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A factor(s) secreted from MIN-6 β-cells stimulates differentiation of definitive endoderm enriched embryonic stem cells towards a pancreatic lineage

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Abstract

In the mouse the developing pancreas is controlled by contact with, and signaling molecules secreted from, surrounding cells. These factors are best studied using explant cultures of embryonic tissue. The present study was undertaken to determine whether embryonic stem (ES) cells could be used as an alternative model *in vitro* system to investigate the role of cell-cell interactions in the developing pancreas. Transwell culture experiments showed that MIN-6 β-cells secreted a factor or factors that promoted differentiation of ES cell derived definitive endoderm enriched cells towards a pancreatic fate. Further studies using MIN-6 condition medium showed that the factor(s) involved was restricted to MIN-6 cells, could be concentrated with ammonium sulphate, and was sensitive to heat treatment, suggesting that it was a protein or peptide. Further analyses showed that insulin or proinsulin failed to mimic the effects of the conditioned media. Collectively, these results suggest that β-cells secrete a factor(s) capable of controlling their own differentiation and maturation. The culture system described here presents unique advantages in the identification and characterisation of these factors.

Introduction

During embryogenesis in the mouse the pancreas first appears as evaginations on the dorsal and ventral surfaces of the primitive foregut at around embryonic day 9.5 (E9.5) [1]. This specification is associated with expression of the transcription factors Pdx1 and Ptf1a, which serve to divert cells away from duodenal, hepatic and duct fates [2]. Once formed the stalk of the pancreatic buds elongates with branching morphogenesis occurring within the apical regions. Multipotent progenitors that give rise to all cell types in the pancreas are present at this branching stage, localised predominantly within the tip regions [3]. At around E12 to E13 the gut rotates, the two buds come into contact with each other and fuse. During this period, termed the secondary transition [4], the multipotent progenitor cells begin to differentiate into the exocrine (acinar and duct) and endocrine cells of the pancreas.

The establishment and expression of the various cell lineages is controlled by the sequential expression of transcription factors [5] in response to signalling molecules emanating from cells within the expanding pancreas and surrounding tissues. There are two possible types of signal, instructive and permissive. In a study that involved grafting blocks from various regions along the anterior-posterior axis from quail embryos into host chick embryos, it was demonstrated that lateral plate endoderm produced instructive signals that could induce pancreatic differentiation in naive endoderm [6]. On the other hand notochord, endothelium and mesenchyme produce permissive signals [7], i.e. signals that do not establish the pancreatic domain but act upon endoderm that has already been pre-patterned towards pancreas.

Experimental approaches aimed at characterising the signalling molecules that control development of the pancreas at present involve explant cultures of embryonic

tissues [1]. Thus growth factors or conditioned media would be added directly to the embryonic pancreatic tissue, or the tissue would be co-cultured with other cell types. An alternative approach might be to use embryonic stem (ES) cells. A number of robust protocols have been developed whereby mouse and human ES cells can be induced to differentiate into β-like cells by mimicking the events that occur in the developing pancreas of the mouse embryo [8]. The cells are first induced to form definitive endoderm (the germ layer which gives rise to the pancreas), and from there sequentially to pancreatic progenitors, endocrine cells, and β-cells. Although much progress has been made in generating insulin- secreting cells from ES cells, it has not been possible to generate a fully functional β-cell using currently available differentiation protocols [9]. However , if pancreatic progenitors generated from ES cells are grafted into mice, then over a prolonged period of time these cells mature into cells that exhibit many of the functional characteristics of β-cells [10] demonstrating that fully functional pancreatic cells can be generated from pluripotent stem cells.

Here we describe how mouse ES cells that have been induced to form an enriched population of definitive endoderm can be used to identify and characterise factors secreted from MIN-6 β-cells that affect their further differentiation towards a pancreatic lineage. We anticipate that this *in vitro* system will have widespread applications in studies on differentiation factors secreted from other cell types, including those present in blood vessels and mesenchyme.

Methods

Cell culture

The mouse embryonic stem cell line CGR8 [11] was maintained in a pluripotent state in maintenance medium supplemented with leukaemia inhibitory factor (LIF) in tissue culture flasks coated with 0.1 % (w/vol) gelatin (Sigma-Aldrich). Maintenance medium consisted of knockout DMEM (Invitrogen) supplemented with 15 % (vol/vol) knockout serum replacement (Invitrogen), 0.1 mM MEM non essential amino acids (Invitrogen), 2 mM L-glutamine (Invitrogen), and 140 μM 2-mercaptoethanol (Sigma-Aldrich). MIN-6, a mouse β-cell line [12] was maintained in high glucose DMEM (Invitrogen) supplemented with 15 % foetal calf serum (FCS) (Invitrogen) and 70 μM 2-mercaptoethanol (Sigma-Aldrich). PANC-1, a human pancreatic carcinoma, epithelial-like cell line (ATCC® Number: CRL-1469™) was maintained in high glucose DMEM supplemented with 10 % FCS and 4 mM L-glutamine (Invitrogen). HEK-293, a human foetal kidney cell line (ATCC® Number: CRL-1573™) and N2A, a rat neuronal cell line (ATCC® Number: CCL-131™), were maintained in low glucose DMEM supplemented with 10% FCS,

Differentiation of CGR8 cells towards definitive endoderm

CGR8 cells were cultured as embryoid bodies (EBs) for 6 days in chemically defined medium (CDM) [13]. Essentially this involved seeding CGR8 cells at a density of 1.3 x 10^6 cells per 6 cm bacterial grade Petri dish (Greiner Bio-one) and gently mixing them on a horizontal shaker for 2 days at 37˚C and 5 % CO_2. The medium was then supplemented with activin A and/or BMP4 (R&D Systems) as indicated in the results section.

Differentiation of definitive endoderm enriched cultures towards pancreatic endoderm

Definitive endoderm enriched embryoid bodies (EB)s were seeded at a density of 30 EBs per well in growth factor reduced Matrigel™ (BD Biosciences) coated 6-well plates. The resultant outgrowths were cultured in CGR8 maintenance medium. In some experiments the outgrowths were co-cultured with MIN-6 cells using 0.4μm membrane transwells (Greiner Bio-One) in CGR8 maintenance medium.

Preparation of conditioned media

Conditioned media were prepared by incubating a 20-30% confluent culture of MIN-6, HEK-293, PANC-1 cells, and N2A cells in CGR8 maintenance medium. Conditioned media were collected after 24 (CM24) or 48 (CM48) hours, filtered through a 0.22 μm filter, and stored at -80˚C until used.

Preparation of RNA and quantitative PCR

Preparation of RNA and quantitative PCR (qPCR) were performed as previously described [14]. Inventoried Taqman© gene expression assays (Applied Biosystems) were used for qPCR. Relative gene expression for each sample was determined using the Pfaffl formula [15]. Randomisation tests to estimate p-values were done with REST© software [16].

Ammonium sulphate precipitation

To generate a 70% fraction, ammonium sulphate powder was added slowly with stirring to conditioned medium at 4˚C. This was left without stirring for 15 – 30 min

to allow all the salt to dissolve. The solution was then centrifuged at 5000 x g for 10 min. The supernatant was decanted, while the pellet was resuspended in 50 mM Tris-acetate buffer, pH 7.0. If a second precipitation step was undertaken, ammonium sulphate powder was slowly added to the supernatant up to the second precipitation concentration. The solution was centrifuged as before, and the pellet dissolved in 50 mM Tris acetate buffer. Both samples were dialysed four times against at least 100 volumes of 50 mM Tris acetate buffer.

Protein assay

Protein concentrations were determined using the DC protein assay (Bio Rad) according to the manufacturer's instructions.

