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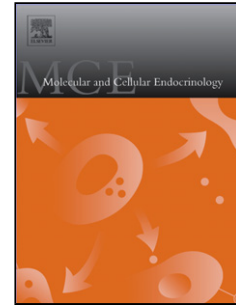
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CXCL12/SDF-1 over-expression in human insulinomas and its biological relevance

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ABSTRACT

This study was performed on the basis of previously obtained investigative gene array data concerning the over-expression of CXCL12/SDF-1 in human insulinomas versus human pancreatic islet preparations. The presence of CXCL12/SDF-1 was studied by RT-qPCR in human insulinomas (n=8) versus pancreatic islets (n=3), and was found to be significantly up-regulated in the former ($p < 0.012$). The mRNA data were confirmed by immunostaining and confocal microscopy of human normal pancreatic islets, which showed the absence of CXCL12 protein and high expression in insulinoma tissue. Individual human insulinoma cells at cytopins stained positive for CXCL12 in the paranuclear region. These morphological data were extended by consecutive immunoblotting for cell-compartment-specific marker proteins of fractions obtained by sucrose gradient fractionation using Rin-5F insulinoma cells. CXCL12-containing fractions were positive for the membrane marker NSF but negative for SNAP-25 and appeared at a lighter density in the gradient than heavy insulin granules, suggesting packaging in specific granules different from insulin.

In order to determine the biological relevance of the protein in insulinomas, we investigated the colony-forming potential of human CXCL12 stable-transfected rat Rin-5F insulinoma cells. These clones secreted human CXCL12 and contained 50- to 1000-fold higher copy numbers compared to its endogenous rat homologue. In colony-forming assays, these transfectant clones developed greater colony numbers, which were larger than wild-type and sham transfectants. To elucidate the mechanism of action, we identified a CXCL12 transfectant-specific increase in the pro-survival factor Mn-SOD, which is considered important for the inactivation of reactive oxygen species, thereby prolonging cell survival. These data demonstrate the importance of CXCL12 in the tumor biology of insulinoma.

1. INTRODUCTION

The investigation of cytokines involved in cell replication is gaining increasing attention in tumor biology. Stromal cell-derived factor 1 (SDF-1, CXCL12) originally cloned by Tashiro et al. (Tashiro et al., 1993) has been linked with tumor progression and metastasis (Homey et al., 2002b; Locati and Murphy, 1999; Tan et al., 2006). CXCL12 belongs to a family of chemokines, which represents a large group of chemotactic cytokines. These small molecules are involved in various biological processes such as leukocyte trafficking (Egorov et al., 1999), angiogenesis, hematopoiesis, organogenesis (Egorov et al., 1999; Sanvito et al., 1994) and immune response (Homey et al., 2002a).

CXCL12 belongs to the CXC chemokine group and possesses two isoforms: SDF1-alpha and SDF1-beta (Yamanaka et al., 1993). In recent years the structure of these two proteins has been clearly described. However, the functional differences between the two have not yet been elucidated. Both of these cytokines are encoded by a single gene and arise from alternative splicing (Shirozu et al., 1995). Their gene is located on chromosome 10q (Shirozu et al., 1995). The gene product was initially characterized as a pre-B-cell growth-stimulating factor (PBSF/SDF-1) (Nagasawa et al., 1994). The mRNA of SDF1-alpha is widely expressed in different types of tissue, such as the liver, lung, kidney, heart, brain, muscle and spleen (Bleul et al., 1996).

Extensive evidence has been found for the fact that CXCL12 possesses only one receptor, namely CXCR4. However, a recent study suggested that CXCR7 is a second receptor for CXCL12 (Balabanian et al., 2005). Various pathways can be activated through ligand receptor interaction, and might be involved in cell proliferation, apoptosis and tumor progression. However, the most relevant of these in tumor biology still is a debated issue. Of the known pathways, ERK1/2 and AKT are most relevant in glioma cell proliferation (Barbero et al., 2003; Sonoda et al., 2001). It has been observed that activation of CXCR4 in transfectants was followed by increased phosphorylation of kinases such as RAFTK/Pyk2 and Crk (Ganju et al., 1998).

Activation of the CXCL12 signaling pathway might mediate transcription, cell proliferation and growth. Recently, several studies tried to identify the role of the CXCL12/CXCR4 receptor ligand system in tumor progression. Koshiba et al. (Koshiba et al., 2000) found mRNA expression of CXCL12 in pancreatic cancer tissue and mRNA expression of CXCR4 in both pancreatic cancer tissue and cell lines. It has been hypothesized that CXCL12/CXCR4 is involved in a paracrine fashion in stimulating the growth of pancreatic cancer. This is confirmed by the fact that pancreatic carcinoma cell lines are frequently found

to test positive for CXCR4 (Marchesi et al., 2004). Important data for the present study were recently obtained by investigations on transgenic mice. Mice expressing CXCL12 in pancreatic beta cells showed increased beta cell survival on treatment with streptozotocin and did not develop diabetes mellitus. Its anti-apoptotic activity and signaling cascade were investigated in two different animal-derived pancreatic beta cell lines, namely MIN6 and INS-1 (Yano et al., 2007).

Little is known thus far about the influence of CXCL12 in insulinoma tumor formation and progression. In the present study, we analyzed the expression of CXCL12 in insulinoma tissues in comparison with human islets. In addition, the over-expression of CXCL12 in the rat insulinoma cell line Rin-5F was mimicked using stable-transfected clones in order to decipher its biological effect. Its intracellular distribution compared to other cell compartment-specific proteins and the role of CXCL12 in colony formation are also addressed.

2. METHODS

2.1. Tissue preparation

Tissues were obtained as described earlier (Nabokikh et al., 2007). Normal human pancreatic tissue was obtained from patients who had undergone resection of the pancreas for malignant disease; pancreatic islet-cell tumors were derived from microsurgical tumor enucleation. Informed consent was taken from each patient in accordance with the guidelines of the local ethics committee. Tissue samples were snap-frozen in liquid N₂ for later use. Human pancreatic islets were obtained from a multi-center European Union-supported program on beta cell transplantation in diabetes, coordinated by Prof. D. Pipeleers, Free University Brussels, Belgium. The program was approved by central and local ethics committees. Single-cell or cell cluster suspensions were obtained by filtering the tumor fragments through a 40- μ m Nylon cell strainer (BD Falcon, Bedford, Mass., USA). The resulting cell suspension was transferred to tissue culture flasks.

2.2. Cell culture and cell lines

Rin-5F cells were obtained from ATCC (CRL-2058) and cultured in RPMI 1640 (10mM glucose) supplemented with 10 % fetal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 IU/ml penicillin, 10 μ g/ml streptomycin in a humidified (95%) atmosphere and 5 % CO₂ at 37 °C. Rin-5F is a secondary clone of the rat islet tumor cell line Rin-m (ATCC CRL-2057). Rin-5F cells (1.5×10^6) were seeded into 25-cm² tissue culture flasks and supplied with fresh medium every third day. Cell cultures were passaged every 5-6 days using trypsin (0.25%) (Wagner et al., 2000). Stable-transfectant cells clones were maintained in the medium as described above. Three groups of cells were used: wild-type Rin-5F cells (WT) as control, CXCL12-transfected Rin-5F, and sham-transfected Rin-5F with pMSCV puro empty vector.

2.3. RNA extraction

About 100 mg of frozen tissue was transferred into 1 ml of TRIzol® Reagent (Roche Diagnostics, Mannheim, Germany) and treated as described earlier (Nabokikh et al., 2007). Homogenization was accomplished using a Polytron power-homogenization unit (Kinematica, Kriens, Switzerland). RNA was extracted by phase separation after the addition of 0.2 ml chloroform. The RNA-containing aqueous phase was precipitated using 500 μ l isopropanol.

