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Karyopherin alpha subtypes and porcine early stage embryo development

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KARYOPHERIN ALPHA SUBTYPES AND PORCINE EARLY STAGE EMBRYO

DEVELOPMENT

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ABBREVIATIONS

Anp32a: Acidic Nuclear Phosphoprotein 32 Family Member A

Arip3: Androgen Receptor Interacting Protein 3

ARM: Armadillo

BRG1: Also referred to as SMARCA4, SWI/SNF Related, Matrix Associated, Actin

Dependent Regulator of Chromatin, Subfamily α, Member 4

BRN2 (POU3F2)：POU domain, Class 3, Transcription Factor 2

CAS: Cellular Apoptosis Susceptibility Protein

Chrp: Cysteine and Histidine Rich Protein

COCs: Cumulus-oocyte Complexes

EDTA: Ethylenediaminetetraacetic Acid

ES cells: Embryonic Stem cells

FG: Phenylalanine Glycine

FUBP1: Far Upstream Element Binding Protein 1

GDP: Guanosine-5'-diphophate

GFP: Green Fluorescent Protein

GTP: Guanosine-5'-triphophate

GV: Germinal Vesicle

HEAT: Huntingtin, Elongation Factor 3, Protein Phosphatase 2A, and the Yeast

PI3-kinase TOR1

Hop2: Homologous Protein 2

Hsf1: Heat Shock Factor Protein 1

IBB: Importin β Binding

ICM: Inner Cell Mass

IPTG: Isopropyl-β-D-1-Thiogalactopyranoside

IVC: *In vitro* Culture

IVF: *In vitro* Fertilization

IVM: *In vitro* Maturation

IVP: *In vitro* Production

KPNAs: Karyopherin α Subtypes

KPNB: Karyopherin β

MII: Metaphase II

MPN: Maternal Pronucleus

mTBM: Modified Tris-buffered Medium

NE: Nuclear Envelope

NES: Nuclear Export Signal

NF-κB: Nuclear Factor kappa-light-chain-enhancer of Activated B Cells

NLS: Nuclear Localization signal

NPC: Nuclear pore complex

Nup: Nucleoporin

OCT4: Octamer-binding Transcription Factor 4

PBS: Phosphate Buffered Saline

POMs: Pore Membrane Domain

PVA: Polyvinyl Alcohol

PZM3: Porcine Zygote Medium 3

RanBP3: Ran Binding Protein 3

RanGTP: Guanosine-5'-triphosphate-binding RAs-related Nuclear Protein

RBD: Ran Binding Domain

RCC1: Ran GTP/GDP Nucleotide Exchange Factor

RNAi: RNA interference

Rnf2: Ring Finger Protein 2

RREB: Ras-responsive Transcriptional Element Binding Protein

Sox2: Sex Determining Region Y-box 2

SP17: Sperm Associated Antigen 17-like Protein

STAT3: Signal Transducer and Activator of Transcription 3

SV40: Simian Virus 40

SWI/SNF: Switch/sucrose Nonfermentable

TE: Trophectoderm

TGFβ: Transforming Growth Factor β

TRβ1: Thyroid Hormone Receptor β1

ZGA: Zygotic Genome Activation

ZP: Zona Pellucida

1PB: First Polar Body

ABSTRACT

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Intracellular communication between the nucleus and cytoplasm is critically important for coordinating cellular events during embryogenesis. The karyopherin α/β heterodimer is an intracellular nuclear trafficking system that mediates nuclear import of proteins that bear classical nuclear localization signals (NLSs). Seven karyopherin α subtypes have been identified in the domestic pig, and while each of these karyopherin α subtypes is able to bind to a nuclear localization signal, individual karyopherin α subtypes have been shown to transport specific NLS-bearing proteins. The objective of this study was to determine the developmental requirements of karyopherin α subtypes (KPNAs) during cleavage development in porcine embryos. The purpose of this dissertation was to test the hypothesis that the karyopherin α/β heterodimer-mediated nuclear trafficking pathway serves regulatory roles during cleavage development by selectively partitioning intracellular cargoes, thereby affecting epigenetic modifications,

