Cloning of porcine signal transducer and activator of transcription 3 cDNA and its expression in reproductive tissues

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Abstract

The signal transducer and activator of transcription 3 (Stat3) protein is a member of the Stat family that has a variety of biological functions including cell growth, anti-apoptosis, and cell motility, depending on the cell type and stimulus. Recent studies have suggested that Stat3 plays an important role in embryo development. Although the Stat3 gene has been cloned in humans, mice, cow, and rats, its sequence in pigs is unknown. In the present study, the 2476 bp Stat3 cDNA was cloned using real time reverse transcriptase (RT)-PCR. Comparison of sequences across species revealed that the porcine Stat3 cDNA is 93 and 90% homologous to human and mouse respectively. To study the expression pattern of Stat3, RNA and protein were isolated from heart, lung, kidney, ovary, oviduct, and uterus tissues. RT-PCR and western blot indicated that Stat3 is expressed in all the tissues tested, and the level of expression is relatively high in tissues from the reproductive system. In addition, immunohistochemistry studies suggested that the Stat3 protein was present in the oocyte, granulosa, theca, and interstitial cells of the ovary, the mucosal folds in the oviduct, and both the epithelium and stromal layers in the endometrium. To study whether Stat3 is functional in responding to growth factor stimulation in the ovary, granulosa cells were isolated from large follicles (> 3 mm) and cultured in the presence of epidermal growth factor (EGF; 10 ng/ml) for 5, 10, 15, 30, and 60 min, following which western blots were performed using an antibody against the phosphorylated Stat3. Phosphorylated Stat3 was upregulated following 5 min of EGF challenge and was sustained during the 15-min stimulation, and decreased back to the control level following 60-min stimulation. The translocation of phosphorylated Stat3 from cytoplasm to nucleus following stimulation of EGF was also detected via immunocytochemistry. Our data suggests that Stat3 may play a role in porcine ovarian function.

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Introduction

The signal transducer and activator of transcription (Stat) proteins are latent cytoplasmic transcription factors that are activated by a series of extracellular signaling proteins, such as cytokines, growth factors, and hormones (Zhong *et al.* 1994, Ihle 1995, Shimoda *et al.* 1997). Seven members of the Stat family (Stat1, 2, 3, 4, 5A, 5B and 6) have been identified in mammals. Each has varying functions and all are important for mediating cellular signaling (for reviews see Darnell 1997, Aaronson & Horvath 2002).

Signal transducer and activator of transcription 3 (Stat3) was originally identified as an interleukin-6 (IL-6) responsive acute-phase factor in the mouse liver, which was capable of selectively interacting with an enhancer element in the promoter sequence of acute-phase genes

(Raz *et al.* 1994, Akira 2000). Similar to other Stat proteins, Stat3 is activated by tyrosine phosphorylation at a single site (Y705) close to the carboxyl terminus, and by serine phosphorylation at a site within the C-terminal transactivation domain (S727). Binding of the IL-6 family cytokines to their specific receptors leads to the heterodimerization of gp130 with cytokine-specific receptors, resulting in the activation of the Janus kinase (JAK) pathway. Subsequently, gp130 is phosphorylated on tyrosine 767, 814, 905, and 915, providing docking sites for the Stat3 Src homology 2 (SH2) domain. Stat3 is then phosphorylated by JAK, which is required for its dimerization, nuclear translocation and transcriptional activity (Greenlund *et al.* 1995, Guschin *et al.* 1995).

Stat3 plays a crucial role in a variety of biological functions including cell growth, survival, and motility

depending on the cell type and stimulus (for review, see Akira 1999). Stat3 is essential during embryogenesis, given that disruption of the mouse Stat3 gene is embryonic lethal at approximately E6.5–7.5 (Takeda *et al.* 1997). Stat3 also plays an important role in mouse and rat reproduction during implantation and decidualization (Teng *et al.* 2004a, 2004b). Additionally, it has recently been reported that leptin regulates porcine ovarian granulosa cell function via the JAK/STAT pathway (Ruiz-Cortes 2003). In addition to IL-6, Stat3 is also activated by other factors, such as the leukemia inhibitory factor (Stewart *et al.* 1992), epidermal growth factor (EGF; Threadgill *et al.* 1995, Chan *et al.* 2004), colony-stimulation factor-1 (Yoshida *et al.* 1990, Stahl *et al.* 1995), and leptin (Takeda *et al.* 1997).