The RNA pellet was washed twice with 75 % ethanol (for 5 min. at 9500 rpm), briefly air dried, re-dissolved in RNase-free H₂O and frozen at -80 °C for later use.

2.4. Reverse transcription

After RNA extraction and purification, the reverse transcription reaction was performed. Briefly, total RNA (2 µg) was treated with DNase I (1 µl) (Invitrogen Carlsbad, CA, USA) for 15 min at room temperature and the reaction was stopped using EDTA (1 µl). The pre-treated RNA was converted into cDNA using SuperScript II™ reverse transcriptase (1 µl) (Invitrogen Carlsbad, CA, USA) according to the manufacturer's instructions. Reaction parameters were as follows: 65 °C 15 min, 4 °C 2 min, 25 °C 4 min, 42 °C 50 min, 65 °C 10 min. The cDNA product was diluted using 80 µl of DNase-free water and frozen at -80 °C.

2.5. Real-time quantitative PCR (RT-qPCR)

Experiments were carried out in duplicate using the ABI PRISM 7000 Cyclor (Applied Biosystems, Forster City, CA, USA). Values of islet and insulinoma tumor gene expression were normalized to FAM/TAMRA-labeled 18S Assay-on-Demand (Applied Biosystems) and calculated by the ΔC_t -method: target amount = $2^{-\Delta C_t}$, where $\Delta C_t = C_t$ (CXCL12 sample x) - C_t (18s sample x) (Livak and Schmittgen, 2001). A probe for 18S (Applied Biosystems, Forster City, CA, USA) was used for internal control. cDNA generated from different human insulinoma tumor samples and human islet preparations was used as template in TaqMan gene expression assays. The original cDNA transcript (1.8 µl) was used together with 2.5 µl of the 20x gene-specific 6-carboxyfluorescein (FAM)-carboxyltetramethyl-rhodamine (TAMRA)-labeled Assay-on-Demand probe (Applied Biosystems, Forster City, CA, USA) and 25 µl of the 2x PCR MasterMix (Roche Diagnostics, Mannheim, Germany) diluted to a final volume of 50 µl with H₂O. Individual tumor- and islet-specific values are presented as means of two individual measurements expressed in comparison to islet number two (taken as 100%). The mean values of different gene transcript levels of the two groups were deemed significant when p-values were below 5 % (p<0.05). Statistical calculations were performed using the Student's t-test.

2.6. Immunofluorescence

Human insulinoma tissue, human normal pancreatic tissue cryosections (4 μm), and cytospin preparations were fixed in acetone for 5 min. This was followed by air-drying and wetting with PBS/BSA (1 $\mu\text{g}/\text{ml}$).

As primary antibodies we used the mouse monoclonal anti-human CXCL12/SDF-1 antibody (R&D Systems, Minneapolis, USA) diluted 1:500 in PBS/BSA (8 $\mu\text{g}/\text{ml}$), and rabbit anti-human SCGN (Wagner et al., 2000) diluted 1:2000, which were incubated for 90 min at room temperature in a moist chamber. Controls were incubated with mouse and rabbit Ig. For detection of primary antibodies, the secondary antibodies Alexa Fluor™ 488 F(ab')₂ labeled Goat Anti-Rabbit Ab (Accurate Chemical & Scientific Corporation, Westbury, NY, USA), or rhodamine (TRITC)-conjugated AffiniPure F(ab')₂ labeled Goat Anti-Mouse IgG (H+L) (Accurate Chemical & Scientific Corporation, Westbury, NY, USA), were used. After the second incubation step for 60 min at RT the slides were washed twice for 10 min at RT with PBS and finally DAPI was applied for nuclear DNA staining. Subsequently the slides were covered with VECTASHIELD® mounting medium (Vector Laboratories, Inc., Burlingame, CA, USA) after a final washing step in PBS for 20 min. Processed slides were evaluated under the Axiovert 200M confocal fluorescence microscope (Zeiss, Jena, Germany). Images were recorded using the LSM510 software and further arranged with Adobe® Photoshop®.

2.7. CXCL12-specific PCR and cloning (human and rat)

cDNA generated with Superscript II™ was used as a template and gene-specific PCR amplification for CXCL12 was performed. For human samples the following settings were used: thermal cycling parameters were set to 35 cycles 94 °C (30 s) for denaturation, 61 °C (30 s) for annealing, and 72 °C (40 s) for synthesis using CXCL12 coding-region-specific primers (forward: 5'-ATGAACGCCAAGGTCGTGG-3', reverse: 5'-GGCTGTGCTTACTTGTTTAAAG-3'). Coding-region specific primers were established using the Gene Runner software, version 3.00 (1994, Hastings Software, Inc., serial number: 38597329). Identical thermal cycling parameters were selected for the rat, with the only difference that the annealing temperature was set to 57 °C. Primers used for amplification of the rat CXCL12-coding region were as follows: forward 5'-ATGGACGCCAAGGTCGT-3' and reverse 5'-TACTTGTTTAAGGCTTTGTC-3'. The PCR products (3 μl) were ligated into the pGEM-T Easy vector (Promega, WI, USA) (1 μl) overnight (12 h) at 4 °C and *E. coli* DH5 α was transformed using heat shock followed by selection on Amp/LB agar plates. Colonies were selected using colony PCR with human and rat CXCL12-specific primers. The

CXCL12 insert encoding clones were isolated by the QIAGEN Plasmid Mini-Column protocol (Qiagen, Hilden, Germany) and sequenced.

2.8. Directional cloning of the CXCL12 coding sequence

In order to insert CXCL12 into the pENTRY/D-TOPO® vector (Invitrogen, Carlsbad, CA) by directional cloning, CACC was added to the 5' end of the CXCL12 forward primer and PCR was performed using pGEM-T Easy CXCL12 as template. Thermal cycling parameters were the same as those for CXCL12 PCR amplification with cDNA as template. For the cloning reaction, the following were mixed and incubated for 5 min at RT: 3 µl PCR product, 1 µl pENTRY/D-TOPO® vector, 1 µl of salt solution, and 1 µl of H₂O. After transformation of the ligation product into E.coli DH5alpha, bacteria were incubated in 500 µl of S.O.C. medium for 1 h at 37°C in a bacterial rotator (CH-4103 Bottmingen, Switzerland). Subsequently they were spread on LB agar plates containing kanamycin (50 µg/ml). Transformants were tested by colony PCR and restriction enzyme digest, and purified by the QIAGEN Plasmid Midi-Protocol.

In order to insert CXCL12 into the pMSCV puro empty vector (Invitrogen, Carlsbad, CA), the clonase reaction was performed as follows: 1 µl of CXCL12 in pENTRY/D-TOPO®, 1 µl of pMSCV puro empty vector, 4 µl of reaction buffer, 10 µl H₂O and 4 µl of clonase were mixed and incubated for 60 min at 25 °C in a water bath. After transformation of the ligation product into DH5alpha, bacteria were incubated in 500 µl of S.O.C. medium for 1 h at 37 °C on the bacteria rotator, followed by spreading on LB agar plates containing ampicillin (50 µg/ml). Transformants were selected by restriction enzyme digest, colony PCR, and purified by the QIAGEN Plasmid Midi-Protocol.