transcription, and embryo developmental potential. We tested our hypothesis via a combination of a series of *in vivo* and *in vitro* assays. Our microinjection assay revealed that POU domain, class 3, transcription factor 2 (BRN2, also referred to as POU3F2) adopts a nuclear localization in all nuclei through the 4-cell stage of development, while only a subset of blastomeres in 8-cell stage embryos possess nuclear BRN2. Octamer-binding transcription factor 4 (OCT4) adopts a nuclear localization in all nuclei prior to the 2-cell stage of development, whereas OCT4 is undetectable in nuclei at the 4-cell stage. *In vitro* binding assays showed that both BRN2 and OCT4 are able to bind with multiple porcine karyopherin α subtypes. Moreover, we tested the impact of KPNA1-depletion in the intracellular localization of BRN2 and the embryo developmental competence via a series of co-microinjection assays. Our results showed that GFP-BRN2 accumulation was significantly reduced in the nuclei of KPNA1-depleted embryos, and KPNA1-depleted embryos possessed significantly fewer nuclei as well as a reduced proportion with the capacity to develop to the 8-cell stage and beyond as compared with the control embryos.

In summary, the data discussed in this dissertation provide more evidence to support the conclusion that discrete classes of NLS-bearing nuclear proteins may

be preferentially imported by individual karyopherin α subtypes. Our data also indicate that KPNA1 might be involved in the transport of transcription factor BRN2 in 4-cell stage embryos. Furthermore, the data suggest that KPNA1 may serve a critical role of partitioning intracellular cargoes that are directly involved in zygotic genome activation, or that direct development immediately after zygotic genome activation, hence affecting early cleavage embryo development.

CHAPTER 1 REVIEW OF THE LITERATURE

Nuclear Trafficking

The nucleus is the defining characteristic of the eukaryotic cells. The nuclear

membrane physically separates the eukaryotic cell into two compartments

between which there is a major shuttling of various molecules including proteins,

nucleic acids and small molecules. This typical double lipid bi-layer membrane is

also called the nuclear envelope (NE). Nuclear pore complexes (NPCs) are

embedded in the NE and form aqueous channels to facilitate the exchange of

molecules between the nucleus and cytoplasm (Peters, 2009). On average, up to

1000 transport cycles, including passive diffusion and energy-dependent

transport are observed through a NPC every second (Fahrenkrog *et al*., 2004;

Peters, 2005). The number of NPCs present in a given cell type can vary widely.

For instance, in comparison with barely 200 NPCs observed per nucleus in yeast,

there are up to 5 x 10^7 NPCs present on the nucleus of a Xenopus oocyte (Fabre and Hurt, 1997; Gorlich and Kutay, 1999).

The NPC is an octamer structure resembling a donut shape (Stoffler *et al.*, 2003), which encloses a central channel (Akey and Radermacher, 1993). The central channel is sandwiched between the cytoplasmic and nuclear rings. Eight cytoplasmic filaments emanate from the cytoplasmic ring to the cytoplasm. In addition, eight nuclear filaments emanate from the nuclear ring and join each other to shape a basket-like structure known as the nuclear basket in the nuclear side of NPC (Elad *et al.*, 2009). Three parts comprise the concentric layers of the NPC. The first is the scaffold layer, which is sandwiched between the other two layers to form the structure of the NPC. The second is the membrane layer, which is the outermost layer that anchors the NPC to the NE. The third layer is another phenylalanine-glycine (FG) repeat layer, which is the innermost layer that coats and facilitates the transport of the cargoes through the channel (Lusk *et al.*, 2007; Walde and Kehlenbach, 2010). The NPC is composed of approximately 30 types of proteins named nucleoporins (Nups), which are highly conserved across eukaryotes ranging from yeast to human (Walde and Kehlenbach, 2010).

Due to the octagonal radial symmetry of the NPC structure, each nucleoporin

functions as multiple copies. Therefore, the total number of Nups per NPC is

estimated to be roughly 500-1000 (Sorokin *et al*., 2007). According to the

localization of nucleoporins within the NPC, they can be divided into six

categories: (1) integral membrane proteins of the pore membrane domain (POMs);

(2) membrane-apposed coat nucleoprins; (3) adaptor nucleoporins; (4) channel

nucleoporins; (5) nuclear basket nucleoporins, and (6) cytoplasmic filament

nucleoporins (Hsia *et al*., 2007). It was well known that nucleoporins play

important functions in various species. It is repeatedly proven that some

Nup-knockout mice are lethal, such as Nup50, Nup214, Nup98, Nup96, Nup133,

and Nup358 (Aslanukov *et al*., 2006; Faria *et al*., 2006; Lupu *et al*., 2008; Okita *et

al*., 2004; Smitherman *et al*., 2000; van Deursen *et al*., 1996; Wu *et al*., 2001).

