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Regulation of integrin α6A by lactogenic hormones in rat pancreatic β-cells: Implications for the physiological adaptation to pregnancy

Short title: Integrin α6 and β-cell proliferation in pregnancy

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ABSTRACT

Aim: During pregnancy the maternal β-cell mass is increased in order to adapt to the physiological changes in insulin demand. Lactogenic hormones stimulate rodent β-cell attachment and proliferation *in vitro*. The aim of the study was to identify adhesion molecules involved in expansion of the β-cell mass during pregnancy in the rat.

Methods: Quantitative RT-PCR was used to evaluate the expression of several integrins and laminins in isolated neonatal rat islets in response to growth hormone and prolactin treatment. Double-immunofluorescence staining of rat pancreas was used to localize the expression of integrin α6β1. β-cell proliferation was evaluated by incorporation of bromodeoxyuridine. The role of STAT5 phosphorylation was tested by addition of STAT5 mutants.

Results: We found that the mRNA level of integrin-α6A, was upregulated 2.5-fold by prolactin or growth hormone. During pregnancy a bi-phasic 3.4-4.5-fold increase of integrin-α6A and B mRNA levels was detected. A disintegrin peptide reduced the hormone-stimulated mitotic activity in neonatal rat β-cells from 2.9 ± 0.4-fold to 1.3 ± 0.3-fold. The hormone-induced expression of α6β1 integrin was shown to be mediated via STAT5 as a dominant negative mutant prevented and a constitutive active mutant augmented the hGH stimulated expression. The disintegrin peptide was found to inhibit hGH-induced transactivation of the PRL receptor promoter 1A and reduce the hGH induced phosphorylation of STAT5.

Conclusion: These results show that integrin-α6 in β-cells is upregulated by lactogenic hormones and is required but not sufficient for the expansion of the β-cell mass in pregnancy in the rat, which may have implications for the understanding and treatment of gestational diabetes.

Keywords: β-cells, lactogenic hormones, integrins, *Itga6*, islets of Langerhans, pregnancy, Signal Transducer and Activator of Transcription 5 (STAT5)

INTRODUCTION

Pancreatic β-cells have a limited capacity for replication, which is thought to be critical for the growth and maintenance of an appropriate β-cell mass during life. Apart from glucose, several growth factors for rodent β-cells have been identified including insulin/IGFs, GLP1/GIP, EGF/PDGF family members and cytokines and hormones signalling through the JAK-STAT pathways [1]. Prolactin (PRL), placental lactogen (PL) and growth hormone (GH) are the most potent mitogens reported for primary rodent β-cells and insulinoma cell lines *in vitro* [2,3]. Physiologically, these hormones are particularly relevant in the adaptive β-cell growth during pregnancy, where the β-cell mass is markedly increased [4-6]. Global depletion of PRL receptors (PRLR) in mice have been shown to impair glucose tolerance and β-cell proliferation during pregnancy [7] and conditional β-cell specific inactivation resulted in gestational diabetes (GDM) [8]. GDM occurs in around 15% of pregnant women [9] and contributes to the global increase of type 2 diabetes (T2D) as the risk of later development of T2D in the offspring is 7-fold [10]. It is therefore pertinent to unravel the molecular mechanisms involved in the compensatory increase of the β-cell mass during pregnancy. The mitogenic effect of GH/PRL/PL *in vitro* is accompanied by enhanced attachment and spreading of rat β-cells[11]. Several studies have indicated that extracellular matrix (ECM), especially the basement membrane components laminins, collagens and fibronectin play a crucial role in the integrin mediated regulation of the growth, differentiation and function of the β-cell [12,13]. Integrins comprise 24 different heterodimers composed of 18 different α-subunits and 8 different β-subunits of which α3, α6, αL, αV, β1, β5, β6 and β8 are abundant in rat β-cells [12]. Several studies have shown that integrin β1 plays a crucial role in β-cell attachment to ECM and influences insulin secretion, survival and proliferation [14-17]. Based on these observations we hypothesized that the potent mitogenic effect of hormones of the GH family on the β-cells may involve regulation of the integrin expression providing an increased competence of the β-cells to progress through the cell

cycle. So far, it has not been shown which integrins that are involved in the increase in the β-cell mass during pregnancy. In the present study, we have identified the integrin α6A subunit as a GH/PRL-regulated gene, which is expressed in mature rat β-cells and plays a role in their compensatory expansion in pregnancy.

RESULTS

Expression of cell adhesion molecules in neonatal rat islets

The expression levels of selected cell adhesion molecules were studied in neonatal rat islets. As seen in Table 1 integrin β1 and α3A mRNAs were most abundant, followed by integrins α1, α6A, α6B and β4. The mRNA expression levels of E-cadherin, N-cadherin and dystroglycan were high and laminin α3, α5 and α1 were detectable. After stimulation for 24 h with 0.5 μg/ml bGH, oPRL, or hGH the expression level of the PRLR increased significantly 3 ± 0.6, 5.8 ± 0.8, and 6.5 ± 1.7 fold, respectively (Fig. 1). The expression level of the integrin α6A subunit was found to be significantly elevated by oPRL and hGH (2.7 ± 0.5 and 2.8 ± 0.4 fold, respectively. The expression levels of the other examined genes were not significantly affected by hormones of the GH family although a tendency to increased expression integrin-α6B was noted.

Regulation of integrin α6 mRNA during pregnancy

To address whether islet cell integrin α6 is prone to regulation by lactogenic hormones *in vivo*, the mRNA expression was examined in freshly isolated islets from adult female rats during pregnancy (Fig. 2). Compared with the expression in non-pregnant animals, the mRNA levels of both integrin α6A and α6B were significantly increased, 3.3 ± 0.9 and 3.3 ± 0.7 fold, respectively on day 13 of pregnancy. At day 16, the expression levels declined to 1.5-1.7 fold, respectively, whereas at day

19, maximal expression of integrin α6A and B was observed (4.4 ± 0.4, and 3.8 ± 0.6 fold, respectively). There was a tendency to an increase in the expression of integrin β1, but it was not significant. The PRLR expression which we have previously found to increase in pancreas from pregnant animals [18,19] was also found to be significantly elevated in isolated islets in the late half of pregnancy with maximal expression on day 16 (4.8 ± 0.4 fold).

Immunohistochemical staining of pancreas for integrin α6β1.

