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Growth differentiation factor 8 down-regulates pentraxin 3 in human granulosa cells

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ABSTRACT

Growth differentiation factor 8 (GDF8), also known as myostatin, is highly expressed in the mammalian musculoskeletal system and plays critical roles in the regulation of skeletal muscle growth. Though not exclusively expressed in the musculoskeletal system, the expression and biological function of GDF8 has never been examined in the human ovary. Pentraxin 3 (PTX3) plays a key role in the assembly of extracellular matrix, which is essential for cumulus expansion, ovulation and *in vivo* fertilization. The aim of this study was to investigate GDF8 expression and function in human granulosa cells and to examine its underlying molecular determinants. An established immortalized human granulosa cell line (SVOG), granulosa cell tumor cell line (KGN) and primary granulosa-lutein cells were used as study models. We now demonstrate for the first time that GDF8 is expressed in human granulosa cells and follicular fluid. All 16 follicular fluid samples tested contained GDF8 protein at an average concentration of 3 ng/ml. In addition, GDF8 treatment significantly decreased PTX3 mRNA and protein levels. These suppressive effects, along with the induction of SMAD2/3 phosphorylation, were abolished by co-treatment with the ALK4/5/7 inhibitor SB431542. Knockdown of ALK5, ACVR2A/ACVR2B or SMAD4 reversed the effects of GDF8-induced PTX3 suppression. These results indicate that GDF8 down-regulates PTX3 expression via ACVR2A/ACVR2B-ALK5-mediated SMAD-dependent signaling in human granulosa cells. These novel findings support a potential role for GDF8 in the regulation of follicular function, likely via autocrine effects on human granulosa cells.

1. Introduction

Growth differentiation factor 8 (GDF8), also known as myostatin, is a member of the transforming growth factor-β (TGF-β) superfamily. In mammals, GDF8 is expressed in both embryonic and adult muscle tissues and is a critical autocrine/paracrine inhibitor of skeletal muscle growth (McPherron et al., 1997). Compared to wild type mice, mice lacking the *GDF8* gene have approximately 25–30% increased muscle mass due to muscle fiber hyperplasia and hypertrophy (McPherron et al., 1997). Interestingly, several spontaneous mutations of the *GDF8* gene have been identified in cattle (McPherron and Lee, 1997; Westhusin, 1997) and humans (Schuelke et al., 2004) that also suggests GDF8 acts as a potent negative regulator of skeletal muscle growth. In addition to its roles in skeletal muscle, GDF8 has also been shown to regulate metabolism, including lipid and protein synthesis (Argiles et al., 2012; Taylor et al., 2001), obesity (Hittel et al., 2009) and insulin resistance (Park et al., 2006). GDF8 is ubiquitously expressed in various tissues during embryogenesis in chickens, including the testis and ovary (Kubota et al., 2007). In bovine developing follicles, GDF8 mRNA has been detected by microarray analysis in granulosa cells from different sizes of antral follicles (Skinner et al., 2008). More recently, the expression and putative functions of GDF8 have been investigated in several reproductive tissues, such as placenta and uterus (Islam et al., 2014; Peiris et al., 2014). However, to date, the expression and physiological function of GDF8 in the human ovary have not been identified and characterized.

Pentraxin 3 (PTX3) is a prototype of the long pentraxin family that plays critical roles in female fertility (Salustri et al., 2004). Studies in knockout mice have shown that depletion of PTX3 results in

Abbreviations: ALK, activin receptor-like kinase; cDNA, complementary DNA; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GDF, growth differentiation factor; hGL, human gralulosa-lutein; PCOS, polycystic ovarian syndrome; PTX, pentraxin; siRNA, small interfering RNA; SMAD, Sma- and Mad-related protein; TGF-β, transforming growth factor-β; TSG-6, TNF-α-stimulated gene.

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infertility, featuring severe abnormalities in cumulus oophorus expansion and failure of *in vivo* fertilization (Salustri et al., 2004). During the periovulatory stage, PTX3 functions as an aggregating reagent by linking the tumor necrosis factor-inducible gene 6 (TSG6) protein, which binds directly to hyaluronan, to form a stable hyaluronan network (Russell and Salustri, 2006; Salustri et al., 2004). This network contributes to extracellular matrix assembly and subsequent cumulus expansion, which is essential for oocyte maturation and ovulation, effective oocyte transportation through the fallopian tubes and successful fertilization (Salustri et al., 2004; Vanderhyden and Armstrong, 1989). Given that optimal cumulus expansion is essential for fertility, the regulation and mechanism of PTX3-coupled cumulus expansion are important research topic in ovarian biology.

Polycystic ovarian syndrome (PCOS) encompasses a broad spectrum of signs and symptoms of ovarian dysfunction. Aside from ovulatory dysfunction, PCOS patients often encounter poor fertilization rates of retrieved oocytes during *in vitro* fertilization treatment (Jabara and Coutifaris, 2003). Indeed, the expression levels of PTX3 in cumulus cells have been shown to positively correlate with the quality and fertilization rates of the corresponding oocytes (Huang et al., 2013; Zhang et al., 2005). Interestingly, serum GDF8 concentrations were shown to be higher in women with PCOS than those without PCOS (Chen et al., 2012). Taken together, these data led us to propose that GDF8 down-regulates PTX3 expression and inhibits cumulus expansion, which contributes to ovulatory dysfunction in PCOS patients. Under normal physiological conditions, GDF8 may be considered a cumulus expansion-inhibiting factor secreted by granulosa cells that is capable of maintaining cumulus oophorus in meiotic arrest prior to ovulation. Therefore, the aim of the present study was to examine GDF8 expression in three types of human granulosa cells. In addition, we investigated the effects of recombinant human GDF8 on PTX3 expression and production, as well as the potential underlying mechanisms.