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde solution (PFA), and permeabilised with 0.2% Triton. The following antibodies were used: guinea pig anti-insulin (Abcam) at 1/125, goat anti-Nkx6.1 (R&D) at 1/100, and rabbit anti-Pdx1 (kindly provided by Dr C. Wright, Vanderbilt University) at 1/400.

Results

ES cells can be induced to form endoderm by treatment with activin A as a surrogate for nodal signalling [17-19]. Thus, when ES cells (CGR8) were cultured as embryoid bodies (EBs), activin A at 30 ng/ml and 100 ng/ml increased the levels of the definitive endoderm markers Sox17, Foxa2 and CXCR4, while decreasing the expression of the mesoderm marker Bry (Fig. 1a). Although BMP-4 is a powerful inducer of mesoderm [20] we found in our previous study [14] and in the present study that prior treatment with BMP-4 before activin A increased the levels of the definitive endoderm markers, while reducing the levels of the extraembryonic endoderm markers Sox7 and Afp, which remained elevated with activin A alone. Treatment with BMP-4 for 3 days followed by activin A for 3 days (B,A) also decreased the levels of the mesoderm marker Bry and the ectoderm marker Zic1, but like activin A alone had little effect on the primitive streak markers Mixl1 and Gsc. We were also able to show by immunocytochemistry that the percentage of Sox17 and CXCR positive cells following B,A treatment was similar to that following treatment with activin A alone [14]. For these reasons all subsequent experiments were performed on a definitive endoderm enriched population generated using BMP-4 and activin A, because these conditions generated the most consistent decrease in non definitive endoderm markers.

To determine whether MIN-6 β-cells secreted factors that could affect the differentiation of ES cells towards a pancreatic lineage, the definitive endoderm enriched EBs were cultured as outgrowths in transwell culture dishes in the presence or absence of MIN6-cells. Following differentiation in the transwell dishes for 7 days (DD7), most of the cells within, and especially towards the periphery of the EB, stained positively for the transcription factor Nkx6.1 (Fig. 2A) with no detectable

staining for Pdx1 (Fig. 2B). At day 14 there were scattered clusters of cells within the outgrowth that co-stained for Nkx6.1 and Pdx1 and for insulin and Pdx1 (Fig 2, E-L). These cell clusters increased in number from day 7 to day 14, and although they could rarely be detected in cultures in the absence of MIN-6 cell, their presence was increased significantly by the MIN-6 cells (Fig. 3). These results suggested that the MIN-6 cells were secreting a factor(s) that was stimulating the production of clusters of pancreatic endocrine cells in the DE–enriched outgrowths.

In order to characterise the pancreatic differentiation factor(s) secreted from MIN-6 cells the DE-enriched outgrowths were cultured in KO DMEM (control media) or the same media that had been exposed to MIN-6 cells for 24 or 48 hours. The results showed that the conditioned media stimulated the expression of the early pancreatic transcription factors Hnf1β, Pdx1 and Ptf1a (Fig. 4), suggesting that CM from MIN-6 cells could stimulate differentiation of endoderm cells into pancreatic progenitors. We also observed that CM from MIN-6 cells was able to increase the expression of β cells markers (insulin 1, insulin 2, islet amyloid polypeptide (IAPP), glucose transporter 2 (GLUT2)), and alpha cell (glucagon), delta cell (somatostatin) and, acinar cell (amylase) markers (Fig. 4), which is in keeping with the stimulated appearance of insulin positive cells in the transwell experiments. To confirm this stimulatory effect EBs were grown for up to 19 days (Fig. 5) in the presence of CM from MIN-6 and Q-PCR showed a strong and robust increase in Pdx1, insulin 1, insulin 2 and glucagon expression. In addition, the effect was dose-dependent (Fig. 6). We were unable to detect insulin or Pdx1 by immunocytochemistry in CM treated cultures, which was in keeping with the low Ct values. This probably relates to differences between the CM and transwell experiments: in the transwell experiments the DE-enriched cells were treated with higher amounts of newly synthesised factor(s)

for longer periods of time. However, taken together, the CM results confirmed that β-cells could produce a factor(s) capable of promoting pancreatic differentiation of ES-derived definitive endoderm. Importantly, these results could not be explained by uptake of mRNA from the conditioned media. Indeed, we could not detect mRNA for insulin 1, insulin 2 and Pdx1 in the conditioned media, suggesting that the filtration step had efficiently removed dead cells and any debris with which nucleic acids might be associated.

The pancreatic differentiation factor(s) appeared to be β- cell specific in that it was absent from HEK-293, Panc-1 and N2A cells (Fig. 7). Interestingly, conditioned media from HEK-293 had a marked stimulatory effect on the insulin 2 gene but not the insulin 1 gene. This may be explained by the fact that, whereas insulin 1 is expressed predominantly in β cells during embryogenesis, insulin 2 is expressed in cells of the nervous system, liver and yolk sac [21,22].

In order to characterise the MIN-6 cell factor(s) the conditioned media was subjected to ammonium sulphate precipitation. Expression of both insulin 1 and insulin 2 was stimulated by the 70% ammonium sulphate fraction (Fig. 8). There was some activity present in the 100% ammonium sulphate fraction and this activity was most pronounced upon the insulin 2 gene. Notably, an 85% ammonium sulphate fraction stimulated the expression of insulin 1 and insulin 2 to the same extent as the conditioned medium (Fig. 8). These results suggested that the factor(s) was a protein or small peptide that could be inactivated by heat treatment at 95^{o} C for 30 minutes (Fig. 8).

Of all the proteins secreted by a MIN-6 cell, insulin is by far and away the most abundant. We therefore tested whether insulin at physiological concentrations, 2.4 and 5.8 ng/ml, or in excess, i.e. 7 μg/ml, could mimic the effects of the MIN-6

conditioned media. The results showed a complete lack of effect of exogenous insulin on the expression of insulin 1 or insulin 2 at either low or high concentrations (Fig. 9). Although the MIN-6 cells can efficiently process proinsulin to insulin, the small amount of proinsulin in the media could be responsible for the differentiation activity. We therefore tested the effect of recombinant human proinsulin. The results showed that, like insulin, the exogenous proinsulin had no effect on the expression of insulin 1, or insulin 2 (Fig. 10). Together, these results demonstrate that neither insulin nor proinsulin is the secreted factors that are capable of increasing pancreatic differentiation of ES-derived definitive endoderm

Discussion

The major finding of the study was that a factor or factor(s) secreted by in MIN-6 β-cells could enhance differentiation of definitive endoderm enriched cells towards a pancreatic lineage, suggesting that β-cells control part of their own development and maturation. This confirms and extends previous findings that pancreatic extracts [23,24] and adult islets cells [25] may provide signals to precursor cells that affect differentiation to endocrine pancreatic lineages.

The presence of the MIN-6 cells or CM stimulated expression of both early and late pancreatic markers, which is in keeping with evidence that β-like (i.e. insulin positive) cells are present in two waves during embryogenesis in the mouse. The first wave begins at about E9.5 with the appearance of insulin^{+}, glucagon^{+}, and cells that stain double positive for both insulin and glucagon [4]. These early insulin^{+} cells lack many of the markers of mature β cells [26], and it is unlikely that these cells contribute to mature β- cells and their fate is unclear [27]. Our results on the effect of

MIN-6 media on early markers of pancreatic differentiation suggest that these "vestigial" β-cells may function to regulate the early stages of pancreatic formation.