Using a monoclonal antibody recognizing the rat integrin α6β1 heterodimer, we examined immunohistochemically its expression in sectioned pancreata from adult, newborn, and fetal rats. Consecutive sections were stained for insulin (Fig. 3 right). Detectable expression of integrin α6β1 was observed in adult, 5- and 2-day- old pancreata (Fig. 3A, C, and E, respectively) and co-localized with the core of insulin-producing cells of the islets (Fig. 3 B, D, and F, respectively), whereas weak/no immunoreactivity was present in the exocrine compartment and in the peripheral part of the islets. No detectable expression of integrin α6β1 was observed in E13, E16 (not shown), and E19 (Fig. 3G and H) fetal rat pancreas. Staining was detected both in the cytoplasm and at the plasma membrane. Double-staining for glucagon and integrin α6β1 (Fig. 4) of sections from adult rat pancreas indicated also mainly staining in the β-cell core of the islets and weaker/no staining in the glucagon positive cells in the periphery.

Double-immunofluorescence staining for insulin (Fig. 5A) and integrin α6β1 (Fig. 5B) of β-cell monolayer cultures promoted by hGH showed expression of integrin α6β1 in most β-cells and revealed a few non-insulin producing cells within the epithelial cell clusters, which were positive for integrin α6β1 (Fig. 5C). The identity of these cells remains to be determined.

Role of STAT5 activation in the expression of integrin α6.

Transduction of INS-1 cells with a recombinant adenovirus expressing a dominant negative (DN) STAT5 mutant abrogated the hGH stimulated expression of integrin α6 mRNA and had a tendency to suppress the basal expression, whereas a constitutive active (CA) STAT5 mutant showed a tendency to increase the expression and did potentiate the hGH stimulated integrin α6 mRNA expression after 4 hours exposure (Fig. 6). Thus, integrin α6 mRNA seems to be positively regulated by hGH via STAT5 signalling.

Effect of disintegrin peptide on hGH-induced β-cell proliferation.

To evaluate the functional significance of integrin α6β1 expression in the β-cells we took advantage of an octapeptide (ANQECDVT) representing the disintegrin domain of rat fertilin β, which at 200-500 μM concentrations has been shown to inhibit binding of laminin to integrin α6β1 [20,21]. Growth hormone-expanded β-cell monolayers were deprived of hGH for 24 h. The culture dishes were not coated with any exogenous matrix. The cells were then incubated for 24 h with 10 μM bromodeoxyuridine (BrdU) in the absence or presence of 0.5 μg/ml hGH and in the absence or presence of 250 μM disintegrin peptide (DP) or 3 control peptides: CP1 (TNCQEADV), containing scrambled amino acids of DP, CP2 (AEDVCDLP), containing the non-functional disintegrin domain of rat fertilin α; CP3 (PECLVADD), containing a scrambled version of CP2. After fixation, the cells were double-stained for BrdU and insulin and the percentage of BrdU-positive β-cells determined by counting. Treatment of the β-cells with hGH for 24 h resulted in a 2.9 ± 0.4 fold (mean ± SEM, n=4) increase of the number of BrdU positive β-cells compared with the non-treated cells (Fig. 7A). Addition of 250 μM DP significantly (P<0.01) reduced the mitotic activity to 1.3 ± 0.3 fold, whereas the three control peptides at the same concentration had no inhibitory

activity. The basal mitotic activity (2-4 % of the total number of β-cells counted) and the attachment of the β-cells were not affected by DP or any of the control peptides at this concentration (data not shown).

Effect of disintegrin peptide on the transactivation of the PRL receptor promoter 1A

We have previously demonstrated that GH and PRL stimulate expression of the PRL receptor (PRLR) via a STAT5-dependent transactivation of the 1A promoter of this gene[22]. Using a promoter-reporter assay in the INS-1E cell line, we investigated the effect of the DP on hGH-induced transactivation of the PRLR 1A promoter after 6 hours exposure (Fig. 7B). The data represent mean ± SEM, n=4 independent experiments in triplicate. The DP inhibited approx. 75 % of the response to hGH in this assay, whereas the control peptides at a similar concentration had no inhibitory activity. Basal activity of the PRL 1A promoter was also inhibited by the DP, but not by the control peptide. Thus, hGH induced STAT5 signalling is decreased when the laminin-integrin interaction is inhibited, suggesting that laminin-induced integrin signalling synergistically increases STAT5 signalling in β-cells upon hGH stimulation.

Effect of disintegrin peptide on hGH-induced phosphorylation of STAT5.

In order to test if the inhibitory effect of DP on β-cell proliferation could be mediated by changes in phosphorylation of STAT5, we measured phosphorylated STAT5 in INS-1E cells exposed to hGH for 15 min in the presence and absence of DP or control peptide (Fig. 7C). Data represent mean ± SEM, n=5 independent experiments. DP resulted in a significant reduction in the hGH-stimulated STAT5 phosphorylation, whereas CP had no significant effect. These data indicate that laminin-integrin signalling is necessary for hGH-induced STAT5 activation.

DISCUSSION

In the present study, we have examined at the mRNA level the expression and hormonal regulation of integrin receptors involved in laminin binding in isolated newborn rat islets. A relatively high abundance of mRNA encoding the integrin subunits α3A and β1 was observed, indicating that integrin α3β1 is a major laminin receptor in insulin producing cells in accordance with a study in the rat insulinoma cell line, RIN-2A, and primary β-cells [23]. In the present study integrin α6 was found to be significantly expressed at the mRNA level in cultured islets (Table 1) and the expression was found to be up-regulated *in vitro* by lactogenic hormones (Fig. 1) and during pregnancy *in vivo* (Fig. 2). In islets from pregnant mice at day 13 we found that integrin α6 was the only integrin subunit found to be markedly upregulated [19]. The low effect of bGH suggest that the signalling via the PRL receptor rather than the GH receptor is responsible for the stimulatory effects as hGH is known to activate both GH and PRL receptors in rodents [24]. In pregnancy, both PRLR and GHR are upregulated in the islets[18] and the placenta produces both placental GH and PL so we have used hGH in order to obtain maximal STAT5 response. The PRLR is upregulated by STAT5 whereas the GHR is not[18].

Integrin α6 has been shown to exist in two alternative splice variants, A and B, in both man, mouse, and hamster differing in the length and amino acid sequence of the encoded proteins [25]. Similar splice variants appear to exist in the rat since RT-PCR amplification using specific primers for integrin α6A and B sequences, respectively, resulted in products with the expected sizes, when run on polyacrylamide gels. The basal mRNA levels of the integrin α6 A and B variants in the cultured islets were similar, whereas oPRL and hGH significantly increased the mRNA level of the A-isoform only (Fig. 1). The expression of the integrin α6 A and B variants has been shown to be differentially regulated during cell differentiation [26,27].