Malfunction of Nup154 in *Drosophila melanogaster* causes defects in both

oogenesis and spermatogenesis (Gigliotti *et al*., 1998). In addition, Nups serve

divergent roles in mammalian development. For instance, Nup50 is specifically

highly expressed in embryonic neuronal tubes and adult testes (Smitherman *et al*.,

2000). Furthermore, homozygous Nup50-knockout mice display aberrant neural

tube formation and high rate of lethality late in gestation (Smitherman *et al*., 2000).

However, Nup133 is specifically highly expressed in neuroepithelium, and

Nup133-null mice are lethal due to defective neuronal differentiation (Lupu *et al*.,

2008). Defects in transport pathways resulting in over-accumulation of molecules in nucleus or cytoplasm or structural alterations in Nups are also highly correlated with many diseases, including several cancers, neurological disorders, and cardiac disease (Cortes *et al*., 2010; Cronshaw and Matunis, 2004; Gika *et al*., 2010).

Karyopherin α/β Mediated Import System

Small molecules and ions (molecular weight around 20-40kDa, diameter around 5-9 nm) are able to travel through the NPC via passive diffusion manner. However, macromolecules (molecular weight larger than 40kDa, diameter of up to 40 nm) can only be transported through an energy-dependent mechanism (Miao and Schulten, 2009; Yang and Musser, 2006). Perhaps the most important transporter is known as the karyopherin α/β heterodimer (Goldfarb *et al*., 2004; Gorlich and Kutay, 1999). A nuclear localization signal (NLS) is an amino acid sequence that tags a protein for import into the cell nucleus by nuclear trafficking. In this well-characterized transport system, karyopherin α acts as an adaptor protein to connect cargo that possesses the NLS to karyopherin β1. Once karyopherin α binds its NLS-bearing cargo, this dimeric complex links with karyopherin β1 afterwards. NPC structural proteins subsequently interact with the trimeric

complex and facilitate its transport into the nucleus (Gorlich *et al.*, 1996). Once the

complex reaches the nucleus, the guanosine-5'-triphosphate-binding RAs-related

nuclear protein (RanGTP) recognizes the Ran-binding domain (RBD) within

karyopherin β1. This interaction detaches NLS-bearing cargo from the complex

via a conformational change of karyopherin β1 (Ribbeck *et al.*, 1998). Following

this, karyopherin α and karyopherin β1 are recycled back separately to the

cytoplasm (Cook *et al.*, 2007; Lee *et al.*, 2005). Karyopherin α is recycled back to

the cytoplasm by the RanGTP-CAS complex composed of RanGTP and cellular

apoptosis susceptibility protein (CAS), and karyopherin β1 is recycled back to the

cytoplasm by the RanGTP-Nup50 (Moore, 2003; Swaminathan and Melchior,

2002). A schematic of the nuclear import pathway mediated by the karyopherin

α/β heterodimers is shown in Figure 1.1.

Nuclear Localization Signals (NLSs)

Both classic and non-classic NLSs exist in cells. Classic NLSs contain basic

charged amino acids like arginine and lysine, whereas nonclassic NLSs lack basic

amino acid residues. There are two types of classic NLSs, which are monopartites

NLSs and bipartites NLSs. Both NLS variants can bind to the armadillo (ARM)

domain within karyopherin α, which contains 10 ARM repeats built as a cylindrical

super-helix (Cook *et al*., 2007; Fontes *et al*., 2000). ARM repeats 2-4 constitutes

the major NLS binding site, while ARM repeats 7 and 8 forms the minor binding

site (Hodel *et al*., 2001; Yang *et al*., 2010). A typical monopartite NLS can be

exemplified by the NLS found in the Simian Virus40 (SV40) T antigen, which

contains a single stretch of basic amino acid stretch "-PKKKRK-". Bipartite NLSs

possess a small N-terminal cluster connected to the larger C-terminal cluster by a

short spacer. For example, the sequence of the bipartite NLS from nucleoplasmin

is shown as "-KRPAATKKAGQAKKKK-" (Dingwall and Laskey, 1991). The

C-terminal cluster binds the major NLS binding site of karyopherin α, while the

N-terminal cluster binds the minor site. The length of the spacer between two

clusters may vary depending on amino acid compositions and is able to reach up

to 29 amino acids (Kosugi *et al*., 2009; Lange *et al*., 2010).