2.9. Quantification of human and rat CXCL12 gene copy numbers:

Human and rat CXCL12 gene containing p-GEMT Easy vectors were used as DNA templates in order to quantify human and rat CXCL12 gene content in stable-transfected clones. Plasmid DNA concentrations of human and rat CXCL12 were measured by a spectrophotometer. Copy numbers of plasmid stock solution were calculated by applying the formula given at the website (http://www.appliedbiosystems.com/support/tutorials/pdf/quant_pcr.pdf) of Applied Biosystems. A serial dilution of both the rat- and the human-specific plasmid stock was applied as standard curve with known copy numbers, together with CXCL12 over-expressing

clones #1, #2 and #3 for RT-qPCR measurement using human- and rat-specific CXCL12 TaqMan probes. TaqMan rat and human CXCL12 probes obtained from Applied Biosystems did not show any inter-species cross-reactivity. CXCL12 gene transcript copy numbers of the clones were calculated using the ABI Prism 7000 SDS software.

2.10. Cell transfection

RIN-5F cells were transfected with puromycin-resistance gene expression vector pMSCVpuro-CXCL12 using the LipofectAMINE™ Plus Reagent (Invitrogen, Carlsbad, CA) method in accordance with the manufacturer's instructions. Briefly, cells were incubated in transfection media for 4 h, which was then changed by addition of 3 ml of complete culture medium. Puromycin (4 µg/ml) was added 24 h after transfection and resistant colonies were picked 14 days later. The colonies were selected under an inverted microscope (Leitz, Laborwert FS, Germany). CXCL12 expression was checked using RNA extraction, reverse transcription and RT-qPCR. Sham transfection of the Rin-5F cells was performed with pMSCV puro empty vector using the LipofectAMINE™ Plus Reagent (Invitrogen, Carlsbad, CA) method described above.

2.11. FACS analysis of intracellular CXCL12

Logarithmically growing Rin-5F clones were fixed by incubation in 100 µl of fixative reagent A (FIX&PERM Kit, An der Grup, Kaumberg, Austria) for 15 min on ice. Cell permeabilization was performed using the FIX&PERM permeabilization reagent B (15 min) on ice, followed by incubation with mouse monoclonal anti-human CXCL12/SDF-1 (1:100 in Beriglobin) for 20 min. After washing the cell population with ice-cold culture medium twice, the secondary antibody rhodamine (TRITC)-conjugated AffiniPure F(ab')₂ labeled Goat Anti-Mouse IgG (H+L) (Accurate Chemical & Scientific Corporation, Westbury, NY, USA), diluted 1:200, was incubated for 20 min on ice. Washed cells were analyzed at the FACS CANTO (Bacton Dickinson) employing the Diva 6 software for data recording and analysis.

2.12. Soft agar growth assay

In order to prepare the bottom layer soft agar, 60-mm culture dishes were used and each dish was coated with 3 ml of 0.75 % agar. For preparing this layer, 1.23 x RPMI supplemented with 10 % fetal calf serum, sodium bicarbonate, 100 µg/ml streptomycin, L-

glutamine (4.28-fold the volume of 4% agarose) were mixed and combined with 4x agarose molten at 70 °C. This bottom layer was permitted to solidify at room temperature. Top agarose (0.4%) was prepared as follows: Rin-5F-CXCL12-transfected cells were trypsinized using trypsin (0.25 %) and 8000 cells to be tested were suspended in 3 ml medium, mixed with 5 ml of 0.75 % soft agar. Two ml of the mixture was added to triplicate dishes on the basal agar. The cell containing agar layer was left to solidify at room temperature for 20 min. Cultures were incubated at 37 °C in a humidified incubator in an atmosphere of 5 % CO₂. Finally, colonies were counted 6 days after plating in an equal-sized quadrant area of each plate (2.5 cm²) using a ruler template under an inverted microscope at 100-fold magnification (Leitz, Laborwert FS, Germany). Pictures were recorded at the same time points using the same microscope and the Leica Image Manager, and further arranged with Adobe® Photoshop®.

2.13. Cell fractionation

CXCL12 over-expressing Rin-5F cells were cultured in 150 cm² dishes. At 75 % confluence, culture dishes were washed twice with pre-cooled PBS and scraped into 2.5 ml of ice-cold homogenization buffer (250 mM sucrose and 3 mM imidazole, pH 7.4) containing protease inhibitors (10 µg/ml aprotinin, 1 µg/ml pepstatin, 10 µg/ml leupeptin and 0.8 mM pepabloc). The cell suspension was then passed five times through a 26-gauge needle and centrifuged at 2500 rpm for 10 min at 4 °C in order to pellet unbroken cells and nuclei. The post-nuclear supernatant was loaded onto a discontinuous sucrose gradient (50% and 20%, respectively) in 14x95 mm poly-allomer centrifuge tubes and centrifuged at 40000 rpm for 3 h at 4 °C in the SW40 TI rotor of an ultracentrifuge (model L-80, Beckman Instruments, Palo Alto, CA). The resulting supernatant was collected, starting at the bottom, in 17 fractions using a peristaltic pump. The fractions were analyzed for CXCL12, SNAP-25 (25-kDa synaptosomal-associated protein), NSF, rat insulin and SCGN (secretagogen) content, using immunoblotting techniques.

2.14. Immunoblotting

Thirty-milliliter aliquots of Rin-5F cell fractions were loaded onto 12 % SDS-polyacrylamide gels, which were run at 200 V using Tris-Glycine as running buffer. A semi-dry blotting device was used for electrophoretic transfer of the protein onto nitrocellulose. The blotted membrane was then blocked with 2 % skim milk for 30 min at room temperature. For antigen detection the membrane was incubated sequentially with the anti-human

CXCL12/SDF1- α (from R&D Systems, Minneapolis, MN), the polyclonal rabbit anti-SCGN (Wagner et al., 2000), mouse monoclonal anti-NSF (BD Biosciences), mouse monoclonal anti-rat insulin (mAb D3E7, Research Diagnostics, Flanders, NJ), the anti-SNAP-25 (mAb SP12, Sigma-Aldrich) and the rabbit polyclonal anti-Mn-SOD antibody (Santa Cruz Biotechnology) overnight at 4°C. The following were used as secondary antibodies: peroxidase-conjugated goat anti-mouse and peroxidase-conjugated goat anti-rabbit antibody (both from Dako, Glostrup, Denmark) (incubation time 40 min). For each antibody incubation step, the membrane was washed twice with PBS containing 0.1 % Tween 20 for 10 min. Antibody binding sites were visualized using BM chemiluminescent reagents (Roche Molecular Biochemicals) and images were recorded with a Lumi-Imager F1 (Roche Molecular Biochemicals). The figures were arranged in Adobe Photoshop CS.

2.15. CXCL12/SDF1- α ELISA

The analysis was performed with a commercially available ELISA kit (Quantikine[®] Human CXCL12/SDF1- α Immunoassay, from R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Briefly, standards were prepared using the provided recombinant protein. One hundred milliliters of assay diluent followed by 50 μ l of either insulinoma-conditioned medium (Gartner et al., 2006) or Rin-5F cell supernatants were added to each well and incubated for 2 hours on a constant shaker at room temperature. Subsequently, the wells were washed 3 times with washing buffer (R&D Systems) using the ELX Auto strip Washer (Biotex Instruments, Winooski, VT, USA), and 200 μ l of conjugate was added to each well. After an additional incubation period of 2 h under constant shaking at room temperature, which was followed by the washing procedure described above, 200 μ l of substrate solution was applied to each well and incubated for 30 min under light protection. The reaction was stopped by addition of stop solution (50 μ l) and the ELISA plate was read at 450 nm within 30 min using the Powerwave ELISA reader (Biotec Instruments, Winooski, VT, USA).