In pregnancy, the A and B variants of integrin α6 exhibited a parallel biphasic increase of expression at days 13 and 19 (Fig. 2). This biphasic pattern coincides with the serum level of placental lactogen I and II in the rat [28]. We have previously observed a similar biphasic expression of preadipocyte factor (pref-1/dlk1) during pregnancy [29]. In contrast, the mitotic activity in the β-cells is maximal at day 14 and declines from day 17 [28]. It has been shown that normalization of blood glucose by insulin treatment in pregnant rats did not change the PL levels, but prevented β-cell proliferation [30]. Thus, in spite of elevated levels of lactogens, PRLR and integrin α6 there is a decline in β-cell proliferation in late pregnancy indicating that the insulin production is sufficient to maintain normoglycemia, which is in accordance with the observation that hyperglycemia is a prerequisite for expansion of the β-cell mass [5,31,32]. Other factors seem also to be involved in the reversal of the β-cell proliferation e.g. progesterone, glucocorticoids and serotonin receptor subtypes [6].

The integrin α6 subunit can dimerize with either integrin β1 or β4 subunits to form a receptor that in contrast to the other laminin receptors belonging to the integrin family (e.g. α3β1, α1β1) is specific for binding of laminins. Since mRNA levels of the β4 subunit were extremely low in the islets, it is conceivable that the major dimerization partner for α6 in islets is β1. The immunohistochemical detection in postnatal pancreatic islets using a monoclonal antibody specific to the α6β1 heterodimer supports this assumption (Fig. 3). The identification of integrin α6 in the endocrine cells of the pancreas has been controversial, but most recent studies have confirmed expression of integrin α6 in human and rodent islets and beta cells[12].

In rat pancreas, the α6β1 immuno-staining was found predominantly in the β-cell core of the islets, whereas glucagon-producing cells at the islet periphery appeared to have no or weak expression (Fig. 4). Islet α6 immuno-staining was observed also in mouse and human tissue (data not shown)

and in all insulin-positive cells of monolayers of rat islet cells as well as a few non-insulin-pro-ducing cells (Fig. 5). Our finding of mainly β-cell expression of integrin α6 was observed in 3 different species using two different antibodies and is in agreement with a previous study[14]. In rat islets, integrin α6β1 has been shown to be up-regulated by glucose and IBMX (3-isobutyl-1-methylxanthine) suggesting that integrin α6β1 also plays a role in the increased β-cell secretory function in pregnancy[14]. Also glucokinase is upregulated by PRL via STAT5 [19] and the cAMP levels are increased in islets during pregnancy [33].

In human islets and β-cells, 11 laminin isoforms each containing an α, β and γ chain have been identified[34]. In the present study, we found consistently a very low and barely detectable expression of the α1 chain, a subunit of laminin-111 (α1β1γ1) and 121 (α1β2γ1). Variable but significant expression of the α3 and α5 chains was observed (Table 1). The α3 chain is a component of laminin 332 (α3β3γ2) and the covalently associated laminin 311 (α3β1γ1) and 321 (α3β2γ1) isoforms. Laminin 511 (α5β1γ1) and laminin 411 (α4β1γ1) have been suggested to involved in human beta cell proliferation, although to a very limited extent[35] and laminin 111 (α1β1γ1) has been reported to induce rat/mouse beta cell differentiation[36,37] and human islet differentiation from duct cells[38,39]. Our study indicates that postnatal islet cells are involved in the synthesis of their own basement membrane and that the laminin constituents of this may be laminin-5 and -10. In mouse islets, the expression of laminin α2, α3, α4, α5, β1, β2, γ1 and γ2 were detected, but were not significantly changed during pregnancy[19].

Dispersed islet cells can form attached monolayers in high serum alone and in low serum in the presence of hGH, presumably via endogenously produced ECM and ECM receptors. To function-ally address the significance of laminin-integrin α6β1 interaction in the β-cells, we employed hGH-promoted β-cell monolayer cultures and inhibited laminin-binding to α6β1 using a disinteg-rin peptide[20,21] based on the sequence of rat fertilin β (ADAM2)[40]. We found that this peptide

almost completely blocked hGH-induced proliferation of the β-cells (Fig. 7A) and inhibited hGH-induced transactivation of the PRLR promoter in the insulinoma cell line, INS-1 (Fig. 7B). The mechanism by which integrins may regulate the growth response of a cell is not clear. Co-precipitation experiments have shown that the activated integrins may co-localize with growth factor receptors and secondary signalling molecules and it has been hypothesized that the integrins act as scaffolds involved in building up efficient growth factor signalling complexes [12].

Moreover, integrins, although devoid of enzymatic activity in their short cytoplasmic domains, has distinct signalling capabilities. A potential candidate as a signal transducer is the tyrosine kinase, focal adhesion kinase FAK, which is located in focal adhesions and can interact with both cyto-skeletal components and other signalling molecules including Grb2 leading to activation of mito-gen-activated protein kinase, MAPK[41]. The integrin α6 A subunit, but not the B subunit, was shown to profoundly activate the p42 and p44 MAPK in P388D1 macrophages in a Ras and protein kinase C-dependent way and this activation was found to correlate with cell migration[42]. The in-tegrin-linked kinase, ILK, is activated by integrin β1, β2, and β3 and leads to nuclear translocation of β-catenin and phoshorylation of glycogen synthase kinase 3 and protein kinase B/AKT[43]. In-tegrin β1 is shown to associate with FAK and activate MAPK/ERK signalling to stimulate human fetal islet cell differentiation[44]. This signalling pathway has been directly linked to regulation of the cell cycle regulatory factor, cyclin D1[45] and in over expression studies using a transgenic approach protein kinase B/AKT has been shown to play a significant role in the regulation of β-cell mass and function[46,47].

Cell attachment and spreading on ECMs are often associated with cell proliferation, but this not always the case. Thus, islet cells can attach and spread into monolayers by addition of high glucose concentration, IBMX[48], trefoil factor-3[49,50] andpreadipocyte factor-1/dlk1[29,51]. The attachment and migration processes do however not alone induce proliferation, but may participate in (de)differ-

entiation processes that under some conditions facilitate proliferation. In the present study, treatment of islets exposed to hGH with DP did not prevent attachment and migration, but impaired proliferation. Expression of integrin α6 is increased in late pregnancy when proliferation has declined (Fig. 2) indicating that integrin α6 does not *per se* stimulate β cell proliferation.