Ran and CAS

Ran is a member of the RAS super family, and is ~25kDa in size. The cellular

function of the RAS family is that it can attach either to GTP

(guanosine-5'-triphophate) or GDP (guanosine-5'-diphophate). Ran has a

guanine nucleotide- binding domain, which stays almost unchanged no matter it

attaches to GTP or GDP (Sheffield *et al*., 2006). CAS contains 20 Huntingtin,

elongation factor 3, protein phosphatase 2A, and the yeast PI3-kinase TOR1 (HEAT) repeats, which are similar to the HEAT repeats of karyopherin β. These HEAT repeats also are composed of two antiparallel α helices, A-helix and B-helix, that build up the internal and external faces of CAS respectively. The most important binding domain is in the HEAT repeat 8, which not only causes the dissociation of cargoes from karyopherin β, but also mediates the attachment of CAS to karyopherin α. These processes are orchestrated via regulating the affinity of karyopherin β and CAS to their partners (Matsuura *et al*., 2003; Zachariae and Grubmuller, 2006).

Karyopherin β (KPNB)

It was reported that there are 14 karyopherin β in yeast and more than 20 in mammalian cells. The molecular weights of karyopherin β family vary from 90kDa to 150kDa (Chook and Suel, 2011; Gorlich and Kutay, 1999). Karyopherin β consists of 19 tandem HEAT repeats, which share only a 20% identity with each other in sequence (Chook and Suel, 2011). Each HEAT repeat contains two α helices, the A-helix and the B-helix. These A-helices form the convex side of karyopherin β, while B-helices construct the concave side. In the unbound condition, karyopherin β has an S-shaped open conformation. When karyopherin

β interacts with karyopherin α, RanGTP, or Nups, this S-shape conformation is closed (Cingolani *et al.*, 1999; Conti *et al.*, 2006). According to the simulation of human karyopherin β dynamics, four different regions can be distinguished in the structure of karyopherin β: the RanGTP binding region, which was highly locked from HEAT repeats 1-9 in the flexible N-terminal; the cargo binding domain, an extended region between HEAT repeats 7 and 19; and the other two nucleoporin binding sites, which present HEAT repeats 5-8 and 13-17 (Cingolani *et al.*, 1999). Although the karyopherin β family has similar isoelectric points and structural domains, the sequence identities across them tend to be only 10-20% (Chook and Suel, 2011). Karyopherin β can bind to a wide variety of NLSs and nuclear export signals (NESs) and participate in the interchange of protein between the nucleus and the cytoplasm. Karyopherin β1, however, is the only subtype known to be involved in the karyopherin α/β mediated import system (Lee *et al.*, 2006). Other than being involved in the karyopherin α/β mediated import system, karyopherin β is also able to bind to cargo directly and execute its import function without karyopherin α (Cingolani *et al.*, 2002). Common transport cargoes of other karyopherin β family widely include ribosomal proteins and histones, as well as transcription factors, splicing regulators, and tRNAs (Chook and Suel, 2011).

Karyopherin α (KPNA) Family

Karyopherin αs are approximately 60kDa in size. They act as adaptor proteins to connect NLSs to karyopherin β. They contain highly structured domains comprised of ten tandem ARM repeats, which are very similar to the HEAT repeats found in karyopherin β. The ARM region is a 40 amino acid motif composed of three α-helices called H1, H2, and H3 respectively (Goldfarb *et al*., 2004; Tewari *et al*., 2010). These helices assemble into a right-handed super-helix, building a slug-like shape. In addition, its flexible N-terminus is composed of 40 amino acids referred to as the importin β binding (IBB) domain. The central part of the ARM repeats is able to bind to NLSs, and the C-terminus part with 10th ARM repeat is the CAS binding site (Goldfarb *et al*., 2004; Stewart, 2006).