3. RESULTS

3.1. Real-time quantitative PCR

Recently elicited gene array data were the principal reason to investigate CXCL12 in human insulinoma tumorigenesis. In this former experiment with top regulated genes, CXCL12 was found to be over-expressed 4.5-fold in three human insulinomas versus one normal pancreatic islet preparation. Besides, in a previous study we found TGF- β 1 to be under-represented in insulinoma tumors (Nabokikh et al., 2007). This is important because TGF- β 1 acts as an expression regulator of the CXCL12 gene (Wright et al., 2003). Furthermore, the CXCL12/CXCR4 axis was reported to be involved in tumor proliferation and growth (Koshiba et al., 2000; Muller et al., 2001). To confirm our initial investigative array data at the mRNA level, we performed RT-qPCR on eight human insulinoma tumors compared with three normal human pancreatic islet preparations. This revealed an over-expression of CXCL12 in insulinomas when compared with islet preparations ($3397\pm 4446\%$ versus $173\pm 81\%$; $p < 0,012$) (**Fig. 1a**). In order to elucidate CXCL12 signaling in insulinoma cells induced by the over-expressed chemokine, we investigated CXCR4. However, CXCR4 expression in human insulinoma tumors was comparable with islet levels (**Fig. 1b**).

3.2. Immunofluorescence

In order to achieve a functional analysis of CXCL12, we focused on characterizing the expression of the CXCL12 protein in human insulinoma tumors as compared with normal pancreatic islet preparation. Using a specific anti-human CXCL12/SDF-1 mAb immunofluorescence staining of cryosections of normal human pancreatic tissue, human insulinoma tissue and human insulinoma cytospin preparations was performed. In order to identify neuroendocrine cells, the antibody directed against SCGN (*green*) was used (Birkenkamp-Demtroder et al., 2005; Gartner et al., 2006; Lai et al., 2006). In accordance with our findings on RT-qPCR, a high level of CXCL12 (*red*) was found in human insulinoma tissue (**Fig. 2, middle panel**) and human insulinoma cytospin preparations (*lower panel*). In contrast, human pancreatic islets were negative for CXCL12 (**Fig. 2, upper panel**). These results confirmed at the protein level our findings obtained by RT-qPCR in human insulinomas, and demonstrated high levels of CXCL12 expression in this tumor entity.

3.3. Directional cloning of the CXCL12 coding sequence

Little is known about the involvement of CXCL12 in human insulinoma tumorigenesis. In order to understand the influence of CXCL12 on insulinoma growth, we transfected Rin-5F rat insulinoma cells with the human CXCL12 gene.

In order to evaluate CXCL12 transgene expression in individual Rin-5F clones, each of these were analyzed by RT-qPCR with a human CXCL12-specific TaqMan probe. Three of human CXCL12 transfectant Rin-5F clones were selected because they showed high CXCL12 expression in comparison to wild-type and sham-transfectant Rin-5F.

3.4. Absolute quantification of rat and human CXCL12 gene copy numbers in Rin-5F cells

To evaluate background CXCL12 expression in the rat insulinoma cell line Rin-5F, we quantified the CXCL12 gene copy number for both human and rat. CXCL12 copy numbers were calculated as described in the Methods section. In human CXCL12 transfectant clones, human CXCL12 copies exceeded the endogenous rat CXCL12 copy number 50- to 1000-fold, depending on the clone (**Fig. 3**). In wild-type Rin-5F cells, the endogenous CXCL12 copy number was comparable with that in stable transfectants, whereas human CXCL12 was absent.

3.5. Measurement of CXCL12 protein expression in transfectants

Logarithmically growing clonally selected Rin-5F-CXCL12 over-expressing clones were fixed and permeabilized for intracellular human CXCL12 staining. Transfectant clones showed at least a 100% increase in mean fluorescence intensity when compared with sham-transfectant or wild-type, confirming human CXCL12 protein expression (**Fig. 4**).

3.6. ELISA

Supernatants taken from Rin-5F cells and three human CXCL12 transfectant clones contained 300-1000 pg/ml/10⁶ cells/24 h CXCL12 (**Fig. 5**). In contrast, supernatants taken from wild-type and sham transfectants were negative. In addition, human insulinoma cell-conditioned medium (the insulinoma cells were derived from three different individuals named here Ins1, Ins2, Ins3) was analyzed. Considerable quantities of CXCL12 were present (360-413 pg/ml/10⁶ cells/24 h), depending on the individual donor (**Fig. 5**).

3.7. Clonogenic assay

In order to obtain a comprehensive idea of the potential role of CXCL12 on colony formation, we investigated the influence of CXCL12 in insulinomas using stable over-expressing Rin-5F clones. Rat Rin-5F insulinoma cells stable-transfected with human CXCL12 (#1 and #3) were used as described above, and compared with sham-transfectant and wild-type Rin-5F (**Fig. 6a**). Performing clonogenic assays with CXCL12-expressing clones #1 and #3, the colony size was found to be about twice as large compared to sham-transfectant Rin-5F (**Fig. 6b**). This phenomenon was in line with significantly higher numbers of colony formation in all transfectant clones (clone #1 $p < 0.003$; clone #3 $p < 0.006$) when compared with colony formation in wild-type Rin-5F and sham transfectants (**Fig. 6c**).

3.8. Activation of an anti-oxidative system in CXCL12 transfectant Rin-5F cell clones:

Reactive oxygen species (ROS) have been associated with apoptotic processes. Enzymes inactivating oxygen radicals such as manganese superoxide dismutases (Mn-SOD) play a major role in anti-apoptosis (Connor et al., 2007). Mn-SOD acts as an oncogene and is believed to protect cancer cells from apoptosis, thus contributing to prolonged survival of cancer cells. In order to evaluate this pro-survival factor in CXCL12 over-expressing Rin-5F cell clones compared with wild-type and sham-transfectant, we performed immunoblots using wild-type, sham-transfected and CXCL12-over-expressing clones #1 and #2 (**Fig. 7a**). Immunoblotting of both CXCL12 over-expressing Rin-5F cell clone #1 and #2 showed about three-fold larger quantities of Mn-SOD (as determined by densitometry and set in relation to actin, **Fig.7b**).

3.9. Growth pattern of CXCL12-transfected Rin-5F cells

Previous studies have reported different growth patterns of human insulinoma cells in vitro (Gartner et al., 2006). Based on our data, we next sought to determine the growth pattern of CXCL12-stable-transfected cells. Rin-5F sham-transfectant cells were taken as controls. Using conventional and phase-contrast light microscopy, we found CXCL12-transfected cells mainly displaying a three-dimensional, cluster-like growth pattern, whereas Rin-5F sham-transfected cells had a largely two-dimensional, monolayer-like growth pattern (**Fig. 8**). Notably, this difference in growth patterns has persisted thus far throughout five passages.

3.10. Sucrose gradient cell fractionation

Given the fact that CXCL12 was found at paranuclear location on immunofluorescence analysis, we characterized the intracellular distribution pattern of CXCL12 and its packaging for secretion. For this purpose, stable-transfected Rin-5F cells were subjected to sucrose gradient-cell fractionation. The individual fractions were then analyzed with regard to their CXCL12 content using immunoblotting. To interpret the location of the protein within the cell, the same blots were re-developed with anti-SNAP-25 and anti NSF antibodies specific for membrane and anti-SCGN antibody, representative of cytosolic fractions. To evaluate potential co-location of CXCL12 with insulin-containing granules, the rat insulin content was investigated at the same fractions (**Fig. 9**).

Rat insulin was predominantly present in fraction 3 and associated with heavy membrane fractions, as documented by the presence of SNAP-25 (**Fig. 9**). Human CXCL12 protein expression was simultaneously found in lighter fractions, using the membrane marker protein NSF. Neuroendocrine differentiation marker protein SCGN was detectable in cytosolic fractions which were negative for CXCL12.