The Stat3 gene has been cloned in humans (Akira *et al.* 1994), mice (Zhong *et al.* 1994), and rats (Ripperger *et al.* 1995), but not in the pigs. EGF is known to stimulate ovarian follicular development in pigs (Mao *et al.* 2004), cows (Gutierrez *et al.* 2000) and rats (Romano *et al.* 1994). It was reported that its action on ovarian granulosa cells occurs via the MAP kinase pathway (Keel *et al.* 1995, Keel & Davis 1999). Whether the Stat3 pathway is also involved in mediating EGF regulation of ovarian function remains unknown. The objective of the present study was to clone the porcine Stat3 coding sequence, study its expression distribution pattern in porcine tissues, and its possible involvement in EGF signaling in porcine granulosa cells.

Materials and Methods

cDNA cloning and sequencing

We have previously reported the expression of Stat3 in porcine skin stem cells (Dyce *et al.* 2004). In the present study, total RNA was isolated from porcine skin stem cells, and reverse transcribed and amplified by PCR using a long template PCR system (Boehringer Mannheim GmbH, Germany) according to the manufacturer's instructions. The primers used in the PCR amplification were: forward 5'-acaggatccttgacgagcac-3' and reverse 5'-ctctctgcagctcctgtcct-3', based on porcine expressed sequence tags (ESTs) (GenBank accession no. CK453710 and BX675510), which are highly homologous (94 and 93% respectively) with human Stat3 mRNA (NM_139276). PCR was performed using Platinum's Taq DNA polymerase (Invitrogen). The PCR product was cleaned and ligated to the pGEM-T Easy vector (Promega). The resulting plasmids with Stat3 cDNA from four independent PCR were sequenced to confirm their identity.

Sample collection

Tissues (lung, kidney, heart, ovary, oviduct, and uterus) were collected from five Yorkshire gilts at slaughter from an

abattoir and frozen immediately using liquid nitrogen. Once frozen, tissues were stored at -80°C until analysis.

RNA isolation and real time RT-PCR

Total RNA was isolated using Trizol reagent (Invitrogen). Approximately, 0.5 μg RNA was DNase I treated and reverse transcriptase (RT)-PCR was performed according to the manufacturer's directions (Invitrogen). Real Time RT-PCR was performed using the SYBR Green PCR kit (Qiagen) and a Smart Cycler thermocycler (Cepheid, Sunnyvale, CA, USA). Briefly, 12.5 μl SYBR Green 2 $\times$ mix (contains HotStar Taq DNA polymerase, QuantiTect SYBR Green PCR buffer, dNTP mix, SYBR Green 1, and 5 mM MgCl_2), 0.3 μM each of primer (Dyce *et al.* 2004; 5'-cccgtacacgaagacaaagt-3' and 5'-gggtcagcagcttcaccatt-3') and 2.5 μl cDNA were included in a 25 μl PCR. Melt curve analysis was performed to confirm the specificity of the products amplified. The relative expression was analyzed using $2^{-\Delta\Delta\text{Ct}}$ method (Livak & Schmittgen 2001). Hypoxanthine-phosphoribosyltransferase (HPRT) was used as a housekeeping control using primers: 5'-ccagtcacggcgcatataa-3' and 5'-cttgaccaaggaaagcaagg-3'. All PCR products were sequenced to confirm their identity.

