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Human IGF1 pro-forms induce breast cancer cell proliferation *via* IGF1 receptor

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Abstract

Purpose IGF1 is a key regulator of tissue growth and development and is implicated in the progression and risk of carcinomas, including breast cancer. The complexity of the *IGF1* gene gives rise to different precursor pro-peptides, namely the IGF1Ea, IGF1Eb and IGF1Ec pro-forms, whose biological role in breast cancer pathogenesis has not been established. The objective of this study is to evaluate the biological activity of IGF1 pro-forms in human breast cancer cell lines.

Methods IGF1 pro-forms were generated by transient transfection of HEK293 cells using specific constructs, and conditioned media were used for MCF7, T47D and ZR751 cells in *in vitro* culture. The affinity between the anti-IGF1 antibody and the IGF1 pro-forms was evaluated by immunoprecipitaion. To determine whether the pro-forms induce cell proliferation, we neutralised the mature IGF1 in conditioned media.

Results The IGF1 pro-forms were the only forms produced intracellularly, whereas both mature IGF1 and IGF1 pro-forms were detected extracellularly. We demonstrated that E peptides impair the accurate measurement of IGF1 pool and the pro-forms were weakly recognised by the anti-IGF1 antibody. We found that the IGF1 antibody completely inhibited the IGF1-induced breast cancer cell proliferation and IGF1R phosphorylation, whereas the same antibody only partially inhibited the pro-form biological activity. Moreover, the IGF1 pro-form activity was completely inhibited neutralising the IGF1R receptor. Finally, we evaluated the bioactivity of IGF1 pro-forms compared to the mature IGF1, demonstrating that the IGF1 pro-forms were less able to phosphorylate IGF1R in breast cancer cells.

Conclusions This study provides the evidence that IGF1 pro-forms induce breast cancer cell proliferation though with lower affinity for IGF1R. These results, together with the low level of specificity of IGF1 antibodies to the pro-forms, underline the importance of an accurate analysis of the proportion of the IGF1 pro-forms produced.

Keywords: IGF1 pro-forms · breast cancer · breast cancer cells · IGF1 receptor

1 Introduction

Insulin-like growth factor-1 (IGF1) plays an important role in tissue growth and development, and several studies have demonstrated the association between circulating levels of IGF1 and breast cancer risk [1-3]. Moreover, since the IGF1 receptor (IGF1R) is over-expressed in about 90% of breast cancer cases and IGF1R levels are higher in cancer cells than in normal breast tissue [4], targeting the IGF1 system remains an active area of clinical investigation.

IGF1 is synthesized as a precursor protein requiring proteolysis at both the N- and C- termini to produce mature IGF-1 [5, 6]. The full-length precursor of IGF1, pre-pro-IGF1, contains an N-terminal signal peptide, the 70 amino acid mature IGF1, and a C-terminal E-peptide extension [7]. The signal peptide is cleaved during translation in the endoplasmic reticulum, resulting in pro-IGF1. The E-peptide can be cleaved from pro-IGF1 by proprotein convertases like furin, resulting in mature IGF1 and E-peptide, but the uncleaved pro-IGF1 is also detectable in conditioned media or *in vivo* in serum [8-13].

The complexity of the IGF1 system is enhanced by alternative splicing of the *IGF1* gene that produces multiple isoforms of IGF1 (IGF1 pro-forms), that, while bearing the same sequence of mature IGF1, have different N- and C-terminal extensions [5]. In humans, the alternative splicing that occurs at the 3' ends of the *IGF1* gene gives rise to three possible IGF1 pro-forms with different C-terminal extensions, called Ea, Eb and Ec domains (Fig. 1A). Another level of complexity in IGF1 activity is the glycosylation of IGF1Ea pro-form, as the human Ea-peptide of IGF-I contains an N-linked glycosylation site at Asn92 [6].

Recent studies in humans have shown that the IGF1 splice variants are differentially transcribed in response to varying conditions and pathologies, such as skeletal muscle damage [14, 15], endometriosis [16] or prostate [17], cervical [18] and colorectal cancer [19]. Moreover, although it is generally assumed that IGF1 exerts its biological actions predominantly through mature peptide,

77 differential biological activities have been reported for the different IGF1 pro-forms or for their E-
78 peptides, exogenously administrated or overexpressed in various *in vitro* models [6, 14, 17, 20, 21].
79 Even though the IGF1 circulating level is affected by physical activity and diet [22], the biological
80 significance of IGF1 pro-forms is currently unknown, and the physiological and molecular
81 mechanisms that regulate their expression are unclear [6]. Moreover, despite the regenerative
82 properties of the IGF-1Ea pro-form in cardiac and skeletal muscle have been extensively
83 documented [21, 29, 30], little is known about the role of pro-forms in cancer.
84 Here we report the biological activity of IGF1 pro-forms in human breast cancer cell lines. We
85 analysed the intracellular and extracellular expression of IGF1 pro-forms in transfected HEK293 cell
86 lysates and conditioned media. We cultured the MCF7, T47D and ZR751 cells in conditioned media
87 to assess whether the IGF1 pro-forms induce cell proliferation and IGF1R phosphorylation. We
88 further evaluated the bioactivity of IGF1 pro-forms compared to the mature IGF1, in terms of cell
89 proliferation, and IGF1R, AKT and ERK1/2 phosphorylation.

2 Materials and Methods

2.1 Cell culture

MCF7, T47D, ZR751 and HEK293 cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Cell lines were cultured in DMEM (MCF7 and HEK293) or RPMI-1640 (T47D and ZR751) media supplemented with 10% fetal bovine serum, 10 mg l^{-1} insulin (MCF7 and T47D), 2 mmol l^{-1} L-glutamine, 1x MEM Non-essential Amino Acid Solution, 0.1 mg/ml streptomycin, 0.1 U l^{-1} penicillin (growth media). Cells were maintained in a humidified incubator (5 % CO_2) at 37 °C. Cells were used in a maximum range of fifteen passages.

