



Decorin differentially modulates the activity of insulin receptor isoform A ligands



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ABSTRACT

The proteoglycan decorin, a key component of the tumor stroma, regulates the action of several tyrosine-kinase receptors, including the EGFR, Met and the IGF-IR. Notably, the action of decorin in regulating the IGF-I system differs between normal and transformed cells. In normal cells, decorin binds with high affinity to both the natural ligand IGF-I and the IGF-I receptor (IGF-IR) and positively regulates IGF-IR activation and downstream signaling. In contrast, in transformed cells, decorin negatively regulates ligand-induced IGF-IR activation, downstream signaling and IGF-IR-dependent biological responses. Whether decorin may bind another member of the IGF-I system, the insulin receptor A isoform (IR-A) and its cognate ligands, insulin, IGF-II and proinsulin, have not been established. Here we show that decorin bound with high affinity insulin and IGF-II and, to a lesser extent, proinsulin and IR-A. We utilized a cell model system mouse embryonic fibroblasts homozygous for a targeted disruption of the *Igf1r* gene (designated R⁻ cells) which were stably transfected with a human construct harboring the IR-A isoform of the receptor. Using these R⁻/IR-A cells, we demonstrate that decorin did not affect ligand-induced phosphorylation of the IR-A but enhanced IR-A downregulation after prolonged IGF-II stimulation without affecting insulin and proinsulin-dependent effects on IR-A stability. In addition, decorin significantly inhibited IGF-II-mediated activation of the Akt pathways, without affecting insulin and proinsulin-dependent signaling. Notably, decorin significantly inhibited IGF-II-mediated cell proliferation of R⁻/IR-A cells but affected neither insulin- nor proinsulin-dependent mitogenesis. Collectively, these results suggest that decorin differentially regulates the action of IR-A ligands. Decorin preferentially inhibits IGF-II-mediated biological responses but does not affect insulin- or proinsulin-dependent signaling. Thus, decorin loss may contribute to tumor initiation and progression in malignant neoplasms which depend on an IGF-II/IR-A autocrine loop.

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1. Introduction

Decorin, the prototype member of the small leucine-rich proteoglycans (SLRPs), modulates the biology of various cancer types by downregulating the activity of several tyrosine-kinase receptors important

for cell growth, motility and survival (Iozzo, 1997; Schaefer and Iozzo, 2008). Decorin interacts with the epidermal growth factor receptor (EGFR) and the Met receptor and downregulates their activity and signaling (Iozzo et al., 1999; Csordas et al., 2000; Santra et al., 2000, 2002; Goldoni et al., 2009; Buraschi et al., 2012). In addition, decorin modulates the insulin-like growth factor (IGF-I) system by binding with high affinity both IGF-I and the insulin-like growth factor receptor 1 (IGF-IR) (Morrione et al., 2013). Notably, a recent *in situ* hybridization study encompassing a large cohort of human urothelial carcinomas has shown that decorin expression is totally absent in non-invasive and invasive bladder carcinomas (Sainio et al., 2013), suggesting that decorin loss might favor the malignant behavior of bladder cancer cells. Moreover, decorin has been implicated in a variety of pathologies including tendon, muscle, bone, cornea and various connective tissues where abnormal signaling and cell/matrix interactions may play an active pathogenetic role (Jarvelainen et al., 2006; Nikitovic et al., 2012; Seidler, 2012; Brandan and Gutierrez, 2013a,b; Chen et al., 2013; Dunkman et al., 2013). Recent evidence indicates that decorin antagonizes the vascular

Abbreviations: SLRP, small leucine-rich proteoglycan; RTK, receptor tyrosine-kinase; R⁻, *Igf1r*^{-/-} cells; IR-A, insulin receptor isoform A; IGF-IR, insulin-like growth factor receptor 1; IGF-II, insulin-like growth factor II; MAPK, mitogen-activated protein kinase; IGF-BP, IGF-binding protein; IRS-1, insulin receptor substrate 1.

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endothelial cell growth factor receptor 2 (VEGFR2) and suppresses angiogenesis (Neill et al., 2012a, 2013a) via induction of endothelial cell autophagy (Buraschi et al., 2013; Neill et al., 2013b).

The type I IGF receptor (IGF-IR) binds with high affinity both insulin-like growth factors I and II (IGF-I and IGF-II) and has a crucial role in the regulation of mammalian growth both *in vitro* (Scher et al., 1979; Stiles et al., 1979) and *in vivo* (Baker et al., 1993; Liu et al., 1993; Eggenschwiler et al., 1997). The IGF-IR and its ligands are frequently deregulated in cancer and may have an important role not only in the early phases of carcinogenesis but also in cancer progression and resistance to a variety of therapies (Baserga, 1995; Baserga et al., 1997; Baserga, 2000; LeRoith and Roberts, 2003). IGF-II, and to a lesser extent IGF-I, binds to a second receptor tyrosine kinase (RTK), the isoform A of the insulin receptor (IR-A), which is highly homologous to the IGF-IR (Krywicky and Yee, 1992; Frasca et al., 1999). The IR-A is considered the fetal form of the IR and primarily mediates mitogenic effects upon IGF-II or insulin binding (Morrione et al., 1997b; Frasca et al., 1999; Pandini et al., 2002), and is also implicated in cancer (Belfiore, 2007; Belfiore et al., 2009). Proinsulin has been recently identified as another IR-A ligand and despite its lower affinity for the IR-A compared to insulin (similar to IGF-II), promotes IR-A phosphorylation and activation of downstream signaling (Malaguamera et al., 2012).

The second IR isoform (IR-B) is involved in glucose metabolism of insulin-responsive organs (Frasca et al., 1999; Belfiore, 2007). Predominant expression of the IR-A over the IR-B has been detected in several cancer models and an autocrine proliferative loop between IGF-II and the IR-A has been demonstrated in malignant thyrocytes and breast cancer cells (Sciacca et al., 1999; Kalli et al., 2002; Sciacca et al., 2002; Vella et al., 2002).

