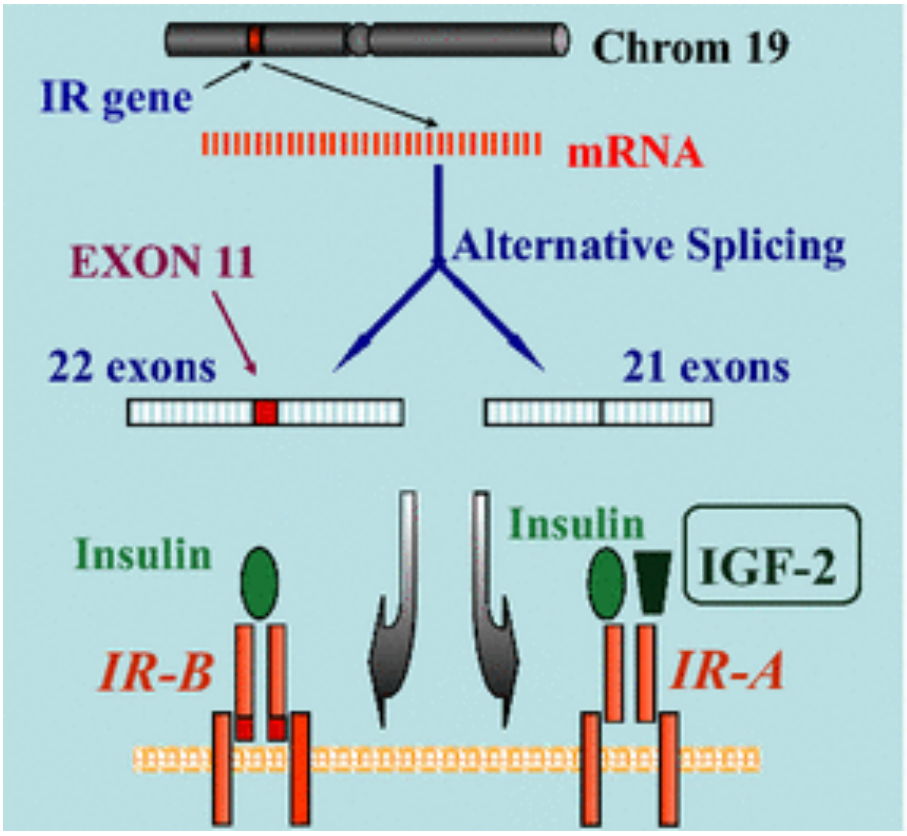

Figure 2: **The IR isoforms** (Vigneri, R., Goldfine, I.D. & Frittitta, L. Insulin, insulin receptors, and cancer. *J Endocrinol Invest*, 2016)

In cells expressing both IR and IGF-IR, IR hemireceptors may heterodimerize with IGF-IR hemireceptors, leading to the formation of hybrid receptors (HRs) [27]. HRs formerly described in both immortalized cell lines and human tissues, have been found in most cells and tissues coexpressing IRs and IGF-IRs, and are believed to result from the random assembly of IR and IGF-IR hemireceptors. Since two isoforms of IR exist (IR-A and IR-B), two combinations of hybrid receptors are possible: IR-A / IGF-IR (HR-A) and IR-B / IGF-IR (HR-B). While the intracellular signaling of HRs is not completely understood, it is well known that IR-A/HRs are activated by both insulin and IGFs although they bind the latter with higher affinity, unlike IR-B/HR that only recognize IGFs. The current hypothesis is that the preferential association of IR-A or IR-B with the IGF-IR essentially depends on the relative abundance of the two IR isoforms in each tissue [23].

1.2.1.1. INSULIN RECEPTOR SIGNALING

Insulin binding to the IR extracellular α-subunit induces a conformational change in the receptor molecule, which brings the two β-subunits in nearness leading to the autophosphorylation of IR. The activation of the tyrosine kinase due to a series of alterations in the β-subunit conformation facilitates the ATP binding, the recruitment of membrane and cytosolic protein substrates, and their subsequent phosphorylation [28, 16]. The IR tyrosine kinase thus phosphorylates several intracellular substrates, including the most extensively characterized IR substrates (IRS-1, -2, -3, and -4), IRS-5/DOK4, IRS-6/DOK5, Shc, Gab1, Cbl, associate protein

substrate (APS), and the signal regulatory protein family members [29, 30]. Each of these phosphorylated proteins provides specific docking sites for effectors containing Src homology 2 (SH2) domains that specifically recognize different phosphotyrosine residues [31, 32]. Some of these molecules contain SH3 domains that bind proline-rich regions with the consensus sequence PXXP and thereby provide additional sites for protein-protein interactions with additional downstream intracellular effectors. In this complex cascade of biochemical signals, two major signaling pathways have been recognized, mediating either prevalent metabolic or mitogenic effects and originating by the activation of PI3K or Ras, respectively.

PI3K produces the phosphaditylinositol 3, 4, 5 triphosphate (PIP3), which levels contribute to the recruitment to the membrane of pleckstrin homology (PH) domain containing proteins including PDK1 and PDK2. PDK1 activates downstream effectors such as the serine/threonine kinase Akt (PKB) and protein kinase C (PKC) [33]. Instead, PDK2 phosphorylates Akt on Ser473 and actives p70S6Kinase, which regulates protein synthesis upon insulin stimulation [34].

Akt is involved in GLUT4 translocation on the cell membrane induced by insulin stimulation [35, 36], the regulation of some anti-apoptotic genes mediated by insulin and IGFs, the inhibition of FKHR (forkhead in human rabdomyosarcoma) that is a transcriptional activator of pro-apoptotic genes [37, 38], and the nuclear translocation of NFkB with consequent activation of pro-inflammatory and survival programs [39]. Moreover, Akt induces mTOR activation through the inhibition of TSC1/2 complex, leading to mTORC1

activation that enhances protein synthesis by the phosphorylation of p70SK6 and 4EBP1 [40].

IRS-1 and IRS-2 can, in addition to p85, bind on the cell membrane the Grb2/Sos complex promoting RAS GTP/GDP exchange. Activated Ras recruits and activates RAF that in turn activates MEK1 and finally ERK, an important regulator of cell cycle. Besides IRS, also SHC is able to positively regulate MAP kinase after insulin and IGFs stimulation [41].

β-catenin/Wnt is another pathway involved in IR mitogenic signaling. GSK3 phosphorylation, via Akt, leads to an increased stability of β-catenin that translocates into the nucleus binding to transcription factors Lef/Tcf complex involved in activation of genes, such as cyclin D1, that regulate cellular proliferation or differentiation programs [42].

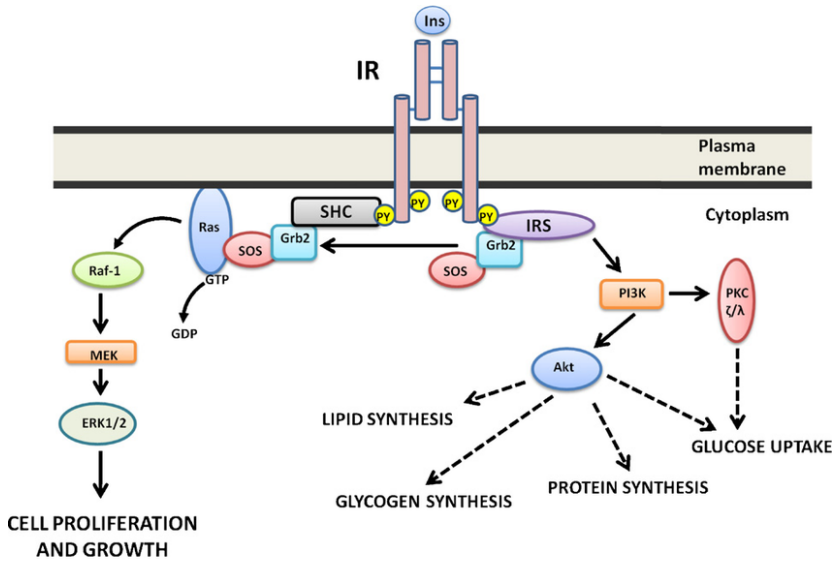

Figure 3: The insulin signaling pathway (Olivares-Reyes, J. A., Arellano-Plancarte, A., & Castillo-Hernandez, J. R. Angiotensin II and the development of insulin resistance: Implications for diabetes. Molecular and Cellular Endocrinology, 2009)

1.2.2. ROLE OF IGF AXIS IN CANCER

Mounting evidences have shown that IGF system is involved in both progression and development of several cancers such as gastrointestinal, gynecological, lung, prostate, thyroid and breast cancers [43]. It has been widely demonstrated that alterations in receptors expression, overexpression of ligands and reduction of circulating IGFBP levels may contribute to tumor promotion [5].

IGF ligands can be delivered and bind to IGF-IR from distant (endocrine) or nearby (paracrine) sources, with circulating free ligand levels regulated by the different IGFBPs [5]. Autocrine production of IGF ligands in some tumor cells which themselves express IGF-IR can also contribute to tumor proliferation [44, 45]. Moreover, in addition to IGF-I and IGF-IR, dysregulation of the IR-A/IR-B ratio has been associated with increased proliferative activity of neoplastic tissues. Indeed, IR-A is frequently overexpressed in cancer [46, 47, 48, 49] where it is constitutively activated by autocrine IGF-II production [23]. Overexpression of IGF-II and IR-A are also associated with a poor prognosis in a wide range of human cancers [50, 51].

Accumulating data demonstrates that activation of the IGF axis not only promotes tumorigenesis, but also confers resistance to standard treatments. Both IGF ligands can function as part of an adaptive mechanism that promotes resistance and tumorigenicity in certain cancers, during or

after treatment [52]. The RTK family encompasses many collaborative members, which share some effectors including PI3K/Akt/mTOR, Ras/Raf/Mek/Erk, Src, and JAK/STAT. Inhibition of one member of RTKs usually triggers the compensation by other members. Of note, in tumor cells where IGF-IR and IR are co-expressed, upregulation of IR can compensate for IGF-IR inhibition, thereby limiting sustained inhibition of downstream signaling and contributing to resistance to IGF-IR targeted therapy [53].

In addition, the IGF axis potentially sustains and contributes to the acquisition of several hallmarks of cancer [54]. For instance, dysregulated IGF signaling might contribute to the imbalance between cancer stem cell self-renewal and differentiation, resulting in the development of non-differentiated cells with stem cell-like properties, and subsequent malignant transformation [55]. Activated IGF-IR can stabilize integrins and promote epithelial mesenchymal transition (EMT) contributing to subsequent progression to metastatic disease [56]. Finally, the IGF pathway has been implicated in DNA damage repairing processes. It has been demonstrated that IGF-IR takes part in the regulation of gene expression by forming transcription complexes, modifying the activity of chromatin remodeling proteins, and participating in DNA damage tolerance mechanisms [57].

Hyperinsulinemia secondary to insulin resistance is a common feature of metabolic syndrome, obesity, and the early stages of type 2 diabetes (T2D). Given that the IR is clearly mitogenic when expressed by cancer cells, it is not surprising that epidemiological and experimental data strongly suggest

that the increased cancer risk and poorer prognosis in these metabolic disorders is related to the hyperinsulinemia. Certainly, as the obesity and diabetes epidemic continues, it is becoming increasingly important to understand the mechanisms that link hyperinsulinemia to cancer and to develop therapeutic strategies to target hyperinsulinemia.

1.3. DDRS STRUCTURE, EXPRESSION AND ACTIVATION

DDRs are extracellular matrix receptors with tyrosine-kinase activity [58]. They are composed of different regions: a N-terminal collagen-binding discoidin domain (DS), a DS-like domain and a transmembrane helix (TM) connected to the C-terminal tyrosine-kinase domain (KD) by a long juxta-membrane region (JM). To date, two DDRs have been identified, DDR1 and DDR2, sharing a sequence identity of roughly 50% [59].

DDR1 gene, located to human chromosome 6, is formed by 17 exons alternatively spliced in order to generate five different DDR1 isoforms (a, b, c, d, e) which differ in the cytoplasmic region, level of glycosylation [60], phosphorylation [59, 61], protein interactions [62], expression patterns and function [61]. However, DDR1-a and -b are the most abundant isoforms and together with DDR1-c are kinase active while the remaining two isoforms originated truncated or kinase inactive receptors. On the other side, DDR2 gene maps on human chromosome 1, it is composed of 19 exons and encodes a single protein.

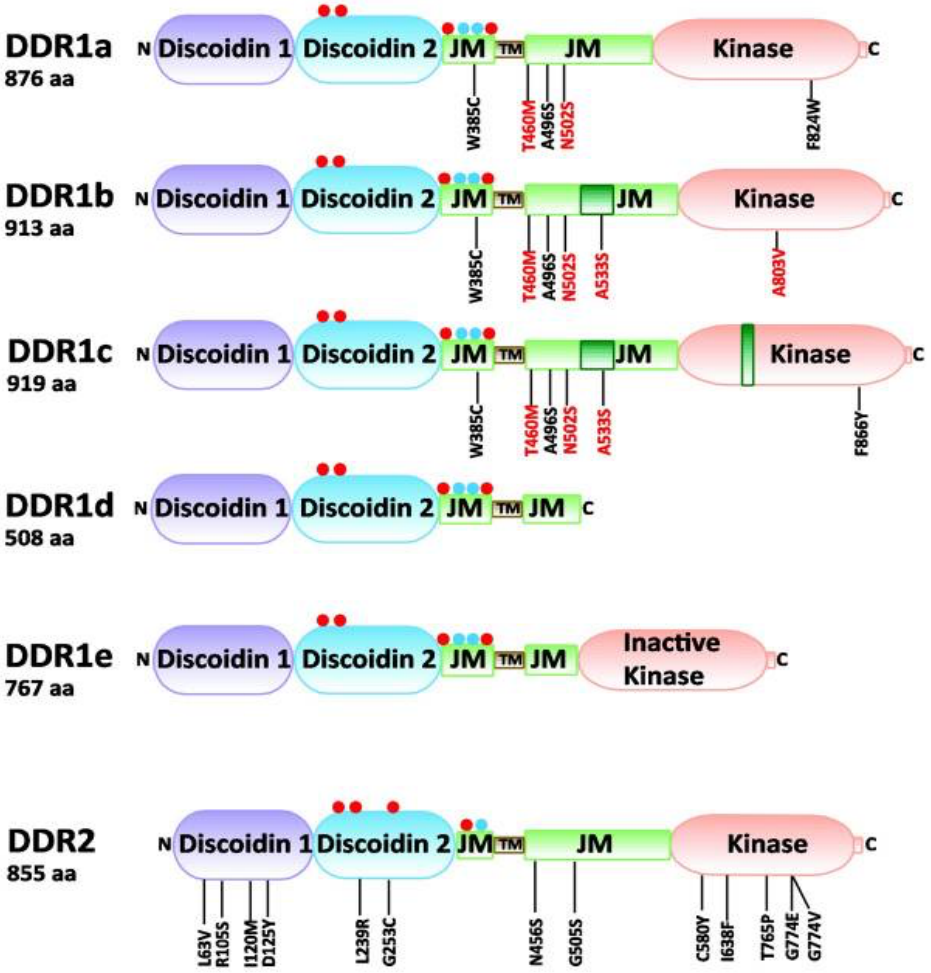

Figure 4: Domain structure of DDRs (Rajeshwari R Valiathan, Marta Marco, Birgit Leitinger, Celina G Kleer, Rafael Fridman; Discoidin domain receptor tyrosine kinases: new players in cancer progression; Cancer Metastasis Rev, 2012)

DDRs are expressed during development and in the adult life; in addition, DDR1 is predominantly expressed in epithelial tissues while DDR2 is expressed in mesenchymal tissues [63]. These receptors are activated by various type of collagen, but they only recognize collagen in its native form or in triple-helical conformation, not in the heat-denatured conformation [64]. DDR1 and DDR2 bind to and are mainly activated by fibrillar collagen

type I but can also bind collagens type II, III and V. Furthermore, collagen type IV interacts with DDR1 but not with DDR2 which has high affinity for collagen X [64].