4. DISCUSSION

CXCL12 belongs to the CXC chemokine group and is found in two isoforms with its ligand receptor CXCR4. Several research groups have focused on the crucial role of CXCL12 in tumor biology. It has been emphasized that this molecule might participate in cell proliferation, transformation and growth, and might contribute to tumor vascularization (Kryczek et al., 2005; Orimo et al., 2005). The CXCL12 chemokine was found to be highly expressed in human ovarian cancer, prostate cancer, pancreatic cancer and other tumor types (Begley et al., 2007; Koshiba et al., 2000; Kryczek et al., 2005).

In the present study we demonstrated over-expression of CXCL12 at both, the mRNA and the protein level, in insulinoma tumors compared with normal islets of Langerhans, and also showed its presence in human insulinoma-conditioned medium. These data add one more tumor type to the previously reported tumor entities over-expressing CXCL12 (Kang et al., 2005; Koshiba et al., 2000); (Sun et al., 2003). Furthermore, not only CXCL12 chemokine, but also its receptor was shown to be highly expressed in tumors. mRNA expression of CXCR4 was significantly up-regulated in primary breast tumors. These findings were confirmed at the protein level by immunofluorescence (Muller et al., 2001). The authors suggest that the CXCL12/CXCR4 axis plays an important role in tumor progression and metastasis. CXCL12 might also serve as an initiator in benign proliferative disease with reference to an aging tissue microenvironment, as has been recently demonstrated (Begley et al., 2007). In the latter study the authors showed that CXCL12 is secreted by aging human prostate fibroblasts and stimulates not only the proliferation of prostate epithelial cells (Begley et al., 2005) but also promotes tumorigenesis and metastasis (Begley et al., 2007).

As a logical consequence, we measured CXCL12 quantitatively and also looked at the expression of CXCR4 mRNA in human insulinoma tumors versus pancreatic islet preparations. This revealed no significant difference between tumors and human islet preparations but demonstrated the susceptibility of both tissues to CXCL12. Using an immunofluorescence method to examine CXCL12 protein expression in human insulinomas, we confirmed its presence in tumor tissue and its absence in normal pancreatic islets. Immunoreactive CXCL12 was found to be dispersed throughout the tumor tissue. Focusing on individual insulinoma cells, we mainly found CXCL12 in the paranuclear region. Employing the rat insulinoma cell line in sucrose gradient fractionation, we were able to delineate the sedimentation of CXCL12 with different granules than that of insulin. Whereas insulin granule-containing fractions are positive for both SNAP-25 and NSF, CXCL12-

containing fractions were only positive for NSF and were separated from insulin granules at a much lower density devoid of SNAP-25. This supports the thesis that secretion of CXCL12 granules is facilitated by a different mode than insulin and might be independent of typical insulin secretagogues.

One of the most informative in vitro techniques to evaluate the potential of individual cells to form proliferative foci is the so-called clonogenic assay (Puck and Marcus, 1956). The assay has been established not only for cells derived from normal tissue, but also for tumor cells (Franken et al., 2006). It enables the investigator to evaluate the colony-forming capability of various tumor cell populations and their descendents such as transfectant clones, which over-express a specific factor.

In the present study we demonstrated that the insulinoma cell line Rin-5F is capable of forming colonies in soft agar. In addition, we identified the potential role of CXCL12 on colony formation using rat Rin-5F insulinoma cells stable-transfected with human CXCL12. The colony-forming capacity of all transfectants was higher than those of wild-type Rin-5F and sham transfectants. This fact confirmed our assumption that CXCL12 might promote insulinoma cell colony formation, which goes in line with the findings of other investigators (Koshiba et al., 2000) who studied other types of tumors. In addition, we observed a difference in colony size between wild-type and transfectants. The colony size was about twice as large in CXCL12 transfectants than in sham transfectants.

TGF- β 1 was also found to participate in the process of tumorigenesis of various tumors. It has been suggested that this cytokine is involved in tumor progression, angiogenesis and metastasis (Bierie and Moses, 2006; Levy and Hill, 2006). The role of TGF- β 1 on CXCL12 expression has been investigated in a previous study (Wright et al., 2003). Using the bone marrow stromal cell line MS-5 expressing CXCL12, the authors showed that TGF- β 1 down-regulates the expression of CXCL12 at the protein as well as the mRNA level. A broad expression of these two cytokines in stromal cells supports the fact that this reciprocal association might not be limited to the bone marrow environment.

In a previous study we demonstrated down-regulation of TGF- β 1 and derangement of its pathway in human insulinoma tumors (Gartner et al., 2007; Nabokikh et al., 2007). The data concerning CXCL12 obtained in the present study are in accordance with previous findings, and demonstrate that insulinoma tumor-specific up-regulation of the chemokine is in line with the reciprocal relationship of these two cytokines. It must be emphasized that changes in the expression of these two molecules could potentially affect the β -cell transformation process and the development of human insulinomas. It might be hypothesized

that the primary step of derangement of this pathway is the expression level of TGF- β 1 and that of CXCL12, resulting in changed transcription rates of other downstream genes in a different mode than in normal pancreatic islets of Langerhans.

In a recent study, Yano and co-workers (Yano et al., 2007) investigated the potential role of CXCL12 in mouse pancreatic islet cells using transgenic mice. They found that CXCL12 over-expression in pancreatic beta cells attenuates diabetes in mice when treated with streptozotocin, which is known to induce beta-cell apoptosis. Furthermore, they demonstrated that CXCL12 signaling through CXCR4 promotes beta-cell survival via activation of Akt. Based on these findings they conclude that CXCL12 agonists might be beneficial in the treatment of diabetes. Our findings are also indicative of a similar mechanism which triggers beta-cell survival in insulinomas. An additional point of evidence for this hypothesis is provided by our Mn-SOD immunoblotting data of CXCL12 stable transfectant Rin-5F clones in comparison to wild-type Rin-5F and sham transfectants. The much larger quantity of Mn-SOD enzyme in stable-transfected clones suggests that CXCL12 influences the activation of anti-oxidant systems, supporting longer cell survival.

In conclusion, we demonstrated high expression of CXCL12 in insulinoma tumors and its secretion into tissue culture media. We deciphered its differential intracellular location in comparison to insulin and elucidated its biological function in inducing pro-survival factors such as Mn-SOD. These findings may enhance our understanding of human insulinoma tumorigenesis and pancreatic stem cell biology. Besides, our results might help to comprehend potential clinical implications of CXCL12.

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6. FIGURE LEGENDS

Figure 1: Comparison of CXCL12 and CXCR4 expression in insulinomas versus human islet preparations at the mRNA level (RT-qPCR):

Eight human insulinoma tumors compared with 3 different human pancreatic islet preparations. Islet #2 was arbitrarily chosen as 100%; tumors and other islet preparations were related to it. CXCL12 expression levels in human insulinomas (**a**) were significantly (*, $p < 0.012$) higher than those in normal islet preparations. The CXCR4 mRNA level (**b**) was comparable with islet levels.

Figure 2: Immunofluorescence of normal human pancreatic and human insulinoma tissues:

Cryosections of normal human pancreatic tissue, human insulinoma and human insulinoma cytopsin preparations were co-immunostained with anti-human CXCL12 antibody and with antibodies directed against the neuroendocrine marker protein secretagogin (SCGN). DAPI was used for nuclear staining (*blue*). Representative results of one pancreatic tissue section (*upper panel*), human insulinoma tissue (*middle panel*), and a human insulinoma cytopsin preparation (*lower panel*) are shown. Confocal immunofluorescence analysis confirmed the marked over-expression of CXCL12 protein (*red*) in human insulinomas when compared with a normal pancreatic islet. Parallel CXCL12 (*red*) and SCGN (*green*) immunostaining demonstrated co-location of both proteins in human insulinomas, which resulted in yellow coloration after electronic picture overlay (magnification 400x).