Decorin regulates the IGF-I system at various levels but there is a surprising dichotomy in the mechanisms of decorin regulation of IGF-IR signaling, which differ between physiological and pathological cellular models (Morrione et al., 2013). In normal endothelial cells, decorin induces IGF-IR phosphorylation and IGF-IR-dependent Akt activation but it also modulates subsequent receptor downregulation (Schonherr et al., 2005). In addition, decorin induces IGF-IR-dependent endothelial cell adhesion and migration on collagen (Fiedler et al., 2008). In renal fibroblasts decorin regulates fibrillin-1 synthesis through an IGF-IR/mTOR/p70S6K signaling cascade (Schaefer et al., 2007). In extravillous trophoblasts, instead, decorin negatively regulates migration by promoting IGF-IR phosphorylation and activation in a dose-dependent manner but the anti-proliferative effect of decorin is IGF-IR-independent (Iacob et al., 2008).

In contrast, in urothelial cancer-derived cells decorin severely inhibits ligand-dependent IGF-IR activation and downstream activation of the Akt and MAPK pathways (Iozzo et al., 2011). In addition, prolonged exposure to decorin did not affect the stability of the IGF-IR in urothelial cancer cells either alone or in the presence of IGF-I but enhances degradation of IRS-1, one of the major downstream effectors of the IGF-IR signaling pathway (Sun et al., 1993; Rose et al., 1994). The effects on IGF-IR signaling led to decorin-evoked inhibition of IGF-I-mediated migration and invasion of urothelial cancer cells (Iozzo et al., 2011). Furthermore, decorin expression inversely correlated with IGF-IR expression in low- and high-grade bladder cancers suggesting that loss of decorin may contribute to bladder cancer initiation and progression of IGF-IR-dependent tumors (Iozzo et al., 2011).

In spite of increasing evidences for the role of the IR-A in cancer (Belfiore, 2007; Belfiore et al., 2009), it has not been established as of yet whether decorin directly binds IR-A ligands and regulate IR-A signaling. In this paper, using mouse embryo fibroblasts cells lacking the *Igf1r* (Sell et al., 1994) and expressing solely the human IR-A ($R^-/IR-A$) (Miura et al., 1995; Morrione et al., 1997b; Morcavallo et al., 2012), we show that decorin binds IGF-II and insulin with high affinity and proinsulin and IR-A with a 3-fold lower affinity. Although decorin did not affect ligand-induced IR-A phosphorylation, it attenuated IGF-II-induced Akt activation, enhanced IR-A downregulation after

prolonged IGF-II stimulation and significantly reduced IGF-II-induced cell proliferation. Notably, decorin did not affect insulin or proinsulin-mediated signaling and biological responses downstream of the IR-A. These results indicate that decorin differentially regulates IR-A ligands and provide a plausible mechanism whereby decorin loss may contribute to tumor formation in cancer systems addicted to an IGF-II/IR-A autocrine loop.

2. Results

2.1. Decorin binds the IR-A and its natural ligands, IGF-II, insulin and proinsulin

Decorin binds the IGF-IR and its cognate ligand IGF-I and regulates IGF-IR action in both physiological and pathological cell models (Schonherr et al., 2005; Morrione et al., 2013). However, whether decorin may bind the IR-A, another receptor member of the IGF-I system, and regulate its action has not yet been established. Thus, we first tested whether decorin core could bind either the IR-A or its ligands IGF-II, insulin and proinsulin in a cell-free system. Decorin protein core bound with high affinity ($K_d = 7.8 \text{ nM} \pm 1.9$) to the IR-A as determined by ELISA assays using recombinant extracellular domain of human IR-A as the immobilized substrate (Fig. 1A). The decorin affinity for the IR-A was about 3-fold lower than the one we previously demonstrated for the IGF-IR using the same experimental approach (Iozzo et al., 2011). Decorin protein core also bound to immobilized IGF-II (Fig. 1B) and insulin (Fig. 1C) with very similar affinities ($K_d = 1.5 \text{ nM} \pm 0.2$ for IGF-II; $K_d = 3.1 \text{ nM} \pm 0.5$ for insulin) while the affinity for proinsulin (Fig. 1D) was lower ($K_d = 9.3 \text{ nM} \pm 1.8$) and more similar to the decorin affinity for the IR-A. As previously shown for IGF-I (Iozzo et al., 2011), decorin proteoglycan bound insulin with high affinity ($K_d 13.36 \text{ nM}$) even though the affinity was about 4-fold lower than decorin protein core (Data not shown).

These results indicate that decorin protein core binds IR-A and its ligands and may therefore regulate ligand-induced IR-A activity.

2.2. Decorin does not affect ligand-mediated IR-A phosphorylation

Given the concurrent binding of decorin core to both ligands and receptor in a cell-free system, we investigated whether decorin could play a role in regulating ligand-induced IR-A activation/phosphorylation. To bypass the possibility that decorin, given its high affinity binding to the IGF-IR (Iozzo et al., 2011), could interfere with its action on the IR-A, we used the unique model of $R^-/IR-A$ cells, which are mouse embryo fibroblasts cells lacking the *Igf1r* (Sell et al., 1994) and expressing solely the human IR-A ($R^-/IR-A$) (Miura et al., 1995; Morrione et al., 1997b; Morcavallo et al., 2012). In addition, this well-defined genetic model allowed us to establish decorin modulation of IGF-II signaling exclusively through the IR-A. Thus, $R^-/IR-A$ cells were first preincubated for 1 h with decorin core (200 nM) and then stimulated for 10 min with physiological concentrations of IGF-II, insulin or proinsulin (10 nM each) (Fig. 2A). In addition, because decorin binds IGF-II, insulin and proinsulin, we performed the same experiments after pre-incubating all ligands with 200 nM decorin core for 1 h and then exposing the cells to the mixture of ligands for 10 min (Fig. 2B). In both experimental conditions, decorin protein core did not affect IR-A phosphorylation at Tyr1150/1151 induced by IGF-II or insulin, but slightly enhanced IR-A phosphorylation mediated by proinsulin (Fig. 2A and B). Decorin core alone had no effect on IR-A activation in both experimental approaches (Fig. 2A and B).

These results suggest that decorin action on IR-A activation substantially differs from its activity on the IGF-IR, where decorin regulates IGF-IR phosphorylation either positively or negatively in non-transformed and transformed cellular models, respectively (Morrione et al., 2013).