When collagen binds DDRs, it induces a slow receptor autophosphorylation that can require hours (up to 18 hours) for maximal tyrosine kinase activity but the receptor activation upon collagen stimulation is sustained and not decreased after receptor endocytosis persisting up to 4 hours [65]. Upon collagen binding, tyrosines in the activation loop of DDRs tyrosine kinase domain are phosphorylated, becoming docking sites for several binding partners including: SH2 proteins [66], proteins containing PTB domain [59], cytoplasmatic protein Nck2, SHP-2 [67], NF-kB [67], ERK1/2-MAPK, AP-1 [68]. In addition, DDRs can cross-talk with other receptors such as Wnt5a/Frizzled [69] and Notch1 for DDR1, and IR for DDR2 [67].

1.3.1. DDRS IMPLICATION IN CANCER

DDRs dysregulation has been linked to multiple forms of cancer. Many studies have shown that DDR overexpression levels and/or mutations can be found in major malignancies including cancer of the breast [70, 71], ovary [72, 73], lung [74, 75], liver [76] and others [77, 78, 79].

In tumor cells, activation and expression of both DDRs can contribute to tumor cell proliferation, EMT, cancer invasion, metastasis formation and modulation of chemotherapy response [65, 80]. Well-differentiated epithelial

cancer cells usually express DDR1 but not DDR2. In contrast, several poorly differentiated epithelial cancer cells co-express both receptors and this co-expression modulates cancer cell migration [80, 81]. Yet, tumor cells undergoing EMT and moving toward a more malignant phenotype switch DDR expression from DDR1 to DDR2 [82, 83]. However, both DDRs can promote EMT contributing to cell invasion and migration depending on cell and microenvironment context. DDR1 crosstalks with integrins and activates signals that promote a mesenchymal phenotype and migratory properties [84]. Both DDRs modulate the expression and activity of MMPs, involved in migration and invasion processes, contributing to tumor cells migratory behavior [85, 86]. Finally, DDR1 was found to control triple-negative BC growth through the modulation of CD4+ and CD8+ T cells [87].

Furthermore, clinical studies have demonstrated a correlation between DDRs expression, metastases formation and reduced survival [65]. A significant correlation between DDR1 overexpression and lymph node metastasis has been shown in patients with non-small-cell lung carcinoma (NSCLC) [88]. Moreover, downregulation of DDR1 in melanoma cell has been able to suppress migration, invasion, and survival [89].

DDR2 also can be also considered a potential biomarker and a molecular target for a variety of cancer disorders. There is strong evidence indicating that DDR2 could be a potential biomarker and a molecular target for a variety of cancer disorders as it contributes to BC metastasis [90] and

appears to be a favorable predictor of recurrence and outcome in primary BCs [91, 92].

1.3.2. DDRs IMPLICATION IN IGF AXIS

Numerous data suggest that a crosstalk between DDR1 and the IGF system occurs in several cancer models with several biological and potential practical implications.

A connection between DDR1 and the IGF signaling was firstly provided by a study with SILAC proteomics aimed to identify novel IR-A molecular partners, which would preferentially interact after stimulation with IGF-II compared to insulin [93]. As mentioned above, the IR-A is frequently overexpressed in cancer where is often constitutively activated by autocrine IGF-II production [46, 47, 48, 49]. Indeed, several molecules, including DDR1 and DDR2, were preferentially recruited by IR containing phosphotyrosine protein complexes after IGF-II stimulation [94]. Interestingly, IGF axis and DDR1 appear to be linked by feed-forward mechanisms as, in breast and thyroid cancer cell models, DDR1 enhances the expression of both the IR and IGF-IR potentiating the biological effects of insulin, IGF-I and IGF-II [95]. In turn, insulin and IGFs were able to induce the expression of DDR1, thus establishing a positive feedback mechanism between DDR1 and the IGF axis contributing to the upregulation of these molecules [95].

As previously mentioned, DDR1 is tyrosine-phosphorylated after collagen binding with a slow kinetics [85, 96]. However, recent data suggested that

the IGF-IR might have a peculiar role in DDR1 phosphorylation. It has been demonstrated that IGF-IR depletion severely impaired collagen-induced DDR1 phosphorylation [97]. Moreover, cell stimulation with IGF-I induced rapid DDR1 phosphorylation in the absence of collagen [97]. Similarly, to what observed with insulin and IGF-II, DDR1 also modulates IGF-I dependent downstream signaling affecting both the Akt and the ERK1/2 pathways and IGF-I dependent biological actions including cell proliferation, migration and colony formation [97]. These data suggest that DDR1 regulates IR and IGF-IR signals with non-canonical actions, which do not require its collagen-induced DDR1 kinase activity. In addition, DDR1 regulates IR expression at multiple levels by modulating protein degradation and stability, gene transcription and post-transcriptional mRNA regulation, whereas it regulates IGF-IR protein expression levels prevalently through post-translational mechanisms [98, 97].

IGF system is an important positive modulator of DDR1 expression via the AKT/miR-199a-5p/DDR1 pathway. In fact, DDR1 induction by miRNA-199a-5p repression contributes to the development and progression of several cancer histotypes by increasing tumor invasion, metastasis and stem-like features [99, 55]. It has been demonstrated in BC cells that IGF system activation inhibits miR199a-5p expression, which promotes DDR1 upregulation and subsequent enhancement of IGF-IR and IR expression [95].

Taken together, these data suggest the existence of a positive feedback loop between DDR1 and IIGFs that amplifies insulin and IGFs signals and

could explain the frequent concomitant overexpression of DDR1, IR or IGF-IR in many cancers.

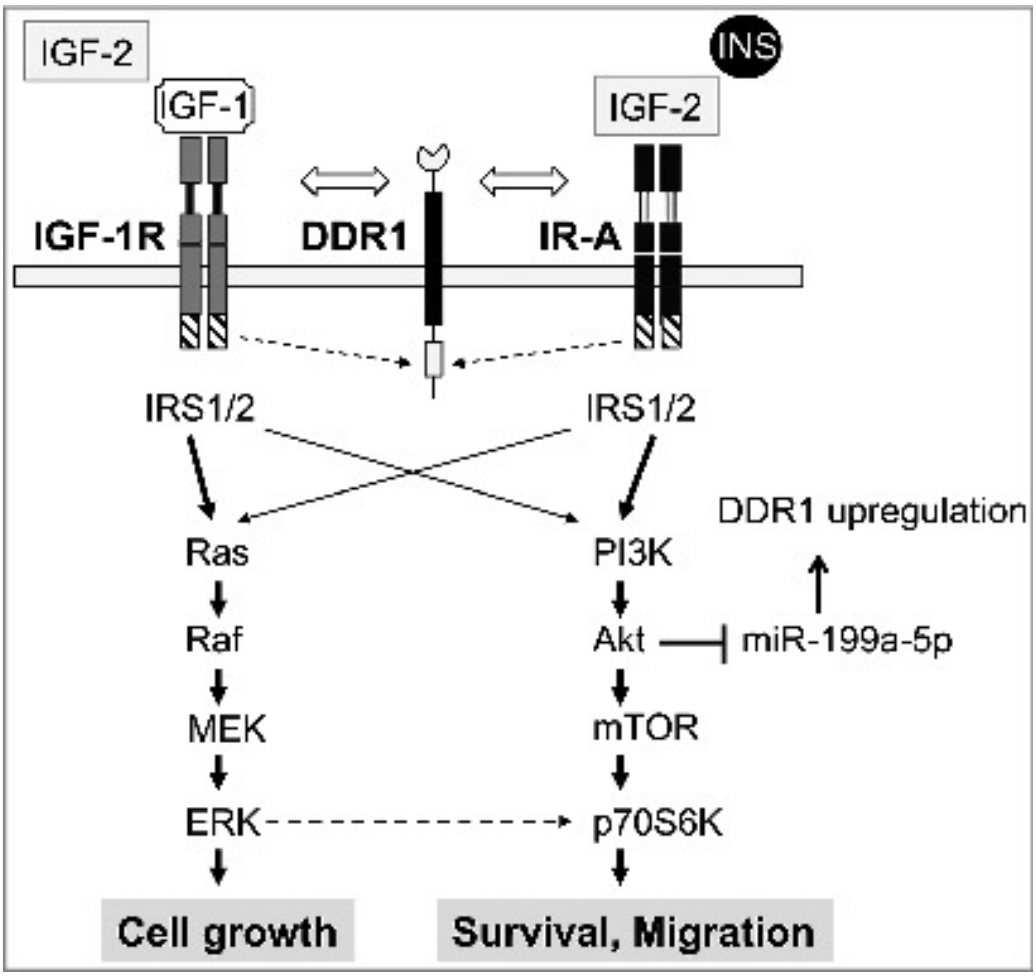

Figure 5: Schematic representation of the crosstalk involving the IIGFs and DDR1 (Antonino Belfiore, Roberta Malaguarnera, Maria Luisa Nicolosi, Rosamaria Lappano, Marco Ragusa, Andrea Morrione, and Veronica Vella. A novel functional crosstalk between DDR1 and the IGF axis and its relevance for breast cancer. Cell Adhesion & Migration 2018)

1.4. CANCER CELL METABOLISM

Metabolic activities are altered in cancer cells relative to normal cells supporting the acquisition and maintenance of malignant properties. Because some altered metabolic features are observed quite generally across many types of cancer cells, reprogrammed metabolism is considered a hallmark of cancer [54]. Reprogrammed activities provide cells with a selective advantage during tumorigenesis. Most of the classical examples of reprogrammed activities either support cell survival under stressful conditions or allow cells to grow and proliferate at pathologically elevated levels.

The classical example of a reprogrammed metabolic pathway in cancer is the Warburg effect or aerobic glycolysis [54]. Otto Warburg in 1920s observed that cancer cells constitutively take up glucose and produce lactate regardless of oxygen availability [100]. Generally, cells in active division, as well as cancer cells, meet their metabolic demands through the process of aerobic glycolysis [101, 100]. The energy generated through aerobic glycolysis is useful not only to compensate for the energy demands associated with the rapid division of cancer cells, but at the same time allows for the accumulation of biosynthetic precursors necessary for anabolic reactions [101, 100], as well ribose phosphate for nucleotides, nicotinamide adenine dinucleotide phosphate (NADPH) for reductive biochemistry, non-essential amino acids such as aspartate, glutamine, serine and glycine for protein and nucleotide synthesis, and fatty acids for assembly of membranes [102]. Simultaneously, cancer cells operate mitochondrial

respiration to obtain part of their ATP [103] and to use intermediates generated by the tricarboxylic acid (TCA) cycle as a lipids, amino acids and nucleotides precursors. Indeed, mitochondrial functions and biogenesis are activated by mitogenic signals and energetic stress has been also implicated in cancer progression and required for cell migration, metastasis and resistance to TK inhibitors (TKI). Moreover, cancer cells have the ability to switch from glycolysis to OxPhos and vice versa (metabolic flexibility), which is a remarkable mechanism of adaptation to the different microenvironments favoring cancer dissemination.

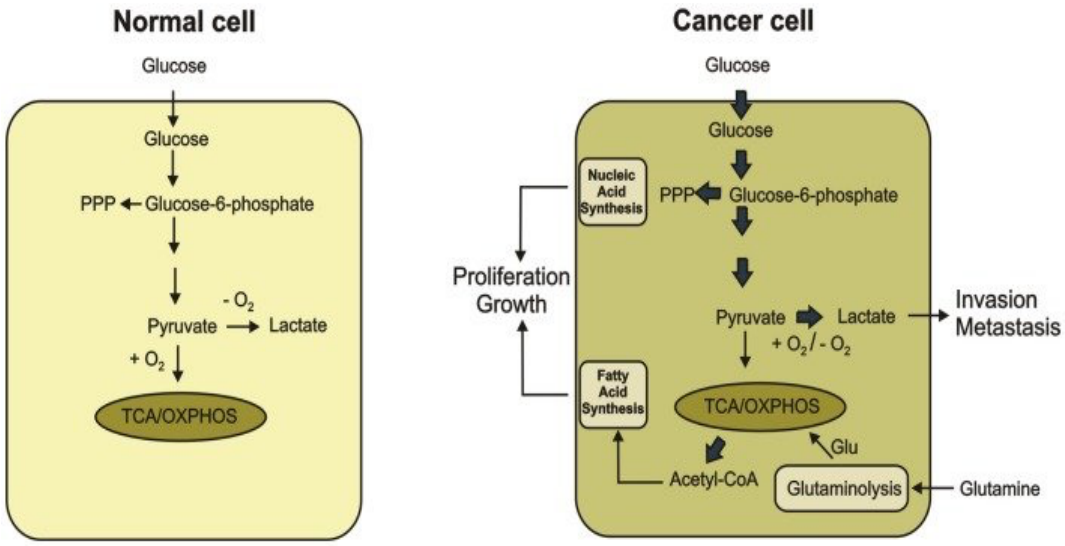

Figure 6: Metabolic differences between normal and cancer cells are shown (PawełJóz´wiak, EwaForma, MagdalenaBrys´ andAnnaKrzes´lak. O-GlcNAcylation and metabolic reprograming in cancer Front. Endocrinol, 2014)

2. RATIONALE AND AIMS

In human BC, the IR and particularly the oncofetal IR-A isoform, is markedly overexpressed [104, 105]. Notably, BCs occur quite often in obese and/or type 2 diabetic patients exposed to high levels of circulating insulin [106]. Accordingly, the exposure to hyperinsulinemia and/or to local IGF-II secretion is associated with constitutive IR autophosphorylation detectable in human BCs and with poor patient survival [107, 108]. The IR homolog IGF-IR is also overexpressed in human BC and believed to contribute to breast oncogenesis [109, 110].

It has been also shown that DDR1 is often dysregulated in cancer being implicated in various aspects of cancer progression, including cell proliferation and invasion, promotion of stem phenotype, metastasis, and modulation of chemotherapy response [65. 111]. Recently, it has been described a feed-forward loop between DDR1 and the IGF signaling where DDR1 enhanced BC cell proliferation, migration, and colony formation in response to insulin and IGFs.

Many lines of evidence have recently suggested that dysregulated RTK signaling in cancer might be implicated in metabolic reprogramming, which is a hallmark of cancer that can be targeted by several therapies [112]. However, whether the IGF-II/IR-A axis and its modulator DDR1 elicit a role in cancer metabolic reprogramming has not been established yet.

Based on these evidences, we propose the following specific aims:

- Find out whether the activation of the loop IR-A/IGF-II is involved in the metabolic reprogramming typical of cancer cells.
- Establish a role of DDR1 in cancer metabolism by modulating IR and IGF-IR.

We expect to clarify the function of IGF axis as well as of the molecular partner DDR1 in the modulation of cancer metabolism in BC.

3. MATERIALS AND METHODS

3.1. CELL CULTURES

The MCF7 human BC cells line, obtained from the American Cell Type Culture Collection (ATCC), were cultured according to the manufacturer's instructions. Cells were grown in complete MEM (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (P/S) and 1% Glutamine and 1% Non-Essential Amino Acid (NEAA).

MCF7/empty vector (EV) and MCF7/IGF-II cells, which overexpress the human IGF-II, were generated by transfection with the pCMV6-Entry vector or the human cDNA containing vector (IGF-II Myc-DDK Tag), respectively, and cells were selected in medium containing 1 μg/mL puromycin.