For the experiments, after overnight incubation in growth media, breast cancer cells were starved overnight in red phenol free DMEM or RPMI without FBS, and medium was changed again with the same medium with or without hormones. All cell culture materials were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2 MTS cell proliferation assay

Triplicate samples of 5×10^3 MCF7, T47D and ZR751 cells in 96-well plates were treated for 4 days with mature IGF1 or IGF1 pro-forms. Cell viability was evaluated using CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI, USA) based on the ability of viable cells to convert a soluble tetrazolium salt (MTS) to a formazan product, as reported previously [23]. The results are expressed as the relative number of viable cells in treated samples respect to controls (untreated cells).

2.3 Plasmid constructs

Plasmid DNA constructs containing the encoding human prepro-IGF1Ea, prepro-IGF1Eb and prepro-IGF1Ec were kindly provided by Dr. Joanne Tonkin and Dr. Tommaso Nastasi, European Molecular Biology Laboratory (EMBL) Monterotondo (Rome, Italy). Each plasmid contained the encoding class 1

IGF1 48-amino acid signal peptide, mature 70-amino acid IGF1, the first 16 amino acids (aa) of the COOH-terminal peptide, and C-terminal sequences encoding either the Ea (19 aa), the Eb (61 aa) or Ec (24 aa) peptide.

2.4 Cell transfection

HEK293 cells were cultured in growth medium without antibiotics at density of $1x10^6$/well in 6 well plates. After overnight incubation cells were transfected with TransIT®-LT1 Transfection Reagent (Mirus Bio, Madison, WI, USA) according the manufacturer's instructions. Briefly, 2.5 µg of plasmid DNA was added to 250 µl of growth medium without FBS and without antibiotics, gently mixed and 7.5 µl of TransIT®-LT1 Reagent was added. After 30 min of incubation at room temperature, the mixture was added drop wise to the cell culture. After 5 hours of incubation, medium was replaced with red phenol free DMEM without FBS. After 24 hours of incubation, supernatants were collected, clarified by 1,000 rpm centrifugation for 5 min, and directly used or stored at -80° C for further experiments. Transfected HEK293 cells were lysate for western immunoblot or real-time PCR analyses. To increase IGF1 pro-forms production, furin convertase inhibitor chloromethylketone (CMK) (Enzo Life Sciences Inc, Farmingdale, NY, USA) was added at 2.5 µmol l^{-1} final concentration during transfection. To E peptides cleavage, supernatants without CMK were treated with 10 nmol l^{-1} recombinant furin (R&D Systems Ltd, Minneapolis, MN, USA) overnight at room temperature with gently shacking [6].

2.5 ELISA assay

For the quantitative determination of human IGF1 concentrations in transfected HEK293 cell culture supernatants, a commercially available ELISA kit was used following the manufacturers' instructions (Quantikine® ELISA DG100, R&D Systems). Data were acquired in duplicate using a microplate reader (Multiskan EX, Thermo Fisher Scientific, Waltham, MA, USA) at 450 nm, and the results were averaged.

142

143 2.6 Western immunoblot analysis

144 MCF7, T47D, ZR751 and HEK293 were processed for western blot analysis as previously reported
145 [24]. Briefly, cells were lysed for 20 minutes on ice with 20 mmol l^{-1} HEPES (pH 7.9), 25 % v/v
146 glycerol, 0.42 mol l^{-1} NaCl, 0.2 mmol l^{-1} EDTA, 1.5 mmol l^{-1} MgCl2, 0.5 % v/v Nonidet P-40, 1 mmol l^{-1}
147 DTT, 1 mmol l^{-1} Naf, 1 mmol l^{-1} Na_3VO_4, and 1× Complete protease inhibitor cocktail (Roche
148 Diagnostics Ltd, Mannheim, Germany). Cell lysate was frozen and thawed twice and clarified by
149 centrifugation at 12,000 rpm for 10 minutes at 4°C. Proteins from HEK293 cell supernatants were
150 concentrated using Amicon Ultra 3K centrifugal filter unit (Merck Millipore, Billerica, MA, USA). Total
151 cell lysates and concentrated supernatants were fractionated by SDS-PAGE and gels were
152 electroblotted onto a nitrocellulose membrane (0.2 µm pore size) (Bio-Rad Laboratories Inc,
153 Hercules, CA, USA). Blots were probed with the following primary antibodies: anti phospho-IGF1
154 Receptor β (3024), IGF1 Receptor β (3027), phospho-p44/42 (ERK1/2) (9101), p44/42 (ERK1/2)
155 (9102), phospho-Akt (Ser473) (9271) and Akt (9272) purchased from Cell Signalling Technology
156 (Beverly, MA, USA); anti IGF1 (I8773) purchased from Sigma-Aldrich. Bands were detected using
157 horseradish peroxidase-conjugated secondary antibody (Bio-Rad Laboratories Inc). Blots were
158 treated with enhanced chemiluminescence reagents (ECL Kit, Amersham Bioscience, Arlington
159 Heights, IL, USA), and the immunoreactive bands were detected and quantified by Chemi-Doc
160 System (Bio-Rad Laboratories Inc) equipped with the Quantity One software.

161

162 2.7 RNA extraction, cDNA synthesis and qRT-PCR

163 Total RNA was extracted and purified using the Omega Bio-Tek E.Z.N.A.™ Total RNA kit (Omega Bio-
164 Tek, Norcross, GA, USA) according to the manufacturer's instructions. After DNA digestion with
165 DNase I enzyme (Qiagen, Hilden, Germany) complementary DNA was synthesized from 1 µg of total
166 RNA using Omniscript RT (Qiagen) and random hexamers. Real time RT-PCR was performed with two
167 microliters of cDNA in a Applied Biosystems StepOnePlus™ Real Time PCR System using TaqMan®

Universal PCR Master Mix No AmpErase® UNG and using premade 6-carboxyfluorescein (FAM)-labeled TaqMan assays for human IGF1 (Hs01547656_m1) and GAPDH (Hs03929097_g1) (Applied Biosystems, Foster City, CA, USA). Gene expression data are expressed using the $2^{-\Delta CT}$ method. The real-time PCR conditions were: 95°C for 10 min followed by 40 cycles of two-steps at 95°C for 15 sec and 60°C for 1 min. The specificity of the amplification products was confirmed by examining thermal denaturation plots and by sample separation in a 4% DNA agarose gel.