The transcription factor STAT5 is a major signalling molecule activated by GH and PRL in insulin-producing cells via the associated tyrosine kinase JAK2[22,52]. Interestingly, studies in primary mammary cells which express integrins α3β1 and α6β1 have shown that laminin binding is required for activation of STAT5, probably involving a phosphatase, which in the absence of laminin dephosphorylates JAK2[53,54]. Studies in breast cancer cells have demonstrated PRL induced complex formation between signal regulatory protein-α (SIRPα) and PRLR, integrin β1 and JAK2 indicating that physical interaction between PRLR and integrins are involved in STAT5 activation[55]. The finding that disruption of laminin binding to integrin by DP in β-cells abrogates hGH-induced transactivation of the STAT5-dependent PRLR promoter suggests a similar cross-talk in these cells. In the mammary gland, activation of STAT5 has been shown to depend on integrin β1 signalling [56]. This was supported by our finding that dominant negative STAT5 reduced the hGH stimulated integrin 6α expression, whereas the constitutive active STAT5 augmented the effect (Fig. 6). The lack of significant effect of hGH and CA-STAT5 alone is probably due the short exposure time (4 hours) as we previously found an increase in cell proliferation after 24h and 48h exposure to oPRL[18]. The marked increase in p-STAT5 by hGH (Fig, 7C) was seen after 15 min whereas the transcription of *Itga6* may require more time. In addition, the reduced STAT5 phosphorylation by the DP supports the pivotal role of STAT5 phosphorylation in the mitogenic effect of lactogenic hormones on β-cells (Fig. 7C). Adult human islets have been shown to have a very low expression of GHR and PRLR compared with mouse islets[57] but very high expression integrin α6[12] and most studies have failed to detect a mitogenic effect of lactogenic hormones[58,59]. In pancreas from pregnant women the increased β-cell mass mainly consists of an increase in the number

of small islets and not larger islets[60] suggesting that the expansion may occur by neogenesis of β-cells from progenitor cells although some proliferation cannot be excluded. Neogenesis from progenitor cells may also depend on expression of ECM molecules that have been shown to promote differentiation from stem cells in the human pancreas[12]. Surprisingly, overexpression of human PRLR and murine STAT5 was reported to induce human β-cell proliferation, while human STAT5 did not [58]. The lactogenic hormones and other factors of fetal or placental origin may be involved in the neogenesis of β-cells in the mothers as well as in the fetus as we have found that serum from pregnant women can induce Ngn3 expression in fetal rat pancreas in vitro[61] . It may be speculated that the same factors that are upregulated during pregnancy, e.g. TFF3[49,50] and dlk-1/Pref-1[29,51], may promote maternal and embryonic β-cell expansion by proliferation or neogenesis or both, depending of species. The establishment of an ECM network may be a crucial process in β-cell formation, expansion and maintenance throughout life.

In conclusion, our results suggest that integrin a6A has a permissive role in the function of the prolactin receptor in the adaptation of the pancreatic β-cells to pregnancy but does not *per se* stimulate beta cell proliferation.

We hypothesize that expression of integrin $\alpha6\beta1$ is a prerequisite for postnatal replication competence of the β-cells. Whereas pancreatic ductal cells have a high mitotic competency[62,63], the mitotic activity during differentiation of the islet cell types is low[37]. The establishment of a basement membrane in late embryonic development may be a critical process in postnatal β-cell maintenance and adaptive β-cell growth in childhood, obesity and pregnancy. Impaired expression of integrin $\alpha6\beta1$ may contribute to the development of GDM.

MATERIALS AND METHODS

Reagents

Recombinant hGH (3 IU/mg) was obtained from Novo Nordisk (Gentofte, Denmark). Bovine GH (0.81 IU/mg) and ovine PRL (30 IU/mg) were purchased from UCB (Brussels, Belgium). Mouse anti-rat $\alpha6\beta1$ integrin antibody (MAB1410) was obtained from Chemicon (ThermoFisher, San Francisco, California, USA). Antibodies to bromodeoxyuridine (BrdU), M744, and to glucagon, A565, were obtained from DAKO (Agilent, Glostrup, Denmark). Guinea pig anti-insulin antiserum was provided by Novo Nordisk (Bagsværd, Denmark). Biotinylated goat anti-mouse-immunoglobulin G (IgG) antiserum, Texas-red-conjugated streptavidin, and fluorescein isothiocyanate (FITC)-conjugated goat anti rabbit IgG antiserum and rabbit anti- guinea pig IgG antibodies were purchased from DAKO. Histostain™ SP (peroxidase) Kits (Mouse and Rabbit) were obtained from ZYMED (ThermoFisher, San Francisco, CA, USA). Three different batches of 4 octapeptides were obtained from Affiniti Research Products Limited (Exeter, UK). The sequences were: disintegrin peptide DP: ANQECDVT, three control peptides CP1: TNCQEADV, CP2: AEDVCDLP and CP3: PECLVADD.

Islet isolation and culture

Pancreatic islets were isolated from 3-5 day-old rats, 2-3 month-old female, and pregnant (gestational day 13, 16, and 19) Wistar rats (Taconic Biosciences, Lille Skensved, Denmark by the collagenase method[64]. Freshly isolated adult islets were processed immediately for RNA isolation as described below. Neonatal rat islets were collected by centrifugation and distributed in 100 mm cell culture dishes (2-3000 islets/dish) and pre-cultured for 7 days in 10 ml RPMI 1640 medium supplemented with 20 mM Hepes, 100 units/ml penicillin, 100 μg/ml streptomycin and 10% newborn calf serum (Gibco, Paisley, Scotland). The islets were then stimulated with bGH, oPRL, or hGH (500 ng/ml) for 24 h in RPMI 1640 supplemented with 0.5% human serum (HS) and then collected by centrifugation and processed for RNA isolation as described below. Monolayer cultures of islet cells were established as previously described[11]. Briefly, islets were dispersed by

trypsin-EDTA treatment and plated in plastic cell culture slide flasks (Nunc, Roskilde, Denmark) in RPMI 1640 medium containing 2 % HS and 500 ng/ml hGH. It should be noted that the cells were cultured without addition of any coating components. The cells were allowed to attach and spread out for 5-7 days, where after the medium was changed. For proliferation experiments, the cells were washed twice in RPMI 1640 supplemented with 2 % HS but without hGH, and then cultured for 24 h in this medium. The medium was changed, and the cells were cultured for further 24 h in the presence of 10 μM BrdU and in the absence or presence of 500 ng/ml hGH and/or 250-500 μM octameric peptide. The slides were washed twice in serum-free medium and the cells then fixed in 1 % paraformaldehyde in 0.1 M phosphate buffer, pH 7.4.

Rat insulinoma cell line INS-1E

INS-1E cells were kindly donated by Dr. C.B. Wollheim, University of Geneva, Switzerland. The cells were cultured in RPMI 1640 supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 10% heat-inactivated fetal calf serum and 50 μM β-mercaptoethanol.

