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Discovery of a novel oocyte-specific Krüppel-associated box domain-containing zinc finger protein required for early embryogenesis in cattle

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ABSTRACT

Zinc finger (ZNF) transcription factors interact with DNA through zinc finger motifs and play important roles in a variety of cellular functions including cell growth, proliferation, development, apoptosis, and intracellular signal transduction. One-third of ZNF proteins in metazoans contain a highly conserved N-terminal motif known as the Krüppel-associated box (KRAB) domain, which acts as a potent, DNA-binding dependent transcriptional repression module. Analysis of RNA-Seq data generated from a bovine oocyte cDNA library identified a novel transcript, which encodes a KRAB-containing ZNF transcription factor (named ZNFO). Characterization of ZNFO mRNA expression revealed that it is exclusively expressed in bovine oocytes and early embryos. A GFP reporter assay demonstrated that ZNFO protein localizes specifically to the nucleus, supporting its role in transcriptional regulation. To test the role of ZNFO in early embryonic development, zygotes were generated by in vitro maturation and fertilization of oocytes, and injected with small interfering RNA (siRNA) designed to knockdown ZNFO. Cleavage rates were not affected by ZNFO siRNA injection. However, embryonic development to 8- to 16-cell stage and blastocyst stage was significantly reduced relative to the uninjected and negative control siRNA-injected embryos. Further, interaction of ZNFO with the highly conserved co-factor, KRAB-associated protein-1 (KAP1), was demonstrated, and evidence supporting transcriptional repression by ZNFO was demonstrated using a GAL4-luciferase reporter system. Results of described studies demonstrate that ZNFO is a maternally-derived oocyte-specific nuclear factor required for early embryonic development in cattle, presumably functioning by repressing transcription.

1. Introduction

Early embryonic development is one of the most critical periods in mammalian development and comprises several important transitions including replacement of maternal RNAs with zygotic RNAs, compaction, the first lineage differentiation into the inner cell mass and trophectoderm and, finally, implantation. Various physiological processes and biosynthetic changes regarding genomic activity take place during this early time. Among these events is the first important developmental transition that occurs following fertilization at which time the embryo switches from using transcripts derived from the maternal genome to those synthesized by the zygote as the result of embryonic genome activation (EGA) (Stitzel and Seydoux, 2007). Mammalian oocytes accumulate a vast collection of mRNA and proteins throughout oogenesis that mediate subsequent embryonic development. During oocyte meiotic maturation and the early stages of embryonic development the transcriptional machinery for this collection of molecules is silent until EGA initiates transcriptional activity within the embryonic nucleus. The onset of EGA is a species-specific event that takes place during the first few cell cycles post-fertilization; around the 8- to 16-cell stage in bovine embryos (Memili and First, 1999; Memili and First, 2000), several cycles later than observed for the mouse (2-cell stage) or human (4- to 8-cell stage) (Telford et al., 1990; Latham and Schultz, 2001). Therefore, any developmental events required for early embryogenesis (i.e. meiotic maturation, fertilization, initial cleavage divisions, and programming of EGA) prior to the onset of EGA are regulated by the translation of pre-existing maternal transcripts (Bettegowda and Smith, 2007).

Zinc finger (ZNF) genes compose one of the largest protein superfamilies in eukaryotic organisms and uphold an essential role in

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transcriptional regulation. In particular, the Cys2His2 (C2H2) class of ZNFs dominate approximately 53% (~700) of the transcription factor repertoire of the mammalian genome (Vaquerizas et al., 2009). Structurally, C2H2 ZNFs are named for the zinc finger motifs, each comprised of 28–30 amino acids, and each stabilized by a zinc ion that coordinates four highly conserved residues, two cysteines and two histidines (Lee et al., 1989). The carboxy-terminal portion of C2H2 ZNF proteins contain from 1 to >30 individual zinc finger motifs arranged in a cluster of tandem repeats. Each individual zinc finger motif is defined by the presence of the consensus sequence Φ-X-Cys-$X_{(2-4)}$-Cys-X_3-Φ-X_5-Φ-X_2-His-$X_{(3-4)}$-His, where X represents any amino acid and Φ represents a hydrophobic residue (Klug and Schwabe, 1995). Transcriptional regulation occurs through sequence-specific DNA binding of these motifs to promoter regions of target genes (Lupo et al., 2013). Although each zinc finger domain is structurally similar, variations of key amino acid residues at particular sites, as well as zinc finger number, create chemical distinctiveness allowing for a great number of possibilities for DNA recognition (Pavletich and Pabo, 1991) and, hence, the variety and presence of ZNFs in nearly all aspects of biological processes (Vaquerizas et al., 2009; Klug and Schwabe, 1995; Urrutia, 2003; Emerson and Thomas, 2009).

One third of the various conserved domains that contribute to C2H2-ZNF protein function contain the Krüppel-Associated Box (KRAB) domain (Urrutia, 2003), making KRAB-ZNFs the single largest group of transcriptional repressors in the genomes of higher organisms. The KRAB domain is known as a potent transcriptional repression module responsible for DNA binding-dependent gene silencing activity and is located at the amino-terminal end of most C2H2 ZNF proteins (Margolin et al., 1994; Witzgall et al., 1994). When tethered to DNA via its zinc finger motifs, the KRAB domain of KRAB-ZNF proteins recruits and interacts with the corepressor protein KRAB-associated protein 1 (KAP1) (Friedman et al., 1996; Moosmann et al., 1996), which is an absolute requirement for KRAB-containing zinc finger proteins to bind and mediate transcriptional repression. Upon binding to DNA, KAP1 functions as a scaffold to form a multi-molecular complex that induces transcriptional silencing by condensing chromatin. As a powerful transcriptional repressor, most members of the KRAB-ZNF family have diverse functional roles in nearly all tissues and a variety of cellular functions, including cell proliferation and differentiation, metabolism, apoptosis, neoplastic transformation, cell cycle regulation, and regulating embryonic development (Lupo et al., 2013; Urrutia, 2003).