MCF7 KO-IGF-IR ($MCF7^{IGF\text{-}IR\text{-}ve}$) and KO-DDR1 ($MCF7^{DDR1\text{-}ve}$) and the control $MCF7^{Cas9}$ cells were purchased from Applied Biological Materials (Richmond, BC, Canada). Cells were grown in complete DMEM (Sigma, St. Louis, MO, USA) supplemented with 10% FBS, 1% P/S and 1% Glutamine. $MCF7^{IGF\text{-}IR\text{-}ve}$ was used for lentiviral transduction and generation of $MCF7^{IGF\text{-}IR\text{-}ve}$/IR-A-FLAG.

All cell lines were grown in a 37 ◦C incubator with 5% CO^2.

3.2. MEASUREMENT OF IGF-II IN CONDITIONED MEDIUM

The conditioned medium (CM) from MCF7/IGF-II cells and MCF7/EV was obtained by incubating cells in a serum-free medium for 48 h. Media were collected and centrifuged at 3500 rpm for 5 min to remove cell debris.

The biological ability of IGF-II released in the medium was assessed by incubating wild-type MCF7 cells with CM from MCF7/IGF-II cells and then measuring IR/IGF-IR phosphorylation by Western blot with anti- pIR/IGF-IR antibodies.

3.3. METABOLIC PROFILING BY SEAHORSE ASSAYS

Cells were seeded into XFp cell culture microplates (Seahorse Biosciences, Santa Clara, CA, USA) and silenced or treated with ligands as indicated. The day before the assay a sensor cartridge was hydrated with XF Calibrant at 37 °C in a non-CO2 incubator overnight.

The day of assay the cell culture miniplate has been removed from 37 °C CO^2, cells have been washed and incubated with Seahorse XF DMEM Medium, pH 7.4 with 10 mM of XF glucose, 1 mM of XF pyruvate, 2 mM of XF glutamine at 37 °C in a non-CO2 incubator for 45-60 minutes. Compounds of the using kit have been loaded into the ports of the hydrated sensor cartridge. The protocol of the assay has been selected from the XFp Extracellular Flux Analyzer (Seahorse Biosciences, Santa Clara, CA, USA) and then the microplate with the cells has been inserted. Using a

spreadsheet, the “Seahorse XFp ATP rate assay kit” was used to calculate ATP production rate and the different ATP from glycolysis and from OxPhos. Using another different spreadsheet, the “seahorse XFp glicolytic rate assay kit” and the “seahorse XFp cell mito stress kit” were used to calculate ECAR (Extracellular Acidification Rate) and OCR (oxygen consumption rate) respectively.

3.4. GENE SILENCING BY SMALL INTERFERING RNA

For small interfering RNA (siRNA) experiments, cells were transiently transfected with a mixture containing Opti-MEM, Lipofectamine RNAiMax, and either a pool of four scramble siRNA oligos (10 nM) or a pool of four specific siRNA oligos for DDR1 (10 nM). The experiment was stopped at 48 hours from transfection.

3.5. RNA EXTRACTION

Total cellular RNA was extracted using TRIzol Reagent according to the manufacturer’s protocol.

3.6. DNASE AND REVERSE TRANSCRIPTION

After reverse transcription was removed double and single stranded DNA according to the manufacturer’s protocol.

Then, total RNA was reversely transcribed using the High Capacity cDNA Reverse Transcription Kit.

3.7. qRT-PCR

Synthesized cDNA was combined with primer sets for the gene of interest and SYBR Green.

Real-time PCR was performed using an ABI 7500 Real-Time PCR System (Applied Biosystems).

qRT-PCR amplification was performed in 17 µL reaction mixture containing 3 µL cDNA sample. qRT-PCR began with a 2 minutes hot start at 50°C, followed at 95°C for 10 minutes, next by 42 cycles at 95°C for 15 seconds and at 60°C for 1 minute.

Data were analyzed using 7500 System software (Applied Biosystems). Finally, relative gene expression levels were normalized to an internal reference gene (β-actin or GAPDH) and mRNA quantification was performed using the comparative CT method (ΔΔCt).

3.8. Western Blot Analysis

Sub-confluent cells were solubilized in radioimmune precipitation (RIPA) buffer at 4°C for 15 min. The cell lysates were centrifuged at 4°C for 15 min at 14000 rpm to separate soluble proteins. Proteins were normalized and

diluted in Laemmli buffer containing β-mercaptoethanol, resolved by 10% SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was blocked with 5% (w/v) non-fat milk or Bovine Serum Albumin (BSA) in Tris Buffered Saline (TBS) containing 0.1% Tween for 1 h and incubated overnight at 4°C with primary antibodies. Then the membrane was incubated for 1 h with appropriate secondary antibodies. Signals were visualized by chemiluminescence.

The following primary antibodies were used: anti-IRβ (C-19, sc-711), anti MCT1 (H-1) (sc-364501) (Santa Cruz Biotechnology); anti-phospho(p)IGF1R (Tyr1135/1136)/ phosphoIR (Tyr1150/1151) (19H7), anti-IGF1R, anti- phospho Tyr100, anti EK2 (C64E5), and anti Myc (9B11), anti-phospho-Akt8 virus oncogene cellular homolog (Akt) (Ser473), anti-AKT, anti- phospho -extracellular signal-regulated kinase (p-ERK)1/2 (T202/Y204), anti-ERK1/2 (Cell Signaling Technology); anti-phospho IR (Y1334), specific for IR (Invitrogen); anti MCT4 (A304-439A) (ThermoFisher Scientific, Inc., Waltham, MA, USA); anti ARALAR, Anti-SLC25A12 (E-AB-63584); anti NRF-1 (E-AB- 16661) (Elabscience, Houston, TX, USA); anti-lactate dehydrogenase A (LDHA) (216-228, SAB1100050), anti-pyruvate kinase M2 (PKM2) (isoform M1, SAB4200094), anti-βactin (Sigma Aldrich). Total OXPHOS human WB Antibody Cocktail (ab110411) was obtained by Abcam (Cambridge, UK).

3.9. PROLIFERATION

Cell proliferation was evaluated by cell counting after 24, 48, 72 and 96 h, using trypan blue for exclusion of dead cells. Briefly, cells were seeded in 48-well plates in triplicates and every 24 h were trypsinized and counted.

3.10. INVASION

The ability of cells to invade the extracellular matrix was measured in Boyden's chamber. Cells were removed from plates with trypsin and placed on polycarbonate filters (8 μm pore size, Corning Costar), coated with 25 μg/mL fibronectin. After 6 h of incubation, cells on the upper surface of filters were removed with a cotton swab and filters were stained for 30 min with crystal violet (0.05% crystal violet in phosphate buffered saline (PBS) plus 20% ethanol). After three washes with water, crystal violet was solubilized in 10% acetic acid for 30 min at room temperature, and its concentration was evaluated by absorbance at 595 nm.

3.11. COLONY FORMATION

Anchorage-independent growth was assessed by seeding the cells in a medium containing agar. In a 6-multiwell plate the mixture of 0.66% agar and medium (hard-agar) was dispensed. Then, cell suspension containing 0.33% agar (soft-agar) was plated on the top of the hard-agar layer. Cells

were cultured in the presence or absence of ligands, as indicated. Colonies were visualized using methyl thiazolyl tetrazolium (MTT) in phosphate buffered saline (PBS), photographed and analyzed with NIH ImageJ.

3.12. DENSITOMETRIC AND STATISTICAL ANALYSIS

Densitometry results were obtained by using Image Studio Lite software (LI-COR Biosciences, Lincoln, NE, USA). The differences between the means were evaluated by Student's t-test. The level of significance was set at $p < 0.05$. Statistical analysis was performed with GraphPad Prism8 software (GraphPad Software, San Diego, CA, USA). Data were expressed as means ± SEM.

4. RESULTS

4.1. CONSTITUTIVE AUTOCRINE IGF-II SECRETION OF HUMAN BC CELLS

To investigate the role of the autocrine signaling loop between IR-A and the IGF-II in metabolic reprogramming, we established human BC cells MCF7 constitutively expressing IGF-II by stably transfecting them with a myc-tagged IGF-II vector (MCF7/ IGF-II). As control we used MCF7 stably transfected with the corresponding empty vector (MCF7/ EV).

We first characterized MCF7/IGF-II cells demonstrating a significantly higher IGF-II mRNA and protein levels as compared to MCF7/EV (Figure 7 A and B). To verify whether MCF7/IGF-II cells were able to secrete biologically active IGF-II in the conditioned medium (CM) we performed a western blot anti c-Myc. Indeed, myc-tagged IGF-II was present not only in cell lysates (Figure 7B) but it was also released in CM (Figure 7C) and could also phosphorylate IR/IGF-IR in wild-type MCF7 cells (Figure 7D).

Notably, MCF7/IGF-II cells showed constitutive activation of both IR and IGF-IR, as revealed by phosphoantibodies recognizing both receptors and by a phosphoantibody specifically recognizing IR (Figure 7E). Downstream signaling molecules such as AKT and ERK1/2 were also clearly phosphorylated in MCF7/IGF-II rather than in MCF7/EV (Figure 7E). Overall, these data indicated that constitutive autocrine IGF-II activated the

two major signaling pathways through activation of both IGF-IR and IR-A (Figure 7E).

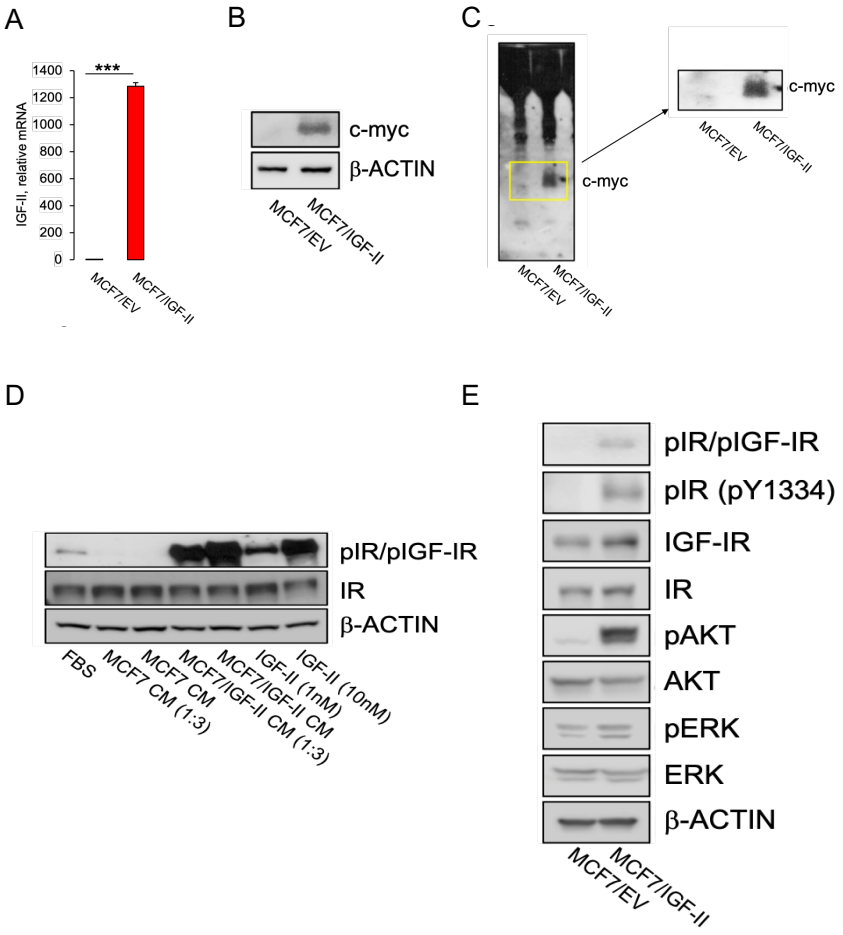

Figure 7: (A) IGF-II mRNA levels were measured in MCF7/IGF-II and MCF7/EV by qRT-PCR. Normalization was done using human β-actin as the housekeeping control gene. Data are presented as means ± standard error of the mean (SEM) (error bars) from three independent. IGF-II (myc-tagged) expression in cell lysates **(B)** and in CM **(C)** from MCF7/IGF2 as compared to MCF7/EV cells by western blot analysis. The yellow box shows the band corresponding to the IGF-II myc-tagged enlarged on the right. A representative blot of three independent experiments is shown. (**D)** CM derived from MCF7/IGF2, but not from MCF7/EV,

induces phosphorylation of IR/IGF-IR in wild type MCF7 cells. A representative blot of three experiments is shown. **(E)** Anti-phospho-(p)IGF1R (Tyr1135/1136)/pIR(Tyr1150/1151) detecting both pIR and pIGF-IR and anti-pIR (Y1334), specific for pIR, were used to assess autophosphorylation of the two receptors. Anti-p Akt8 virus oncogene cellular homolog (Akt) (Ser473) and anti-p extracellular signal-regulated kinase (ERK)1/2 (T202/Y204), were used to measure the activation of both AKT and ERK1/2. β-actin was used as control for protein loading. Myc blotting was used to determine myc-tagged IGF-II levels in transfected cells. Blot is representative of three independent experiments.

4.2. MCF7/IGF-II CELLS SHOWED INCREASED GLYCOLYTIC AND MITOCHONDRIAL ACTIVITIES

To assess whether constitutive IGF-II expression affects the metabolic phenotype of BC cells, we evaluated the expression of glycolytic and mitochondrial genes.

Glucose transport into cells is regulated by membrane translocation of glucose transporters (GLUTs), and expression of GLUTs is often increased in various glycolytic cancers including BC. Therefore, we evaluated the expression of GLUT1 (Figure 9) showing higher mRNA expression levels in MCF7/IGF-II as compared to control cells.

However, in cancer cells, intracellular lactic acidosis produced by glycolysis activity is regulated by extracellular lactate transport through proton-linked monocarboxylate transporters (MCTs), that are important regulators of

intracellular pH. Cancer cells require the activity of MCTs, membrane carriers involved in transporting lactate, pyruvate and ketone bodies. Indeed, MCTs are often up-regulated in highly glycolytic tumors in order to counteract apoptosis driven by cellular acidosis. Thus, we assessed the expression of transporters for lactate such as MCT1, which supports lactate entry, and MCT4, which favors lactate cell efflux. As expected, MCF7/IGF-II cells showed lower expression of MCT1, as compared to control cells, while expressing higher expression levels of MCT4 (Figure 8), suggesting a predominant requirement of MCT4 aimed at reducing intracellular lactate by promoting its efflux.

We also evaluated the expression of key players of energy metabolism in glycolytic cells, such as lactate dehydrogenase A (LDHA) enzyme that synthesizes lactate from pyruvate, pyruvate kinase M2 (PKM2) isoenzyme that catalyze the last step within glycolysis, and hexokinase-2 enzyme, responsible on the first step of the glycolytic pathway. We found that MCF7/IGF-II expressed significantly higher levels of LDHA, PKM2 and hexokinase-2 (EK2) as compared to control cells (Figure 8).