2.8 Immunoprecipitation

To prepare magnetic beads for immunoprecipitation, Dynabeads® Protein G (Life Technologies, Monza, Italy) were washed twice with PBS/0.1% Tween-20 and incubated with 5 µg of IGF1 antibody (Sigma) for 1 hour at room temperature with end-over-end rotation. Bead-antibody complex was washed with PBS/0.1% Tween-20 and the IGF1 monoclonal antibody was covalently bound to the beads using BS^3 as cross-linkers according to the manufactures instructions (Thermo Scientific, Milano, Italy). Subsequently, beads were washed three times with PBS/0.1% Tween-20 to remove non-covalently bound antibodies and were incubated with 1 ml of tissue culture supernatant for 1 hour at room temperature with end-over-end rotation. The beads were washed three times with washing buffer and bound proteins were eluted by heating the beads for 10 minutes at 70°C in 20 µl of elution buffer and 10 µl of SDS-PAGE sample buffer.

2.9 IGF1 and IGF1R neutralisation

To neutralise IGF1 activity, culture medium containing IGF1 or IGF1 pro-forms was incubated with 3 µg/ml of anti-IGF1 (Sigma) for 1 hour at 37°C. MCF7 and ZR751 cells were then cultured in IGF1-neutralised media for the evaluation of cell proliferation and IGF1R phosphorylation. To neutralise IGF1R, cells were pre-incubated with 5 µg/ml of anti-IGF1R (R&D System) for 1 hour at 37°C and treated with IGF1 or IGF1 pro-forms for the evaluation of cell proliferation and IGF1R phosphorylation.

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195 2.10 Statistical analysis

196 Statistical analysis was performed using one-way or two-way ANOVA as appropriated followed by

197 Bonferroni's multiple comparison post hoc tests (GraphPad Software, Inc., La Jolla, CA, USA).

3 Results

3.1 Analysis of IGF1 pool in HEK293 transfected cells

IGF1 pro-forms were obtained by transiently transfection of HEK293 cells with specific constructs for each pro-form. Transfected cells lysates and supernatants were analysed by western immunoblotting using an IGF1 antibody directed against the mature region of IGF1. The amount of IGF1 in the supernatants were quantified by ELISA, concentrated with filter columns, and 50 ng of IGF1 were loaded in the gel. Our results showed that IGF1 pro-forms are the only forms produced intracellularly by transfected HEK293 cells, whereas both mature IGF1 and IGF1 pro-forms were detected extracellularly (Fig. 1b). Notably, both the glycosylated and non-glycosilated IGF1Ea were detected in the cell lysate, while only the glycosylated IGF1Ea pro-form (gly-IGF1Ea) was secreted. Moreover, IGF1Eb and IGF1Ec showed additional bands at higher molecular weight, suggesting that also these pro-forms are subjected to extensive post-translational modifications.

3.2 E peptides impair the accurate quantification of the IGF1 pool

As previously described, the quantity of gly-IGF1Ea pro-form is partially underestimated in non-denaturating conditions such as ELISA assays, suggesting that the E peptide could impair the affinity between IGF1 and IGF1 antibodies [13]. In order to check if ELISA assay gives an accurate measure of the IGF pool, HEK293 cells were transfected with IGF1Ea, IGF1Eb and IGF1Ec expression vectors with or without the furin inhibitor CMK. The same efficiency of IGF1 mRNA expression was obtained in CMK-treated and untreated cells (Supplementary Fig. S1). The supernatants were analysed in both ELISA and western immunoblot after filter column concentration for the IGF1 quantification. As shown in Fig. 2a, western blot analysis of conditioned media from HEK293 treated or untreated with CMK did not show a variation in total IGF1 pool (mature IGF1 and pro-forms). The ELISA quantification of gly-IGF1Ea enriched media did not differ after CMK treatment, whereas there was a significant reduction of IGF1, after CMK treatment, in IGF1Eb and IGF1Ec enriched media (Fig. 2b).

Therefore, the E peptides in the IGF1 pro-forms could impair the affinity to the anti-IGF1 antibody under non-denaturating conditions and hence the accuracy of the ELISA result.

To further confirm this hypothesis, conditioned media obtained from HEK293 transfected with IGF1 pro-forms were immunoprecipitated with Dynabeads coupled with an anti-IGF1 antibody. The proteins bound to the bead-antibody complex were subsequently recovered and analysed by western blotting. As shown in Fig. 2c, the gly-IGF1Ea pro-form was, at least partially, recognized by the anti-IGF1 antibody, whereas there was only a weak immunoprecipitation of IGF1Eb and IGF1Ec pro-forms. These results confirm that E peptides, in the IGF1 pro-forms, impair the accurate measurement of IGF1 pool.

3.3 Biological activity of the IGF1Ea, IGF1Eb and IGF1Ec enriched media

The activity of each IGF1 pool in MCF7 and ZR751 human breast cancer cells has been evaluated in term of cell proliferation and IGF1R phosphorylation.

Cell proliferation was evaluated using the MTS cell proliferation assay, an indirect assay that evaluates cell metabolic activity. MCF7 cells were firstly grown with increasing concentration of mature IGF1, and after 4 days of culture, cell proliferation was evaluated with both MTS assay and cell count. As shown in supplementary Fig. 2S, both methods give similar results.

Cells were then cultured in IGF1 pro-form-enriched media, previously normalised to 10 ng/ml using an ELISA kit. Importantly, at this point we were unable to accurately quantify the total IGF1 pool (mature IGF1 and pro-forms) in IGF1 pro-form-enriched media using ELISA assay and therefore, were unable to directly compare the effects between each IGF1 pool.

The IGF1 pro-form-enriched media significantly induced both MCF7 and ZR751 cell proliferation compared to the control (unstimulated cells) (Fig. 3a and b). It was not possible to evaluate the proliferation in T47D cells because of their poor growth in serum free medium (not shown). Supernatants of HEK293 cells not-transfected or transfected with empty vector did not induce cell proliferation (not shown). The IGF1 antibody was then used to neutralize the activity of mature IGF1.