Protein isolation and western blot analysis

Protein was isolated using a previously described method (Matsuoka *et al.* 1999). Approximately, 0.5 g of each tissue was homogenized in 1 ml ice-cold buffer using a homogenizer. Protein was quantified using Bio-Rad protein quantification reagent, and approximately 30 μg protein was subjected to 10% SDS-PAGE. Protein was transferred to a PVDF membrane (Millipore), which was blocked overnight in 5% skim milk at 4°C . The membranes were incubated with antibody against Stat3 (1:1000, rabbit anti-human, Santa Cruz Biotechnology), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:40 000, rabbit anti-human, Santa Cruz Biotechnology) and phosphorylated Stat3 (1:1000, p-Stat3 Y705, rabbit anti-human, Santa Cruz Biotechnology) respectively, diluted in PBS, 0.1% Tween-20 and 5% skim milk powder for 1 h at room temperature, and followed by incubation with the secondary antibody (1:8000, goat anti-rabbit IgG horse-radish peroxidase; Cell Signaling Technology) for 1 h at room temperature. Proteins were detected using the ECL Advance Western Blotting Detection Kit (Amersham). Digital images of X-ray films were obtained using GeneSnap and densitometry of band intensities was performed using GeneTools (Syngene).

Granulosa cell isolation, culture and epidermal growth factor stimulation

Porcine ovaries (approximately 70 ovaries were used per experiment) were collected from pre-pubertal gilts

at a local slaughterhouse and transported to the laboratory in PBS (37 °C). Granulosa cells were aspirated from large-sized (> 3 mm) follicles, pelleted, and cultured as previously described (Li *et al.* 1995). Briefly, viable cells ($7\text{--}9 \times 10^6$ cells/ml) in minimum essential medium (Gibco BRL) containing 1 mg/l insulin (Sigma), 0.1 mM non-essential amino acids (Gibco BRL), 5×10^4 IU/l penicillin (Gibco BRL), 50 mg/l streptomycin (Gibco BRL), 0.5 mg/l fungizone (Gibco BRL), and 10% fetal calf serum (FCS; Gibco BRL) were plated in 2 ml on a 3.5 cm cell culture dish (Corning) for 24 h at 37 °C. EGF (10 ng/ml) challenges were performed at the time of 0, 5, 10, 15, 30, and 60 min, and cells were immediately lysed for western blot analysis or fixed for immunocytochemistry.

Immunofluorescence

For tissue sections: tissues were fixed with 10% formalin for 20–24 h and paraffin-embedded. Tissue sections of 5 µm were deparaffinized in Xylene (Fisher) and rehydrated in decreasing concentrations of ethanol (100, 90, and 70%). Following 0.2% sodium borohydride treatment, a non-specific binding was blocked by incubation in 5% non-immune rabbit serum (Invitrogen). Slides were incubated overnight at 4 °C in a humidified chamber with the primary antibody (1:300, rabbit anti-human Stat3, Santa Cruz Biotechnology). After two washes in PBS, the secondary antibody (1:500, goat anti-rabbit IgG FITC, fluorescein isothiocyanate, Sigma) was incubated with the slides for 2 h in the dark. Slides were counter stained with 4,6-diamidino-2-phenylindole (DAPI), and coverslips were mounted using fluorescent mounting medium (Dako-Cytomation). Negative control slides were subjected to the same method, in which the first antibodies were substituted with an equivalent dilution of non-immune rabbit serum (Invitrogen). Fluorescence was detected using an Olympus BX-UCB microscope and MetaMorph image analysis software (Universal Imaging Corporation).

For cultured cells: granulosa cells were fixed in 4% paraformaldehyde for 20 min followed by treatment in 0.1% Tween 20 for 10 min. Cells were then blocked and permeabilized in PBS supplemented with 0.1% Triton X-100 and 5% BSA for 1 h. Rabbit anti-human Stat3 (1:500) or p-Stat3 (1:500, Santa Cruz Biotechnology) was incubated with the blocked cells for 2 h, followed by goat anti-rabbit FITC secondary antibody incubation (1:1000, Sigma) for 1.5 h at room temperature. Cells were counterstained with DAPI, and mounted on slides with fluorescent mounting medium. For negative controls, slides were subjected to the same method, except the primary antibodies were substituted with equivalent dilution of non-immune rabbit serum (Invitrogen). Fluorescence was examined using an Olympus BX-UCB microscope and MetaMorph image

analysis software (Universal Imaging Corporation). The percentage of nuclei p-Stat3 positive cells (p-Stat3 positive nuclei/total p-Stat3 positive cells) was determined by counting at least 150 p-Stat3 positive cells per group.