The second wave of β-cell formation occurs during the secondary transition after E13. A recent study has shown that β-cells present during this stage of development may interact with other endocrine cells to establish the correct proportion of endocrine cells [28]. Thus a conditional knockout of Pdx1 at late gestation prior to islet formation led to a dramatic reduction in insulin$^+$ cells and an increase in glucagon$^+$ and somatostatin$^+$ cells in the neonatal islets. These paracrine effects of the β-cell on the adjacent endocrine cells are not particularly rapid, since in another study that involved toxin-mediated ablation of β- cells no effect on the development of glucagon$^+$ and somatostatin$^+$ cells was observed at E16.5 [29]. Taken together these results clearly demonstrate the existence of a factor(s) produced by β-cells themselves that influence their differentiation and maturation.

Importantly, the *in vitro* culture system described here presents unique advantages toward identifying these factors. It allows one to undertake large scale experiments impossible to perform *in vivo,* and also to screen efficiently for the effects of potential candidates. As an example, this system already allowed us to demonstrate that the factor(s) was absent from other cells lines that were tested and was likely to be a protein or small peptide based on ammonium sulphate precipitation and heat stability. In addition, the factor was not insulin since exogenous insulin had no effect on the differentiation of the definitive endoderm enriched cells. This is not unexpected since high concentrations of insulin are normally present in media used to culture and differentiate ES cells, and there is no evidence that insulin can enhance formation of pancreatic lineages. The lack of effect of proinsulin further suggests that the factor is unlikely to be a product of the insulin gene, which would be in keeping

with previous observations that a double mutation for insulin 1 and insulin 2 had no major developmental defects, although the islets were slightly larger [30,31].

Interestingly, it was previously shown that conditioned media from pancreatic fibroblasts were able to induce β-cell proliferation and formation of islet-like structures. These effects were specifically blocked using an anti-HGF antibody [32]. Therefore, it would be interesting to test if HGF can mimic the effect of MIN-6 conditioned media and if this effect can be blocked by anti-HGF antibodies.

In summary, our results suggest that the developing β- cell may secrete a factor(s) that affects the development of the pancreas. The culture system described here will facilitate the purification and characterisation of this factor(s) and allow us to determine whether it is present and capable of functioning during the first and/or second waves of β-cell expression. Such a factor(s) would have important applications in generating a replenishable supply of β- cells from ES or iPS cells. The ES differentiation system may also be of use in characterising pancreatic differentiation factors from endothelial and mesenchymal cell populations that are known to secrete factors that control cell fate during pancreatogenesis.

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Figure Legends

Figure 1. Effect of activin A and BMP-4 on the differentiation of mouse ES cells.

ES cells were cultured as EBs in CDM for 6 days with 30 ng/ml activin A (30 A), 100 ng/ml activin A (100A), 1 ng/ml BMP-4 for 3 days followed by 100 ng/ml activin A for 3 days (B,A), and 10 ng/ml BMP-4 and 100 ng/ml activin for 3 days followed by 100 ng/ml activin A for 3 days (AB,A). The untreated ES cells (ESC) are also shown. The cells were harvested, RNA prepared and the indicated mRNA levels were measured by qPCR. The data are expressed as fold change in gene expression relative to GAPDH and normalised to an untreated control (N/A). The results represent mean ± SD where n=3 (* $p<0.05$, ** $p<0.01$, *** $p<0.001$ relative to the untreated control).

Figure 2. Min-6 cells secrete a factor(s) that stimulates differentiation towards a pancreatic lineage.

DE-enriched outgrowths were co-cultured with MIN-6 cells in 0.4 μM transwell dishes. After 7 or 14 days of differentiation (DD7 or DD14) the cells were stained with the indicated antibodies. The staining in panels A-D are from DD7 cultures and show an attached EB with some outgrowth. Panels E-L show staining within the outgrowth which now covers a substantial portion of the dish. The data shown here are from a single experiment that was repeated on three separate occasions with similar results.

Figure 3. The presence of MIN-6 cells stimulates the formation of cell clusters.

DE-enriched outgrowths were co-cultured with MIN-6 cells in 0.4 μM transwell dishes. After 7 or 14 days of differentiation (DD7 or DD14) the cells were stained with the indicated antibodies and number of antibody positive cell clusters counted.

The results represent the mean ± SEM of three separate wells of a 6- well dish. The experiment was repeated 3 times with a similar result. * $p<0.05$ relative to cells incubated without MIN6 cells.

Figure 4. MIN-6 conditioned media induces differentiation of definitive endoderm enriched cells towards pancreas.

Definitive endoderm enriched cells were cultured for 10 days as a monolayer in DMEM (N/A) or condition medium from 24h (CM24) or 48h (CM48) cultures of MIN-6 cells. The cells were harvested, RNA prepared and the indicated mRNA levels were measured by qPCR. The data are expressed as fold change in gene expression relative to GAPDH and normalised to an untreated control (N/A). The results represent mean ± SD where n=3 (* $p<0.05$, ** $p<0.01$, *** $p<0.001$ relative to the untreated control).

Figure 5. Prolonged exposure to MIN-6 conditioned medium enhances the stimulatory effect on pancreatic markers.

Definitive endoderm enriched cells were cultured as a monolayer for 10, 15 or 19 days in DMEM (N/A) or conditioned medium from 48-hour cultures of MIN-6 cells (CM). The cells were harvested, RNA prepared and the indicated mRNA levels were measured by qPCR. The data are expressed as fold change in gene expression relative to GAPDH and normalised to an untreated control (N/A). The results represent mean ± SD where n=3 (* $p<0.05$, ** $p<0.01$, *** $p<0.001$ relative to the untreated control).

Figure 6. The effect of MIN-6 conditioned media is dose dependent.

Definitive endoderm enriched cells were cultured as a monolayer for 10 days in DMEM (N/A) or neat (1x) or four times concentrated conditioned medium (4x) from 48-hour cultures of MIN-6 cells. The cells were harvested, RNA prepared and insulin 1 (ins1) and insulin 2 (ins2) mRNA levels were measured by qPCR. The data are expressed as fold change in gene expression relative to GAPDH and normalised to an untreated control (N/A). The results represent mean ± SD where n=3 (* $p<0.05$, ** $p<0.01$, *** $p<0.001$ relative to the untreated control).

Figure 7. The pancreatic differentiation factor(s) is restricted to MIN-6 cells.

Definitive endoderm enriched cells were cultured as a monolayer for 10 days in DMEM (N/A) or conditioned medium from 48-hour cultures of MIN-6, Panc-1, HEK-293 and N2A cells. The cells were harvested, RNA prepared and the indicated mRNA levels were measured by qPCR. The data are expressed as fold change in gene expression relative to GAPDH and normalised to an untreated control (N/A). The results represent mean ± SD where n=3 (* $p<0.05$, ** $p<0.01$, *** $p<0.001$ relative to the untreated control).

Figure 8. The pancreatic differentiation factor(s) can be concentrated using ammonium sulphate.

Definitive endoderm enriched cells were cultured as a monolayer for 10 days in DMEM (N/A) or conditioned medium from 48-hour cultures of MIN-6 cells, or with a 70% (F70), 85% (F85) or 100% (F100) ammonium sulphate fraction from the condition medium. F70 + F100 contains a combination of the F70 and F100 fractions. The effect of the heat-treated F70 fraction is also shown. The cells were harvested,

RNA prepared and the indicated mRNA levels were measured by qPCR. The data are expressed as fold change in gene expression relative to GAPDH and normalised to an untreated control (N/A). The results represent mean ± SD where n=3 (* $p<0.05$, ** $p<0.01$, *** $p<0.001$ relative to the untreated control).

Figure 9. The pancreatic differentiation factor(s) is not insulin.

Definitive endoderm enriched cells were cultured as a monolayer for 10 days in DMEM (N/A) or conditioned medium from 48-hour cultures of MIN-6 cells (CM), or supplemented with exogenous insulin at the indicated concentrations. The cells were harvested, RNA prepared and the indicated mRNA levels were measured by qPCR. The data are expressed as fold change in gene expression relative to GAPDH and normalised to an untreated control (N/A). The results represent mean ± SD where n=3 (* $p<0.05$, ** $p<0.01$, *** $p<0.001$ relative to the untreated control).