As shown in Fig. 3a and b, the IGF1 antibody completely inhibited the IGF1-induced cell proliferation, whereas the same antibody only partially inhibited MCF7 (Fig. 3a) and ZR751 (Fig. 3b) cell proliferation induced by HEK293 supernatants containing the IGF1 pro-forms. Moreover, the IGF1 antibody markedly inhibited the IGF1R phosphorylation induced by mature IGF1 but not the phosphorylation induced by IGF1 pro-forms (Fig. 3c and d). These results suggest that the IGF1 pro-forms induced breast cancer cell proliferation and IGF1R phosphorylation.

The activity of IGF1 pro-forms is IGF1R dependent. In fact, by inhibiting IGF1R activation with an IGF1R antibody, neither cell proliferation nor IGF1R phosphorylation induced by mature IGF1 or IGF1 pro-forms were detected (Fig. 3).

3.4 Biological activity of mature IGF1 vs. IGF1 pro-forms

To evaluate IGF1 pro-form activity compared to mature IGF1, supernatants containing different ratios of mature IGF1 and pro-forms were produced. Recombinant furine was used to induce E peptide cleavage and increase the amount of mature IGF1. Furin convertase inhibitor CMK was used to inhibit E peptide cleavage and increase IGF1 pro-form amounts during transfection. Supernatants were concentrated with filter columns and analysed in western immunoblot using an IGF1 antibody. Our results showed that CMK markedly increased the IGF1 pro-form amount, while in supernatants treated with furine, the IGF1 pro-forms were not detectable (Fig. 4a, 4b and 4c).

MCF7 cells were then cultured in two-fold diluted conditioned media (from 1:4 to 1:32) containing different ratios of mature IGF1 and pro-forms, and cell proliferation and phosphorylation of IGF1R, AKT and ERK1/2 were evaluated at the indicated times. No significant differences in MCF7 cell proliferation were detected (supplementary Fig. S3a-b-c). On the other hand, by increasing the amount of gly-IGF1Ea pro-form in cell culture, the IGF1R phosphorylation was found to be markedly reduced (Fig. 5a), suggesting a minor affinity of gly-IGF1Ea for IGF1R. While increasing the amount of gly-IGF1Ea also reduced AKT phosphorylation in MCF7 cells, it did not affect ERK1/2 phosphorylation (Fig. 5a).

Lower levels of IGF1R phosphorylation were also observed in MCF7, cultured with higher amounts of IGF1Ec and partially with IGF1Eb, while no differences of AKT and ERK1/2 phosphorylation were observed (Fig. 5b and 5c).

The activity of IGF1 pro-forms compared to mature IGF1 was also evaluated in T47D and ZR751 breast cancer cell lines (Fig. 6). Confirming the results obtained in MCF7 cells, we found that the glycosylated IGF1Ea and the IGF1Ec pro-forms were less able to phosphorylate IGF1R in both T47D (Fig. 6a) and ZR751 (Fig. 6b) cells.

Furin and CMK did not alter the phosphorylation of IGF1R, AKT and ERK1/2 induced by mature IGF1 (supplementary Fig. S3d).

5 Discussion

The IGF pathway has a well-documented role in the development and/or progression of breast carcinomas [2]. The complexity of *IGF1* gene splicing and post-translational modifications gives rise to a different precursor of IGF1 polypeptides, namely the IGF1Ea, IGF1Eb and IGF1Ec pro-forms in humans, that share mature peptide, but differ by the structure of their extension peptides, or E-peptides, on the C-terminus [5, 6].

The IGF1 pro-forms also undergo posttranslational modifications, such as glycosylation and proteolytic processing by proprotein convertases such as furin [6]. Convertase-mediated cleavage generally occurs intracellularly [25], but it has also been reported that there are potential proprotein convertases that could process pro-IGF1 extracellularly, resulting in secretion of unprocessed IGF1 pro-forms [10, 13]. Our data confirm this evidence, revealing that the IGF1 pro-forms are the predominant forms inside the transfected HEK293 cells, and they are also abundantly secreted in cell culture media. Our results also show that the non-glycosylated IGF1Ea is detectable in the cell lysate only, whereas only the glycosylated form was secreted. The Ea-peptide of human IGF1 is the unique E peptide that contains an N-linked glycosylation site, and it has been hypothesized that glycosylation may play a role in IGF1 biological activity modulation, such as bioavailability [26]. Interestingly, our data revealed that both IGF1Eb and IGF1Ec are subject to posttranslational modification, which still need to be completely identified.

As reported by Durzyńska *et al.* [13], the ELISA measurements are more sensitive to mature IGF1 than the pro-forms, suggesting that the presence of the E-peptide impaired the ability of the IGF1 antibody to recognize the native protein. Our results support this hypothesis, showing that the IGF1 antibody has higher affinity for the mature IGF1 with respect to the IGF1 pro-forms, especially for IGF1Eb and IGF1Ec. Moreover, according to the literature [13], the ELISA quantification appears impaired in supernatants with a large amount of pro-forms obtained with CMK during transfections, even though immunoblotting did not show a decrease in total IGF1. Thus, it is difficult to compare