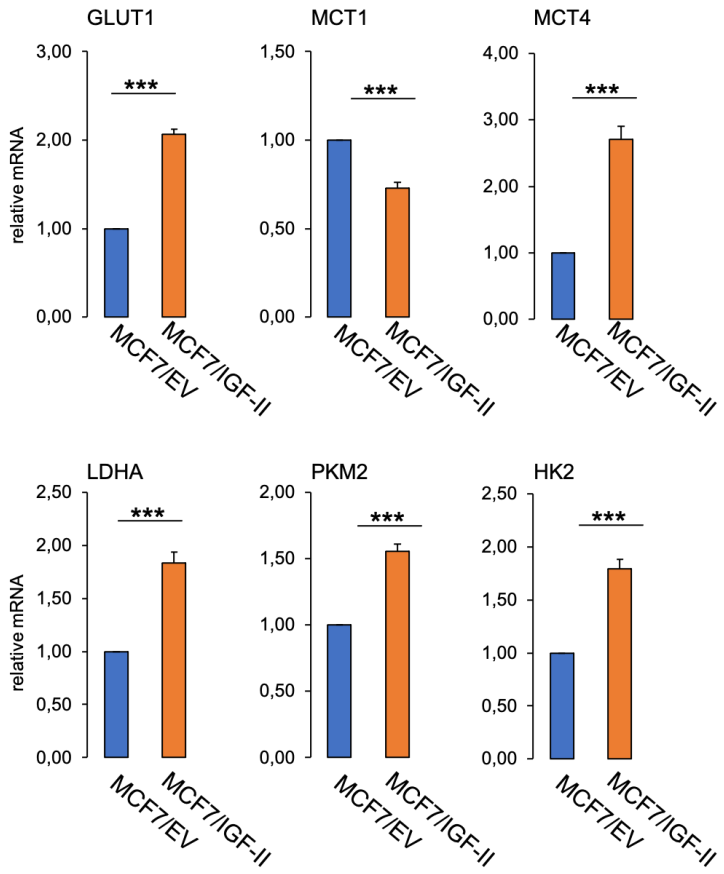

Figure 8: Cells were processed to evaluate mRNA expression, for glucose transporter 1 (GLUT1), monocarboxylate transporters (MCT1 and MCT4), glycolytic enzymes lactate dehydrogenase A (LDHA), pyruvate kinase M2 (PKM2), hexokinase-2 (EK2), by qRT-PCR analysis. Values are means ± SEM of three separate experiments.

Increased protein expression for LDHA and PKM2 were confirmed by Western blot and evaluated by densitometric analysis (Figure 9).

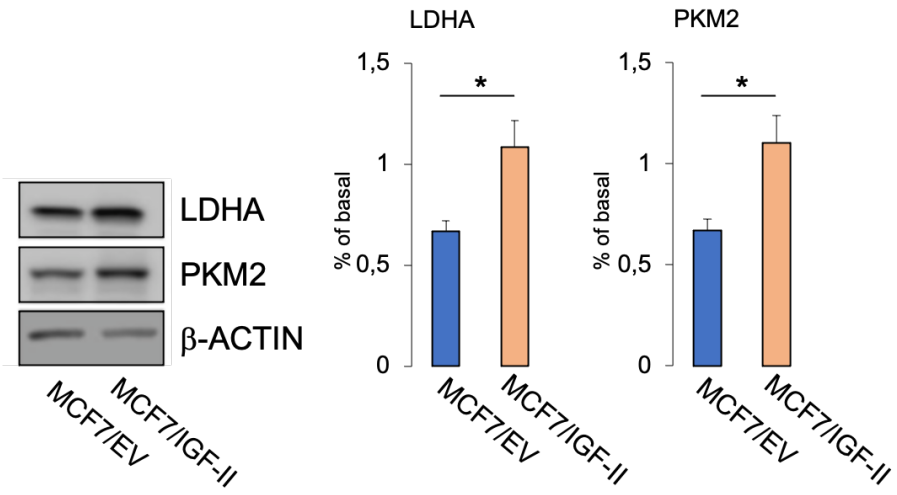

Figure 9: MCF7/IGF-II cells and MCF7/EV grown in medium containing 10% charcoal stripped- fetal bovine serum (FBS) for 48 h, were lysed and immunoblotted with primary antibodies for LDHA and PKM2. Graphs represent the mean ± SEM of densitometric analysis of three independent experiments.

In order to evaluate mitochondrial function in MCF7/IGF-II cells, we investigated the expression of the peroxisome proliferator-activated receptor gamma coactivator-1 (PGC1) family of transcriptional co-activators, involved in mitochondrial biogenesis. Moreover, mitochondrial biogenesis requires the expression of genes encoded by both mitochondrial and nuclear genomes under the control of PGC1 isoforms and activated by mitogenic and energetic stress. Accordingly, in MCF7/IGF-II cells we observed increased expression of PGC1α, PGC1β and PGC1α-related coactivator (PRC) genes. We also evaluated the expression of the nuclear-encoded mitochondrial carrier protein aspartate–glutamate mitochondrial carrier 1 (ARALAR/AGC1), and the nuclear respiratory factor 2 (NRF2). To

further characterize the mitochondrial activity in MCF7/IGF-II cells, we measured the levels of cytochrome C oxidase 1 (COX1) (Figure 10). Similarly, the expression of markers of mitochondrial mass marker, TOMM20, was analyzed (Figure 10).

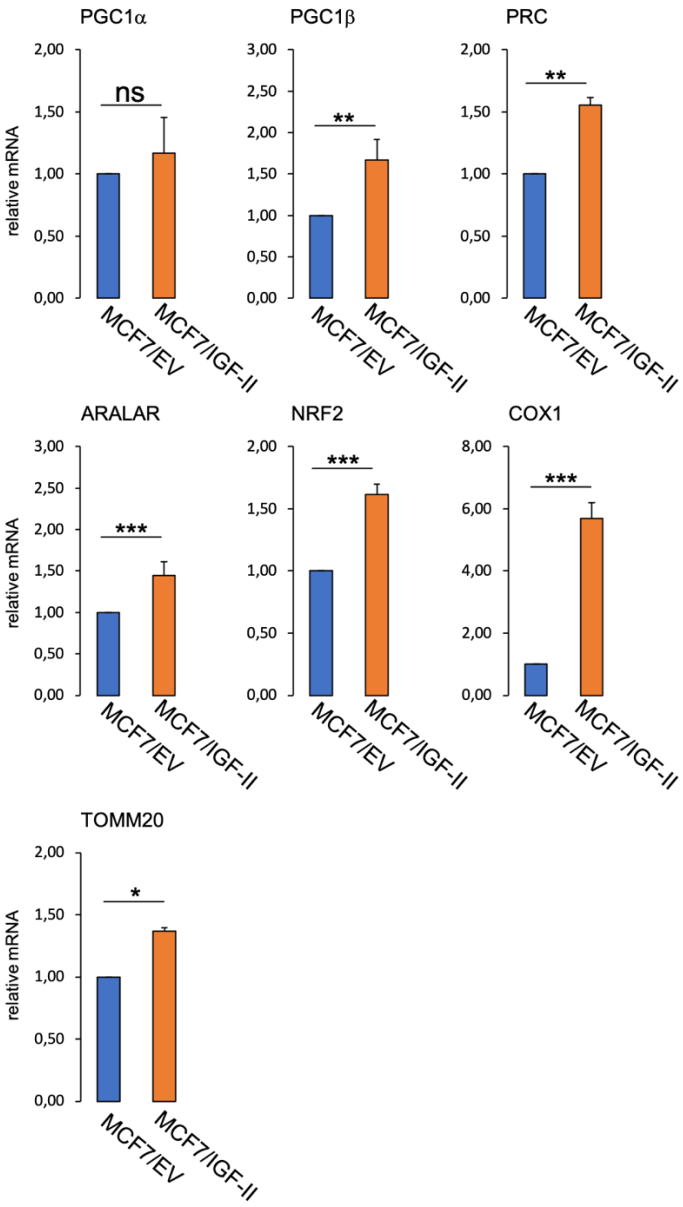

Figure 10: Cells were processed to evaluate mRNA expression, in MCF7/IGF-II as compared to MCF7/EV, for peroxisome

proliferator-activated receptor gamma coactivator-1 isoforms (PGC1α-β), PGC1α-related coactivator (PRC), aspartate-glutamate mitochondrial carrier 1 (ARALAR/AGC1) and nuclear respiratory factor 2 alpha (NRF-2α), cytochrome C oxidase 1 (COX1), translocase of outer membrane (TOMM20)

Additionally, we assessed ATP Real-Time rate assay using the Seahorse XF® technology. Our results showed that total ATP production was enhanced by more than two-fold, in MCF7/IGF-II as compared to MCF7/EV cells (Figure 11). Specifically, the increase in ATP production was mostly due to enhanced glycolysis. These results showed that MCF7/IGF-II cells were significantly more metabolically active than control cells suggesting a switch towards a more energetic metabolic phenotype.

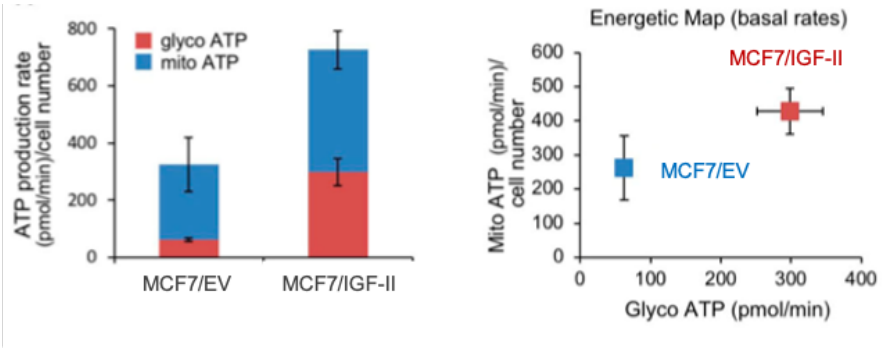

Figure 11: ATP production rate in MCF7/IGF-II cells. In particular, the glycolytic ATP (glycoATP) and the mitochondrial (mitoATP) production rates were evaluated in MCF7/IGF-II cells and MCF7/EV cells. The histogram presented (left panel) and the energetic map (right panel) show the mean and range from two independent experiments.

4.3. BIOLOGICAL EFFECT OF IGF-II OVEREXPRESSION

In order to investigate whether the production of IGF-II would affect the cells, MCF7/IGF-II were biologically characterized. Firstly, cells were seeded, grown and counted after 24, 48, 72 and 96h. Notably, as compared to MCF7/EV, they proliferated more (Figure 12A). Afterwards, we demonstrated that they have enhanced ability to migrate through fibronectin-coated filters (Figure 12B) and to form colonies in semi-solid agar (Figure 12C). Overall, the autocrine secretion of IGF-II enhanced BC cell growth and protumorigenic activities.

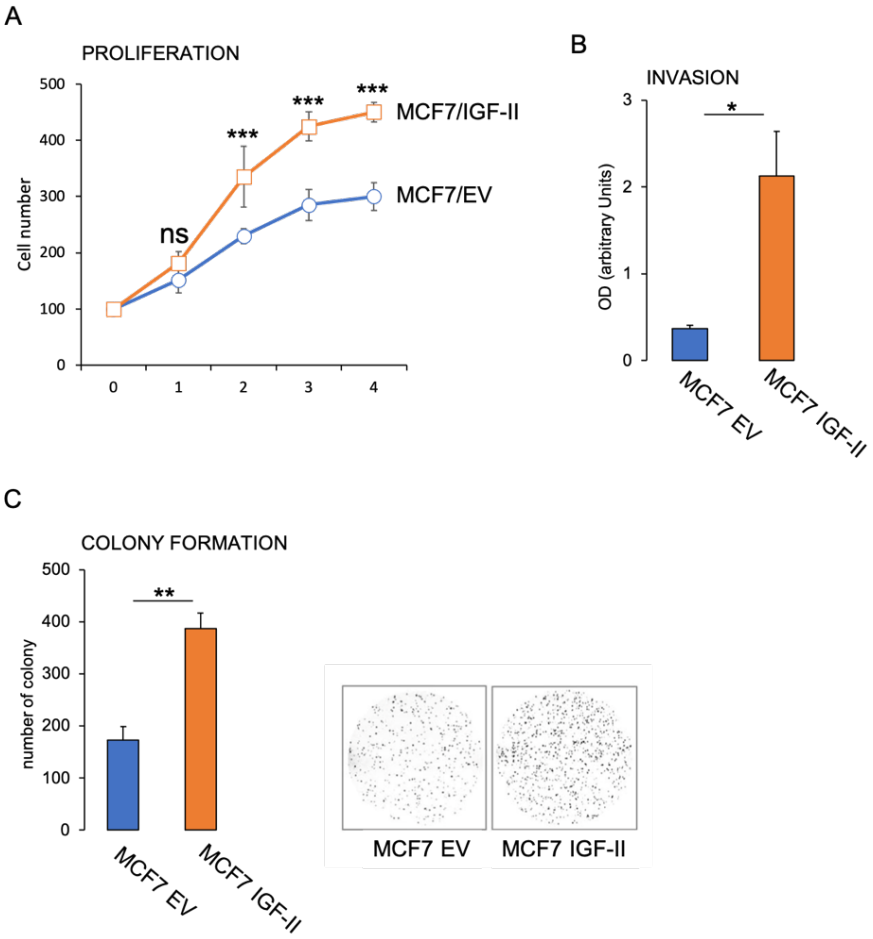

Figure 12: (**A**) Cell proliferation. Cell was counted by trypan blue exclusion assay at the indicated time points. Values are means ± SEM of three independent experiments. (**B**) Cell invasion. Cells were seeded on polycarbonate filters and allowed to migrate for 6 h to the lower chamber. Values show means of two independent experiments done in duplicate. (**C**) Colony formation. Cells were seeded in soft-agar and grown in 5% charcoal stripped-serum. Colonies were stained with methyl thiazolyl tetrazolium (MTT) and then photographed. The histogram represents the mean of number colonies from two independent experiments, each run in quadruplicate wells.

4.4. METABOLIC EFFECTS OF INSULIN AND IGF2 IN MCF7IGF^{-IR-ve}/IR-A CELLS

To better define the metabolic effect of IR-A, which is the receptor principally involved in IGF-II action in cancer cells, we used MCF7 cells where the IGF-IR was stably knocked-out by CRISPR/Cas technology and stably overexpressed the IR-A (MCF7$^{IGF\text{-}IR\text{-}ve}$/IR-A).

As expected, MCF7$^{IGF\text{-}IR\text{-}ve}$/IR-A cells lacked the IGF-IR and showed high IR levels compared to the controls MCF7$^{IGF\text{-}IR\text{-}ve}$/EV and MCF7/^{Cas9} cells (Figure 13, A and B).

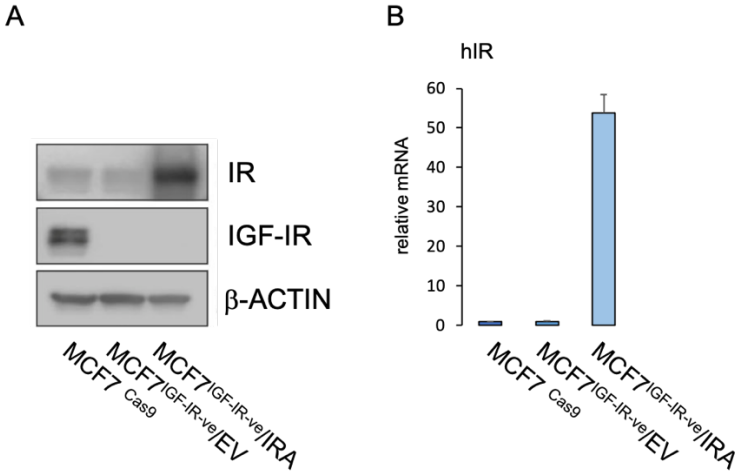

Figure 13: (A) MCF7$^{IGF\text{-}IR\text{-}ve}$/IR-A, control MCF7$^{IGF\text{-}IR\text{-}ve}$/EV cells and parental MCF7$^{/Cas9}$ cells were grown in 10% FBS, lysed and analyzed by immunoblot for IR and IGFI-R expression. (**B**) The same cells as in (A) were then analyzed for IR expression by qRT-PCR using human β-actin as the housekeeping control gene for normalization. Results are shown as means ± SE of three independent experiments.