Transduction of INS-1E cells with recombinant adenovirus expressing STAT5 mutants.

Generation of recombinant adenovirus was carried out using the AdEasy kit from Q-BIOgene (Carlsbad, CA, USA) according to the manufacturer's instructions. The cDNA encoding WT mouse STAT5b and constitutive active STAT5b (also known as STAT5B 1*6) and dominant negative STAT5 (also known as STAT5Δ749) as described[22,65]. INS-1E cells were incubated with the virus for 24 h prior to the experiments.

RNA isolation and cDNA synthesis

Cultured islets and freshly isolated fetal pancreata were lysed in RNAzol (Sigma-Aldrich, Copenhagen, Denmark) and total RNA was extracted using the single step acid-guanidium-thiocyanate-

phenol-chloroform method, as described by the manufacturer. The RNA preparations were subjected to DNase treatment and the final RNA concentrations were determined from optical absorbance at A_{260} nm. Complementary DNA was synthesized from 1 µg of RNA by denaturation in 10 µL H_2O containing 0.5 µg random primers at 65°C for 1 min followed by incubation for 1 h at 42 C in a volume of 20 µl 130 mM Tris-HCl, pH 8.3, containing 5 mM $MgCl_2$, 20 mM KCl, 250 µM dNTP, 0.5 µg random primers (ThermoFisher) and 17 units AMV Reverse Transcriptase (Agilent, La Jolla, CA). The cDNA samples were stored at -20°C in 50 µL 0.1 % Triton X-100. For RT-qPCR, cDNA was synthesized using 500 ng RNA and the SuperscriptIII RT PCR system using hexamer primers (ThermoFisher).

Quantitative RT-PCR analysis

Primer sets complementary to cDNA encoding cell adhesion molecules and internal controls were purchased from ThermoFisher. The sequences of these primers and the expected lengths of the generated PCR products are given in Supplementary Table S1.

PCR was carried out in 50-µl reactions using 3 uL of cDNA as template basically as described [66]. The reaction mixture contained 50 mM KCl; 10 mM Tris-HCl, pH 9.0; 1.5 mM MgCl2; 40 mM dATP, dGTP, and dTTPs; 20 mM dCTP, 2.5 µCi of 3,000 Ci/mmol (α–^{32}P)dCTP (Amersham Pharmacia Biotech); 10 pmol of each primer and 2.5 units of AmpliTaq Gold polymerase (Perkin-Elmer/Roche). A single denaturing step at 94 C/10 min was followed by 25-30 cycles as given: 94 C/30 s; 55 C/1 min; 72 C/1.5 min. PCR products were analyzed by electrophoresis on 6 % denaturing polyacrylamide gels containing 7 M urea, 130 mM Tris, 80 mM boric acid and 0.15 mM EDTA. The gels were dried and exposed to PhosphorImage storage screens that were scanned by Molecular Dynamics Phosphorimager series 400 (GE Healthcare, Copenhagen, Denmark) and band intensities were quantified using the program Image-Quant (Molecular Dynamics). The expression of the reference genes G6PDH and TBP did not change by the hormone treatment or

during pregnancy. RT-Q-PCR on RNA from adenovirus-transduced cell samples was performed using Quantitect SYBR reagent (Qiagen, Ballerup, Denmark) with melting curve detection using a MX3005P instrument (Agilent Technologies) and TFIIB was used for normalization.

Immunocytochemistry

Pancreatic tissue from fetal, neonatal, and adult Wistar rats was immersion-fixed in 4 % (wt/vol) Para formaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) overnight at 4°C. The tissue was routinely embedded in paraffin and 4 μm sections were cut using a Leica 2055 Autocut microtome. The sections were treated with 1 % H_2O_2 for 20 min to remove endogenous peroxidase activity. Antigen retrieval of de-paraffinized and hydrated sections was performed in a Polar Patent (PP-780) precision pulsed laboratory microwave oven (Ax-Lab, Copenhagen, Denmark) at 600 Watt in 0.01 M citrate buffer pH 6.0 three times for 5 min followed by 10 min at room temperature. For integrin α6β1 peroxidase staining of rat; primary antibodies were added to the sections as indicated and incubated overnight at 4°C. Washing procedures and incubations with secondary biotinylated antibodies and enzyme conjugate were performed as recommended by the manufacturer of the Histostain-SP Bulk Kit. In some sections, nuclei were counter-stained with hematoxylin. Mounting of the slides was performed in Aquatex (Merck, Darmstadt, Germany). For integrin α6β1 and glucagon double-immunofluorescence staining the primary antibodies (1:25, and 1:200 dilutions, respectively) were added to the sections and incubated for 30 min at room temperature. Secondary biotinylated goat anti mouse IgG antibody and FITC-conjugated pig anti-rabbit IgG antibody were incubated for 30 min followed by incubation with Texas-red-conjugated streptavidin for a further 30 min. The fluorescence staining for integrin α6β1was enhanced using the TSA™ Biotin System (Perkin Elmer, Skovlunde, Denmark) according to the instruction by the manufacturer. Mounting of the slides was performed in Fluorescence Mounting Medium (DAKO, Agilent). For staining of dispersed rat islets in monolayer culture the fixed cells were washed twice

in PBS for 10 min. Slides to be stained for BrdU and insulin were exposed to 1.5 M HCl for 1 h and washed to neutrality. Incubation with primary antibodies was carried out in PBS containing 0.3 % Triton X-100 and 0.1 % human serum albumin (HSA) for 1 h at room temperature. BrdU and insulin antibodies were visualized using Texas-red conjugated goat anti-mouse-immunoglobulin G (IgG) and fluorescein isothiocyanate-conjugated goat anti-guinea pig IgG, respectively.

Transient transfection and Dual Luciferase Reporter (DLR) Assay.

INS-1E cells were transiently transfected with the PRLR 1A promoter firefly luciferase reporter construct as previously described[22] . Briefly, the cells were seeded in 24-well plates (~300,000 cells per well) and cultured overnight. The cells were transfected with reporter plasmid, internal standard Renilla luciferase plasmid, and carrier plasmid overnight using Lipofectamine 2000 Reagent (ThermoFisher) and incubation in OptiMem (ThermoFisher). The medium was changed to RPMI 1640 containing 0.25 % Bovine serum albumin and the cells were pre-incubated for 0.5 h in the absence or presence of peptides and further incubated in the absence or presence of hGH for 6 hours before harvesting and dual luciferase measurement. Cells were harvested using Passive Lysis Buffer and firefly and renilla luciferase activities determined using the Dual Luciferase Reporter System (Promega).