311 the bioactivity of the different pro-forms, as the E-peptide in pro-IGF1 impaired the ability to
312 accurately measure and subsequently normalise the IGF1 content under non-denaturing conditions.
313 Since it was unclear whether pro-IGF1 is bioactive or simply an inactive precursor or source for
314 mature IGF1 [7], we cultured MCF7 and ZR751 cells in IGF1-neutralised conditioned media. As
315 expected, the IGF1 antibody is able to completely neutralise the activity of mature IGF1 in terms of
316 cell proliferation and IGF1R phosphorylation. On the contrary, the IGF1 antibody is ineffective in the
317 inhibition of cell proliferation and IGF1R phosphorylation of cells cultured in conditioned media
318 containing the IGF1 pro-forms. These results suggest that the IGF1 pro-forms are able to induce
319 breast cancer cell proliferation. *In vitro* studies have suggested that the E-peptides of the human
320 IGF1 precursors may act as independent growth factors inducing mitosis, independently from IGF1R
321 [6]. In contrast, our results show that by neutralising IGF1R, the induction of cell proliferation
322 induced by mature IGF1 or pro-forms was completely inhibited, suggesting that IGF1 pro-hormones
323 induce cell proliferation *via* IGF1R activation.
324 Despite the large amount of evidence regarding the biological activity of E peptides, little is known
325 about IGF1 pro-form bioactivity [6, 13, 21, 27]. In this study, the biological activity of the IGF1 pro-
326 forms compared to mature IGF1 has been evaluated. We generated a set of conditioned media
327 containing different ratios of mature IGF1 and pro-forms using proprotein convertase furin to induce
328 pro-form cleavage and increase mature IGF1 amounts, and using convertase inhibitor CMK during
329 transfection to inhibit pro-form cleavage and increase IGF1 pro-form amounts. Results show that
330 culturing MCF7, T47D and ZR751 cells with increasing amounts of IGF1 pro-forms, the
331 phosphorylation of IGF1R markedly decreased. This correlates with recent evidence showing that
332 glycosylated pro-IGF1Ea is less efficient at receptor activation than pro-IGF1 and mature IGF1 [13].
333 Despite the fact that pro-forms decreased the activation of IGF1R, no significant differences were
334 revealed in cell proliferation and ERK1/2 phosphorylation compared to the mature IGF1.
335 Interestingly, the phosphorylation of AKT in MCF7 cells seems to be affected by gly-IGF1Ea. It has
336 been suggested that IGF1Ea could activate alternative IGF1R downstream pathways [6], as the

canonical PI3K/AKT/mTOR signaling pathway was not induced in the transgenic mice overexpressing IGF1Ea [28, 29]. However, the effect of gly-IGF1Ea on AKT phosphorylation in breast cancer cells need to be confirmed.

In conclusion, our results provide new evidence that IGF1 pro-forms induce breast cancer cell proliferation *via* IGF1R phosphorylation. There are data supporting the role of IGF1 pro-forms in cancer, such as prostate [17], cervical [18] and colorectal cancer [19]; however, the biological activity of IGF1 variants in breast cancer pathogenesis has not been established, and no analytical methods are available to detect and quantify IGF1 pro-forms. In fact, the available methods imply the use of antibodies, which recognise the mature IGF1 peptide, underestimating the pro-forms. The lower level of specificity of IGF1 antibodies to the pro-forms could also have implications in breast cancer therapy because a targeted strategy includes the use of the IGF1 antibody to neutralise the IGF system [31].

The lower affinity of pro-IGF1s for IGF1R, together with the poor prognosis associated with higher IGF1R expression, make the search for regulatory mechanism(s) and potentially specific bioactivity of the various IGF1 peptides an area of particular interest, and further studies will focus on the identification of the pro-IGF1s as candidates for positive prognostic factors.

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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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Figure legends

Fig. 1

Human IGF1 pro-forms. (**a**) Schematic presentation of mature IGF1 and IGF1 pro-forms. (**b**) Western blot analysis of HEK293 cells transfected with specific constructs. Cell lysates and supernatants (SN) were analysed after 24 hours post-transfection using an anti-IGF1 antibody. Images are representative of three replicates giving similar results.

Fig. 2

Mature IGF1 and IGF1 pro-forms affinity with anti-IGF1 antibody. Quantification of mature IGF1 and IGF1 pro-forms from a representative set (n = 3) of transfected HEK293 cells using (**a**) western blot analysis and (**b**) ELISA. Furin convertase inhibitor chloromethylketone (CMK) was used to increase IGF1 pro-form production. **c** Representative western blot of immunoprecipitation of mature IGF1 and HEK293 supernatants containing IGF1 pro-forms using Dynabeads-anti-IGF1 complex. Arrows indicate IGF1 pro-forms.

Fig. 3

IGF1 pro-forms induced cell proliferation *via* IGF1R. (**a**) MCF7 and (**b**) ZR751 cell proliferation cultured 4 days with mature IGF1 (10 ng/ml) or HEK293 supernatants containing IGF1 pro-forms (means ± SEM; n=3). Cell proliferation was evaluated by MTS assay. Data are expressed as relative proliferation *vs*. unstimulated cells. *** Significantly different, P<0.001; ns: not significantly different; 1-way ANOVA followed by Bonferroni's multiple comparison test. Representative western blot (n = 3) of phospho-IGF-R levels in (**c**) MCF7 and (**d**) ZR751 cells stimulated for 10 minutes with mature IGF1 or HEK293 supernatants containing IGF1 pro-forms. IGF1R was used as a loading control. Densitometry values for specific proteins presented relative to unstimulated cells (set as

491 onefold), are included below the lanes. An IGF1 antibody was used to neutralise IGF1 biological
492 activity. An IGF1R antibody was used to inhibit IGF1R phosphorylation/activation.
493
494 **Fig. 4**
495 IGF1 pro-forms production and E peptides cleavage. Representative western blot on supernatants of
496 HEK293 cells transfected with specific constructs for (**a**) IGF1Ea, (**b**) IGF1Eb and (**c**) IGF1Ec pro-forms.
497 Mature IGF1 and IGF1 pro-forms were detected using an IGF1 antibody. Furin convertase inhibitor
498 CMK was used to increase IGF1 pro-form production. Recombinant furin was used to E peptides
499 cleavage.
500
501 **Fig. 5**
502 Phosphorylation of IGF1R, AKT and ERK1/2 in MCF7 cells. Representative western blot (*n* = 3) of
503 phospho-IGFR, phospho-AKT and phospho-ERK1/2 levels in MCF7 cells stimulated for the indicated
504 times with HEK293 supernatants containing mature IGF1 and (**a**) IGF1Ea, (**b**) IGF1Eb and (**c**) IGF1Ec
505 pro-forms. IGF1R, AKT and ERK1/2 were used as loading controls. Densitometry values for specific
506 proteins presented relative to unstimulated cells (set as onefold), are included below the lanes.
507
508 **Fig. 6**
509 Phosphorylation of IGF1R, AKT and ERK1/2 in (**a**) T47D and (**b**) ZR751 cells. Representative western
510 blot (*n* = 3) of phospho-IGF1R, phospho-AKT and phospho-ERK1/2 levels in cells stimulated for 60
511 minutes with HEK293 supernatants containing mature IGF1 and IGF1Ea, IGF1Eb and IGF1Ec pro-
512 forms. IGF1R, AKT and ERK1/2 were used as loading controls. Densitometry values for specific
513 proteins presented relative to unstimulated cells (set as onefold), are included below the lanes.
514
515 **Fig. 7**

Schematic presentation of IGF1 pro-form biological activity in breast cancer cells. (a) IGF1 induces IGF1R phosphorylation that is completely inhibited neutralizing IGF1 or IGF1R. (b) E peptide decreases the IGF1R phosphorylation induced by IGF1. The IGF1 neutralisation is ineffective to inhibit IGF1R phosphorylation that is completely inhibited by IGF1R neutralization.