Figure 10. The pancreatic differentiation factor(s) is not proinsulin.

Definitive endoderm enriched cells were cultured as a monolayer for 10 days in DMEM (N/A) or conditioned medium from 48-hour cultures of MIN-6 cells (CM), or supplemented with exogenous proinsulin at the indicated concentrations. The cells were harvested, RNA prepared and the indicated mRNA levels were measured by qPCR. The data are expressed as fold change in gene expression relative to GAPDH and normalised to an untreated control (N/A). The results represent mean ± SD where n=3 (* $p<0.05$, ** $p<0.01$, *** $p<0.001$ relative to the untreated control).

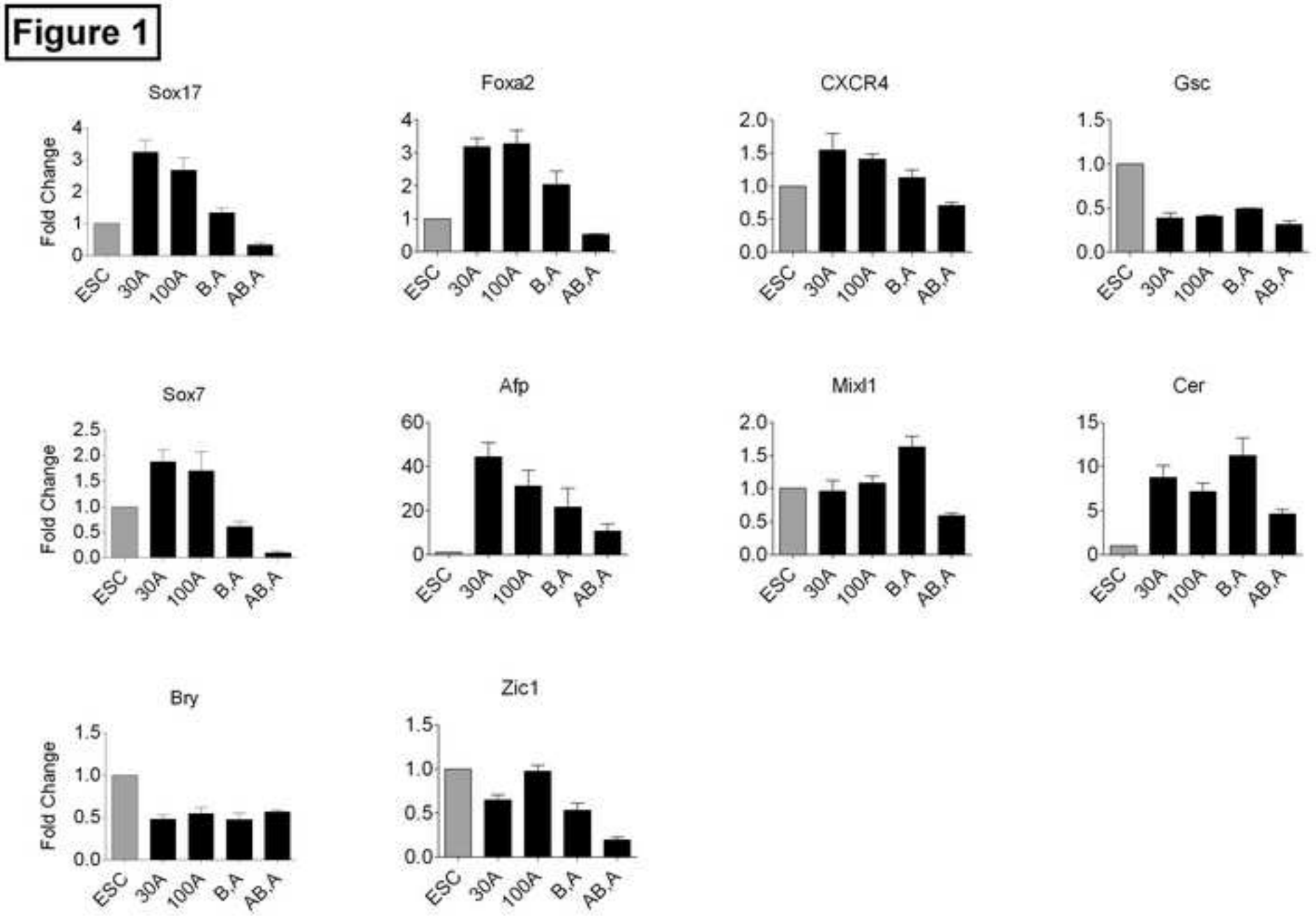
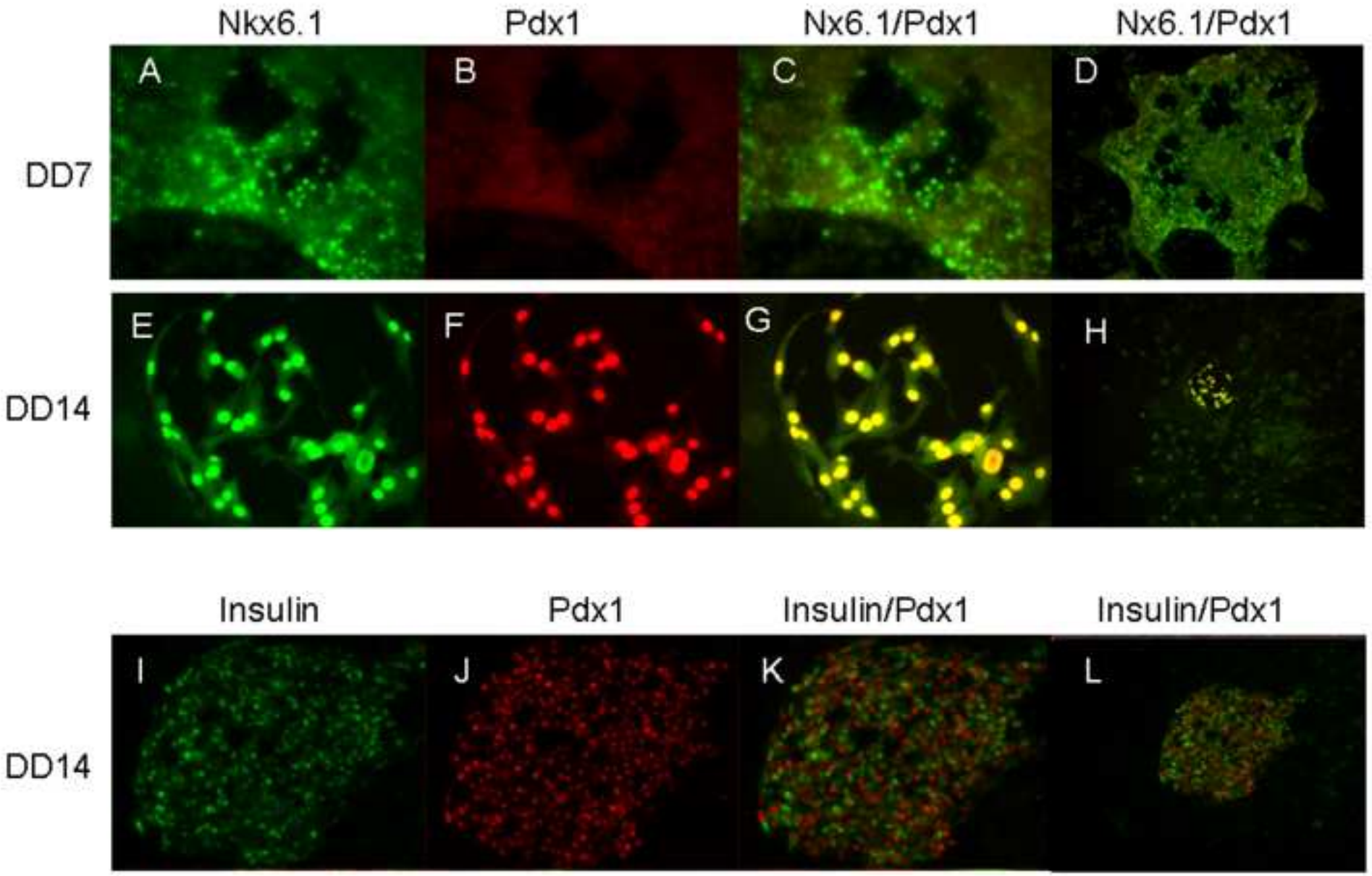