Figure 3: Rat and human CXCL12 gene copy number quantification in Rin-5F cells:

Rat and human CXCL12 gene copy numbers were defined using RT-qPCR and rat and human CXCL12-encoding plasmids as standards. Rat CXCL12 was present in wild-type Rin-5F cells as well as in transfectant clones. However, the copy number of human CXCL12 exceeded that of endogenous rat CXCL12 50- to 1000-fold in transfectant clones.

Figure 4: Intracellular FACS for human CXCL12 expression:

Fixed and permeabilized sham-transfectant Rin-5F cells (gray field) and stable transfectant clone #1 (white field) and #3 (black field) stained using human specific CXCL12 mAb were analyzed by FACS. Fluorescence intensity is shown at the x-axis and cell number at the y-axis.

Figure 5: CXCL12 concentration in human insulinoma-conditioned medium and CXCL12 over-expressing Rin-5F cell supernatants:

Human CXCL12 secretion was evaluated in the supernatant of stable transfected Rin-5F clones and human insulinoma-cell-conditioned medium using a commercially available ELISA kit. Human CXCL12 was absent in supernatants derived from wild-type and sham-transfected Rin-5F cells. High CXCL12 levels were found in insulinoma-conditioned medium and supernatants taken from Rin-5F transfectant clone #1, #2 and #3. (WT: wild type, pMSCV: pMSCV sham-transfected Rin-5F cells, Ins: human insulinoma cell-conditioned media).

Figure 6: Clonogenic assay:

Human CXCL12 stable-transfected rat insulinoma Rin-5F cell clones, sham-transfectant and wild-type Rin-5F cells were cultured in soft agar for 6 days. **(a)** Equal size areas of culture dishes taken from wild-type, sham-transfectant and stable-transfected clones are demonstrated on phase-contrast light microscopy (magnification 100x, upper panel). Size differences of individual colonies were evaluated at higher magnification (magnification 400x, lower panel). **(b)** Colony formation numbers were significantly higher in stable-transfected clones #1 (* $p < 0.02$) and #3 (** $p < 0.0006$) when compared with wild-type and sham transfectant. Wild-type colonies were taken as 100% in each assay. Values are shown as means \pm SD. **(c)** The colony diameters were quantified in wild-type and stable-transfectant clone #3. Over-expression of CXCL12 induced a larger colony size when compared with WT Rin-5F (* $p < 0.04$).

Figure 7: Immunoblot analysis of the Mn-SOD levels in the Rin-5F cells:

(a) Rin-5F WT (lane 1), sham transfectant (lane 2), CXCL12 transfectant clone #1 (lane 3) and transfectant clone #2 (lane 4) were probed with rabbit polyclonal anti-Mn-SOD antibody capable of detecting the monomer form of the Mn-SOD enzyme (23 kDa). The same blot was re-probed with rabbit polyclonal anti-actin antibody. **(b)** Densitometric measurement of Mn-SOD expression in individual clones set in relation to beta actin.

Figure 8: Growth pattern of CXCL12-transfected compared with sham-transfected Rin-5F:

Rat Rin-5F insulinoma cells stable-transfected with human CXCL12 gene and sham-transfected Rin-5F insulinoma cells were cultured in Petri dishes. Using phase-contrast microscopy, we observed a monolayer-like growth pattern in Rin-5F wild-type and sham-transfectant cells **8a**). In contrast, CXCL12 stable-transfected cells **8b**) showed a three-dimensional cluster-like growth pattern.

Figure 9: Immunoblotting of sucrose gradient-cell fractions:

CXCL12 stable-transfected Rin-5F cells were fractionated into 17 aliquots using sucrose gradient sedimentation. Aliquots of individual fractions were loaded onto 12 % SDS-PAGE gel and blotted to nitrocellulose. Sequential evaluation of the membrane markers SNAP-25 and NSF as well as the cytosolic protein SCGN together with rat insulin permitted delineation of different cellular compartments in individual fractions. CXCL12 was demonstrated in lighter fractions devoid of SNAP-25 rather than in heavy insulin-containing granules. Whereas insulin was detected in heavy granules together with the presence of the membrane marker SNAP-25, CXCL12-containing fractions showed the presence of NSF but the absence of SCGN.

Figure 1a)

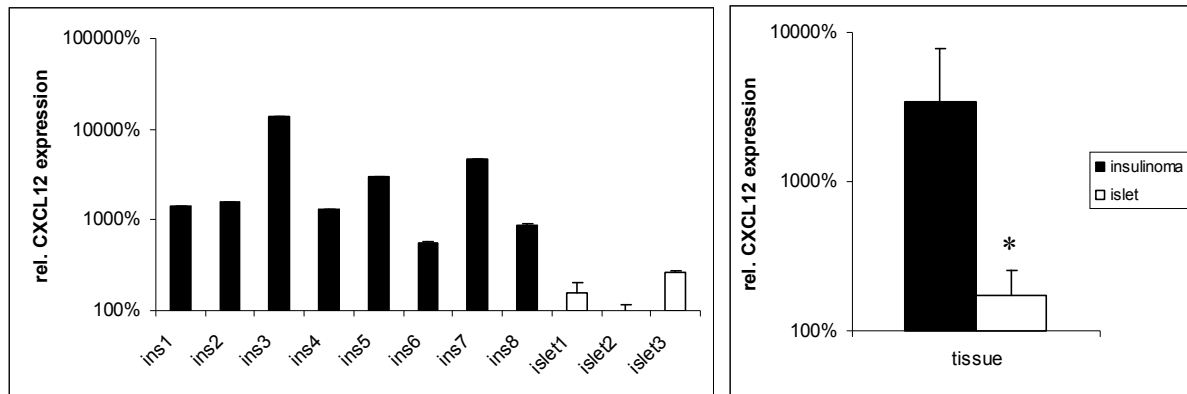


Figure 1b)