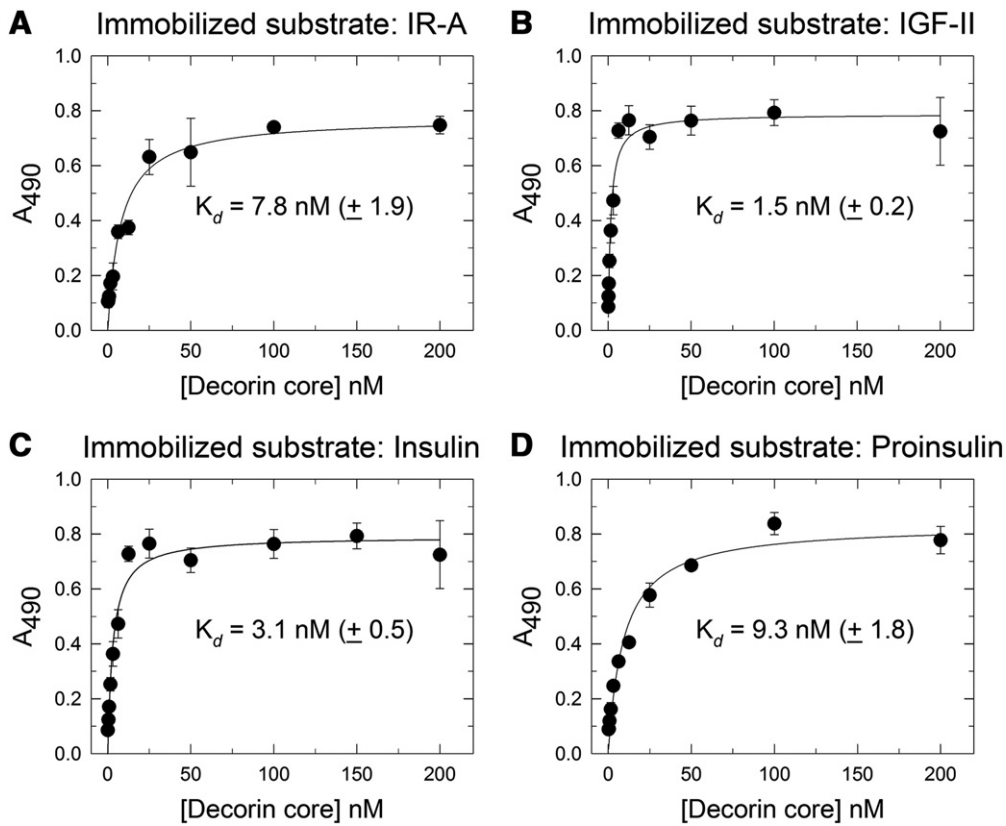


Fig. 1. Decorin protein core binds to the IR-A, IGF-II, insulin and proinsulin. [A–D] soluble decorin protein core binds to immobilized human recombinant IR-A and IR-A ligands, IGF-II, insulin and proinsulin in a saturable fashion. Solid phase ELISA assays were performed as described in details in Experimental Procedures. Values represent the mean \pm SEM of three independent experiments run in triplicates.

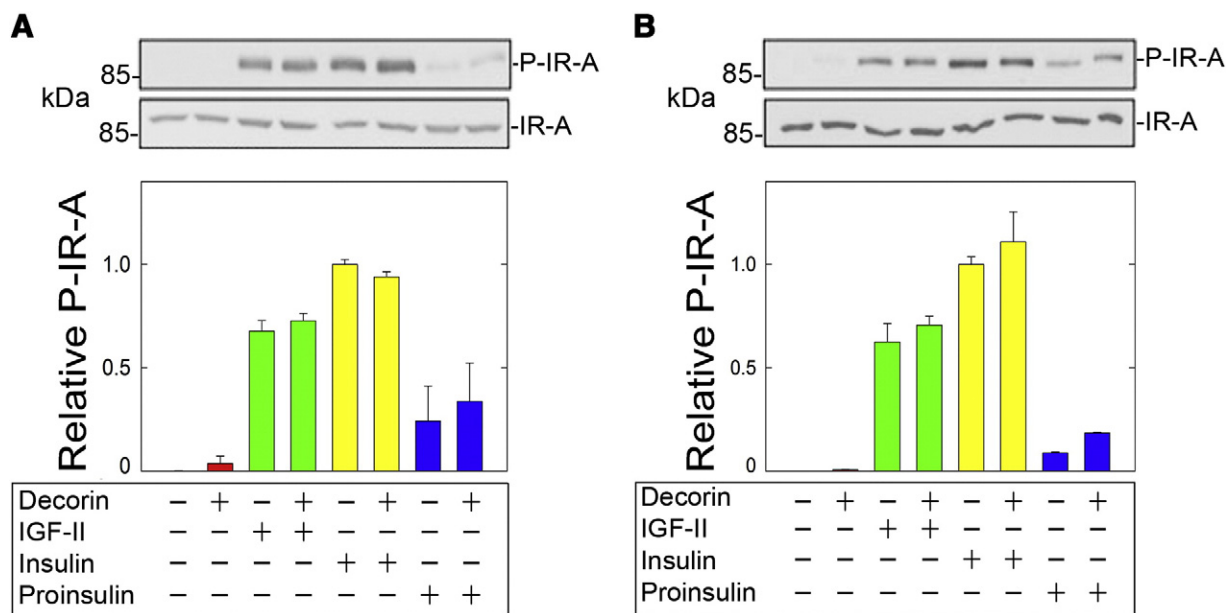


Fig. 2. Decorin does not affect IR-A phosphorylation. [A] Serum-starved R^- /IR-A cells were preincubated for 1 h with decorin (200 nM) and then stimulated with decorin alone (200 nM), IGF-II, insulin or proinsulin (10 nM) or decorin supplemented with growth factors for 10 min. Phosphorylation of the IR-A was determined by immunoblot using anti-phospho-IGF-IR β (Tyr1135/1136)/IR β Tyr1150/1151 rabbit monoclonal antibodies (Cell signaling Technology). Total IR-A levels were detected using anti-IR polyclonal antibodies, which recognize the β subunit of the IR-A. [B] To test the effect of decorin core protein on ligands, IGF-II, insulin and proinsulin were preincubated with 200 nM decorin core and then supplemented to R^- /IR-A cells. IR-A phosphorylation and total levels were determined as above. Blots are representative of three independent experiments. [C, D] Densitometric analysis was performed using the ImageJ program (rsbweb.nih.gov/ij/) and the values are reported as relative densitometric arbitrary units. Values represent the mean \pm SD of three independent experiments run in duplicates.