Determination of STAT5 phosphorylation by Western blot

INS-1E cells were seeded in 24-well plates and cultured for 48 h in RPMI 1640 supplemented with 10% FBS followed by 24 h in RPMI 1640 supplemented with 0.5 % FBS. Then the cells were exposed to 250 μM DP or CP and 0.5 μg hGH for 15 min. The cells were lysed and the proteins were separated by NuPAGE and blotted onto a PVDF membrane The membranes were stained with mouse anti-GAPDH (Abcam) and rabbit anti-phosphoSTAT5 (Cell Signalling Technology) using HRP conjugated goat anti-mouse antibody (Santa Cruz Biotechnology) and goat anti-rabbit

antibody (DAKO, Agilent), respectively, and ECL detection system (Pierce, ThermoFisher) for quantification.

Statistical analyses

Statistical analyses were performed using GraphPad Prism (vers. 7) and the specific statistical test is given in the legend for each figure. A p-value of <0.05 was considered significant. All data conform with good publication practice in physiology [67].

CONFLICTS OF INTEREST

The authors have no competing interests,

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Table 1. Basal mRNA levels of cell adhesion molecules in isolated neonatal rat islets.

	% of G6PDH mRNA expression	% of TBP mRNA expression
Prolactin receptor	63 ± 19 (6)	*nd
E-cadherin	18 ± 4.9 (5)	nd
N-Cadherin	31± 2.7 (3)	nd
Integrin α1	3.6 ± 0.8 (4)	nd
Integrin α3	109 ± 26 (3)	2472 ± 512 (5)
Integrin α6A	7.5 ± 1.7 (5)	183 ± 45 (4)
Integrin α6B	6.6 ± 1.8 (3)	235 ± 80 (4)
Integrin β1	252 ± 85 (4)	nd
Integrin β4	nd	4.7 ± 2.6 (4)
Dystroglycan	3.8 ± 0.5 (3)	nd
Laminin α1	nd	1.4 ± 0.8 (4)
Laminin α3	nd	146 ± 92 (5)
Laminin α5	nd	58 ± 33 (6)

*nd=not determined. Data are calculated as percentage of the mRNA levels of G6PDH and TBP, respectively, and are presented as means ± SEM. The number of independent RNA isolations analyzed are given in brackets.

LEGENDS TO FIGURES

Fig. 1. RT-PCR analysis of the hormonal regulation of cell adhesion molecules in neonatal rat islets.

Quantification of the effects of bGH, oPRL, and hGH on the mRNA levels of PRLR and cell adhesion molecules. The quantified PCR products were normalized to G6PDH or TBP, and the results are expressed as fold increase (mean $\pm$ SEM, n=3-6) over the basal mRNA levels. Statistical significance was analysed using Students t-test, * $p<0.05$, †$p<0.01$.

Fig. 2. RT-PCR analysis of islet integrin $\alpha 6$ mRNA expression during rat pregnancy.

Total RNA isolated from freshly isolated islets from adult female and pregnant animals (n = 4-6 within each group) was subjected to cDNA synthesis and RT-PCR as described above. Data are expressed as fold increase over the basal mRNA levels in the non-pregnant animals and represent the mean $\pm$ SEM of 3-4 independent cDNA preparations and RT-PCR reactions. Statistical significance was analysed using Students t-test, * $p<0.05$, †$p<0.01$, ‡$p<0.001$.

Fig. 3. Immunohistochemical detection of integrin $\alpha 6\beta 1$ in pancreatic rat β-cells at different ages.

Immunostaining of adult (A and B), 5 day-old (C and D), 2 day old (E and F), and fetal E19 (G and H) rat pancreata with mouse anti-rat integrin $\alpha 6\beta 1$ antibody (1:500 dilution) on the left hand side (A, C, E, G). Consecutive sections stained with an anti-insulin antiserum (1:1000 dilution) are shown on the right hand side (B, D, F, H). Bar size represents 50 μm.

Fig. 4. Immunohistochemical detection of integrin α6β1 in pancreatic rat islets.

Double-immunostaining of adult rat pancreas for glucagon and integrin α6β1. A) Staining for integrin α6β1 (using MAB1410) in a representative islet visualised in red using Texas Red-conjugated streptavidin and biotinylated goat anti-mouse IgG secondary antibody. B) Staining for glucagon (using A565) visualized in green using FITC-conjugated pig anti-rabbit IgG secondary antibody. C) Superimposition of the images shown in A and B. D) Hoechst staining showing total cell nuclei of the section in blue.

Fig. 5: Immunohistochemical detection of integrin α6β1 in dispersed pancreatic rat islet cells.

Double immunofluorescence staining of hGH-promoted dispersed rat islets cells in monolayer culture for insulin (A), and integrin α6β1 (B) using FITC- and Texas-red conjugated secondary antibodies, respectively. The two fluorophore images are combined in C. The arrowhead identifies a cell that is insulin-negative, but integrin α6β1-positive. Bar size represent 10 μm.

Fig. 6: STAT5 activation increases expression of integrin α6 mRNA in INS-1E cells.

Transduction of INS-1E cells with recombinant adenovirus expressing dominant negative STAT5 (DN-STAT5) or constitutively active STAT5 (CA-STAT5) mutants. Cells were transduced for 24hrs, cultured with 0.5% FCS for 24h, followed by 4h of stimulation using hGH or PBS as control, before RNA extraction and RT-qPCR. The values are normalized to the negative control, C. Statistical significant differences were evaluated by one-way ANOVA followed by Tukey's multiple comparison test. *$p<0.05$, n=5-9.