Figure 1
Sox17
Foxa2
CXCR4
Gsc
Sox7
Afp
Mixl1
Cer
Bry
Zic1
Fold Change
ESC
30A
100A
B,A
AB,A

Figure 2


Nkx6.1
Pdx1
Nx6.1/Pdx1
Nx6.1/Pdx1
A
B
C
D
DD7
E
F
G
H
DD14
Insulin
Pdx1
Insulin/Pdx1
Insulin/Pdx1
I
J
K
L
DD14

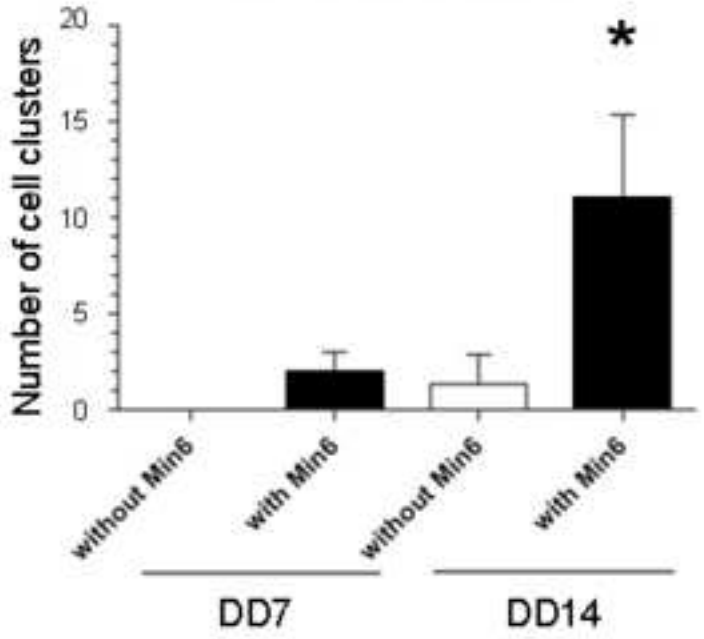
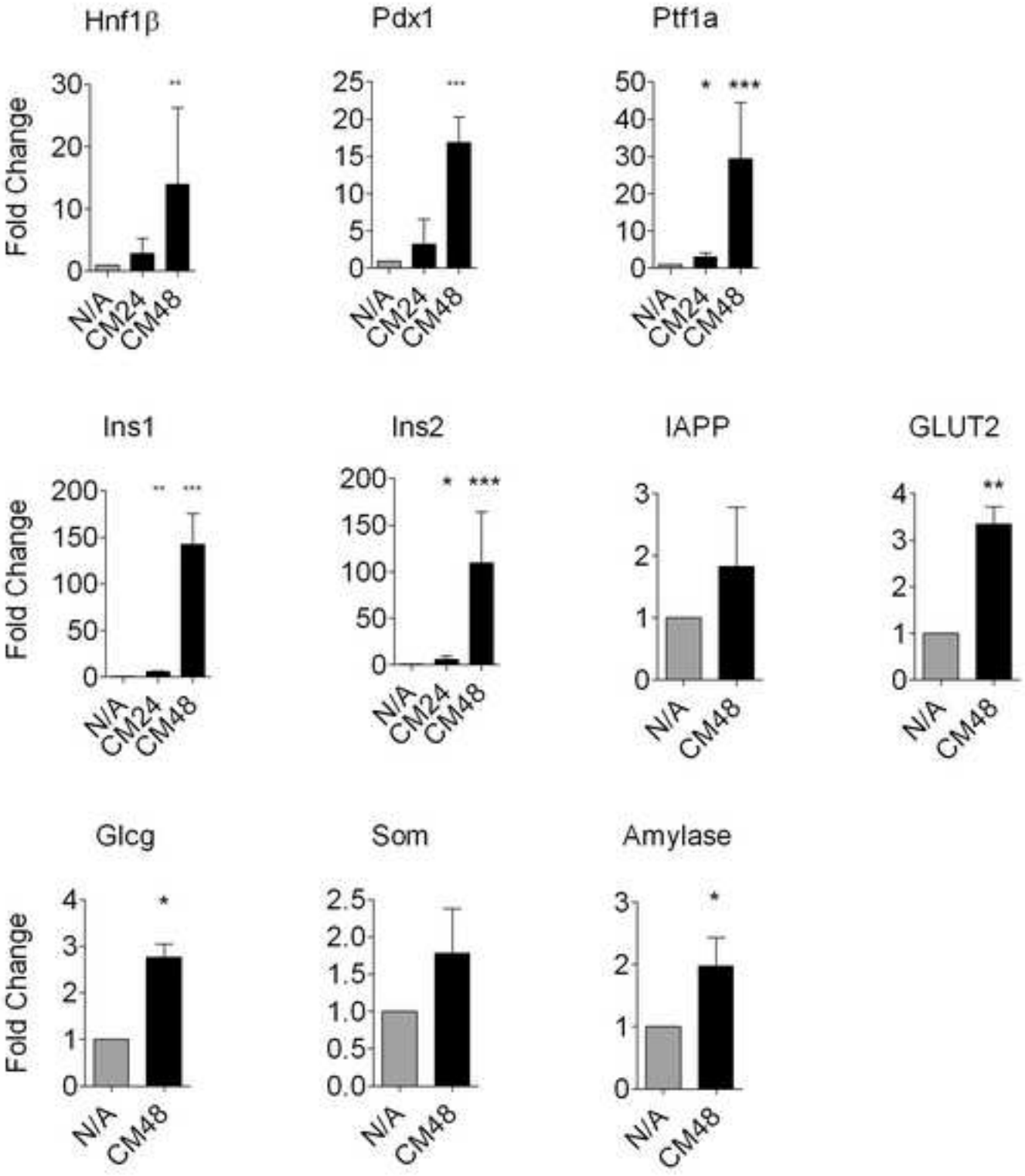

Number of cell clusters
20
15
10
5
0
*
without Min6
with Min6
without Min6
with Min6
DD7
DD14