2. Materials and methods

2.1. Preparation of primary human granulosa-lutein (hGL) cells and follicular fluid

Primary hGL cells were obtained with informed patient consent following approval from the University of British Columbia Research Ethics Board. Samples were anonymized immediately after collection and none of the investigators were permitted to access patient information. The controlled ovarian stimulation protocol for *in vitro* fertilization (IVF) patients consisted of either luteal-phase naferelin acetate (Synarel, Pfizer, Kirkland, Quebec, Canada) or follicular phase GnRH antagonist (Ganirelix; Merck Canada) down-regulation. Gonadotropin stimulation began on menstrual cycle day 2 with human menopausal gonadotropin (hMG; Menopur, Ferring, Canada) and recombinant FSH (Puregon, Merck, Canada), and was followed by human chorionic gonadotropin administration 34–36 h before oocyte retrieval, based on follicle size. Granulosa cells were purified by density centrifugation from follicular aspirates collected from women undergoing oocyte retrieval as previously described (Chang et al., 2013c). Immediately after follicular aspiration, the cumulus oocyte complex was separated from follicular fluid. After 20 min of centrifugation at 2400 × *g*, the follicular fluid was stored at –20 °C until it was assayed.

2.2. Simian virus 40 large T antigen-immortalized human granulosa cell (SVOG) and KGN cell culture

A non-tumorigenic immortalized human granulosa-lutein cell line (SVOG), previously produced by transfecting human granulosa-lutein cells with the SV40 large T antigen (Lie et al., 1996), and a human granulosa cell tumor-derived cell line, KGN were used in this study. SVOG or KGN cells were counted with a hemocytometer, and cell viability was assessed by trypan blue (0.04%) exclusion. The cells were seeded ($4–8 \times 10^5$ cells per well in 6-well plates) and cultured in a humidified atmosphere containing 5% CO_2 and 95% air at 37 °C in Dulbecco's ,modified Eagle medium/nutrient mixture F-12 Ham (DMEM/F-12; Sigma-Aldrich Corp., Oakville, ON) supplemented with 10% charcoal/dextran-treated fetal bovine serum (HyClone, Logan, UT), 100 U/ml penicillin (Invitrogen, Life Technologies, NY), 100 μg/ml streptomycin sulfate (Invitrogen) and 1X GlutaMAX (Invitrogen). The culture medium was changed every other day in all of the experiments, and cells were maintained in serum-free medium for 24 h prior to growth factor treatment.

2.3. Antibodies and reagents

Polyclonal rabbit anti-phospho-SMAD2, anti-phospho-SMAD3 and anti-SMAD3 antibodies were obtained from Cell Signaling Technology (Beverly, MA). These antibodies do not cross-react with other SMAD-related proteins. Monoclonal mouse anti-SMAD2 antibody was obtained from Cell Signaling Technology. Polyclonal goat anti-actin (C-11; sc-1615) antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated goat anti-rabbit, goat anti-mouse and donkey anti-goat IgGs were obtained from Bio-Rad and Santa Cruz Biotechnology, respectively. Mouse myeloma cell-derived recombinant human GDF8 obtained from R&D Systems (Minneapolis, MN) was >90% pure (SDS–PAGE) and supplied lyophilized from a 0.2 μm filtered solution in of HCl with bovine serum albumin as a carrier protein. Dorsomorphin dihydrochloride (dorsomorphin) and SB431542 were obtained from R&D Systems and Sigma-Aldrich Corp., respectively.

2.4. Reverse transcription quantitative real-time PCR (RT-qPCR)

Cells were washed with cold PBS and total RNA was extracted with TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. RNA (2 μg) was reverse-transcribed into first-strand cDNA with random primers and MMLV reverse transcriptase (Promega, Madison, WI). TaqMan gene expression assays for MSTN (GDF8), PTX3, ACVR1B (ALK4), TGFBR1 (ALK5), ACVR1 (ALK2), BMPR1A (ALK3), BMPR1B (ALK6), BMPR2, ACVR2A, ACVR2B, SMAD4 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Hs00976237_m1, Hs00173615_m1, Hs00244715_m1, Hs00610320_m1, Hs00153836_m1, Hs01034913_g1, Hs01010965_m1, Hs00176148_m1, Hs00155658_m1, Hs00609603_m1, Hs00929647_m1 and Hs02758991_g1, respectively; Applied Biosystems) were performed in triplicate on corresponding cDNA samples. For each 20 μl TaqMan reaction, 4 μl cDNA was mixed with 5 μl RNase-free water, 10 μl 2X TaqMan gene expression master mix (Applied Biosystems) and 1 μl 20X TaqMan gene expression assay. qPCR was performed on an Applied Biosystems 7300 Real-Time PCR System equipped with 96-well optical reaction plates. The PCR parameters for the reaction were 50 °C for 2 min, 95 °C for 10 min and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Three separate experiments were performed on different cultures, and each sample was assayed in triplicate. A mean value was used for the determination of mRNA levels by the comparative Cq ($2^{-\Delta\Delta Cq}$) method with GAPDH as the reference gene.

2.5. Western blot analysis

After treatment, cells were washed with cold PBS and lysed in lysis buffer (Cell Signaling) containing protease inhibitor cocktail (Sigma-Aldrich). Extracts were centrifuged at 20,000 × *g* for 15 min at 4 °C to remove cellular debris, and protein concentrations were quantified using the DC Protein Assay (Bio-Rad). Equal amounts of