2.3. Decorin preferentially affects IGF-II-modulated IR-A stability and signaling

We have previously characterized IR-A endocytosis and trafficking and demonstrated that chronic insulin stimulation of R⁻/IR-A cells induces IR-A downregulation, which is instead only modestly affected after IGF-II or proinsulin stimulation (Malaguarnera et al., 2012; Morcavallo et al., 2012). To further investigate decorin's mechanism of action, we tested by immunoblotting IR-A levels and determined whether decorin core could affect IR-A stability after prolonged stimulation with IGF-II, insulin or proinsulin. In agreement with our previous results, IR-A levels in R⁻/IR-A cells were considerably decreased after a 24-h stimulation with insulin (10 nM) but only modestly affected after incubation with equimolar concentrations of either IGF-II or proinsulin (Fig. 3A, B). Significantly, decorin core protein enhanced IR-A downregulation after prolonged IGF-II stimulation but had no effect on IR-A stability either alone or in combination with insulin or proinsulin (Fig. 3A, B).

Because decorin enhanced IGF-II-induced IR-A downregulation we next determined whether decorin might affect IR-A levels at the cell surface and/or promote receptor endocytosis. Thus, we assessed ligand-induced IR-A internalization from the cell surface by ELISA, as described in previous work from our laboratories (Morcavallo et al., 2012). In agreement with our previous results (Morcavallo et al., 2012), insulin stimulation of R⁻/IR-A cells induced a clear reduction in the level of cell surface IR-A proteins compared to IGF-II while proinsulin promoted only a modest internalization of the IR-A which was not statistically significant (Fig. 3C). Decorin alone did not affect cell surface IR-A levels and did not modulate IGF-II- and proinsulin-induced IR-A internalization. On the contrary, decorin significantly reduced insulin-mediated internalization of the IR-A (Fig. 3C). Collectively, these results demonstrate that decorin binds the IR-A at cell surface and may modulate cell surface IR-A levels.

One of the major downstream effectors of the IGF-IR signaling pathway is the docking protein insulin receptor substrate 1 (IRS-1), which is recruited in ligand-dependent manner to the IGF-IR thereby regulating

the activation of the PI3K and Akt pathways. IRS-1 is critical for IGF-IR-dependent biological effects, including cell proliferation and transformation (Sun et al., 1993; Rose et al., 1994). Because we have recently shown that in urothelial cancer cells decorin enhanced IGF-I-mediated down-regulation of IRS-1 (Iozzo et al., 2011), we assessed in R⁻/IR-A cells IRS-1 protein levels after prolonged exposure to IGF-II, insulin, proinsulin and/or decorin. We discovered that decorin core had no appreciable effects in modulating IRS-1 stability either alone or in combination with all IR-A ligands (data not shown).

Next, we determined whether decorin could affect ligand-dependent IR-A signaling by analyzing the activation of the Akt and MAPK pathways, which are critical for IR-A-dependent cell proliferation (Morrione et al., 1997b; Frasca et al., 1999; Malaguarnera et al., 2012). To this end, we tested the activation of Akt and ERK1/2 kinases and downstream effectors S6 ribosomal protein using the PathScan Multiplex Western Cocktail I (Cell Signaling). This method allows testing the activation of multiple pathways in one single immunoblot, utilizing a mixture of well-defined phospho-specific antibodies targeting different activated proteins. Decorin core alone had no effect in activating both Akt and ERK1/2 but enhanced the activation of S6 ribosomal protein (Fig. 4A). In agreement with our previously published results, all biological ligands induced the activation of ERK1/2, Akt and S6 ribosomal protein. However, insulin and IGF-II were more effective than proinsulin in inducing Akt activation, while insulin was more potent than IGF-II and proinsulin in mediating ERKs activation (Morrione et al., 1997b; Frasca et al., 1999; Malaguarnera et al., 2012; Morcavallo et al., 2012). Importantly, decorin inhibited early time points of IGF-II-dependent activation of Akt without affecting either insulin or proinsulin-induced Akt signaling (Fig. 4A, B). In contrast, in three independent experiments decorin did not affect IGF-II-mediated ERK1/2 activation (Fig. 4C). While decorin core alone enhanced S6 activation at levels comparable to IGF-II and proinsulin, it did not significantly affect ligand-modulated S6 activity (Fig. 4D). Collectively, these results demonstrate that decorin specifically regulates IR-A stability and activation of downstream signaling effectors after IGF-II stimulation but has no effect on IR-A levels or signaling after insulin or proinsulin stimulation.