4.5. COMBINATION TREATMENT WITH INHIBITORS OF GLYCOLYSIS AND MITOCHONDRIAL RESPIRATION IN STIMULATED MCF7$^{IGF\text{-}IR\text{-}VE}$/IR-A CELLS

To assess whether insulin or IGF-II stimulation affected the metabolic phenotype of MCF7$^{IGF\text{-}IR\text{-}ve}$/IR-A cells, we analyzed glycolysis and mitochondrial bioenergetics. Real-time measurements of extracellular acidification rates (ECAR) showed significantly higher basal ECAR in cells stimulated with either insulin or IGF-II (Figure 14A). When mitochondrial respiration was suppressed by injecting rotenone/antimycin A, glycolysis

was stimulated to compensate for lack of energy production and this stimulation (glycolytic reserve) was also higher in cells exposed to both insulin and IGF-II (Figure 14A). Similarly, we assessed the effects of insulin and IGF-II stimulation on mitochondrial respiration (Figure 14B). Cells stimulated with both insulin and IGF showed higher oxygen consumption rates (OCR) and ATP linked respiration as compared to untreated cells. This difference was maintained after treatment with metabolic inhibitors, as indicated (Figure 14B). Taken together, these data indicated that, in MCF7$^{IGF\text{-}IR\text{-}ve}$/IR-A cells, insulin and IGF-II stimulated both glycolysis and OxPhos, both in basal and high energy demand conditions.

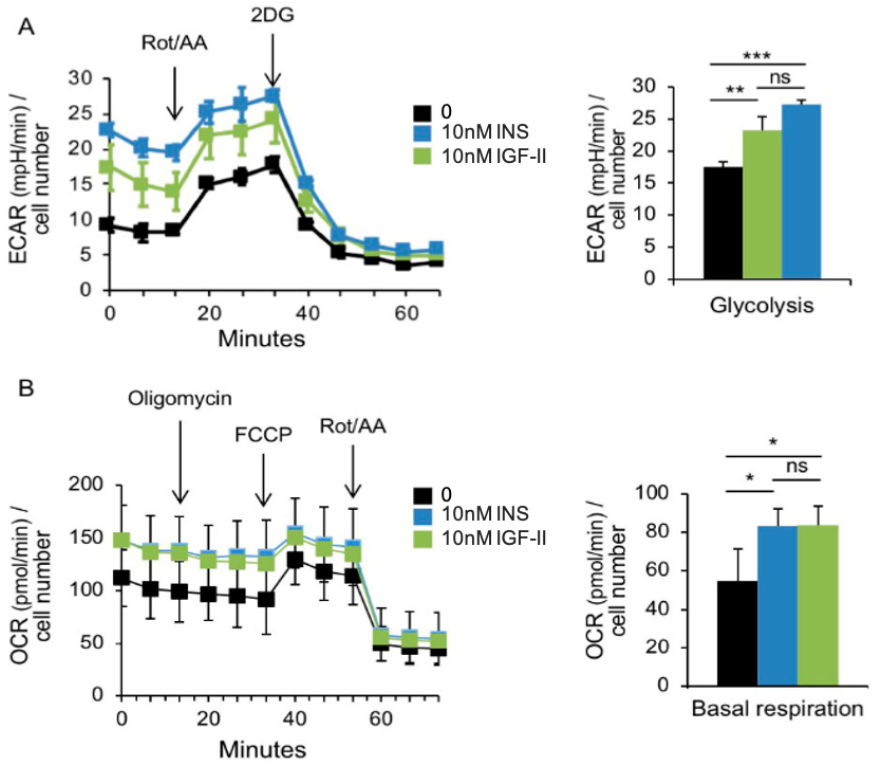

Figure 14: (**A**) Glycolytic rate assay. Analysis of ECAR after serial injections of metabolic modulators in cells serum starved for 4 h and stimulated with 10 nM insulin or IGF-II for further 20 h or untreated (NT). The bar chart on the right shows basal glycolysis. (**B**) Mitochondrial stress test. OCR was determined in cells treated as in (A) in addition of the indicated modulators. The bar chart shows mitochondrial respiration. (A–B) Data are presented as means ± SEM of three independent experiments.

Having observed an increase of both glycolysis and mitochondrial respiration in MCF7$^{IGF\text{-}IR\text{-}ve}$/IR-A cells stimulated by insulin or IGF-II, we evaluated the effects of 2-deoxyglucose (2DG, inhibitor of glycolysis) and metformin (MET, inhibitor of mitochondrial complex I) on IR-A-driven biological responses of migration and colony formation.

As expected, stimulation with insulin or IGF-II makes MCF7$^{IGF\text{-}IR\text{-}ve}$/IR-A cells migrate significantly more as compared to untreated cells. Notably, MET and 2DG at a dose of 16 mM significantly reduced migration induced by stimulation with the combined treatment showing an additive effect (Figure 15A).

Similar results were observed in colony formation after insulin stimulation, in fact the number of colonies was higher in cells stimulated by insulin but significantly reduced upon 2DG compared to MET treatments (Figure 15B).

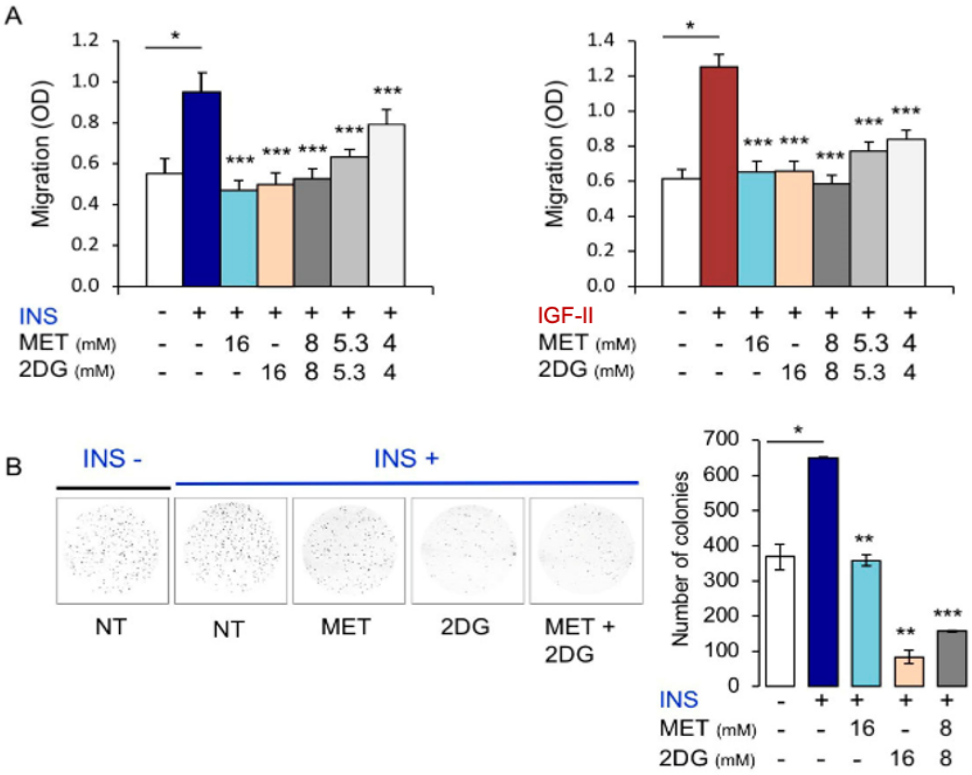

Figure 15: (A) Cell invasion. Serum starved MCF7$^{IGF\text{-}IR\text{-}ve}$/IRA were seeded on polycarbonate filters and treated with either metformin or 2DG alone or in combination. Cells were then allowed to migrate in the absence (NT) or presence of 10 nM INS or IGF-II and after 6h were stained and quantified. Values are means ± SEM of three done in duplicate. **(B)** Colony formation. MCF7$^{IGF\text{-}IR\text{-}ve}$/IRA cells were seeded in soft agar and treated with either MET or 2D alone or in combination in the absence (NT) or presence of 10 nM INS. Colonies were then stained, photographed and measured. The histogram represents the number of colonies from two independent experiments run in quadruplicate wells.

4.6. DDR1 AFFECTS THE METABOLIC REPROGRAMMING OF BC CELLS CONSTITUTIVELY OVEREXPRESSING IGF-II

Based on these results, we hypothesized that DDR1, by regulating IIGF, might control the metabolic reprogramming of BC cells. To this purpose we used the MCF7/IGF-II cell line that showed a higher expression of DDR1 than the wild-type cell line.

First, we knocked down DDR1 in MCF7/IGF-II cells with a pool of four specific small interfering RNA (siRNA) oligos against DDR1 (siDDR1). As shown in Figure 16, DDR1 silencing showed reduction mRNA expression levels of IR (Figure 16), while IGF-IR mRNA levels were unaffected.

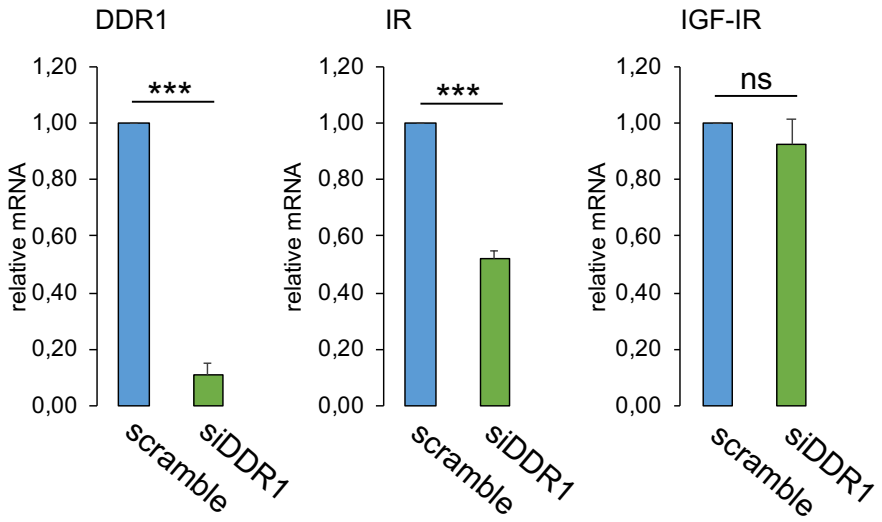

Figure 16: Expression of DDR1, IR, and IGF-IR mRNAs in MCF7/IGF-II cells transiently transfected with siRNA to DDR1 or scramble siRNAs, as measured by qRT-PCR. GAPDH was used as housekeeping control gene. Data are presented as the means ± SEM of three independent experiments.

However, both IR and IGF-IR protein levels were significantly decreased (Figure 17). Constitutive phosphorylation of IR/IGF-IR and specific IR phosphorylation in MCF7/IGF-II cells were also reduced by DDR1 depletion (Figure 17) according to reduced receptor levels.

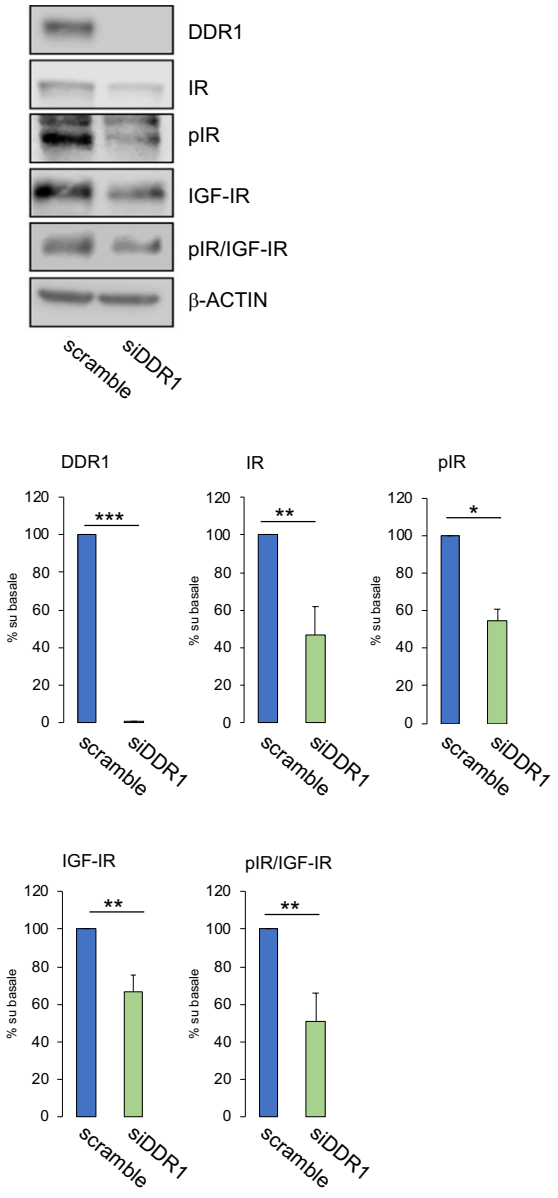

Figure 17: MCF7/IGF-II cells transfected with siDDR1 or scramble oligonucleotides were subjected to immunoblot

analysis with the primary antibodies for anti-phospho-(p)IGF1R (Tyr1135/1136)/pIR (Tyr1150/1151), antibody detecting both pIR and pIGF-IR and anti-pIR (Tyr1334) antibody, specific for pIR. Blots are representative of three independent experiments. The bar chart shows densitometric analysis.

To evaluate the effect of DDR1 depletion in bioenergetics we investigated total ATP production as well as glycolysis–derived ATP (glycoATP) and mitochondrial OxPhos–derived ATP production (mitoATP) using the Seahorse technology. In DDR1-silenced MCF7/IGF-II cells, we observed that total ATP production was significantly decreased as compared to control cells with the reduction primarily due to mitoATP production than glycoATP production rate (Figure 18).

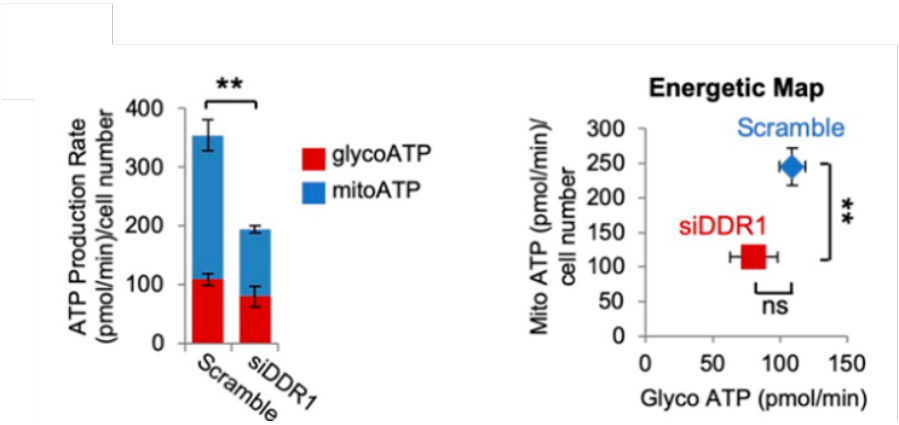

Figure 18: ATP production rate in MCF7/IGF-II cells silenced for DDR1 and in control cells treated with scramble siRNAs. Glycolytic ATP (red columns- glycoATP) and the mitochondrial

(blue columns- mitoATP) production rates were evaluated according to the manufacturer's

As previously mentioned, glycolysis produces substantial quantities of lactate and acidification of the extracellular microenvironment is associated with tumor progression [113]. Therefore, in MCF7/IGF2 cells silenced for DDR1 we evaluated the mRNAs expression of GLUT1, MCT1 and MCT4, and of the glycolytic enzymes EK2, LDHA and PKM2 as compared to control cells. Interestingly, after DDR1 silencing cells showed reduction of MCT4, EK2 and LDHA mRNA levels (Figure 19).