Figure 3

Figure 4



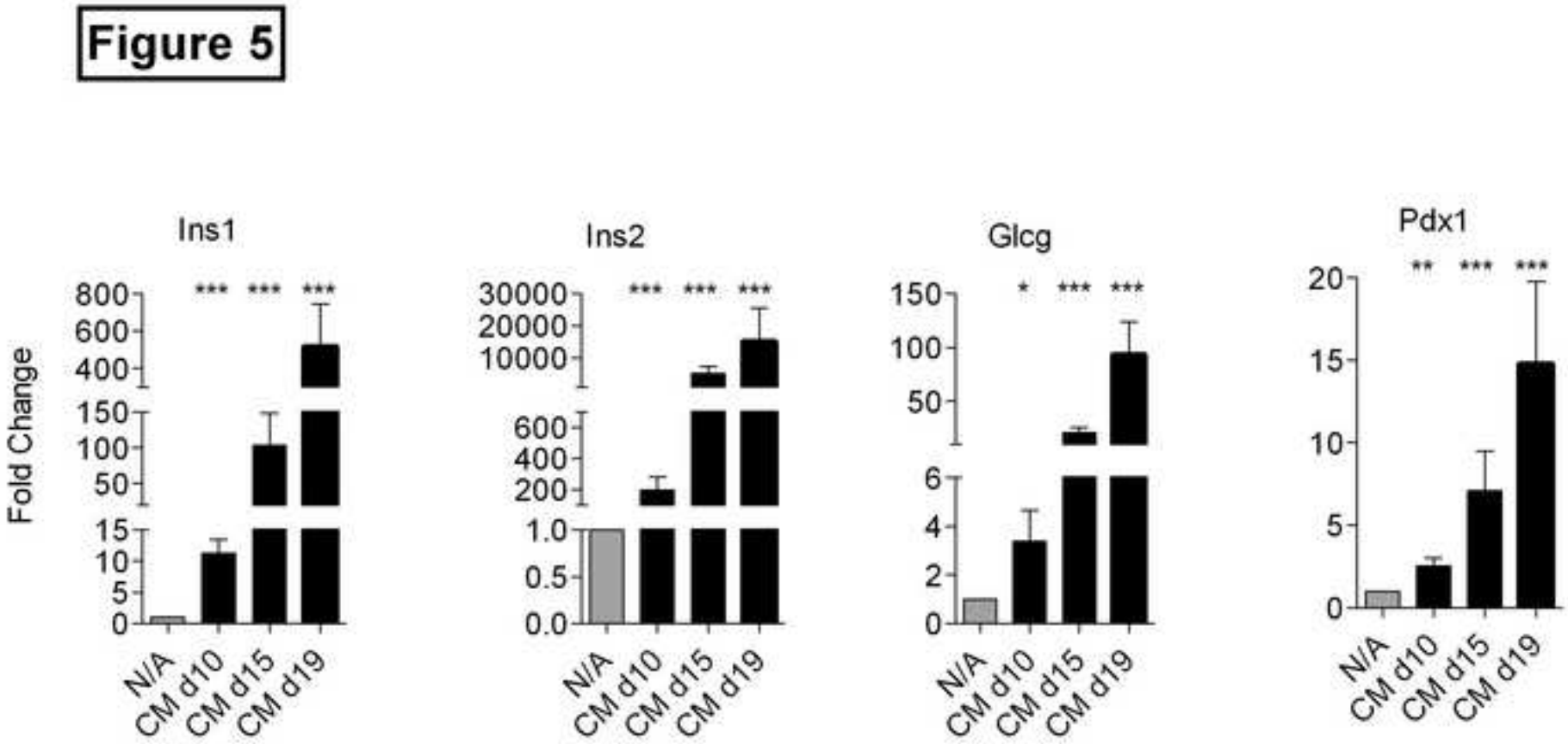


Figure 5
Ins1
Ins2
Glcg
Pdx1
Fold Change
800
600
400
150
100
50
15
10
5
0
30000
20000
10000
600
400
200
1.0
0.5
0.0
6
4
2
20
***
**
*
N/A
CM d10
CM d15
CM d19

Figure 6

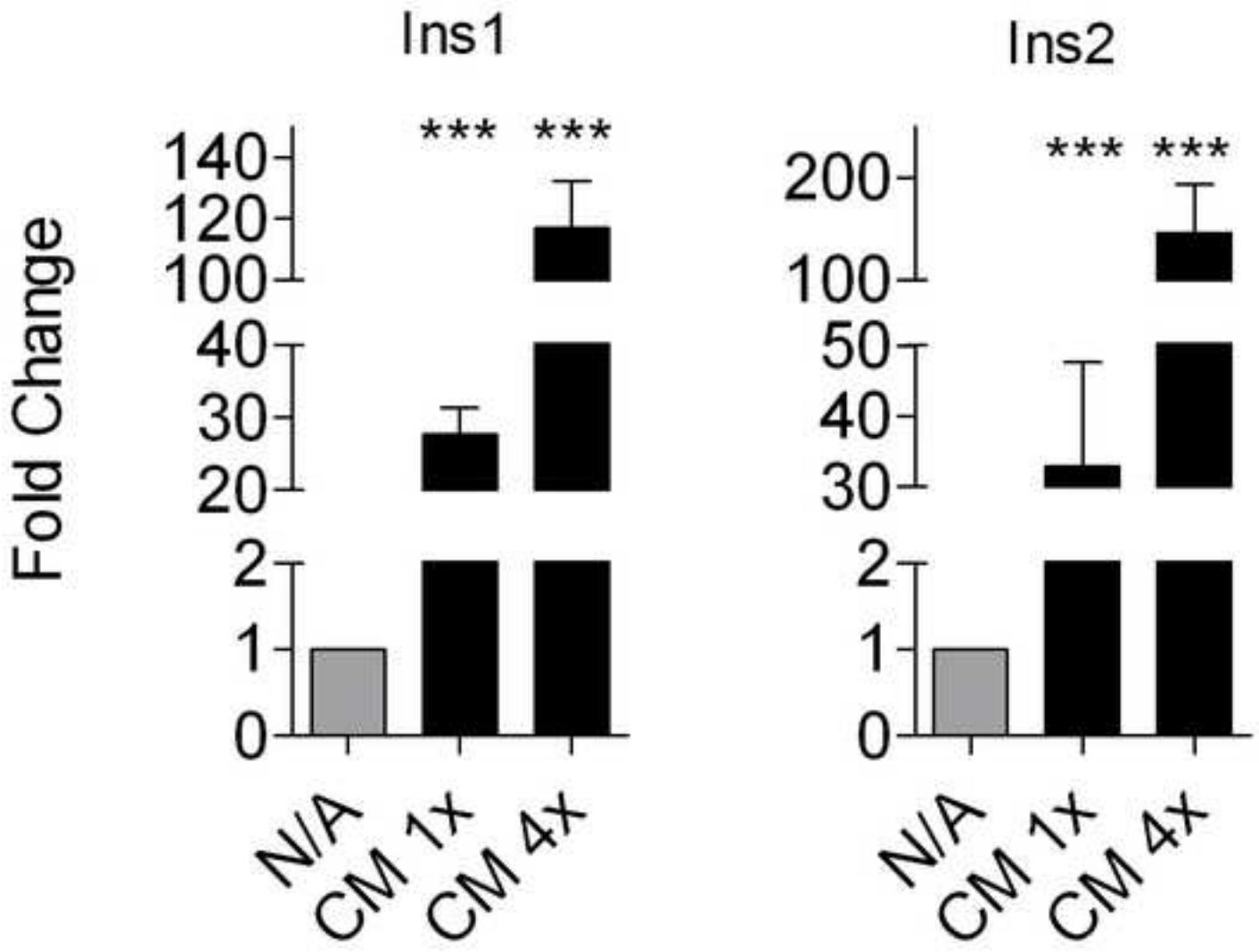

Ins1
Ins2
Fold Change
***
***
140
120
100
40
30
20
2
1
0
200
100
50
40
30
2
1
0
N/A
CM 1x
CM 4x