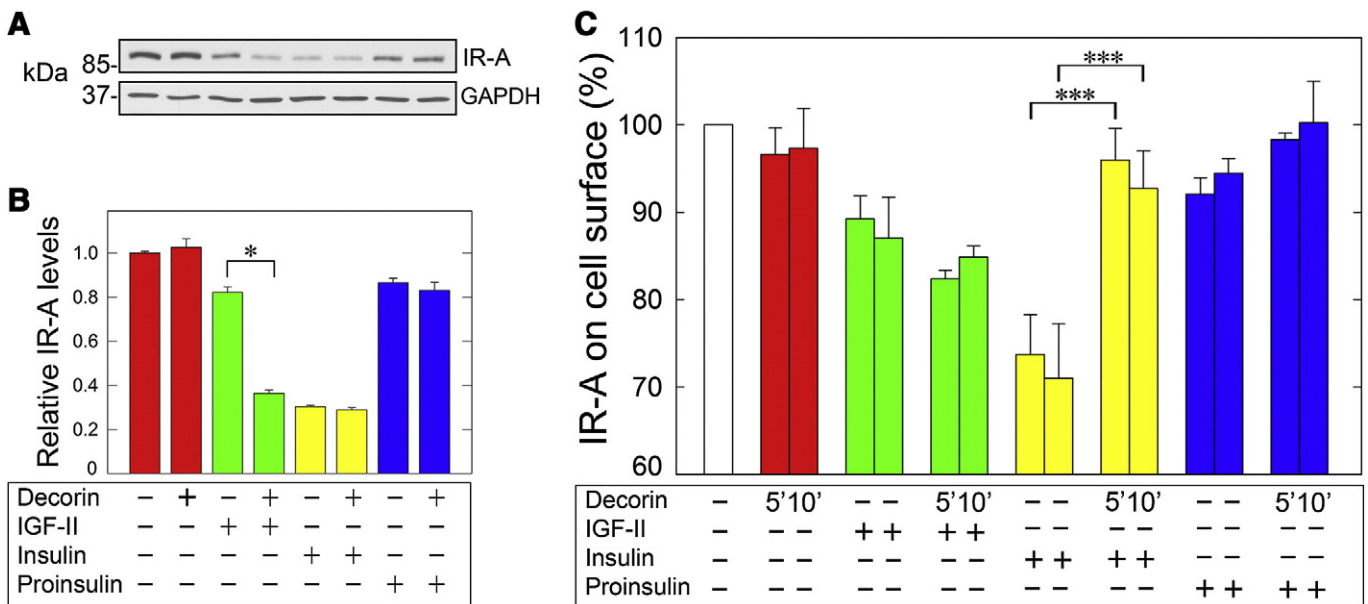


Fig. 3. [A] Decorin enhances IGF-II-dependent IR-A downregulation. Serum-starved R⁻/IR-A cells were incubated with either decorin alone (200 nM), IGF-II, insulin, proinsulin (10 nM) or ligands supplemented with decorin for 24 h and IR-A levels were detected by immunoblot using anti-IR polyclonal antibodies, which recognize the β subunit of the IR-A. Protein loading was monitored using anti-GAPDH monoclonal antibodies. [B] Densitometric analysis was performed using the ImageJ program (rsbweb.nih.gov/ij/) and the values are reported as relative densitometric arbitrary units. Values represent the mean \pm SD of three independent experiments run in duplicates and we present the statistically significant differences between critical samples (* $p < 0.05$). [C] Cell surface IR-A levels were measured by ELISA assay in unstimulated (basal cell surface level), decorin alone (200 nM) or ligand-stimulated cells (IGF-II, insulin and proinsulin at 10 nM) supplemented or not with decorin core (200 nM). Densitometric analysis was performed using the ImageJ program (rsbweb.nih.gov/ij/) and the values are reported as relative densitometric arbitrary units. Values represent the mean \pm SD of three independent experiments run in duplicates (** $p < 0.001$).