Fig. 7: Effects of disruption of laminin-integrin α6β1 interaction on hGH induced β-cell proliferation, PRLR transcription and STAT5 activation. A: Dispersed neonatal rat islet cells in monolayer culture were deprived of hGH for 24 h and then incubated with 10 μM BrdU in the absence or presence of hGH and in the absence or presence of 250 μM octamer peptide for 24 h. The cells were fixed and double immunofluorescence stained for insulin and BrdU and double-positive cells were counted under the microscope. Data were calculated as the percentage of BrdU positive cells per total number of insulin-positive cells counted (1000-2000/dish) and are given as the fold increase over the basal level (without hGH). The basal number of BrdU-positive β-cells was 2-4 %. DP: disintegrin peptide; CP1: control peptide 1; CP2: control peptide 2; CP3: control peptide 3. Data represent mean ± SEM of 4 independent experiments. Statistical significant differences were evaluated by one-way ANOVA followed by Tukey's multiple comparison test. *, p<0.05 between DP and C or CP1-3, respectively. B: INS-1 cells were transiently transfected with a PRLR-promoter-luciferase construct overnight. Cells were pre-incubated for 0.5 h in the absence or presence of 250 μM DP or CP1 and then further incubated for 6 h in the absence or presence of 500 ng/ml hGH. Cells were extracted and subjected to Dual-luciferase assay. Data represent mean ± SEM of 4 independent experiments performed in triplicate. The values are normalized to the negative control C. Statistical significant difference was evaluated by one-way ANOVA followed by Tukey's multiple comparison test *p<0.05, ‡p<0.0001. C: Phosphorylation of STAT5 was measured in INS-1E cells incubated with 250 μM DP or CP1 for 15 min followed by addition of 0.5 μg/ml hGH for 15 min. Data represent mean+/- SEM of 5 independent experiments. The values are normalized to the negative control C. Statistical significant difference was evaluated by one-way ANOVA followed by Tukey's multiple comparison test. *p<0.05, ‡p<0.001.
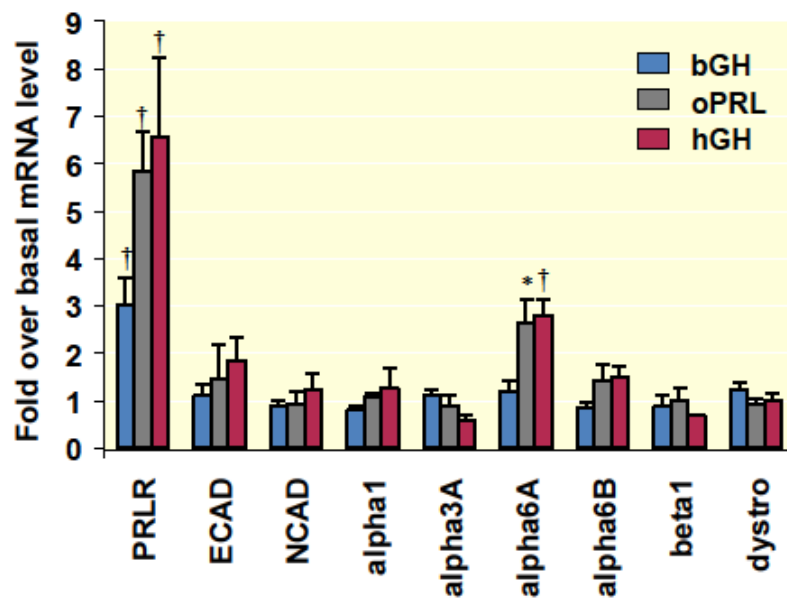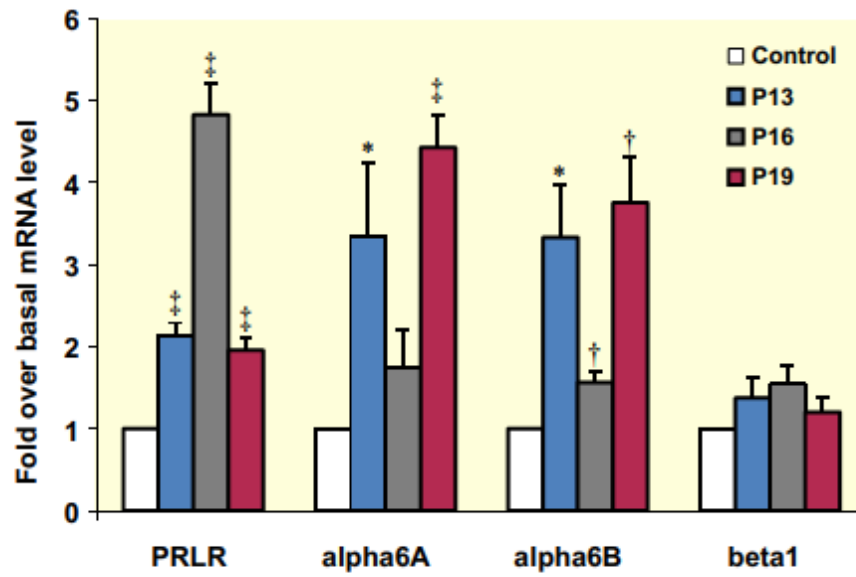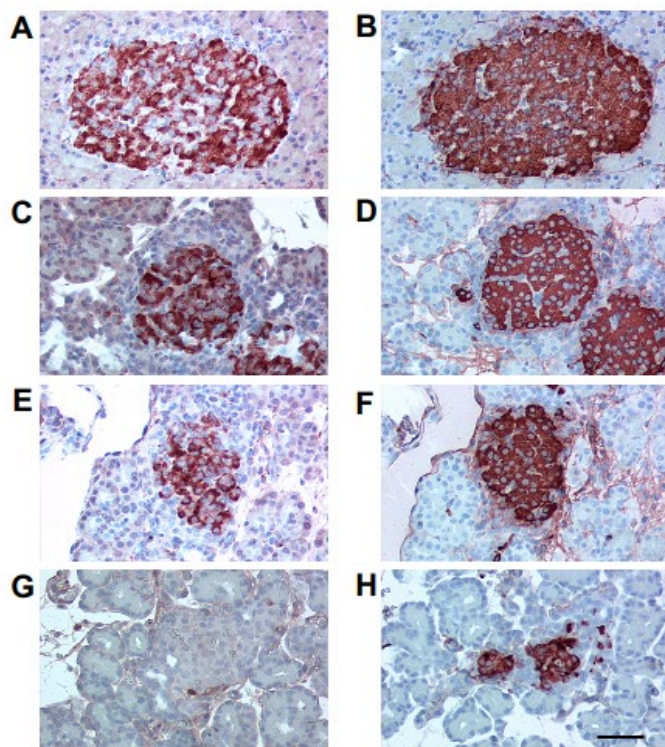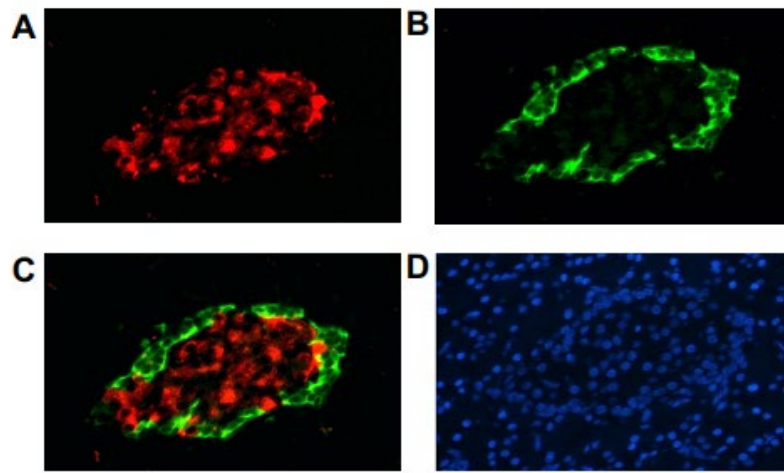
Figure 1