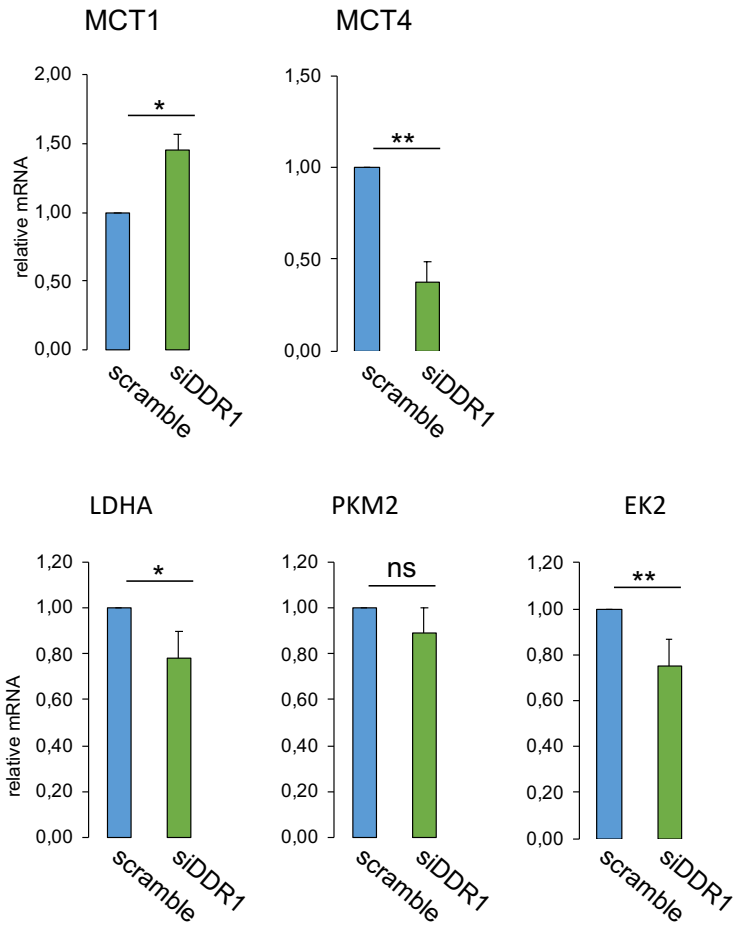

Figure 19: MCF7/IGF-II cells transiently transfected with siRNA for DDR1 or scramble siRNAs were processed for mRNA expression of glycolysis related transporters: MCT1 and MCT4 and enzymes LDHA, PKM2, EK2.

Then we measured, under the same conditions, the protein expression of MCT1 and MCT4, EK2, LDHA and PKM2 and the results obtained indicated that DDR1 depletion was associated with upregulation of MCT1 protein and downregulation of MCT4, PKM2, and EK2 protein (Figure 20).

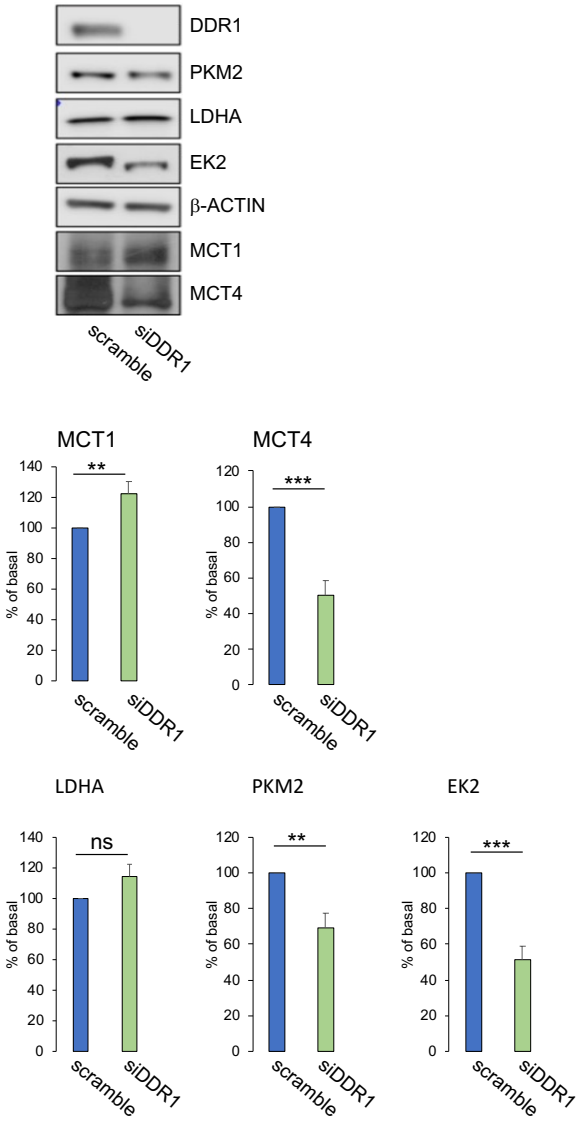

Figure 20: Western blot of selected glycolysis related molecules (PKM2, LDHA, MCT1, MCT4, EK2) in MCF7/IGF-II cells silenced for DDR1 or treated with scramble siRNAs. The bar chart shows densitometric analysis of results obtained in three independent experiments.

Moreover, in MCF7/IGF-II cells silenced for DDR1, we evaluated the expression of genes related to mitochondria biogenesis (PGC1α, PGC1β, PRC), activity (ARALAR, NRF-1, NRF-2a, COX1), and mass. Specifically, we observed increased mRNA levels of PGC1α, while PGC1β gene expression was unaffected. On the contrary, a significantly decreased gene expression of PRC was detected in DDR1-silenced cells compared to control cells (Figure 21, upper graphs). In addition, we also found reduced levels of ARALAR, NRF-1 and NRF-2α (Figure 21, middle graphs). COX1 and TOMM20 were not significantly affected in DDR1-silenced MCF7/IGF-II compared to control cells (Figure 21, lower graph).

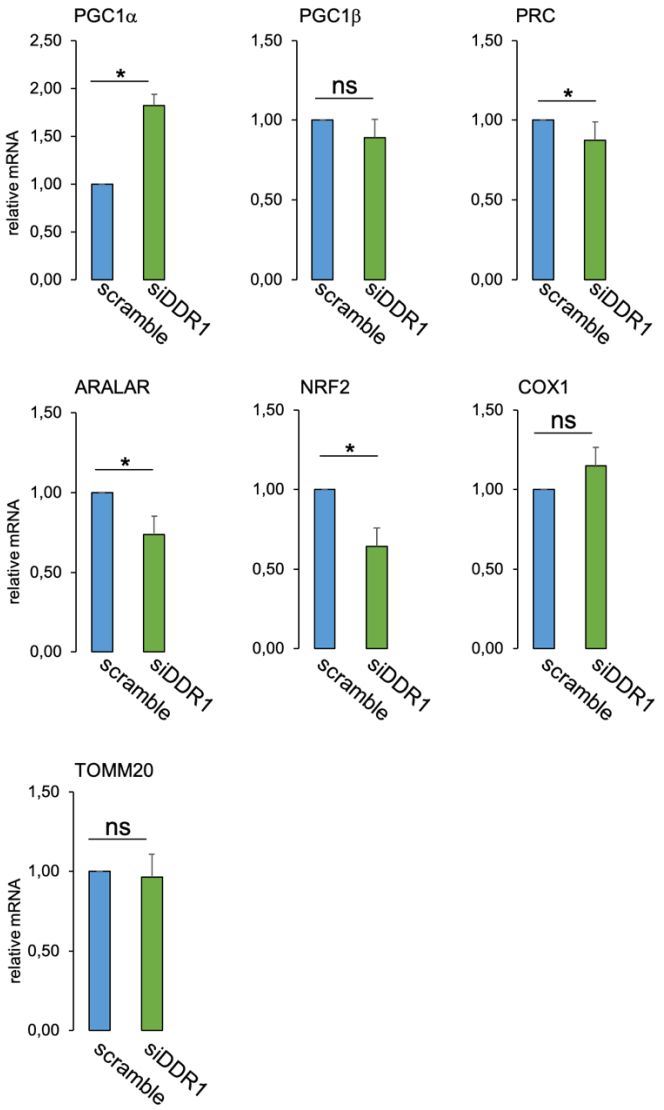

Figure 21: OxPhos related markers in MCF7/IGF-II cells silenced for DDR1 or treated with scramble siRNAs. Markers of mitochondrial biogenesis (PGC1α, PGC1β and PRC) and mitochondrial markers (ARALAR and NRF-2) were measured by qRT-PCR analysis. Mitochondrial activity was evaluated by mRNA expression levels of COX1 and mitochondrial mass was measured by mRNA levels of TOMM20. Values are expressed as the means ± SEM of three separate experiments.

To confirm these data, we measured the protein expression of NRF1, ARALAR and of mitochondrial complexes. The results obtained indicated that DDR1 silencing was associated with downregulation of mitochondrial complex IV and I and NRF1, while ARALAR was unaffected compared to control cells (Figure 22).

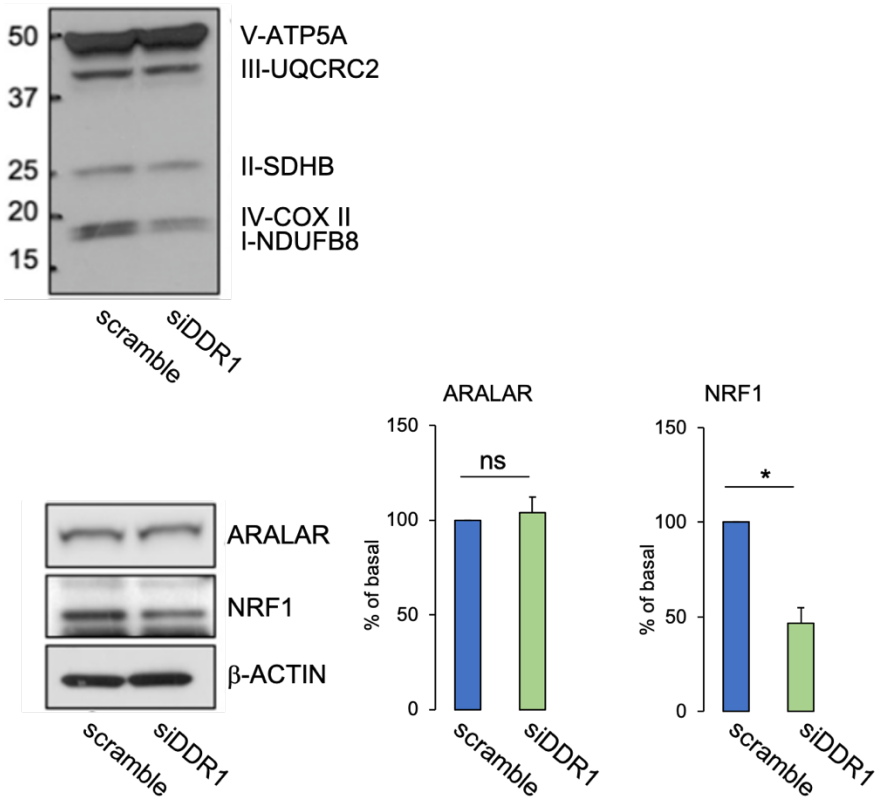

Figure 22: Western blot with OxPhos antibodies cocktail against mitochondrial complexes showing a decrease of mitochondrial complex IV and I. NRF1 protein was also markedly decreased whereas ARALAR did not show significant changes. Graphs represent the mean ± SEM of densitometric analysis of three independent experiments.

4.7. MCF7$^{DDR1\text{-}ve}$ CELLS SHOW IMPAIRED METABOLIC ACTIVITY

Moreover, to establish the role of DDR1 in BC cell metabolic reprogramming we studied several parameters involved in cell metabolism in MCF7$^{DDR1\text{-}ve}$ cells as compared to control MCF7/^{Cas9} cells. Indeed, MCF7$^{DDR1\text{-}ve}$ cells showed upregulation of MCT1 and downregulation of MCT4 and hexokinase-2 in protein expression (Figure 23).

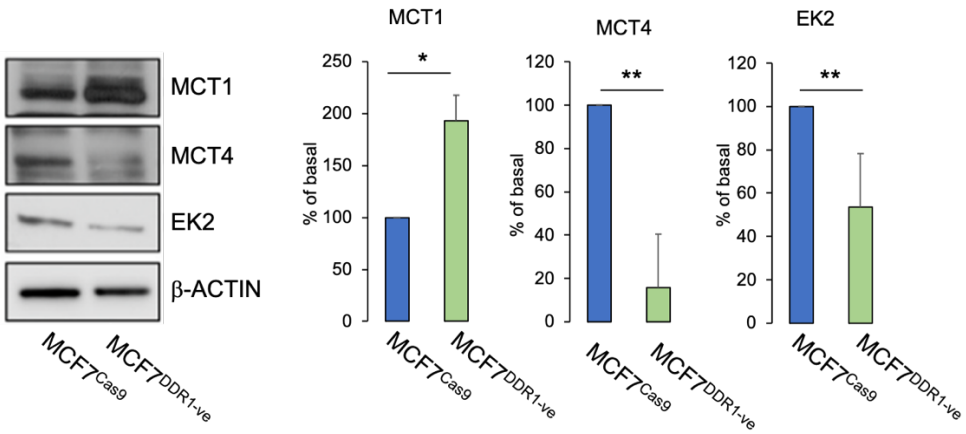

Figure 23: MCF7$^{DDR1\text{-}ve}$ cells and parental MCF7/ ^{CAS9} cells were grown in 10% FBS, lysed and analyzed by immunoblot for selected glycolysis related molecules (MCT1, MCT4, EK2). β-actin antibody was used as control for protein loading. Graphs represent the mean ± SEM of densitometric analysis of three independent experiments.

Moreover, NRF1 and ARALAR and mitochondrial complexes were also significant reduced (Figure 24).

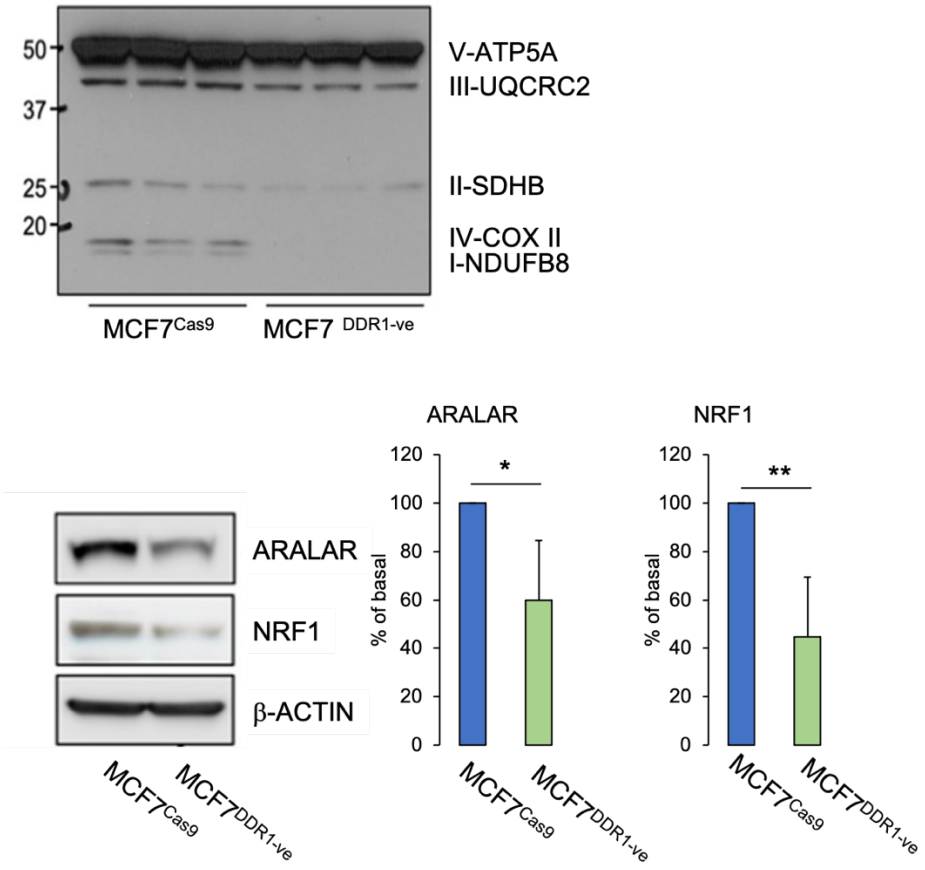

Figure 24: MCF7$^{DDR1\text{-}ve}$ cells and parental MCF7/^{CAS9} cells were grown in 10% FBS, lysed and analyzed by immunoblot for OxPhos antibodies cocktail to mitochondrial complexes and mitochondrial markers (ARALAR and NRF-2). Graphs represent the mean ± SEM of densitometric analysis of three independent experiments.