Throughout the course of eukaryotic evolution, the karyopherin α gene family has undergone considerable expansion. Yeast encodes a single karyopherin α, *D. melanogaster* and *C. elegans* have three, whereas mammals encode multiple karyopherin α subtypes (Goldfarb *et al*., 2004; Yoneda, 2000). Six karyopherin α subtypes have been identified in the mouse, while seven have been identified in the human and the pig, which are referred to as karyopherin α1-α7 (KPNA1, KPNA2 KPNA3, KPNA4, KPNA5, KPNA6, and KPNA7) (Cabot and Prather, 2003;

Hu *et al*., 2010; Kelley *et al*., 2010). Based on their sequence identity, karyopherin α subtypes can be divided into three subfamilies. The first subfamily consists of KPNA1, KPNA5, and KPNA6, the second subfamily contains KPNA3 and KPNA4, and KPNA2 and KPNA7 belong to the third subfamily (Kelley *et al*., 2010). Subtypes of each group share a high degree of sequence identity (40%-80%). The degrees of shared identity among the karyopherin α family in pig are shown in Table 1.1.

Despite their structural similarities, the seven karyopherin α subtypes display tissue-specific expression; developmental stage-specific expression has also been observed. In mouse, KPNA3 and KPNA4 are only detectable in Leydig cells, whereas KPNA1, KPNA5 and KPNA6 are detectable at similar levels in all cells of the seminiferous tubule, as well as the Leydig cells (Kamei *et al*., 1999). KPNA1 is predominantly detectable in mouse brain, while KPNA2 is undetectable. In addition, in mouse embryonic stem cells, expression switching from KPNA2 to KPNA1 induced neural differentiation (Yasuhara *et al*., 2007). In human, KPNA2 and KPNA3 are greatly repressed in differentiated HL-60 cells, while KPNA6 is weakly down-regulated (Kohler *et al*., 2002). In contrast to the stable expression level of KPNA4 in cells differentiating into granulocyte-like cells, KPNA4 is predominantly down-regulated in cells differentiating towards macrophage-like

cells. Conversely, KPNA1 has been shown to be up-regulated in cells differentiating into macrophage-like cells (Suzuki *et al*., 2008).

It has also been reported that the transcripts abundance of seven karyopherin α subtypes exhibits significant changes in the process of development from germinal vesicle (GV) stage oocyte to blastocyst stage embryo in porcine (Wang *et al*., 2012). For instance, KPNA1 transcript levels are highest in abundance in blastocyst stage embryo among these early developmental stages. The KPNA2 transcript becomes undetectable in the blastocyst stage embryo, even though it is detectable in other stages. The KPNA4 transcript increases in abundance in 4-cell stage embryos, but is reduced in blastocyst stage embryos. At a given stage of development individual transcript levels of KPNA subtypes can vary widely. For example, in GV- and MII-stage oocytes, KPNA7 transcript abundance is significantly greater than that of all other known karyopherin α subtypes, whereas, in blastocyst stage embryos, transcript abundance of KPNA1, KPNA3, and KPNA4 is higher than that of KPNA7. In addition, intracellular localization of KPNA subtypes show developmental changes in porcine cleavage stage embryos. For example, KPNA7 adopts a nuclear localization in porcine GV-stage oocytes and 2-cell and 4-cell stage embryos, but is not detectable in blastocyst stage embryos. The developmental changes in both transcript and protein levels of karyopherin α

subtypes strongly suggest that karyopherin α subtypes serve critical functions during porcine embryogenesis.

Although each karyopherin α subtype shares a high degree of sequence identity and is able to transport NLS-bearing substrates, certain NLS-bearing substrates are preferentially transported by specific karyopherin α subtypes. For example, murine importin α5 (KPNA1) is able to bind with several transcription factors *in vitro* such as acidic nuclear phosphoprotein 32 family member A (Anp32a), thyroid hormone receptor β1 (TRβ1), and far upstream element binding protein 1 (FUBP1), while androgen receptor interacting protein 3 (Arip3), homologous protein 2 (Hop2), and cysteine and histidine rich protein (Chrp) can interact with murine importin α2 (KPNA2) (Fukumoto *et al*., 2011; Ly-Huynh *et al*., 2011). Sperm associated antigen 17-like protein (SP17) and Ras-responsive transcriptional element binding protein (RREB) are able to interact with multiple porcine KPNA subtypes (Park *et al*., 2012). In addition, Ran binding protein 3 (RanBP3) and Ran GTP/GDP nucleotide exchange factor (RCC1) have been shown to be preferentially imported by KPNA3 (Talcott and Moore, 2000; Welch *et al*., 1999; Yeung *et al*., 2006), and KPNA6 and KPNA7 have been shown to be carriers of signal transducer and activator of transcription 3 (STAT3) (Ma and Cao, 2006), while nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)

is transported into the nucleus by KPNA3 and KPNA4 (Fagerlund *et al*., 2005).