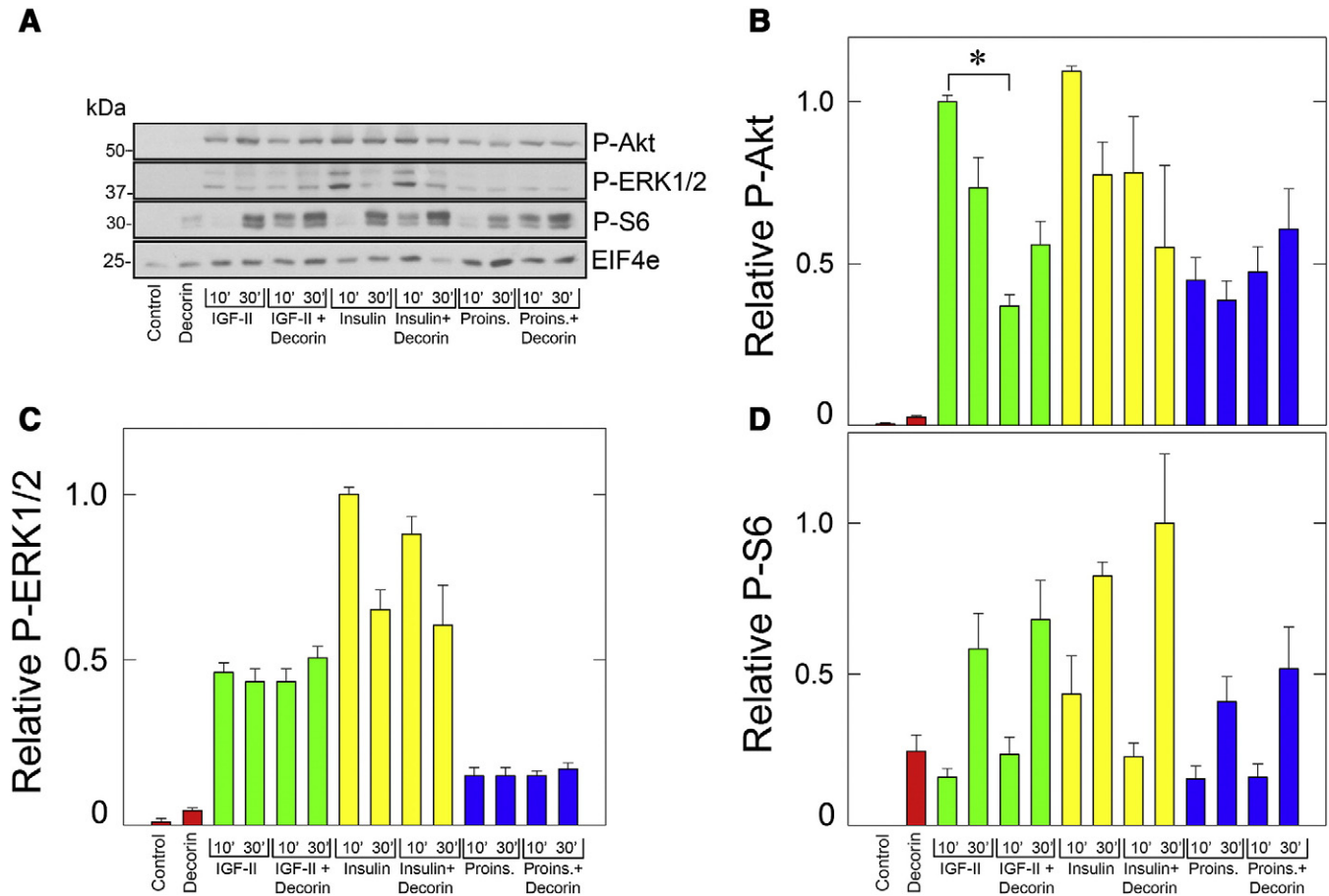


Fig. 4. Decorin affects IGF-II-induced signaling. [A] Western immunoblotting of serum-starved $R^{-}/IR-A$ treated for 10 and 30 min with the various ligands as indicated at the bottom. The various bands were visualized using the PathScan Multiplex Western Cocktail I (Cell Signaling Technology). EIF4e protein provides a control to monitor protein loading. Blots are representative of three independent experiments. [B–D] Quantification of three independent experiments similar to that shown in panel A. Densitometric analysis was performed using the ImageJ program (rsbweb.nih.gov/ij/) and the values are reported as relative densitometric arbitrary units. Values represent the mean \pm SD of three independent experiments run in duplicates and we present the statistically significant differences between critical samples ($*p < 0.05$). The PathScan includes also antibodies for activated p90RSK, which was not detectable in these experimental conditions (data not shown).

2.4. Decorin affects IGF-II-induced proliferation of $R^{-}/IR-A$ cells

Cell proliferation induced by IGF-II and insulin through the IR-A requires the simultaneous activation of the Akt and MAPK pathways (Morrione et al., 1997b; Frasca et al., 1999). Because decorin core protein preferentially enhanced IGF-II-mediated IR-A downregulation and inhibited IGF-II-induced IR-A signaling to Akt, we performed growth curves in $R^{-}/IR-A$ cells. The expected results should clarify whether decorin would differentially affect IR-A-dependent cell proliferation induced by IGF-II, insulin or proinsulin. While decorin alone (200 nM) had no effect on $R^{-}/IR-A$ cell proliferation, it significantly inhibited cell growth induced by IGF-II without significantly affecting insulin- or proinsulin-mediated mitogenesis (Fig. 5).

Altogether, these results provide the first evidence of a role for decorin in the regulation of ligand-dependent IR-A action and demonstrate that decorin specifically modulates IGF-II-dependent IR-A signaling and biological responses without affecting insulin or proinsulin-dependent IR-A function.

3. Discussion

Decorin is a stromal proteoglycan synthesized by fibroblasts, stressed vascular endothelial cells and smooth muscle cells (Neill et al., 2012b). The role of decorin in regulating the biology of various types of cancer is well-established as in fact decorin modulates the activity of several receptor tyrosine-kinases critical for cell growth and survival of malignant

cells (Iozzo, 1997; Schaefer and Iozzo, 2008; Neill et al., 2012b). The mechanism of action has been partially elucidated and occurs via a transient activation of the EGFR and the Met receptor (Moscatello et al.,

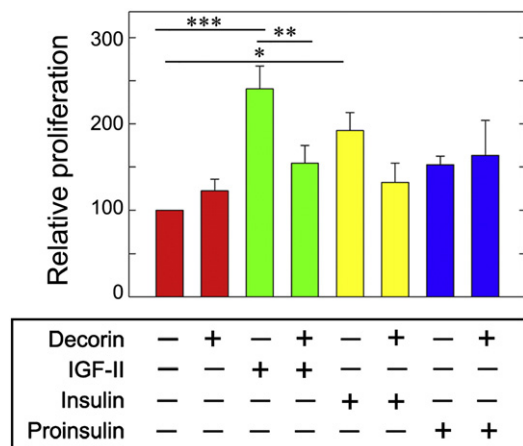


Fig. 5. Decorin inhibits IGF-II-induced proliferation in $R^{-}/IR-A$ cells. Quantification of $R^{-}/IR-A$ cell growth was performed as previously described (Morcavallo et al., 2012) and detailed in Section 4. Cells were incubated with decorin core alone (200 nM), IGF-II, insulin, proinsulin (10 nM) or ligands supplemented with decorin core protein. Cells were counted after 48 h in a hemocytometer. Values represent the mean \pm SD of three independent experiments run in triplicates ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$).

1998; Iozzo et al., 1999; Goldoni et al., 2009; Buraschi et al., 2010), which is associated to caveolin-mediated endocytosis, downregulation of the receptors in lysosomes and subsequent inhibition of cell growth (Csordas et al., 2000; Zhu et al., 2005). Decorin action on the IGF-I system, and in particular on the IGF-IR, is more complex as it differs between non-transformed (normal) and transformed cells (Morrione et al., 2013). Moreover, the insulin receptor A isoform (IR-A), together with autocrine production of its ligand IGF-II, is emerging as an important mechanism of normal and cancer cell expansion and is a key feature of several malignancies (Belfiore and Malaguarnera, 2011; Bartella et al., 2012).

In this study we investigated whether decorin may regulate IR-A activity mediated by its ligands IGF-II, insulin and proinsulin using mouse embryo fibroblasts lacking the *Igf1r* and expressing exclusively the human IR-A (Miura et al., 1995). We found that: (i) Decorin binds with high affinity both the IR-A and IR-A ligands, although the affinity for the IR-A and proinsulin is 3-fold lower than the affinity for IGF-II and insulin; (ii) Decorin does not affect ligand-mediated IR-A phosphorylation; (iii) Decorin enhances downregulation of the IR-A after prolonged IGF-II stimulation but does not affect IR-A stability after insulin or proinsulin stimulation; (iv) Decorin regulates cell surface IR-A levels by affecting insulin-dependent internalization; (v) Decorin inhibits IGF-II-mediated Akt activation without affecting insulin- and proinsulin-dependent signaling, and (vi) Decorin negatively regulates IGF-II-dependent mitogenesis but not cell proliferation induced by insulin or proinsulin. A schematic drawing summarizing our results and decorin action on IR-A activity is shown in Fig. 6.