4.8. DDR1 SILENCING DECREASES THE METABOLIC ACTIVITY OF BC CELLS OVEREXPRESSING IR-A

We then investigated whether DDR1 silencing could inhibit the metabolic activity of BC cells that overexpress the oncofetal IR-A using the Seahorse technology. To avoid the possible interfering activity of IGF-IR, we used

MCF7$^{IGF\text{-}IR\text{-}ve}$/IR-A cell line. As shown in Figure 25, DDR1 silencing significantly inhibited the ATP production both in the presence and in the absence of IGF-II stimulation. The reduction was especially evident on the mitochondrial ATP production rate in samples DDR1-depleted (Figure 25). Taken together these data indicate that DDR1 silencing also effectively reduced ATP production in MCF7 cells overexpressing IR-A, a cell model that mimics the IR expression pattern often found in human BCs.

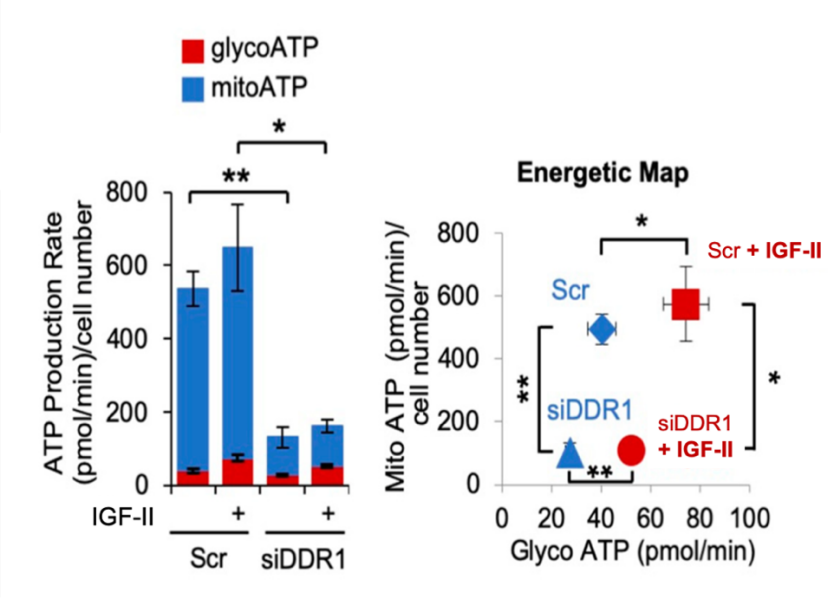

Figure 25: ATP production rate in MCF7$^{IGF\text{-}IR\text{-}ve}$/IR-A cells silenced for DDR1 and in control cells treated with scramble siRNAs, upon stimulation with 100nM IGF-II for 48h. Glycolytic ATP and mitochondrial ATP production rates were evaluated according to the manufacturer's instructions. In the right panel the energetic map from three independent experiments is shown.

5. DISCUSSION

In this study we demonstrated that the insulin/IGF-II-IR-A axis might have a role in the metabolic reprogramming of BC cells and that DDR1 potentiate this effect, thus representing a suitable therapeutical target.

Previous studies had established that IR-A overexpression in cancer cells is implicated in tumor promotion, metastatic spread, stem-like cell phenotype, dedifferentiation and resistance to cancer therapies [23, 116] and that both insulin and IGF-II bind to and activate IR-A [114**,** 115]**.** IGF-IR, also frequently expressed in BCs, contributes to transduce IGF-II effects. To investigate the metabolic potential of the activated insulin/IGF axis we studied various cell models. First, we studied MCF7 human BC cells stably engineered to overexpress and secrete IGF-II (MCF7/IGF-II). These cells showed stronger capacity to proliferate, migrate and form colonies as compared to control MCF7/EV cells. Furthermore, these cells exhibited increased metabolic activity due to both enhanced glycolysis and mitochondrial respiration. In fact, MCF7/IGF-II cells showed an increase of glucose transporter GLUT1, glycolytic enzymes LDHA and PKM2, as well as lactate transporter MCT4, which extrudes lactate from the cell. Specifically, LDHA is often upregulated in neoplastic tissues and support cancer cell proliferation. PKM2 expression is an important metabolic signature of tumor cells. However, MCF7/IGF-II cells showed increased expression of various markers of mitochondrial biogenesis and mitochondrial activity associated with augmented mitochondrial mass.

Taken together, these findings indicate that MCF7/IGF-II cells have acquired increased metabolic activity that includes higher glycolytic and mitochondrial activity as compared to MCF7/EV cells. However, in MCF7/IGF-II cells, both the PI3K/AKT and the ERK1/2 cascades were constitutively activated.

To investigate more specifically the effects of IR-A in the absence of IGF-IR, we used MCF7 cells knocked out for IGF-IR and overexpressing IR-A (MCF7$^{IGF\text{-}IR\text{-}ve}$/IR-A). As expected, MCF7$^{IGF\text{-}IR\text{-}ve}$/IR-A cells, in response to both insulin and IGF-II stimulation, showed increase glycolysis and mitochondrial respiration. Consistent with these results, in ligand stimulated MCF7$^{IGF\text{-}IR\text{-}ve}$/IR-A cells, both a glycolysis inhibitor (2DG) and an OxPhos inhibitor (metformin) were able to block cell invasion and colony formation. The two compounds had additive effects in combination treatment. This first set of results indicates that constitutive IGF-II expression contribute to the metabolic reprogramming and increased metabolic flexibility of human BC cells [117] and that insulin and IGF-II have similar metabolic effects in BC cells lacking IGF-IR and overexpressing IR-A [117].

Pharmacological approaches able to specifically target the IR-A in cancer are still unavailable [94]. Based on previous studies indicating an effective functional crosstalk between insulin/IGF signaling and DDR1, we evaluated whether DDR1 targeting could be an alternative strategy to inhibit IR-A-driven metabolic reprogramming in BC cells. For this reason we used DDR1 silencing in MCF7/IGF-II cell model. We observed a downregulation of both IR and IGF-IR proteins and a reduction of IR/IGF-IR phosphorylation

associated with DDR1 silencing. Notably, we observed that DDR1 depletion is associated with a reduction in ATP production, both glycoATP and mitoATP and with a decrease of glycolytic markers, including MCT4, EK2, PKM2, and of markers of mitochondrial biogenesis and mitochondrial activity, including PRC, ARALAR, NRF-1 and NRF-2a.

DDR1 silencing also caused a reduction in ATP production in MCF7 cells overexpressing IR-A in the absence of IGF-IR, suggesting that the metabolic effect of DDR1 silencing does not require IGF-IR activation.

To further corroborate these data, we showed that, compared to control cells, MCF7 cells knocked out for DDR1 ($MCF7^{DDR1\text{-}ve}$) exhibit a downregulation of key molecules related to both glycolysis and OxPhos. Taken together our results indicate that DDR1 has a role in BC cells metabolic reprogramming.

6. CONCLUSION

Overall, our study indicate that the upregulation of IGF-II and IR-A is a novel mechanism contributing to metabolic reprogramming and increased metabolic flexibility in human BC cells and that insulin also elicits similar metabolic effect than IGF-II by acting through the IR-A. Moreover, we also found that DDR1, a molecular partner of IRA, regulates metabolic reprogramming partially through IR/IGF-IR activation.

Therefore, the present results identify DDR1 as a suitable target for inhibiting the metabolic effects of insulin / IGF axis hyperactivation and, in particular, IR-A overexpression in BC cells.

7. REFERENCES

1. Sung, H., et al., *Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries.* CA Cancer J Clin, 2021. **71**(3): p. 209-249.
2. Kaminska, M., et al., *Breast cancer risk factors.* Prz Menopauzalny, 2015. **14**(3): p. 196-202.
3. Winters, S., et al., *Breast Cancer Epidemiology, Prevention, and Screening.* Prog Mol Biol Transl Sci, 2017. **151**: p. 1-32.
4. Gonzalez-Angulo, A.M., F. Morales-Vasquez, and G.N. Hortobagyi, *Overview of resistance to systemic therapy in patients with breast cancer.* Adv Exp Med Biol, 2007. **608**: p. 1-22.
5. Farabaugh, S.M., D.N. Boone, and A.V. Lee, *Role of IGF1R in Breast Cancer Subtypes, Stemness, and Lineage Differentiation.* Front Endocrinol (Lausanne), 2015. **6**: p. 59.
6. Payne, S.J., et al., *Predictive markers in breast cancer--the present.* Histopathology, 2008. **52**(1): p. 82-90.
7. Dillon, D., A.J. Guidi, and S.J. Schnitt, *Pathology of invasive breast cancer*. 2014.
8. Cao, J. and D. Yee, *Disrupting Insulin and IGF Receptor Function in Cancer.* Int J Mol Sci, 2021. **22**(2).
9. LeRoith, D., J.M.P. Holly, and B.E. Forbes, *Insulin-like growth factors: Ligands, binding proteins, and receptors.* Mol Metab, 2021: p. 101245.
10. Rinderknecht, E. and R.E. Humbel, *The amino acid sequence of human insulin-like growth factor I and its structural homology with proinsulin.* J Biol Chem, 1978. **253**(8): p. 2769-76.
11. Laron, Z., *Insulin-like growth factor 1 (IGF-1): a growth hormone.* Mol Pathol, 2001. **54**(5): p. 311-6.
12. Holly, J.M.P., K. Biernacka, and C.M. Perks, *The Neglected Insulin: IGF-II, a Metabolic Regulator with Implications for Diabetes, Obesity, and Cancer.* Cells, 2019. **8**(10).

13. Baral, K. and P. Rotwein, *The insulin-like growth factor 2 gene in mammals: Organizational complexity within a conserved locus.* PLoS One, 2019. **14**(6): p. e0219155.
14. Pessin, J.E. and A.R. Saltiel, *Signaling pathways in insulin action: molecular targets of insulin resistance.* J Clin Invest, 2000. **106**(2): p. 165-9.
15. Whittaker, J., et al., *Alanine scanning mutagenesis of a type 1 insulin-like growth factor receptor ligand binding site.* J Biol Chem, 2001. **276**(47): p. 43980-6.
16. Ullrich, A., et al., *Insulin-like growth factor I receptor primary structure: comparison with insulin receptor suggests structural determinants that define functional specificity.* EMBO J, 1986. **5**(10): p. 2503-12.
17. Jones, J.I. and D.R. Clemmons, *Insulin-like growth factors and their binding proteins: biological actions.* Endocr Rev, 1995. **16**(1): p. 3-34.
18. Boone, D.N. and A.V. Lee, *Targeting the insulin-like growth factor receptor: developing biomarkers from gene expression profiling.* Crit Rev Oncog, 2012. **17**(2): p. 161-73.
19. Brahmkhatri, V.P., C. Prasanna, and H.S. Atreya, *Insulin-like growth factor system in cancer: novel targeted therapies.* Biomed Res Int, 2015. **2015**: p. 538019.
20. Scott, C.D. and W. Kiess, *Soluble M6P/IGFIIR in the circulation.* Best Pract Res Clin Endocrinol Metab, 2015. **29**(5): p. 723-33.
21. Bleach, R., et al., *Growth Hormone/Insulin Growth Factor Axis in Sex Steroid Associated Disorders and Related Cancers.* Front Cell Dev Biol, 2021. **9**: p. 630503.
22. Moller, D.E., et al., *Tissue-specific expression of two alternatively spliced insulin receptor mRNAs in man.* Mol Endocrinol, 1989. **3**(8): p. 1263-9.
23. Belfiore, A., et al., *Insulin Receptor Isoforms in Physiology and Disease: An Updated View.* Endocr Rev, 2017. **38**(5): p. 379-431.
24. Malaguarnera, R., A. Morcavallo, and A. Belfiore, *The insulin and igf-I pathway in endocrine glands carcinogenesis.* J Oncol, 2012. **2012**: p. 635614.

25. Frasca, F., et al., *Insulin receptor isoform A, a newly recognized, high-affinity insulin-like growth factor II receptor in fetal and cancer cells.* Mol Cell Biol, 1999. **19**(5): p. 3278-88.
26. Frasca, F., et al., *The role of insulin receptors and IGF-I receptors in cancer and other diseases.* Arch Physiol Biochem, 2008. **114**(1): p. 23-37.
27. Yang, Y. and D. Yee, *Targeting insulin and insulin-like growth factor signaling in breast cancer.* J Mammary Gland Biol Neoplasia, 2012. **17**(3-4): p. 251-61.
28. Ebina, Y., et al., *Expression of a functional human insulin receptor from a cloned cDNA in Chinese hamster ovary cells.* Proc Natl Acad Sci U S A, 1985. **82**(23): p. 8014-8.
29. Liu, F. and R.A. Roth, *Binding of SH2 containing proteins to the insulin receptor: a new way for modulating insulin signalling.* Mol Cell Biochem, 1998. **182**(1-2): p. 73-8.
30. Kanzaki, M. and J.E. Pessin, *Signal integration and the specificity of insulin action.* Cell Biochem Biophys, 2001. **35**(2): p. 191-209.
31. Tamemoto, H., et al., *Insulin resistance and growth retardation in mice lacking insulin receptor substrate-1.* Nature, 1994. **372**(6502): p. 182-6.
32. Withers, D.J., et al., *Disruption of IRS-2 causes type 2 diabetes in mice.* Nature, 1998. **391**(6670): p. 900-4.
33. Mora, A., et al., *PDK1, the master regulator of AGC kinase signal transduction.* Semin Cell Dev Biol, 2004. **15**(2): p. 161-70.
34. Sarbassov, D.D., S.M. Ali, and D.M. Sabatini, *Growing roles for the mTOR pathway.* Curr Opin Cell Biol, 2005. **17**(6): p. 596-603.
35. Sano, H., et al., *Insulin-stimulated phosphorylation of a Rab GTPase-activating protein regulates GLUT4 translocation.* J Biol Chem, 2003. **278**(17): p. 14599-602.
36. Zeigerer, A., M.K. McBrayer, and T.E. McGraw, *Insulin stimulation of GLUT4 exocytosis, but not its inhibition of endocytosis, is dependent on RabGAP AS160.* Mol Biol Cell, 2004. **15**(10): p. 4406-15.
37. Kops, G.J., et al., *Direct control of the Forkhead transcription factor AFX by protein kinase B.* Nature, 1999. **398**(6728): p. 630-4.