POU domain, class3, transcription factor 2 (BRN2 also referred to as POU3F2)

has been reported to specifically require murine KPNA1 for nuclear import but has

been shown to be able to bind multiple porcine KPNA subtypes *in vitro* (Li *et al*.,

2013).

Although little detail is known about the requirements of specific karyopherin α

subtypes during spermatogenesis or oogenesis, existing data also support the

claim that karyopherin α subtypes play important roles in reproductive

competence in mammals. Compared to wild-type mice, KPNA1 knockout mice

exhibit depressed reproductive organ function, including fewer maturing follicles,

thinner uterine layers, and less serum progesterone. Furthermore, they have

abnormal parturition and a high rate of pup mortality (Moriyama *et al*., 2011).

Porcine embryos injected with interfering RNAs that target KPNA3 display

aberrant development compared with noninjected counterparts (Cabot and

Prather, 2003). Female mice homozygous for a loss-of-function mutation of

KPNA6 are unable to produce offspring. In addition, fertilized oocytes of these

females display an arrest at the pronuclear or 2-cell stage of development (Rother

et al., 2011). KPNA7, a karyopherin α subtype identified in mouse (Kelley *et al*.,

2010), human (Hu *et al*., 2010) and bovine (Tejomurtula *et al*., 2009) has been

found to be expressed almost exclusively in female tissues, including ovaries and

oocytes, and early stage embryos prior to 4-cell stage. In addition, KPNA7

appears to be required for cleavage development in both bovine (Tejomurtula *et al*., 2009) and porcine (Wang *et al*., 2012) embryos; female mice lacking

functional KPNA7 exhibit decreased litter sizes and imbalanced sex ratios of pups

delivered (Hu *et al*., 2010).

Oogenesis and Early Stage Embryos Development

The ovary is endowed at birth with a fixed stock of oocytes enclosed in primordial

follicles. The number of oocytes declines as a result of atresia and recruitment

towards ovulation (Faddy, 2000). Primary oocytes are arrested in the diplotene

stage of the first meiotic prophase until after the LH surge, which precede

ovulation (Guzeloglu-Kayisli *et al*., 2012). Before ovulation, primary oocytes are

not fertile, which have to grow to become competent to resume nuclear

maturation. During growth, oocytes undergo a burst in transcription and

translation, stockpiling mRNA, proteins, metabolic substrates and organelles,

which will support subsequent events, like fertilization, unpacking of the paternal

genome, and early cleavage division of the zygote (Akam, 1987; Duval *et al*.,

1990). Besides, oocytes form an extracellular matrix called the zona pellucida

(ZP). Human, rat and porcine ZP consists of four ZP glycoproteins, whereas mouse oocyte has three (Bleil and Wassarman, 1980; Hoodbhoy *et al*., 2005; Lefievre *et al*., 2004). Full oocyte growth must be supported by surrounding granulosa cells, which proliferate and form multiple layers of cumulus cells. In the meantime, oocytes resume meiotic division and begin the process of maturation. Nuclear maturation comprises resumption of meiosis I, progression to metaphase II (MII), and extrusion of the first polar body (1PB) (Tripathi *et al*., 2010). Cytoplasmic maturation consists of acquisition of competence for sperm penetration, calcium release and preparation for preimplantation development (Ajduk *et al*., 2008). After ovulation, oocytes are picked up by the adhesion between the extracellular matrix of cumulus cells and oviductal cells and are subsequently transported into the ampulla of the oviduct, where they await spermatozoa for fertilization (Talbot *et al*., 2003).