Or…

Schematic presentation of IGF1 pro-form biological activity in breast cancer cells. (a) IGF1R phosphorylation induced by mature IGF1, with or without the IGF1 or IGF1R neutralization. (b) IGF1R phosphorylation induced by IGF1 pro-forms, with or without the IGF1 or IGF1R neutralization.

figure 1

Click here to download line figure: De Santi_FIG.1.pdf **Fig. 1**

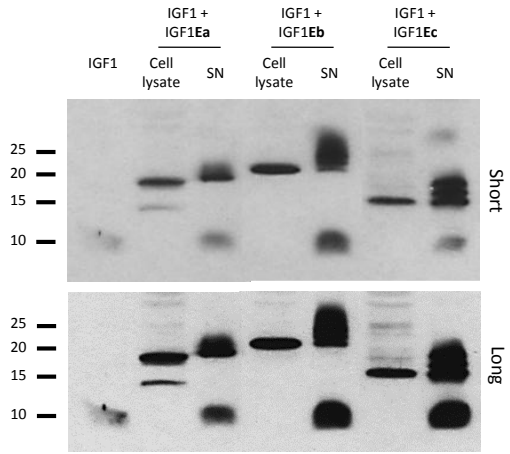


figure 2

Click here to download line figure: De Santi_FIG.2.pdf **Fig. 2**

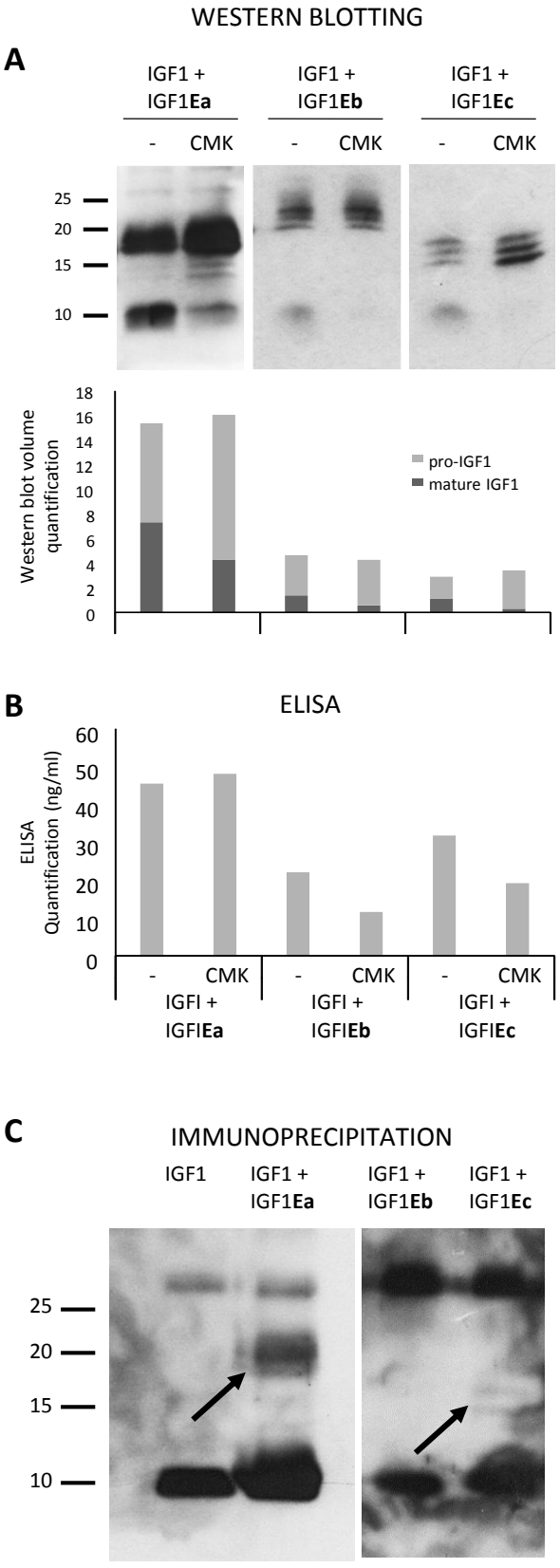


figure 3

Click here to download line figure: De Santi_FIG.3.pdf **Fig. 3**

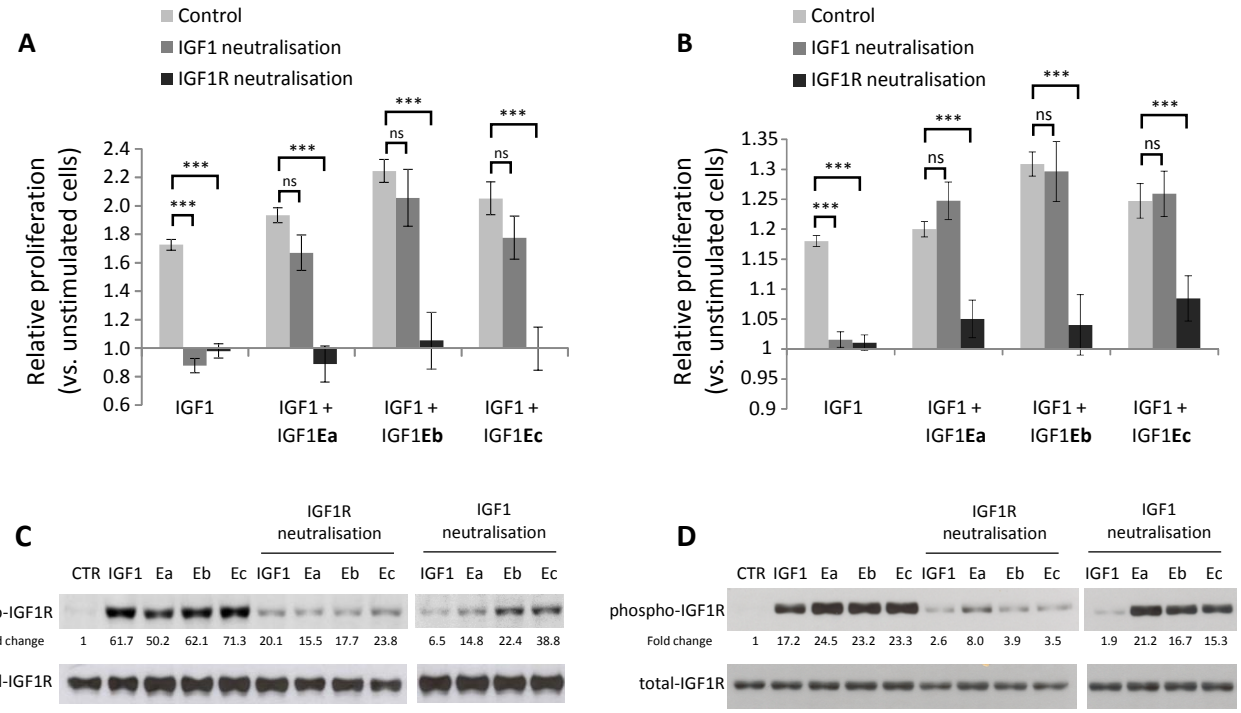


figure 4

Click here to download line figure: De Santi_FIG.4.pdf **Fig. 4**

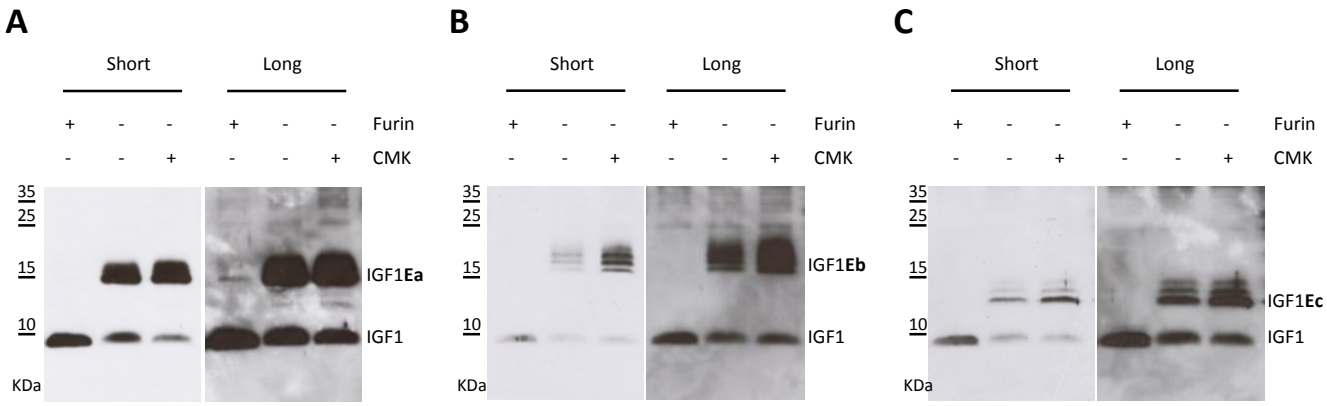


figure 5

Click here to download line figure: De Santi_FIG.5.pdf **Fig. 5**