Statistical analysis

Relative levels of Stat3 mRNA and protein, and p-Stat3 proteins were analyzed by ANOVA. Significant results were analyzed by Tukey's multiple comparison test. Results were considered significant at $P < 0.05$. Each experiment was repeated at least three times.

Results

Cloning and sequencing of porcine Stat3 cDNA

Porcine ESTs homologous with human Stat3 mRNA sequence were obtained using NCBI's Blastn database search. ESTs CK453710 and BX675510 were 94 and 93% homologous to the human 5' and 3' Stat3 mRNA sequence including the translational start and stop codon respectively. Primers were designed based on these ESTs and used to obtain a 2476 bp porcine Stat3 coding sequence (DQ470570, Fig. 1a) via RT-PCR. The porcine sequence was compared to the human (NM_139276) and the mouse (NM_213659) Stat3 cDNA sequences using NCBI's Align Two Sequences Tool. Overall, the porcine cDNA sequence shows 93 and 90% homology to human and mouse mRNA sequences respectively. Individual functional domain encoding sequences show variable homology, ranging from 89 to 96%. As shown in Fig. 1b, the N-terminal domain encoding sequence is 93 and 92% homologous to human and mouse respectively. The DNA binding domain-encoding sequence is 92 and 90% homologous to human and mouse respectively. The SH2 domain-encoding sequence is 94 and 89% homologous to human and mouse respectively, while the C-terminal domain is 96 and 94% homologous respectively. The predicted pig Stat3 amino acid sequence is highly homologous (>99%) with human and mouse Stat3 protein.

Stat3 mRNA expression in porcine tissues

To study the expression pattern of Stat3 mRNA, total RNA was isolated from porcine heart, lung, kidney, and female reproductive tissues (ovary, oviduct, and uterus). The relative levels of Stat3 mRNA were determined using Real Time RT-PCR. As shown in Fig. 2, the 129 bp Stat3 PCR products were detectable in all the tissues tested. Quantitation using the $2^{-\Delta\Delta C_t}$ method (Livak & Schmittgen 2001) suggested that the expression is higher in the reproductive organs and lung, when compared to that in the heart and kidney.

Figure 1 (a) Porcine Stat3 cDNA sequence (DQ470570) and predicted amino acid sequence. Both the start and stop codons are shown in boxes. The conserved domains are underlined: solid, N-terminal domain; square dot, coiled-coil domain; long dash, DNA-binding domain; round dot, SH2 domain; and dash dot, C-terminal domain. (b) Comparison of Stat3 sequences encoding functional domains from the pig, human, and mouse. The porcine Stat3 cDNA sequence was compared with that of human (NM_139276) and mouse (NM_213659) sequences using NCBI's blastn program. The functional domains of the predicted Stat3 are shown in bars: horizontal, N-terminal domain; vertical, coiled-coil domain; solid, DNA-binding domain; open, SH2 domain; and dot, C-terminal domain. The sequence was from four independent reverse transcriptase (RT)-PCRs.

Stat3 protein expression and localization

Consistent with the mRNA study, Stat3 protein was detected in all tissues analyzed (Fig. 3a), however, the relative expression level in the kidney and heart was

significantly lower ($P < 0.05$) than in the other tissues tested (Fig. 3b).

To further study where Stat3 is present and may exert its physiological role within the reproductive tissues,

Figure 2 Detection of Stat3 mRNA in adult pig tissues. Total RNA was isolated from porcine tissues and reverse transcribed to cDNA. Ten percent of the RT reaction was used as a template for real time RT-PCR amplification of Stat3 and HPRT. Expression of HPRT was used for normalization of Ct values, and the $2^{-\Delta\Delta C_t}$ methods were used to calculate the relative expression between tissues. Relative levels of Stat3 mRNA are shown in this figure. Data are the mean $\pm$ S.E.M. of four independent experiments using four animals. Different alphabetical letters indicate statistical differences ($P < 0.05$).

immunofluorescence was performed on paraffin embedded porcine ovarian, oviduct, uterine tissues, and cultured granulosa cells. In the ovary, Stat3 was localized in the oocyte, granulosa, theca, and interstitial cells, with a similar localization pattern from preantral and antral follicles (Fig. 4a, g), while in the oviduct Stat3 was detected primarily in the mucosal folds (Fig. 4c).