After fertilization, fusion of the sperm and oocyte plasma membrane results in the deposition of the sperm chromatin into the ooplasm. The oocyte-derived chromatin and sperm-derived chromatin each form a separate pronucleus that moves to the center of the oocytes. The first mitosis of the embryo begins approximately 24 hours after fertilization. During the process of first mitosis, the pronuclear envelopes breakdown and the sperm-derived and oocyte-derived

chromatin mix to form the chromosomes aligning on the metaphase plate and subsequently segregate to the two poles of the cell, and the cytoplasm and proteins are divided into these two smaller cells known as blastomeres (Prather *et al.*, 1996). Compaction, the first event of morphogenic and cellular differentiation event, occurs during this period of cleavage development. The embryo continues to develop to the blastocyst stage, which possesses two distinct cell populations, the outer cells, being flatten, which form the trophectoderm (TE), and the inner cells, remaining round and apolar, which form the inner cell mass (ICM). The TE will contribute to the chorion. In contrast, the ICM will develop into the embryo, yolk sac, amnion, and allantois (Dyce *et al.*, 1987; Fleming, 1987).

Zygotic Gene Activation (ZGA)

In *Drosophila* and *Xenopus*, mRNAs stored in the oocyte are stable and able to control many aspects of embryonic development (Akam, 1987; Duval *et al.*, 1990). However, in mammals, oocyte-derived mRNA are degraded shortly after fertilization (Thompson *et al.*, 1998), and maternal control is subsequently replaced by zygotic genome control. Zygotic gene activation (ZGA) is an essential event that governs the transition from maternal to embryonic control of development (Piko and Clegg, 1982). In this transition, maternal mRNAs are

quickly degraded and embryonic transcription is initiated. In the meantime,

embryos become more sensitive to environmental conditions (Tadros and Lipshitz,

2009). The initiation of ZGA varies depending on the species. In human and pig,

this event takes place at 4-cell stage; in mouse, at 2-cell stage; and in cattle, at

8-cell stage (Braude *et al*., 1988; Flach *et al*., 1982; King *et al*., 1988; Norberg,

1970; Telford *et al*., 1990).

ZGA is regulated by multiple molecular mechanisms including DNA replication,

expression of maternal effect genes, and chromatin remodeling (Minami *et al*.,

2007). Many maternal factors, including Mater, heat shock factor protein 1 (Hsf1),

ring finger protein 2 (Rnf2), as well as the maternal pluripotency transcription

factors Oct4 and sex determining region Y-box 2 (Sox2) have been shown to play

a key role in regulation of ZGA (Christians *et al*., 2000; Foygel *et al*., 2008; Pan

and Schultz, 2011; Posfai *et al*., 2012; Tong *et al*., 2000). Depletion of the gene

encoding any one of these maternal factors leads to embryonic arrest at cleavage

stage development (Li *et al*., 2010). Accurate chromatin structure is critical for the

initial of ZGA. Evidence has been reported that abnormal expression of chromatin

remodeling factors tends to result in prevention of ZGA. For instance, SWI/SNF

related, matrix associated, actin dependent regulator of chromatin, subfamily α,

member 4 (SMARCA4, also referred to BRG1), a catalytic subunit of

switch/sucrose nonfermentable (SWI/SNF) chromatin remodeling complex, has been indicated to be involved in the event of ZGA (Bultman *et al*., 2006). DNA replication has also been implicated to influence ZGA by selectively establishing a transcription repression structure, which would further participate in the regulation of gene expression (Wolffe, 1991).

In Vitro Production of Porcine Embryos

An *in vitro* embryo production (IVP) system comprises *in vitro* maturation (IVM), *in vitro* fertilization (IVF) of oocytes, and *in vitro* culture (IVC) of fertilized oocytes (Somfai and Hirao, 2011). The establishment of a reliable porcine IVP system enables researchers to generate large quantities of porcine embryos, reducing the need for live animals dedicated solely to research. In addition, because of their high physiological similarities to humans, pigs are an ideal species targeted for use as potential xenograft donors. Despite the fact that porcine embryos produced via IVP are able to develop to viable piglets (Abeydeera *et al*., 1998; Funahashi *et al*., 1997; Funahashi *et al*., 1996; Kikuchi *et al*., 2002), their developmental competence is rather low compared with *in vivo* counterparts (Kikuchi *et al*., 1999).