Figure 2

Figure 3

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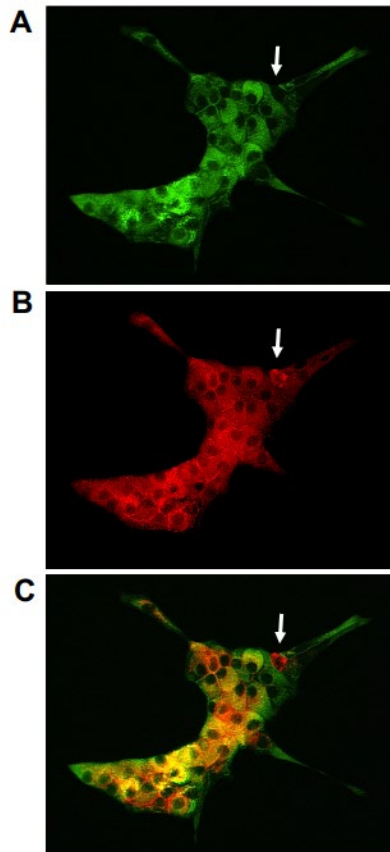
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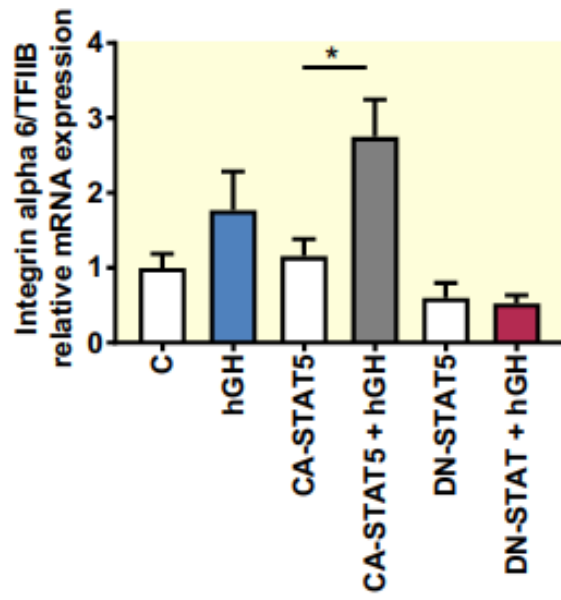
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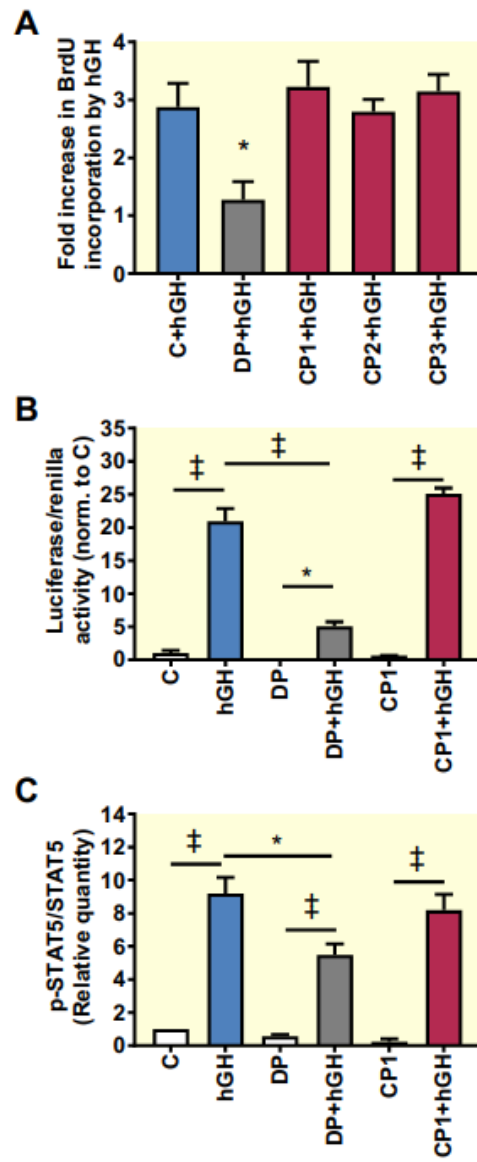
Supplementary material

Table S1. Sequences of primers for RT-PCR

Gene	5' primer	3' primer	Product size (bp)
Rat glucose-6-phosphate dehydrogenase (G6PDH)	gacctgcagagctccaatcaac	cacgaccctcagtaccaaaggg	214
Mouse tata-binding protein (TBP)	acccttcaccaatgactcctatg	atgatgactgcagcaaaccgc	190
Rat prolactin receptor (PRLR)	gctgatgtgtgcaagctagccggaa	gacaccttggcatactccttactgg	340
Mouse epithelial cadherin (ECAD)	tattactatgatgaagaaggaggtg	ccgtacatgtccgccagcttcttg	373
Mouse neural cadherin (NCAD)	caggactatgacttgagccagctcc	agtcattcaggtagtcatagtcctg	320
Rat integrin α1	ctgaggcagatatcacggagctttt	ttgttgcacttaagtgaatgattgc	585
Mouse integrin α3	gagctggtggaggagctgcccgctg	gtgggtagcgtgctccctggaggtg	A: 355 B: 211
Mouse integrin α6	<u>gcacagcaaccttgaatatacagtg</u>	<u>ctcagccttgtgataggtggcatcg gaatgctgtcatcgtacctagagcg</u>	A: 634 B: 615
Rat integrin β1	acactggcagtgcatgtgactgttc	ggattgaccacagttgtcacggcac	624
Mouse integrin β4	gattcatccaacatcgtggagctgc	attgcacacacagtgtccacacatg	490
Mouse dystroglycan	ctgagtaccccaaccagagtatgcc	actgcctgcgagttcattcacggtt	543
Mouse laminin α1	agccctggaggcccggaaccatttc	ctccagcataacctttcctacatgg	374
Rat laminin α3	cgagacatcaaaagcttccctgcgg	agttcctcacccatggggaccaagt	499
Mouse laminin α5	tgattgcaatgctgcagggacccag	cggccagcctcgtcacagccttcag	330
Rat integrin α6A	tcactgtccagagtcaaggtc	gactcaggacgtaacaccttcc	126