Figure 7

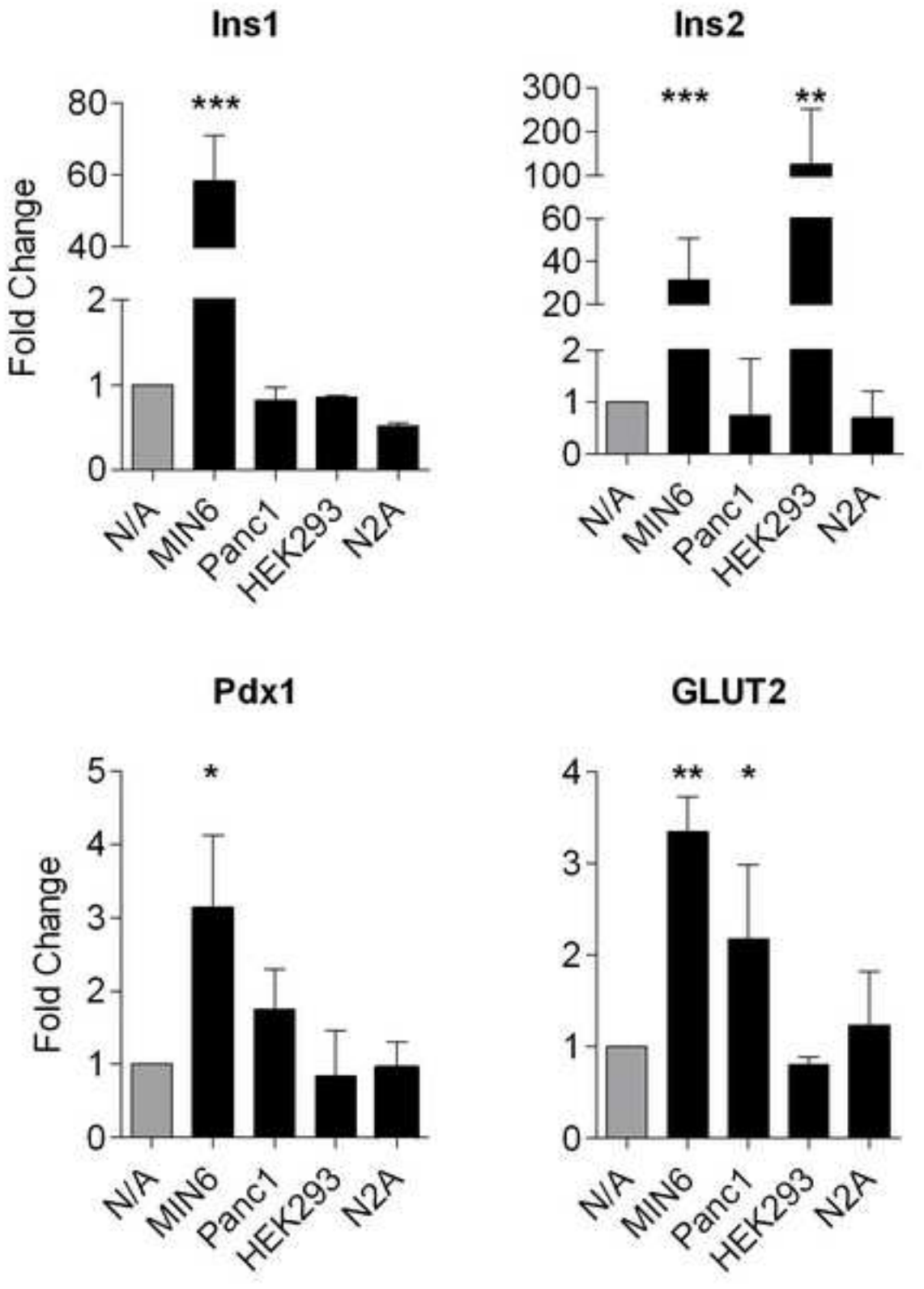
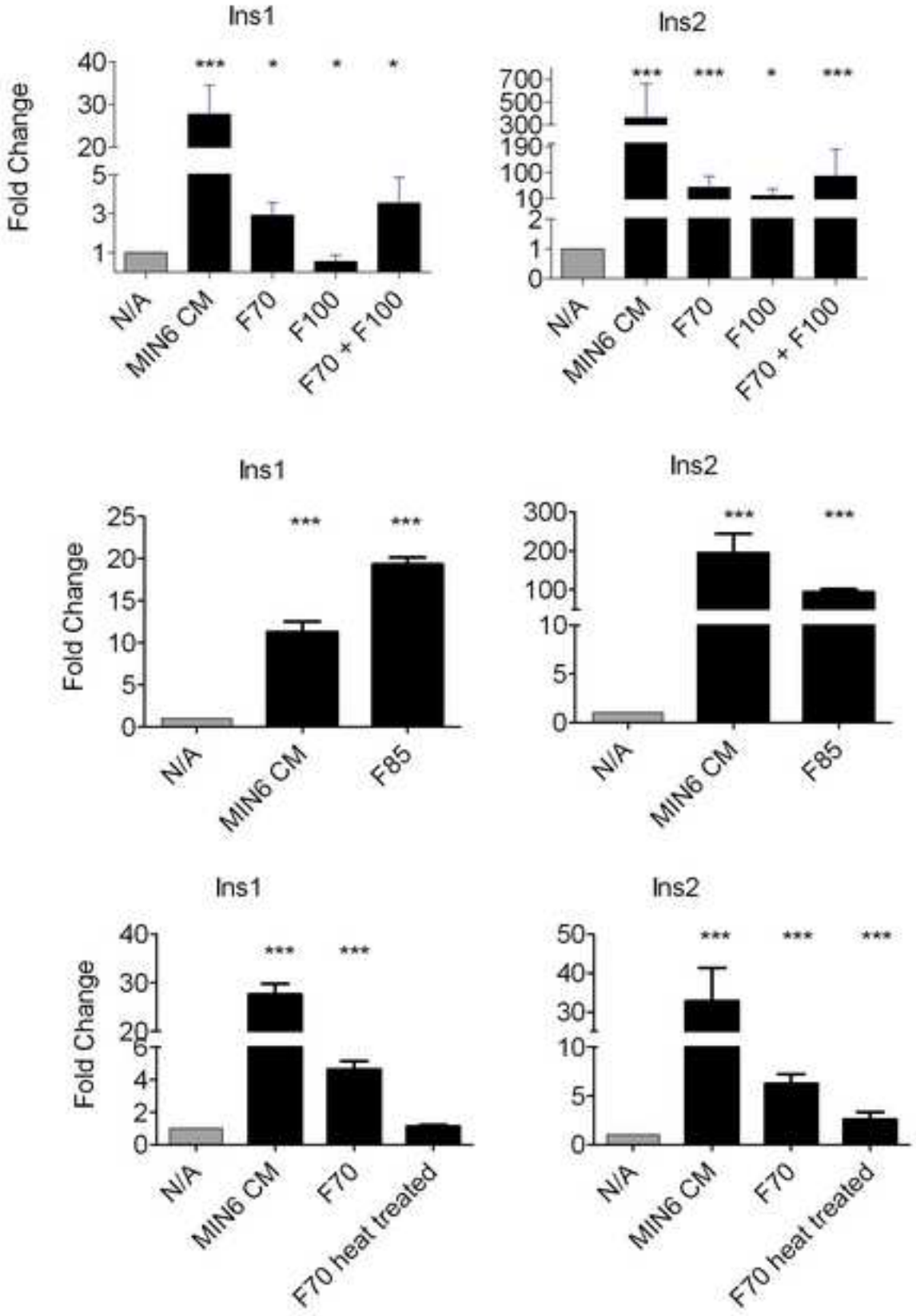