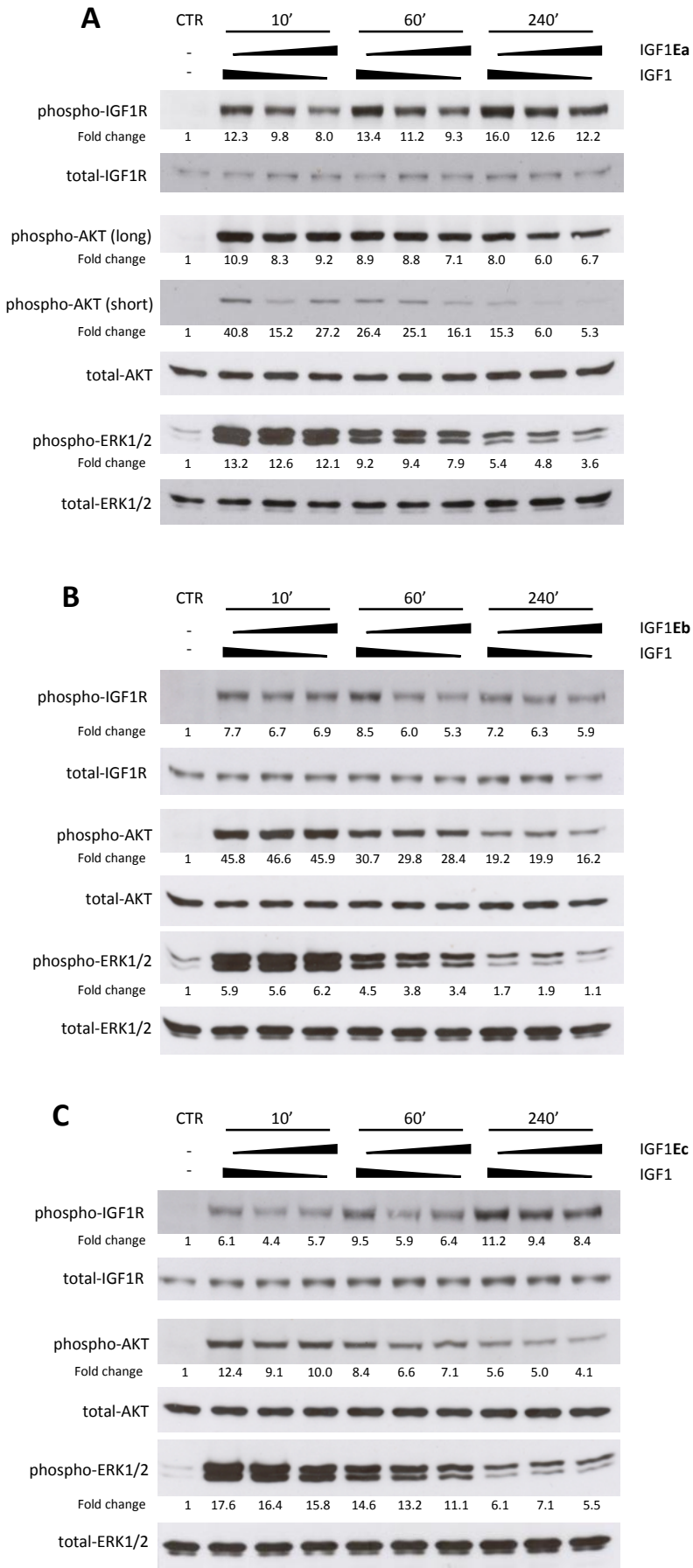
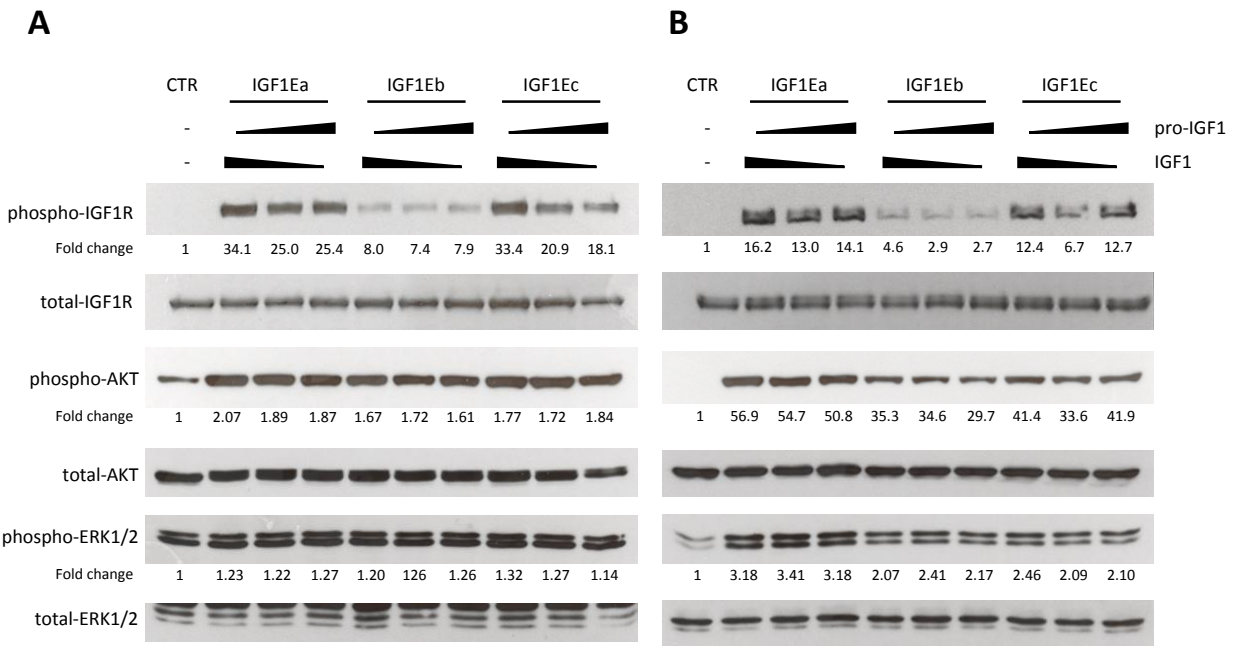
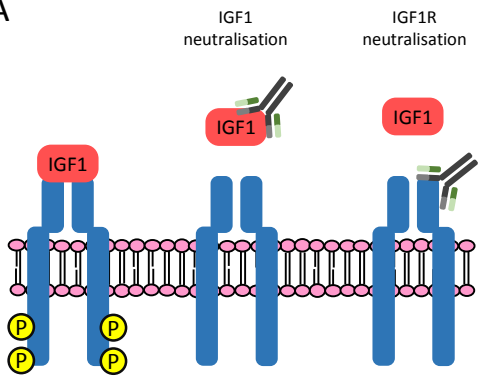


figure 6

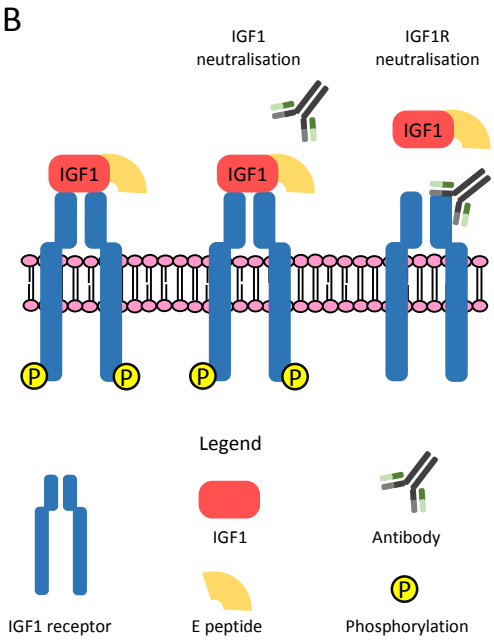
Click here to download line figure: De Santi_FIG.6.pdf **Fig. 6**



A


IGF1
neutralisation
IGF1R
neutralisation
IGF1
IGF1
IGF1
P
P
P
P

B


IGF1
neutralisation
IGF1R
neutralisation
IGF1
IGF1
IGF1
P
P
P
P
Legend
IGF1 receptor
IGF1
Antibody
E peptide
P
Phosphorylation