We have recently demonstrated that, in urothelial cancer cells, decorin binding to IGF-I and the IGF-IR inhibits receptor phosphorylation and downstream activation of the Akt and MAPK pathways, which is associated with a significant reduction in motility and invasion capabilities of these cells (Iozzo et al., 2011). Surprisingly, decorin did not affect IGF-IR stability (Iozzo et al., 2011) providing the first evidence in support of a very different mode of decorin action between transformed and non-transformed cells, where decorin promotes

transient IGF-IR activation followed by IGF-IR degradation (Morrione et al., 2013). $R^{-}/IR-A$ mouse embryo fibroblast cells are able to grow in serum-free media supplemented solely with growth factors (Morrione et al., 1997b; Frasca et al., 1999; Morcavallo et al., 2012) but they are not fully transformed as in fact an overexpressed human IR-A in the absence of the *Igf1r* is not able to promote colony formation in soft-agar and anchorage-independent growth (Miura et al., 1995). Notably, our results suggest that decorin plays a role in regulating ligand-induced IR-A action in a way that mechanistically differs from both non-transformed and transformed cells. In fact decorin does not affect receptor phosphorylation but enhances IR-A downregulation and negatively modulates downstream signaling. More significantly, decorin specifically regulates IGF-II-mediated IR-A function and signaling without interfering with insulin and proinsulin-induced IR-A pathways in spite of the similar affinity of decorin for IGF-II, insulin and proinsulin.

IGF-binding proteins (IGF-BPs), which either positively or negatively modulate the IGF-I system, depending on cell and tissue models (Jogie-Brahim et al., 2009) bind IGF-I and IGF-II but not insulin or proinsulin (Forbes et al., 2012). We can speculate therefore that IGF-BPs may complex with decorin, potentiate decorin binding to IGF-II thereby promoting prolonged occupancy and enhancing decorin action on IGF-II but not on insulin and proinsulin. This different binding capacity would therefore differentially regulate IR-A signaling and biological responses. Whether decorin may complex IGF-II in the presence of IGF-BPs remains to be determined and further experiments are required to clarify this issue. However, there is ample evidence that decorin is synthesized and released in the circulation by activated macrophages during sterile and bacterial sepsis in both human and mouse blood (Merline et al., 2011). Thus, there is the possibility of a direct interaction between decorin and IGF-BP to modulate IGF system in peripheral tissues.

Our recent work (Iozzo et al., 2011) has shown an affinity of decorin for IGF-I very similar to the one we demonstrated in the present work for IGF-II and insulin, but while decorin strongly affects IGF-IR activation (Iozzo et al., 2011), it has no effect in modulating either IGF-II- or insulin-induced phosphorylation of the IR-A. However, our experiments on the IGF-IR were conducted in bladder cancer cells (Iozzo et al., 2011), which do not express decorin, while fibroblast cells used in these studies produce decorin as previously shown (Neill et al., 2012b).

We have recently demonstrated that insulin and IGF-II affect IR-A biological responses by differentially regulating IR-A trafficking (Morcavallo et al., 2012). Using $R^{-}/IR-A$ cells, we discovered that insulin significantly promotes IR-A internalization, a process only modestly affected by IGF-II. Notably, prolonged stimulation of $R^{-}/IR-A$ cells by insulin, but not by IGF-II, targets the receptor for degradation (Morcavallo et al., 2012). Decorin alone or in combination with either insulin or proinsulin has no effect on regulating IR-A stability but significantly enhances IGF-II-mediated IR-A downregulation. These results would suggest that decorin could play a role in regulating IR-A endocytosis by enhancing IGF-II-mediated IR-A internalization from the cell surface. However, decorin did not significantly affect IGF-II-mediated IR-A internalization from the cell surface indicating that decorin may play a more relevant role in regulating later stages of receptor endocytosis such as IGF-II-dependent sorting of the IR-A into lysosomal compartments. Significantly, decorin protein core reduced early time points of insulin-mediated IR-A internalization but it did not affect insulin-modulated IR-A stability. These results suggest the hypothesis that decorin may contribute to the regulation of cell surface IR-A levels by modulating receptor recycling to the membrane.

We also demonstrated that upon ligand stimulation the IR-A is internalized via both clathrin-dependent and -independent pathways (Morcavallo et al., 2012). However, clathrin-dependent endocytosis is absolutely necessary for ligand-induced IR-A degradation as siRNA-mediated depletion of endogenous clathrin severely reduced IR-A degradation, which was not affected instead by targeting caveolin-1 (Morcavallo et al., 2012). Decorin, therefore, may contribute to enhance the pool of IR-A internalized upon IGF-II stimulation through clathrin-

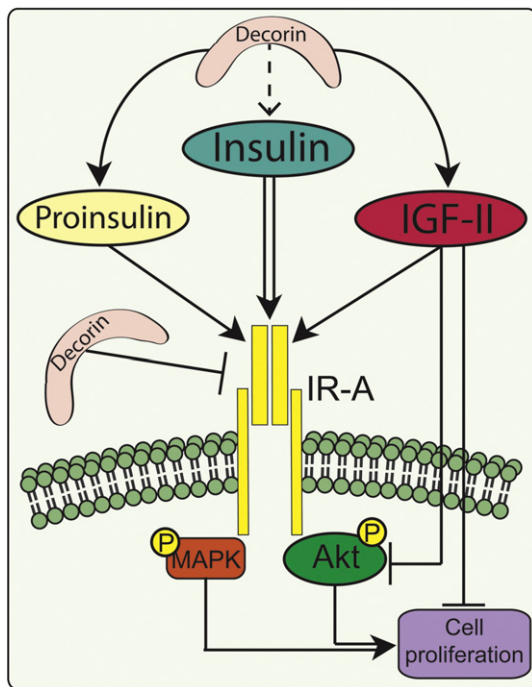


Fig. 6. Schematic drawing summarizing decorin effects on the regulation of ligand-dependent IR-A activity. Decorin binds IGF-II, insulin, proinsulin and the IR-A and enhances IGF-II-induced IR-A downregulation, Akt activation and IGF-II-induced cell proliferation. In addition decorin may regulate IR-A levels at the cell surface. For more information consult Section 3.

dependent pathways thereby increasing the fraction of the IR-A sorted for degradation. Experiments are currently under way to determine the molecular details of decorin action on IR-A endocytosis and trafficking.