Figure 8



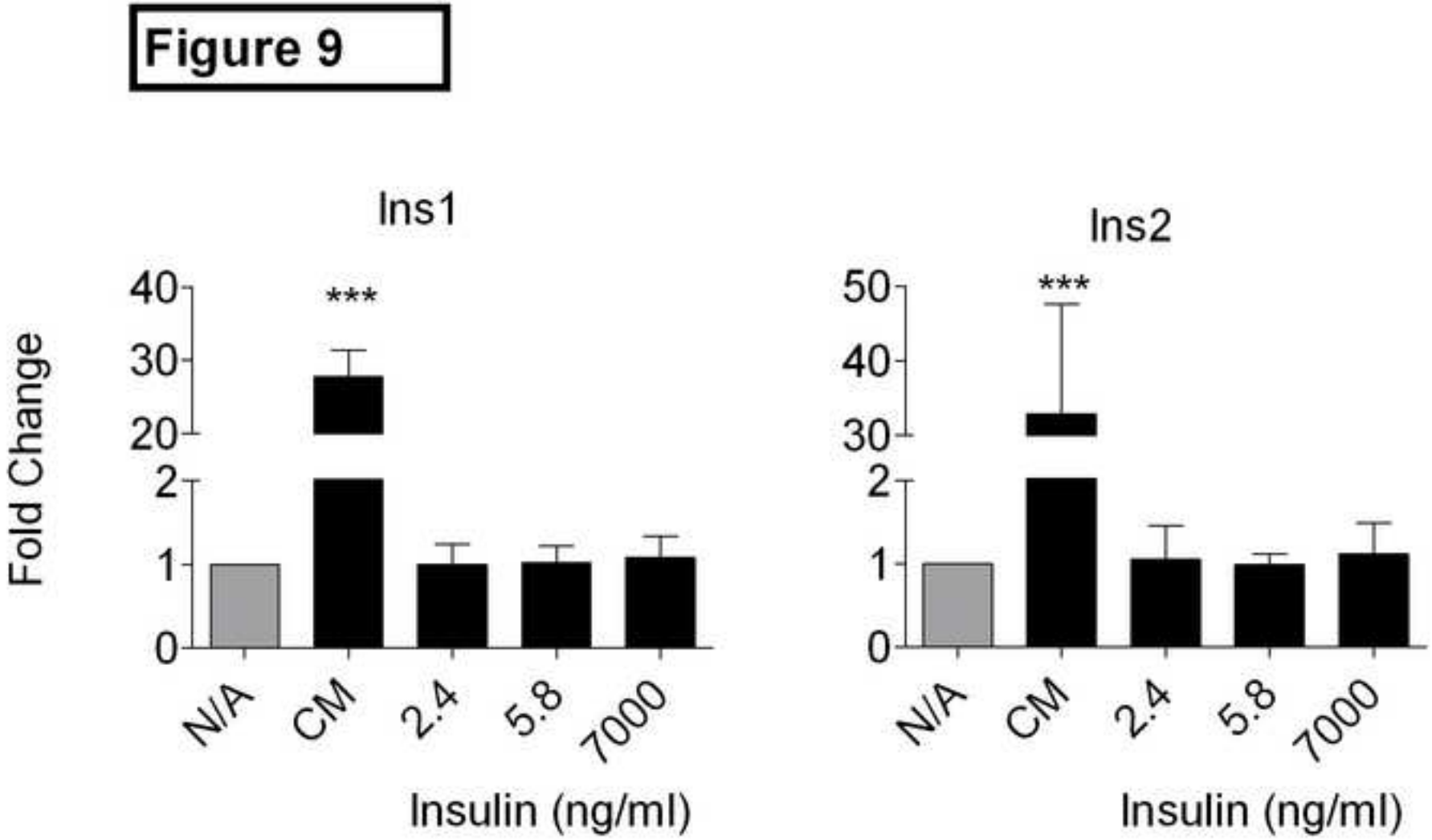
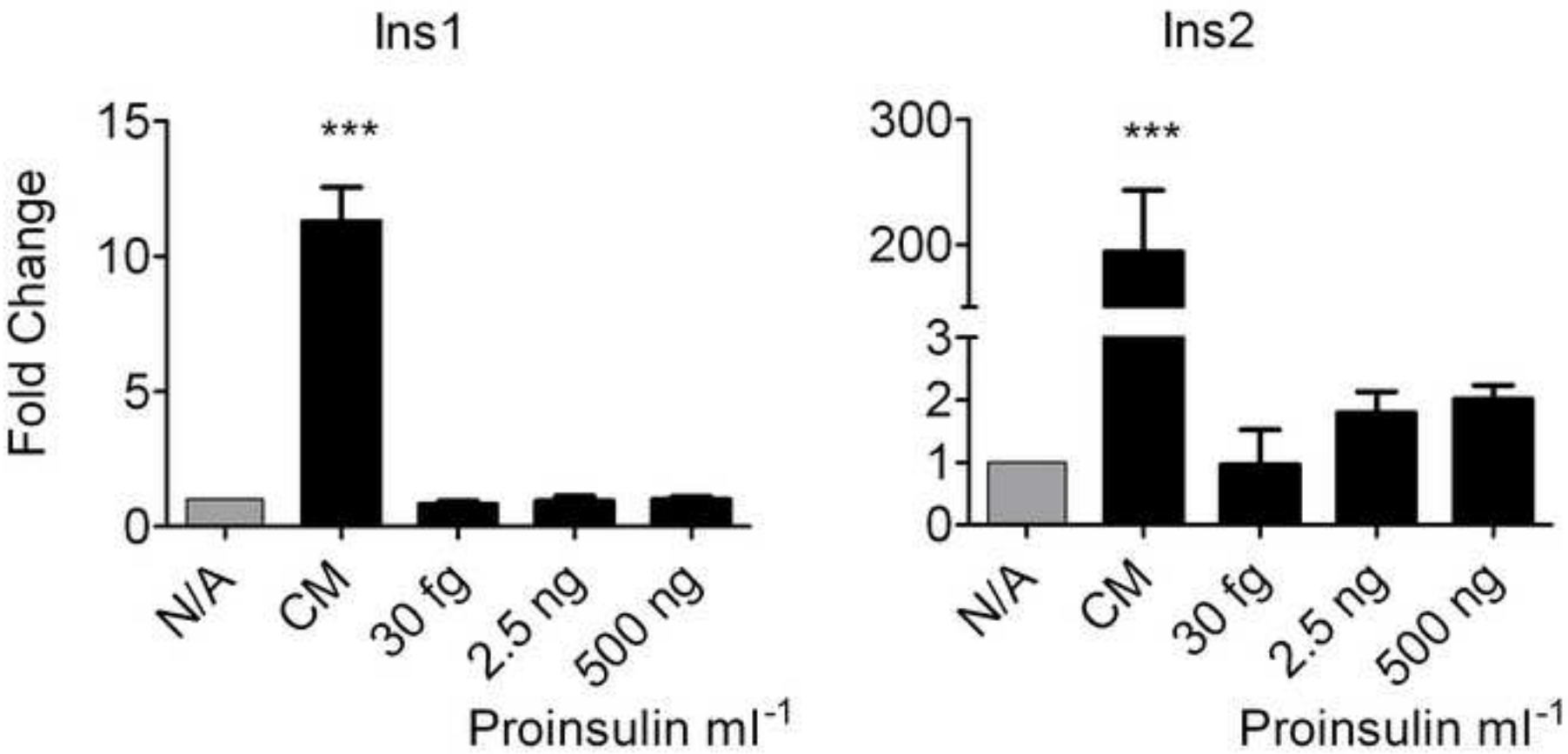


Figure 9
Ins1
Fold Change
40
30
20
2
1
0
***
N/A
CM
2.4
5.8
7000
Insulin (ng/ml)
Ins2
50
40
30
2
1
0
***
N/A
CM
2.4
5.8
7000
Insulin (ng/ml)

Figure 8


Ins1
Fold Change
15
10
5
0
***
N/A
CM
30 fg
2.5 ng
500 ng
Proinsulin ml-1
Ins2
300
200
3
2
1
0
***
N/A
CM
30 fg
2.5 ng
500 ng
Proinsulin ml-1