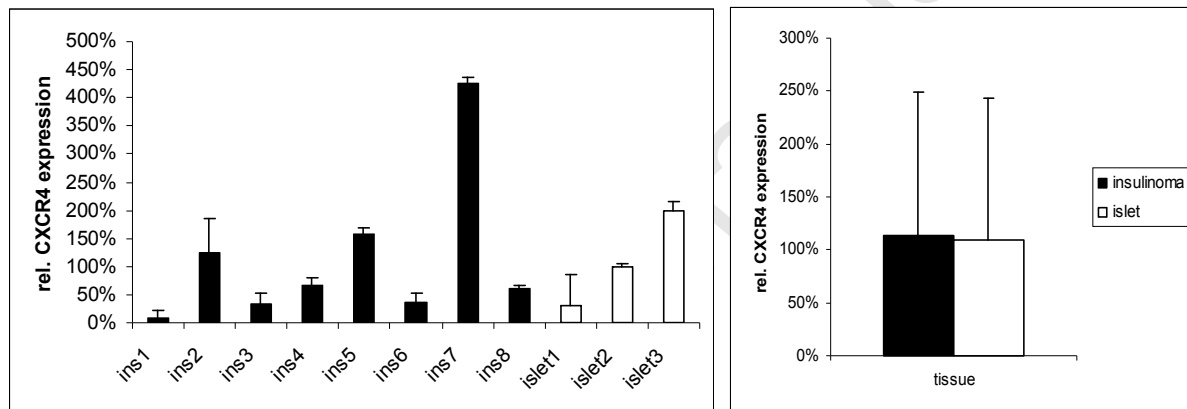
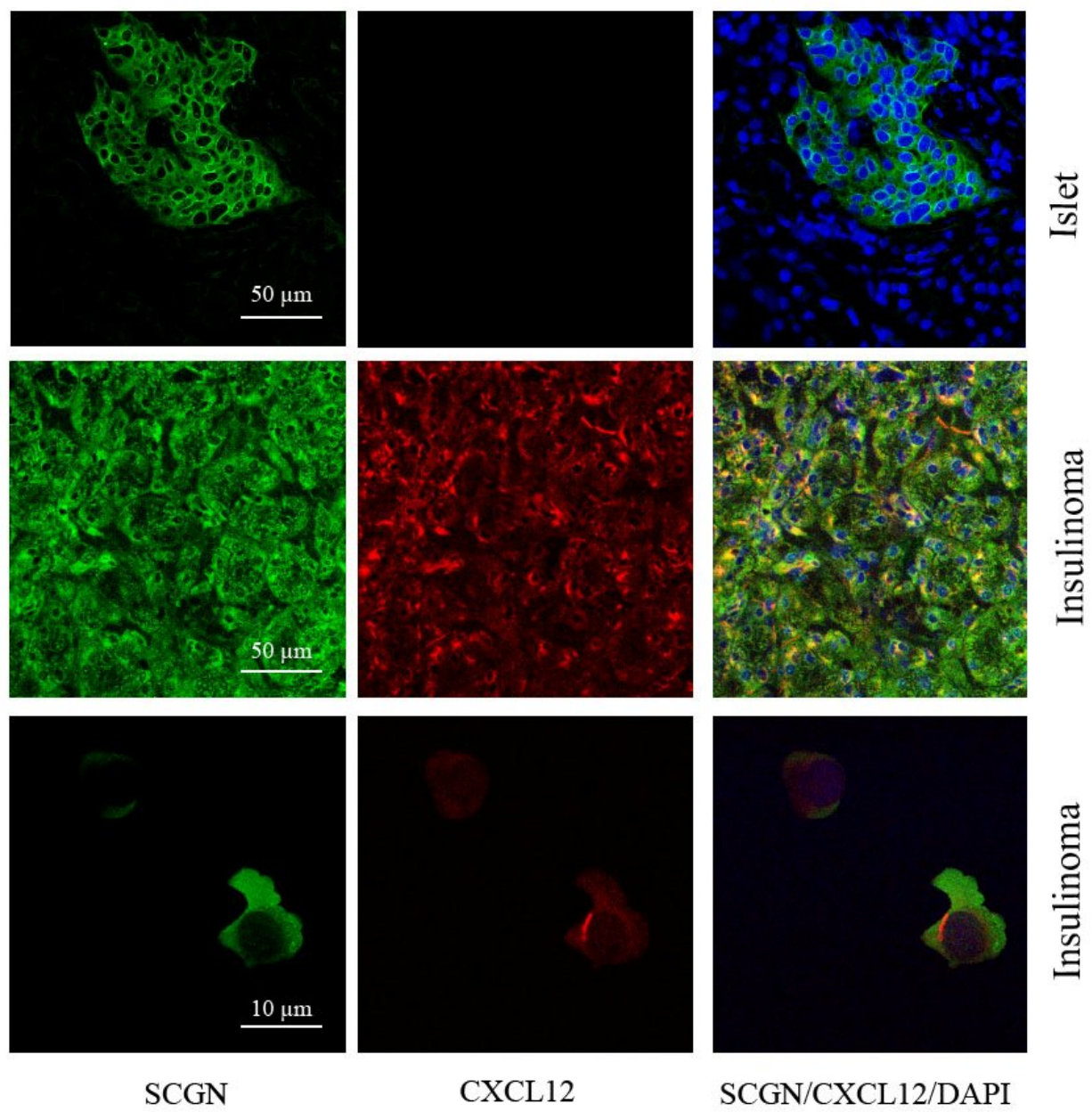


Figure 2)



SCGN

CXCL12

SCGN/CXCL12/DAPI

Figure 3)

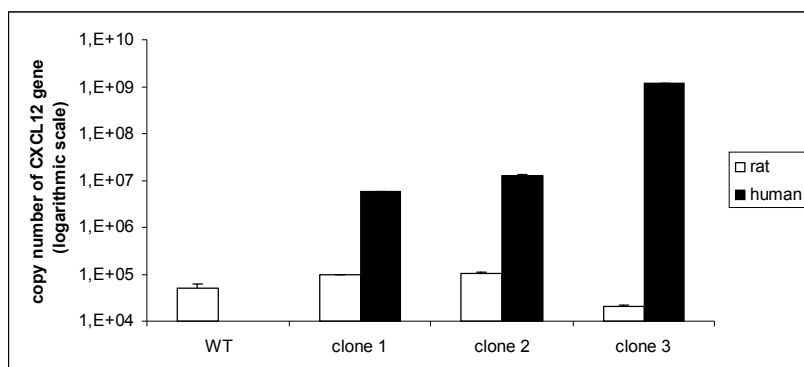


Figure 4)

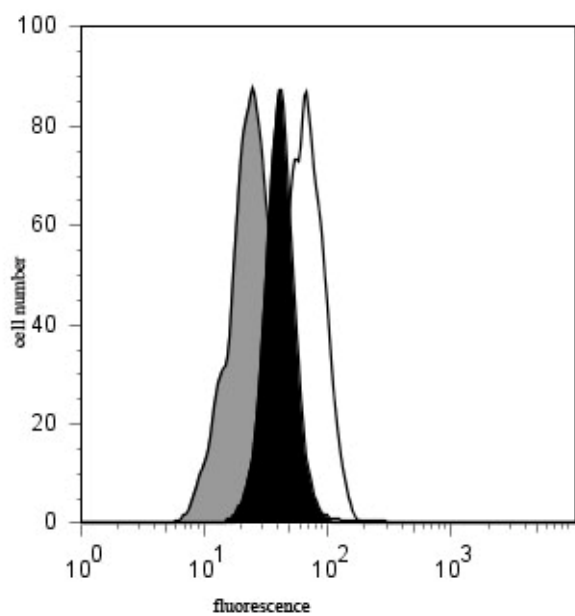


Figure 5)

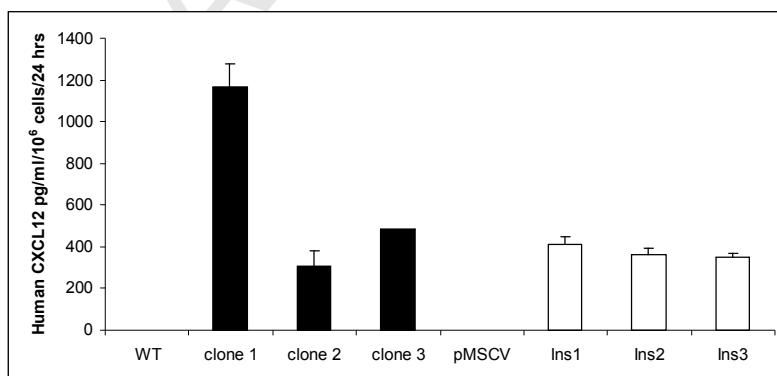


Figure 6a)