In conclusion, we have investigated the role of soluble decorin in modulating the bioactivity of various ligands for the insulin receptor isoforms, utilizing a well-defined genetic cell system lacking the endogenous *Igf1r* but expressing the insulin receptor isoform A (IR-A). We discovered that decorin binds with high affinity insulin and IGF-II and, to a lesser extent, proinsulin and IR-A, and that it does not affect ligand-induced IR-A phosphorylation. However, decorin evokes IR-A downregulation after prolonged IGF-II stimulation without affecting insulin- or proinsulin-dependent effects on IR-A stability. Moreover, soluble decorin attenuates IGF-II-mediated cell proliferation of R⁻/IR-A cells but affects neither insulin- nor proinsulin-dependent mitogenesis. These results suggest that decorin differentially regulates the action of IR-A ligands by preferentially inhibiting IGF-II-mediated biological responses without affecting insulin- or proinsulin-dependent signaling. Our findings corroborate the notion that decorin's regulation of a wide network of tyrosine-kinase receptor signaling could play a critical role in the regulation of many aspects of mammalian biology in physiology and disease.

4. Experimental procedures

4.1. Cell lines and recombinant proteins

R⁻/IR-A cells are mouse embryo fibroblasts derived from *Igf1r*^{-/-} mice (Sell et al., 1994) and expressing solely the human IR-A isoform (Miura et al., 1995). Cells are maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 2 µg/ml of puromycin. These cells were previously well characterized for their mitogenic response to insulin and IGF-II (Morrione et al., 1997b; Frasca et al., 1999). Serum-free medium (SFM) is DMEM supplemented with 0.1% bovine serum albumin and 50 µg/ml of transferrin (Sigma-Aldrich). The production of human recombinant decorin protein core has been previously described (Merline et al., 2011). Human recombinant IR-A was purchased from Sino Biological Inc. (11086-H08H) and consisted of the IR-A extracellular domain (Met 1-Lys 944) fused with a polyhistidine tag at the C-terminus. Recombinant human IGF-II was from Peprtech, insulin from porcine pancreas was from Sigma-Aldrich while human proinsulin was purchased from R&D System.

Decorin protein core binding to the IR-A was assessed by ELISA. Briefly, wells were coated with recombinant IR-A (100 ng/well) overnight at room temperature in the presence of carbonate buffer, pH 9.6. Plates were then washed with PBS and incubated for 2 h with serial dilutions of decorin core (up to 200 nM). Plates were then extensively washed with PBS, blocked with PBS/1% BSA, and incubated with primary antibody against the N-terminus of decorin and HRP-conjugated secondary antibody. Signal was detected using Sigma-Fast tablets (Sigma-Aldrich) and read at 450 nm OD. Decorin protein core binding to IGF-II, insulin or proinsulin was performed as described above but plates were coated with IGF-II, insulin or proinsulin (100 ng/well) instead. All experiments on IR-A phosphorylation, stability and downstream signaling were performed using decorin core protein at 200 nM, which is the optimal concentration previously established to affect IGF-IR activity and signaling (Iozzo et al., 2011).

4.2. Detection of IR-A activation and IR-A levels

Serum-starved R⁻/IR-A cells were preincubated with decorin (200 nM) for 1 h and then incubated with SFM or physiological concentration (10 nM) of IGF-II, insulin or proinsulin and decorin or decorin alone for 10 min. To test the effect of decorin binding to the ligand, IGF-II, insulin and proinsulin were preincubated with 200 nM decorin

core and then supplemented to cells. IR-A phosphorylation was detected by immunoblot using anti-phospho-IGF-IRβ (Tyr1135/1136)/IRβ (Tyr1150/1151) rabbit monoclonal antibodies (Cell Signaling Technology). Total IR-A was assessed using anti-IR polyclonal antibodies, which recognize the β subunit of the IR-A (Santa Cruz Biotechnology). IR-A stability was assessed by testing IR-A levels by immunoblot after 24 h of IGF-II, insulin or proinsulin (10 nM) stimulation with or without 200 nM decorin. Protein loading was monitored by using anti-GAPDH monoclonal antibodies (GeneTex). Blots are representative of three independent experiments.

4.3. Analysis of cell surface IR-A levels

The level of IR-A on the cell surface of R⁻/IR-A cells was determined by ELISA assays (Morcavallo et al., 2012) using a monoclonal antibody against the IR α-subunit (Novus Biologicals, diluted 1:1000), and goat anti-mouse alkaline phosphatase-conjugated antibody (diluted 1:1000; Sigma-Aldrich). Antibody binding was visualized by adding 0.25 ml of alkaline phosphatase substrate (Bio-Rad). The reaction was stopped by transferring 0.1 ml of the substrate to a 96-well microtiter plate containing 0.1 ml of 0.4 M NaOH. Plates were read at 405 nm in a microplate reader (Dynex Technologies) by using Microplate Manager Software.

4.4. Detection of activated MAPK and Akt and cell growth experiments

Serum-starved R⁻/IR-A cells were preincubated with decorin for 1 h and then stimulated with IGF-II, insulin or proinsulin (10 nM), and decorin or decorin alone for 10 min. The activation of p90RSK, Akt, ERK1/2 and pS6 ribosomal protein was analyzed by Western immunoblot using the PathScan Multiplex Western Cocktail I (Cell Signaling Technology) (Monami et al., 2006, 2008, 2009). EIF4E protein is provided in the kit as control to monitor the loading of the samples. Densitometric analysis was performed using the ImageJ program (rsbweb.nih.gov/ij/).

Growth curves of R⁻/IR-A cells in the presence of either IGF-II, insulin or proinsulin alone (10 nM) or supplemented with decorin core (200 nM) were performed as previously described from our laboratories (Morrione et al., 1997a,b; Morcavallo et al., 2012). Briefly 3 × 10⁴ cells were plated in serum-containing media and counted after 24 h (time 0). At that point cells were shifted to SFM or SFM supplemented with decorin alone, ligands alone or the combination of the two. Cells were counted after 48 h with a hemocytometer.

4.5. Statistical analysis

Experiments were carried out in triplicate and repeated at least three times. Results are expressed as mean ± SEM. All statistical analyses were carried out with SigmaStat for Windows version 3.10 (Systat Software, Inc., Port Richmond, CA). Results were compared using the two-sided Student's *t* test. Differences were considered statistically significant at *p* < 0.05.

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