Figure 3 Protein expression of Stat3 in porcine tissues. Protein was isolated from gilts tissues and subjected to SDS-PAGE. (a) Representative western blot of Stat3 and GAPDH. Lane 1, lung; lane 2, kidney; lane 3, heart; lane 4, ovary; lane 5, oviduct; and lane 6, uterus. (b) Relative levels of Stat3 expression. Data are expressed as the fold of heart Stat3 expression and are the mean $\pm$ S.E.M. of five independent experiments using five animals. Different alphabetical letters indicate a statistical difference ($P < 0.05$).

In the uterus, high levels of expression were observed in the endometrium both in the epithelium and stromal layers (Fig. 4e).

EGF induced Stat3 phosphorylation, translocation in granulosa cells

Since Stat3 is expressed in ovarian granulosa cells (Fig. 4a, g), we then studied whether Stat3 is phosphorylated in response to growth factor stimulation. Granulosa cells were isolated from porcine ovaries cultured in medium with 10% FCS, and challenged with EGF (10 ng/ml) for 0 (control), 5, 10, 15, 30, and 60 min following 24 h of culture. Protein was then isolated from these granulosa cells and western blots were performed to assess the content of the phosphorylated form of Stat3. As shown in Fig. 5a, although the Stat3 protein was present in all the groups tested at a similar level, the p-Stat3 was upregulated following 5 min of EGF stimulation and remained high during 15 min of the challenge. The amount of p-Stat3 decreased following 30 min of EGF stimulation and was in at a very low level when compared to 0 min following 60 min of EGF stimulation. To study whether p-Stat3 is also translocated to the nucleus after phosphorylation, immunocytochemistry was performed using specific antibody against the p-Stat3. p-Stat3 was not detectable in granulosa cell nuclei prior to stimulation. From 5 min of EGF stimulation, p-Stat3 was observed to be present in the nuclei of some granulosa cells (Fig. 6 d, f, h). The percentage of nuclear p-Stat3 positive cells was approximately 16, 35, and 38% for 5, 10, and 15 min respectively.

Discussion

The present study cloned the porcine Stat3 coding sequence and demonstrated that Stat3 is expressed in various pig tissues, with relatively high levels in reproductive tissues, and can be phosphorylated in response to EGF stimulation in ovarian granulosa cells.

Comparison of human (Akira *et al.* 1994), mouse (Zhong *et al.* 1994), and rat (Ripperger *et al.* 1995) Stat3 coding sequences revealed that the homology between human and mouse is 90% and between human and rat is 90%. Our present study shows that porcine Stat3 cDNA is 93 and 90% homologous to human and mouse Stat3 mRNA sequences respectively. This finding confirms that there is high homology over the region of coding sequence among mammalian species. Stat3 has several conserved domains that are critical for function. This includes a N-terminal domain, coiled-coil domain, DNA-binding domain, SH2 domain, and C-terminal transactivation domain. The N-terminal domain allows Stat3 dimerization and is highly conserved within the Stat family (Calo *et al.* 2003). The Stat3 DNA-binding domain is responsible for

Figure 4 Localization of Stat3 protein in reproductive tissues and granulosa cells. Stat3 protein was detected (green) in porcine ovarian (A), oviduct (C), uterine (E) tissues, and isolated granulosa cells (G). In the ovary, Stat3 was noted in the oocyte (a), granulosa cells (b), theca cells (c) and interstitial cells (d) in an antral follicle. A similar expression pattern was observed in a preantral follicle (Fig. a, inset). In the oviduct Stat3 was detected in the mucosal folds (e). In the uterus, high levels of expression were noted in the endometrium, in both the epithelium (f) and stromal (g) layers. B, D, F and H are negative controls for ovary, oviduct, uterus and granulosa cells respectively, in which primary antibody was substituted with non-immune rabbit serum. Magnification: A and insert box, C, B, D, F, G, H 100 $\times$; E, 200 $\times$.

binding of Stat3 to target gene sequences, which is usually a conserved DNA sequence TT(N)₄AA (Seidel *et al.* 1995). Both the coiled-coil domain and the SH2 domain are involved in protein–protein interactions. The SH2 domain is required for the recruitment of Stats to phosphorylated receptors and the SH2-phosphotyrosine interactions between monomeric Stats to form dimers (Shuai 1999). The function of the C-terminal domain is to communicate with the transcriptional complex, which has a conserved serine (Calo *et al.* 2003). The high homology among pig, human, and mouse Stat3 sequences coding for functional domains suggests that the function(s) of these domains may also be conserved among mammalian species.