In an effort to characterize the bovine oocyte transcriptome in search of oocyte-specific factors essential for the regulation of folliculogenesis and early embryonic development in cattle, a bovine oocyte cDNA library was previously constructed. Analysis of RNA-Seq data from this library identified a novel transcript that matches an uncharacterized KRAB-containing zinc finger gene (named ZNFO) which is explicitly expressed in the bovine oocyte. We hypothesized that this novel KRAB-containing zinc finger protein has a distinct and essential role in the development of the early bovine embryos. Herein, we report the identification and cloning of cDNA encoding ZNFO, the temporal expression of ZNFO mRNA during oocyte maturation and early embryonic development, and evidence supporting a critical role for ZNFO in early embryogenesis in cattle. We also show that ZNFO interacts with the highly conserved co-factor, KAP1, and functions as a transcriptional repressor.

2. Materials and methods

2.1. Tissue collection

Bovine tissue samples, including adult lung, spleen, stomach, brain, muscle, kidney, liver, heart, intestine, ovary, adult testis, fetal testis, and fetal ovaries were collected from a local slaughterhouse. Age of fetuses from which fetal ovaries were collected was estimated by measuring crown-rump length (Richardson et al., 1990). Granulosa and theca cells were isolated from antral follicles as described previously (Murdoch et al., 1981). All samples were frozen in liquid nitrogen following collection and stored at −80 °C until use.

2.2. RNA isolation, cDNA synthesis, and RT-PCR analysis

Total RNA was extracted using Tri-reagent (Ambion, Inc., Austin, TX) according to the manufacturer's instructions. Isolated total RNA was treated with TURBO™ DNase I (Ambion) before cDNA synthesis. Approximately 2 μg of DNase-treated total RNA was used for first strand cDNA synthesis in a 20 μl reaction including Oligo $(dT)_{18}$ primer and SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA). Concentrations of isolated RNA were determined by measuring absorbance at 260 nm. Purity of RNA was determined by calculating the ratio of absorbance at 260 nm and 280 nm, and integrity of RNA was determined by agarose gel electrophoresis. The cDNA was used as a template for PCR amplification of ZNFO mRNA fragments using gene-specific primers (Supplemental Table S1). The RT-PCR was performed by denaturation at 95 °C for 3 min followed by 35 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 90 s and final extension at 72 °C for 10 min. The amplified products were separated through a 1% agarose gel containing RGB. Amplification of cDNA for bovine ribosomal protein L19 (*RPL19*) was used as a positive control for RNA quality and RT.

2.3. Plasmid construction

For preparation of glutathione S-transferase (GST) fusion recombinant protein, the open reading frame (ORF) of ZNFO cDNA was amplified and cloned into pGEX-4T1 (GE Healthcare, Salt Lake City, UT) using primers containing *Sma*I and *Xho*I sites (pGEX-4T1-ZNFO). The KAP1 ORF was amplified and cloned into pcDNA3.1 (Invitrogen, Carlsbad, CA) using a forward primer containing a Kozak sequence and *Hind*III site and a reverse primer containing a FLAG-tag sequence and *Bam*HI site (pcDNA3.1-KAP1-FLAG). The ZNFO expression construct used in the luciferase reporter assay was generated by cloning the PCR-amplified ZNFO ORF in frame with the GAL4 DNA-binding domain (GAL4DBD) sequence into pBIND vector (Promega, Madison, WI) using *Sal*I and *Kpn*I restriction sites (pBIND-ZNFO). For expression of green fluorescent protein (GFP) fused to ZNFO, the ZNFO ORF sequence was amplified by PCR from the pGEX-4T1-ZNFO plasmid and inserted into pcDNA3-EGFP expression vector (Addgene, Cambridge, MA) (pcDNA3-ZNFO-EGFP). The mutant construct encoding ZNFO-EGFP with the predicted NLS (RHRK) changed to RHAA was generated by cloning the mutant ZNFO ORF (produced by two-step PCR using primers designed to introduce the desired mutations) into pcDNA3-EGFP vector (pcDNA3-ZNFO-RHAA-pEGFP). Deletion constructs expressing ZNFO-EGFP proteins lacking various zinc fingers were generated by PCR amplification of ZNFO fragments using the same gene-specific forward primer in combination with different reverse primers that target different sites in the zinc finger region followed by cloning into pcDNA3-EGFP expression vector. All clones were confirmed by sequencing. All primers used for plasmid construction are listed in Supplemental Table S1.

2.4. Quantitative real-time PCR (RT-qPCR)

Oocyte and embryo samples analyzed for mRNA expression included germinal vesicle (GV) and metaphase II (MII) stage oocytes ($n = 4$ pools of 10 oocytes), and pronuclear, two-cell, four-cell, eight-cell, 16-cell, morula and blastocyst stage embryos ($n = 4$ pools of 10 embryos/stage) generated by in vitro fertilization of abattoir-derived oocytes as previously described (Bettegowda et al., 2006). Quantitative real-time PCR set-up and standardization conditions were carried out as previously described (Wang et al., 2013). Before RNA extraction, each sample was spiked with 250 fg of *GFP* synthetic RNA (polyadenylated) as an exogenous control. The quantity of ZNFO mRNA or *GFP* RNA in each sample was determined using respective standard curves. The quantity