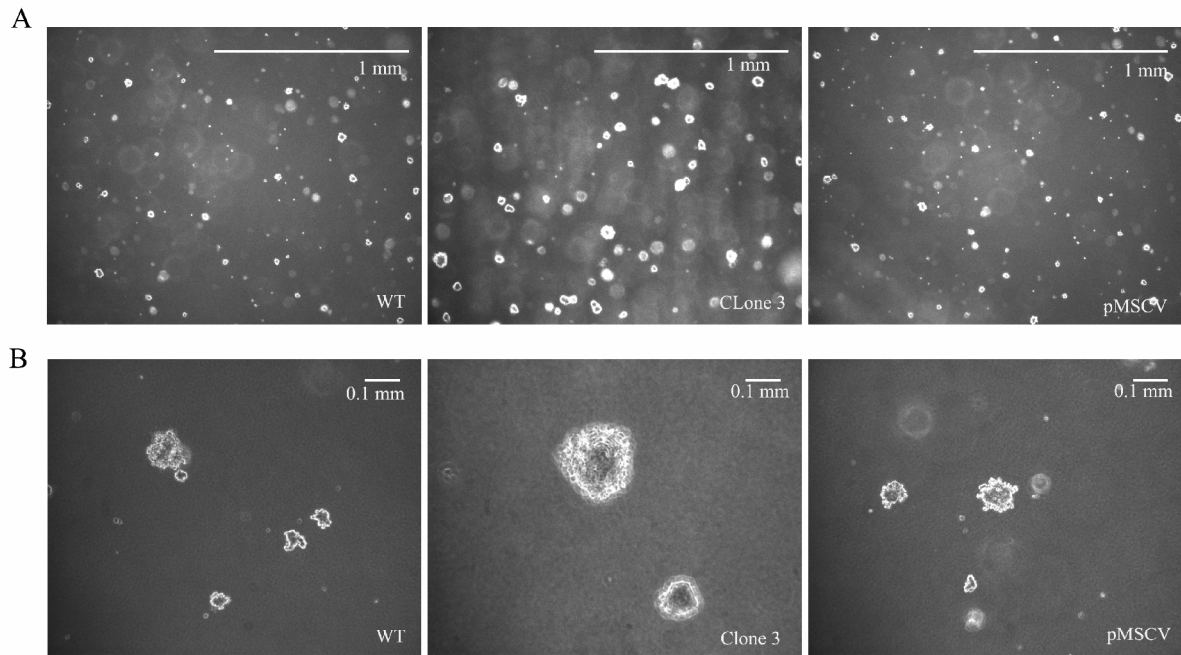


Figure 6b)

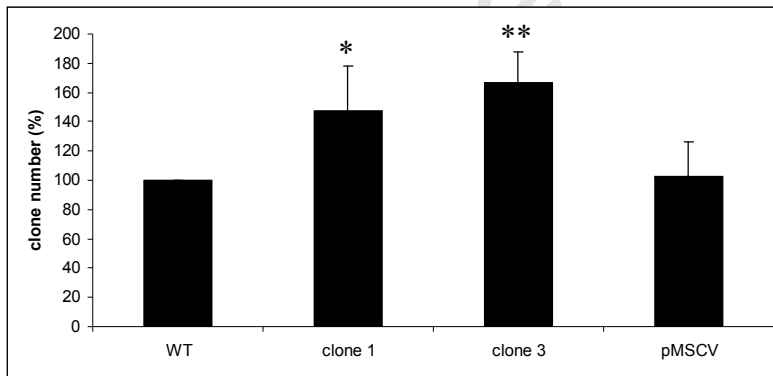


Figure 6c)

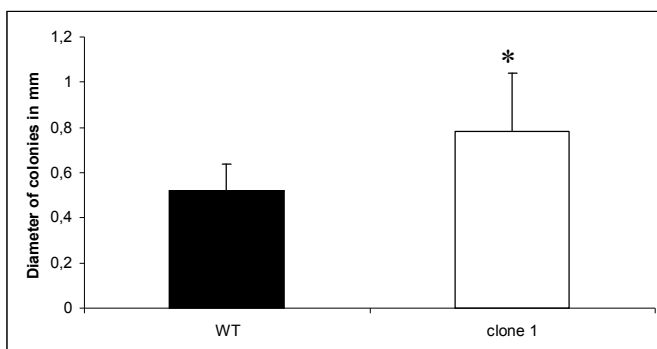


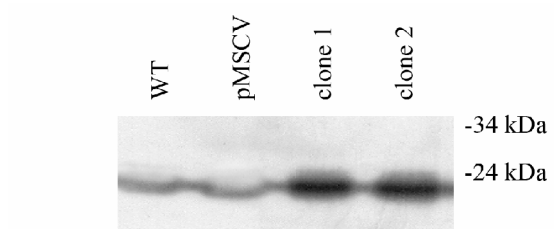
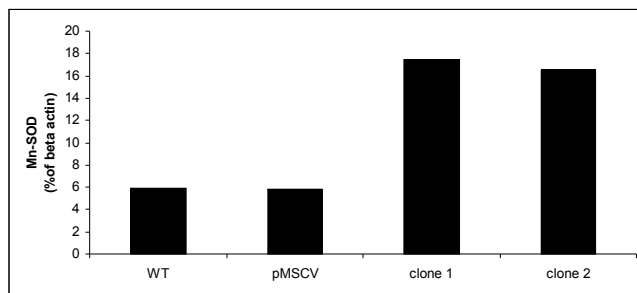
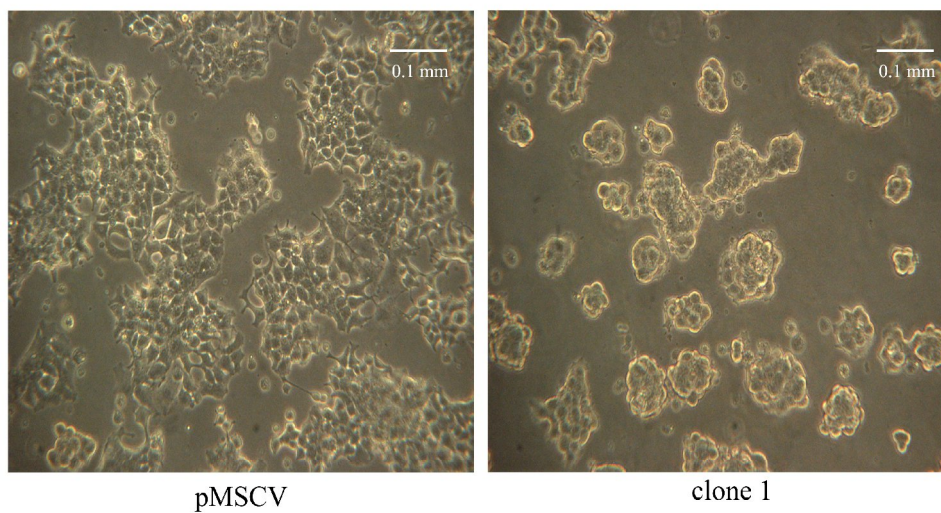
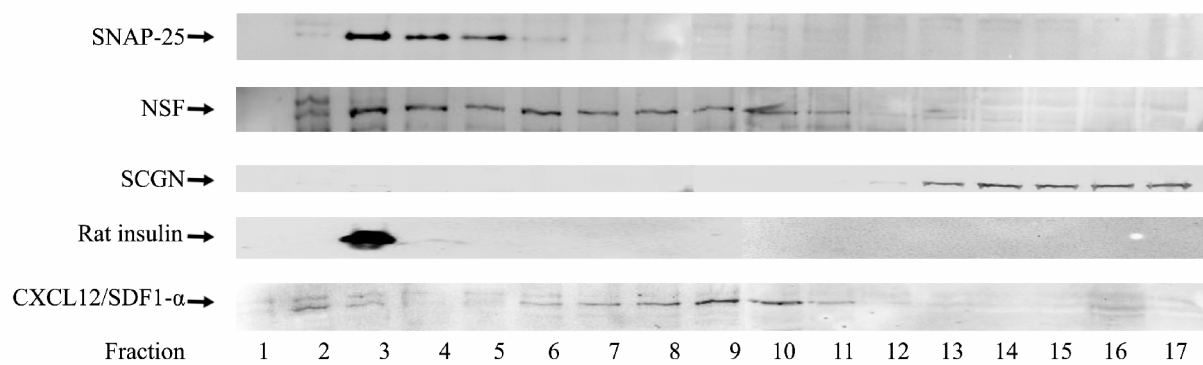
Figure 7a)**Figure 7b)****Figure 8)**

Figure 9)

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