Stat3 plays an important role in the regulation of cell proliferation, apoptosis and cell migration in a cell specific manner (Akira 1999). Our data showed that Stat3 is expressed, both at the mRNA and protein level, in various tissues in the pig. Our findings that Stat3 is expressed at high levels in the reproductive tissues suggest that Stat3 may play a role in the regulation of reproductive function. The presence of Stat3 protein in the mucosal folds of the oviduct, and the endometrium of the uterus is consistent with the previously reported role of Stat3 during early embryogenesis (Takeda *et al.* 1997), implantation and development (Teng *et al.* 2004a, 2004b). It is possible that the expression of Stat3 in the oviduct and uterus is involved in upregulating the expression of genes that are important for early embryo development and implantation. In addition, we have also observed that Stat3 is expressed in oocytes,

Figure 5 The phosphorylation of Stat3 in granulosa cells in response to EGF stimulation. Granulosa cells were challenged with EGF (10 ng/ml) for 0 (control), 5, 10, 15, 30, and 60 min respectively, in the presence of 10% FCS. Protein was then isolated and western blot was performed. (a) Representative western blot of phosphorylated and non-phosphorylated Stat3. (b) Relative level by densitometry quantitation of the phosphorylated Stat3 normalized by the non-phosphorylated Stat3. Data are the mean $\pm$ s.e.m. of four independent experiments. Different alphabetical letters indicate a statistical difference ($P < 0.05$).

Figure 6 Phosphorylated Stat3 (p-Stat3) localization in granulosa cells in response to EGF stimulation. (a) p-Stat3 was minimum in granulosa cells in the absence of EGF; (c–h) p-Stat3 level was elevated in granulosa cells at 5 (C, D), 10 (E, F), and 15 (G, H) min of EGF stimulation. From 5, 10, and 15 min of EGF stimulation, p-Stat3 was observed to be present in the nuclei of some granulosa cells (d, f, h arrow pointed). (b) Negative control in which primary antibodies were substituted with non-immune rabbit serum. Cells were counterstained with DAPI (blue) to show nuclei. Magnification: A, C, E, G: 200 $\times$; B, D, F, H: 400 $\times$.

granulosa, and theca cells within the ovary. The expression of Stat3 in theca cells has not been reported previously and future studies may investigate the role of Stat3 in this cell type. The role of Stat3 in the regulation of granulosa cell functions remains largely unclear, although it has been recently shown that Stat-3 mediates leptin's modulation of steroidogenesis in granulosa cells (Ruiz-Cortes *et al.* 2003). Interestingly, Stat3 is also involved in the regulation of oocyte maturation by leptin (Matsuoka *et al.* 1999). However, whether the Stat3 pathway can be triggered by growth factors or cytokines in the ovary was unknown. This study demonstrated that EGF induced the phosphorylation of Stat3 in ovarian granulosa cells. Meanwhile the phosphorylated Stat3 translocated to the nuclei in response to EGF stimulation. These results suggest that EGF may be another activator of the Stat3 pathway in granulosa cells. Interestingly, EGF is known to control the initial growth of granulosa cells (Driancourt & Thuel 1998), apoptosis (Oliver *et al.* 1999), and the initiation of steroidogenesis (Li *et al.* 1995, Hernandez & Bahr 2003). The regulation of granulosa cell function by EGF was previously shown to be mediated via the MAP kinases pathway (Keel *et al.* 1995, Keel & Davis 1999). To our knowledge, this is the first report on the possible involvement of Stat3 in the EGF signaling in ovarian cells, irrespective of the animal species. Activation of the Stat3 pathway may be a novel mechanism of EGF action within the ovary.

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