38. Kino, T., et al., *HIV-1 accessory protein Vpr inhibits the effect of insulin on the Foxo subfamily of forkhead transcription factors by interfering with their binding to 14-3-3 proteins: potential clinical implications regarding the insulin resistance of HIV-1-infected patients.* Diabetes, 2005. **54**(1): p. 23-31.
39. Liu, T., et al., *NF-kappaB signaling in inflammation.* Signal Transduct Target Ther, 2017. **2**.
40. Holland, E.C., et al., *Signaling control of mRNA translation in cancer pathogenesis.* Oncogene, 2004. **23**(18): p. 3138-44.
41. Ceresa, B.P. and J.E. Pessin, *Insulin regulation of the Ras activation/inactivation cycle.* Mol Cell Biochem, 1998. **182**(1-2): p. 23-9.
42. Liang, J. and J.M. Slingerland, *Multiple roles of the PI3K/PKB (Akt) pathway in cell cycle progression.* Cell Cycle, 2003. **2**(4): p. 339-45.
43. Motallebnezhad, M., et al., *The insulin-like growth factor-I receptor (IGF-IR) in breast cancer: biology and treatment strategies.* Tumour Biol, 2016. **37**(9): p. 11711-11721.
44. Burrow, S., et al., *Expression of insulin-like growth factor receptor, IGF-1, and IGF-2 in primary and metastatic osteosarcoma.* J Surg Oncol, 1998. **69**(1): p. 21-7.
45. Steller, M.A., et al., *Overexpression of the insulin-like growth factor-1 receptor and autocrine stimulation in human cervical cancer cells.* Cancer Res, 1996. **56**(8): p. 1761-5.
46. Sciacca, L., et al., *Insulin receptor activation by IGF-II in breast cancers: evidence for a new autocrine/paracrine mechanism.* Oncogene, 1999. **18**(15): p. 2471-9.
47. Vella, V., et al., *A novel autocrine loop involving IGF-II and the insulin receptor isoform-A stimulates growth of thyroid cancer.* J Clin Endocrinol Metab, 2002. **87**(1): p. 245-54.
48. Sciacca, L., et al., *In IGF-I receptor-deficient leiomyosarcoma cells autocrine IGF-II induces cell invasion and protection from apoptosis via the insulin receptor isoform A.* Oncogene, 2002. **21**(54): p. 8240-50.

49. Belfiore, A. and R. Malaguarnera, *Insulin receptor and cancer.* Endocr Relat Cancer, 2011. **18**(4): p. R125-47.
50. King, H., et al., *Can we unlock the potential of IGF-1R inhibition in cancer therapy?* Cancer Treat Rev, 2014. **40**(9): p. 1096-105.
51. Livingstone, C., *IGF2 and cancer.* Endocr Relat Cancer, 2013. **20**(6): p. R321-39.
52. Simpson, A., et al., *Insulin-Like Growth Factor (IGF) Pathway Targeting in Cancer: Role of the IGF Axis and Opportunities for Future Combination Studies.* Target Oncol, 2017. **12**(5): p. 571-597.
53. Garofalo, C., et al., *Efficacy of and resistance to anti-IGF-1R therapies in Ewing's sarcoma is dependent on insulin receptor signaling.* Oncogene, 2011. **30**(24): p. 2730-40.
54. Hanahan, D. and R.A. Weinberg, *Hallmarks of cancer: the next generation.* Cell, 2011. **144**(5): p. 646-74.
55. Malaguarnera, R. and A. Belfiore, *The emerging role of insulin and insulin-like growth factor signaling in cancer stem cells.* Front Endocrinol (Lausanne), 2014. **5**: p. 10.
56. Heerboth, S., et al., *EMT and tumor metastasis.* Clin Transl Med, 2015. **4**: p. 6.
57. Poreba, E. and J. Durzynska, *Nuclear localization and actions of the insulin-like growth factor 1 (IGF-1) system components: Transcriptional regulation and DNA damage response.* Mutat Res, 2020. **784**: p. 108307.
58. Johnson, J.D., J.C. Edman, and W.J. Rutter, *A receptor tyrosine kinase found in breast carcinoma cells has an extracellular discoidin I-like domain.* Proc Natl Acad Sci U S A, 1993. **90**(12): p. 5677-81.
59. Carafoli, F. and E. Hohenester, *Collagen recognition and transmembrane signalling by discoidin domain receptors.* Biochim Biophys Acta, 2013. **1834**(10): p. 2187-94.
60. Vogel, W., *Discoidin domain receptors: structural relations and functional implications.* FASEB J, 1999. **13 Suppl**: p. S77-82.

61. Vogel, W.F., R. Abdulhussein, and C.E. Ford, *Sensing extracellular matrix: an update on discoidin domain receptor function.* Cell Signal, 2006. **18**(8): p. 1108-16.
62. Vella, V., et al., *Insulin/IGF signaling and discoidin domain receptors: An emerging functional connection.* Biochim Biophys Acta Mol Cell Res, 2019. **1866**(11): p. 118522.
63. Alves, F., et al., *Distinct structural characteristics of discoidin I subfamily receptor tyrosine kinases and complementary expression in human cancer.* Oncogene, 1995. **10**(3): p. 609-18.
64. Leitinger, B. and A.P. Kwan, *The discoidin domain receptor DDR2 is a receptor for type X collagen.* Matrix Biol, 2006. **25**(6): p. 355-64.
65. Valiathan, R.R., et al., *Discoidin domain receptor tyrosine kinases: new players in cancer progression.* Cancer Metastasis Rev, 2012. **31**(1-2): p. 295-321.
66. Koo, D.H., et al., *Pinpointing phosphotyrosine-dependent interactions downstream of the collagen receptor DDR1.* FEBS Lett, 2006. **580**(1): p. 15-22.
67. Rammal, H., et al., *Discoidin Domain Receptors: Potential Actors and Targets in Cancer.* Front Pharmacol, 2016. **7**: p. 55.
68. Su, J., et al., *Discoidin domain receptor 2 is associated with the increased expression of matrix metalloproteinase-13 in synovial fibroblasts of rheumatoid arthritis.* Mol Cell Biochem, 2009. **330**(1-2): p. 141-52.
69. Dejmek, J., et al., *Wnt-5a and G-protein signaling are required for collagen-induced DDR1 receptor activation and normal mammary cell adhesion.* Int J Cancer, 2003. **103**(3): p. 344-51.
70. Barker, K.T., et al., *Expression patterns of the novel receptor-like tyrosine kinase, DDR, in human breast tumours.* Oncogene, 1995. **10**(3): p. 569-75.
71. Neuhaus, B., et al., *Migration inhibition of mammary epithelial cells by Syk is blocked in the presence of DDR1 receptors.* Cell Mol Life Sci, 2011. **68**(22): p. 3757-70.

72. Heinzelmann-Schwarz, V.A., et al., *Overexpression of the cell adhesion molecules DDR1, Claudin 3, and Ep-CAM in metaplastic ovarian epithelium and ovarian cancer.* Clin Cancer Res, 2004. **10**(13): p. 4427-36.
73. Quan, J., et al., *Identification of receptor tyrosine kinase, discoidin domain receptor 1 (DDR1), as a potential biomarker for serous ovarian cancer.* Int J Mol Sci, 2011. **12**(2): p. 971-82.
74. Yang, S.H., et al., *Discoidin domain receptor 1 is associated with poor prognosis of non-small cell lung carcinomas.* Oncol Rep, 2010. **24**(2): p. 311-9.
75. Miao, L., et al., *Discoidin domain receptor 1 is associated with poor prognosis of non-small cell lung cancer and promotes cell invasion via epithelial-to-mesenchymal transition.* Med Oncol, 2013. **30**(3): p. 626.
76. Shen, Q., et al., *Role of microRNA-199a-5p and discoidin domain receptor 1 in human hepatocellular carcinoma invasion.* Mol Cancer, 2010. **9**: p. 227.
77. Couvelard, A., et al., *Identification of potential therapeutic targets by gene-expression profiling in pancreatic endocrine tumors.* Gastroenterology, 2006. **131**(5): p. 1597-610.
78. Shimada, K., et al., *Prostate cancer antigen-1 contributes to cell survival and invasion though discoidin receptor 1 in human prostate cancer.* Cancer Sci, 2008. **99**(1): p. 39-45.
79. Gadiya, M. and G. Chakraborty, *Signaling by discoidin domain receptor 1 in cancer metastasis.* Cell Adh Migr, 2018. **12**(4): p. 315-323.
80. Itoh, Y., *Discoidin domain receptors: Microenvironment sensors that promote cellular migration and invasion.* Cell Adh Migr, 2018. **12**(4): p. 378-385.
81. Henriet, E., et al., *Multitasking discoidin domain receptors are involved in several and specific hallmarks of cancer.* Cell Adh Migr, 2018. **12**(4): p. 363-377.
82. Maeyama, M., et al., *Switching in discoid domain receptor expressions in SLUG-induced epithelial-mesenchymal transition.* Cancer, 2008. **113**(10): p. 2823-31.

83. Toy, K.A., et al., *Tyrosine kinase discoidin domain receptors DDR1 and DDR2 are coordinately deregulated in triple-negative breast cancer.* Breast Cancer Res Treat, 2015. **150**(1): p. 9-18.
84. Shintani, Y., et al., *Collagen I-mediated up-regulation of N-cadherin requires cooperative signals from integrins and discoidin domain receptor 1.* J Cell Biol, 2008. **180**(6): p. 1277-89.
85. Vogel, W., et al., *The discoidin domain receptor tyrosine kinases are activated by collagen.* Mol Cell, 1997. **1**(1): p. 13-23.
86. Roberts, M.E., et al., *Discoidin domain receptor 1 regulates bronchial epithelial repair and matrix metalloproteinase production.* Eur Respir J, 2011. **37**(6): p. 1482-93.
87. Zhong, X., W. Zhang, and T. Sun, *DDR1 promotes breast tumor growth by suppressing antitumor immunity.* Oncol Rep, 2019. **42**(6): p. 2844-2854.
88. Ford, C.E., et al., *Expression and mutation analysis of the discoidin domain receptors 1 and 2 in non-small cell lung carcinoma.* Br J Cancer, 2007. **96**(5): p. 808-14.
89. Reger de Moura, C., et al., *Discoidin domain receptors: A promising target in melanoma.* Pigment Cell Melanoma Res, 2019. **32**(5): p. 697-707.
90. Zhang, K., et al., *The collagen receptor discoidin domain receptor 2 stabilizes SNAIL1 to facilitate breast cancer metastasis.* Nat Cell Biol, 2013. **15**(6): p. 677-87.
91. Ren, T., et al., *Increased expression of discoidin domain receptor 2 (DDR2): a novel independent prognostic marker of worse outcome in breast cancer patients.* Med Oncol, 2013. **30**(1): p. 397.
92. Ren, T., et al., *Discoidin domain receptor 2 (DDR2) promotes breast cancer cell metastasis and the mechanism implicates epithelial-mesenchymal transition programme under hypoxia.* J Pathol, 2014. **234**(4): p. 526-37.
93. Morcavallo, A., et al., *Insulin and insulin-like growth factor II differentially regulate endocytic sorting and stability of insulin receptor isoform A.* J Biol Chem, 2012. **287**(14): p. 11422-36.
94. Malaguarnera, R. and A. Belfiore, *The insulin receptor: a new target for cancer therapy.* Front Endocrinol (Lausanne), 2011. **2**: p. 93.

95. Belfiore, A., et al., *A novel functional crosstalk between DDR1 and the IGF axis and its relevance for breast cancer.* Cell Adh Migr, 2018. **12**(4): p. 305-314.
96. Shrivastava, A., et al., *An orphan receptor tyrosine kinase family whose members serve as nonintegrin collagen receptors.* Mol Cell, 1997. **1**(1): p. 25-34.
97. Malaguarnera, R., et al., *Novel cross talk between IGF-IR and DDR1 regulates IGF-IR trafficking, signaling and biological responses.* Oncotarget, 2015. **6**(18): p. 16084-105.
98. Vella, V., et al., *Discoidin domain receptor 1 modulates insulin receptor signaling and biological responses in breast cancer cells.* Oncotarget, 2017. **8**(26): p. 43248-43270.
99. Mata, R., et al., *IGF-I induces upregulation of DDR1 collagen receptor in breast cancer cells by suppressing MIR-199a-5p through the PI3K/AKT pathway.* Oncotarget, 2016. **7**(7): p. 7683-700.
100. Vander Heiden, M.G., L.C. Cantley, and C.B. Thompson, *Understanding the Warburg effect: the metabolic requirements of cell proliferation.* Science, 2009. **324**(5930): p. 1029-33.
101. Warburg, O., *On the origin of cancer cells.* Science, 1956. **123**(3191): p. 309-14.
102. Ghergurovich, J.M., et al., *Local production of lactate, ribose phosphate, and amino acids within human triple-negative breast cancer.* Med (N Y), 2021. **2**(6): p. 736-754.
103. Ward, P.S. and C.B. Thompson, *Metabolic reprogramming: a cancer hallmark even warburg did not anticipate.* Cancer Cell, 2012. **21**(3): p. 297-308.
104. Belfiore, A., et al., *Insulin receptors in breast cancer.* Ann N Y Acad Sci, 1996. **784**: p. 173-88.
105. Papa, V., et al., *Elevated insulin receptor content in human breast cancer.* J Clin Invest, 1990. **86**(5): p. 1503-10.
106. Kang, C., D. LeRoith, and E.J. Gallagher, *Diabetes, Obesity, and Breast Cancer.* Endocrinology, 2018. **159**(11): p. 3801-3812.

107. Gallagher, E.J., et al., *Insulin receptor phosphorylation by endogenous insulin or the insulin analog AspB10 promotes mammary tumor growth independent of the IGF-I receptor.* Diabetes, 2013. **62**(10): p. 3553-60.
108. Frittitta, L., et al., *Structural and functional studies of insulin receptors in human breast cancer.* Breast Cancer Res Treat, 1993. **25**(1): p. 73-82.
109. Kim, J.H., et al., *Prognostic significance of insulin growth factor-I receptor and insulin growth factor binding protein-3 expression in primary breast cancer.* Oncol Rep, 2010. **23**(4): p. 989-95.
110. Peiro, G., et al., *Increased insulin-like growth factor-1 receptor mRNA expression predicts poor survival in immunophenotypes of early breast carcinoma.* Mod Pathol, 2011. **24**(2): p. 201-8.
111. Vogel, W.F., et al., *Discoidin domain receptor 1 tyrosine kinase has an essential role in mammary gland development.* Mol Cell Biol, 2001. **21**(8): p. 2906-17.
112. Lanning, N.J., et al., *Metabolic profiling of triple-negative breast cancer cells reveals metabolic vulnerabilities.* Cancer Metab, 2017. **5**: p. 6.
113. Rofstad, E.K., et al., *Acidic extracellular pH promotes experimental metastasis of human melanoma cells in athymic nude mice.* Cancer Res, 2006. **66**(13): p. 6699-707.
114. Aljada, A., et al., *Quantification of insulin receptor mRNA splice variants as a diagnostic tumor marker in breast cancer.* Cancer Biomark, 2015. **15**(5): p. 653-61.
115. Bjorner, S., et al., *Combined and individual tumor-specific expression of insulin-like growth factor-I receptor, insulin receptor and phospho-insulin-like growth factor-I receptor/insulin receptor in primary breast cancer: Implications for prognosis in different treatment groups.* Oncotarget, 2017. **8**(6): p. 9093-9107.
116. Belfiore, A., et al., *Insulin receptor isoforms and insulin receptor/insulin-like growth factor receptor hybrids in physiology and disease.* Endocr Rev, 2009. **30**(6): p. 586-623.

117. Vella, V., et al., *Insulin Receptor Isoform A Modulates Metabolic Reprogramming of Breast Cancer Cells in Response to IGF2 and Insulin Stimulation.* Cells, 2019. **8**(9).