In the *in vitro* embryo production process in the domestic pig, immature oocytes are typically obtained from ovaries collected at commercial slaughterhouses. Oocytes are subsequently selected according to well-defined criteria, such as uniformity of the cytoplasm and the numbers of surrounding cumulus cells (Somfai *et al*., 2004). The aim of IVM is to lead the oocytes mature to MII stage, such that they can be successfully fertilized in the *in vitro* environment. Although great improvements have been achieved in porcine IVM culture media, approximately 10-30% of pig oocytes still fail to reach MII stage at the end of IVM (Kikuchi *et al*., 2009). A small portion of oocytes remains at the GV stage, whereas most of them permanently arrest at an immature stage characterized by metaphase chromosomes and the lack of extrusion of the first polar body. However, the successful extrusion of the first polar body is not a guarantee for normal chromosome numbers in the resultant embryos since many oocytes that possess a first polar body show aneuploidy (Sosnowski *et al*., 2003). Even the oocytes proceed to maturation normally *in vitro*, they exhibit a reduced development competence compared with the oocytes matured *in vivo* (Nagai *et al*., 2006). The oocyte maturation includes both nuclear maturation and cytoplasmic maturation. Nuclear maturation leads resumption and progression to MII stage, and extrusion of the1BP, while the cytoplasmic maturation involves a series of processes that required for oocytes to acquire form maternal pronucleus

(MPN), which is crucial for the early stage embryonic development (Nagai *et al.*, 2006). Therefore, the main issue of the comprised developmental competence of *in vitro* matured oocytes might be caused by poor cytoplasmic maturation.

In porcine IVF systems, matured oocytes and sperm are co-cultured in the IVF medium droplet. Sperm penetration takes place around 3 hours after insemination and is generally thought to be completed by 6 hours after gamete mixing (Funahashi *et al.*, 2000). Major difficulties encountered with IVF of porcine oocytes are poor maternal pronucleus formation, a low frequency of sperm penetration, and a high incidence of polyspermy. Therefore, an optimal setting for sperm concentration and gamete co-culture duration is essential for successful embryo production by IVF. Based on the advantage for selection of MII oocytes with the presence of 1 BP and facilitation of sperm penetration, IVF was usually performed on denuded oocytes. However, early research had demonstrated that cumulus cells play an important role during fertilization. Therefore, the removal of cumulus cells might also be another factor of comprised developmental competence of embryos produced by IVF (Cox *et al.*, 1993).

In comparison with embryos produced *in vivo*, the IVP embryos have a rather low developmental competence in terms of the proportion that develop to the

blastocyst stage and cell numbers in blastocyst stage embryos (Dang-Nguyen *et al.*, 2011). In addition, IVP embryos exhibit high rates of DNA fragmentation and apoptosis (Pomar *et al.*, 2005). Cytogenetic analysis of porcine IVP embryos showed that approximately 45% of these embryos have chromosomal aberrations compared to 7.3% in embryos obtained *in vivo*. Besides, IVP embryos have lower rates of cleavage, and asynchronous pronucleus development compared to *in vivo* counterparts (McCauley *et al.*, 2003; Ulloa Ullo *et al.*, 2008). Overall, the transfer of IVP embryos results in developmental abnormalities such as increased frequency of embryo mortality, extended gestation, lower growth rate and body weight of offspring (Cameron *et al.*, 2006; Rath *et al.*, 1995).

It is elemental to produce embryos with normal development competence for the success of application of porcine IVP system. Based on previous studies, the decreased incidence of abnormal chromosome formation and aberrant gene expression found in *in vitro* produced embryos can significantly impair the normality of the porcine embryos produced *in vitro.* Since many nuclear proteins, including transcription factors and chromatin remodeling enzymes, are required in the process of embryonic development, it is important to further explore the mechanisms by how these intracellular proteins are partitioned between nuclear and cytoplasmic compartments. Understanding how these crucial nuclear

proteins gain access to the nucleus to exert their regulatory functions could help

us understand how the epigenetic state of chromatin is regulated and how

alteration in chromatin structure occurs, due to *in vitro* manipulation, which

subsequently could bring some insight to the development of methods to improve

the quality of embryos following *in vitro* manipulation.

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Table 1.1: Karyopherin α family identity matrix

	KPNA1	KPNA2	KPNA3	KPNA4	KPNA5	KPNA6	KPNA7
KPNA1	100%	46%	49%	48%	79%	81%	41%
KPNA2		100%	49%	54%	49%	46%	53%
KPNA3			100%	83%	49%	48%	45%
KPNA4				100%	50%	49%	47%
KPNA5					100%	84%	42%
KPNA6						100%	42%
KPNA7							100%
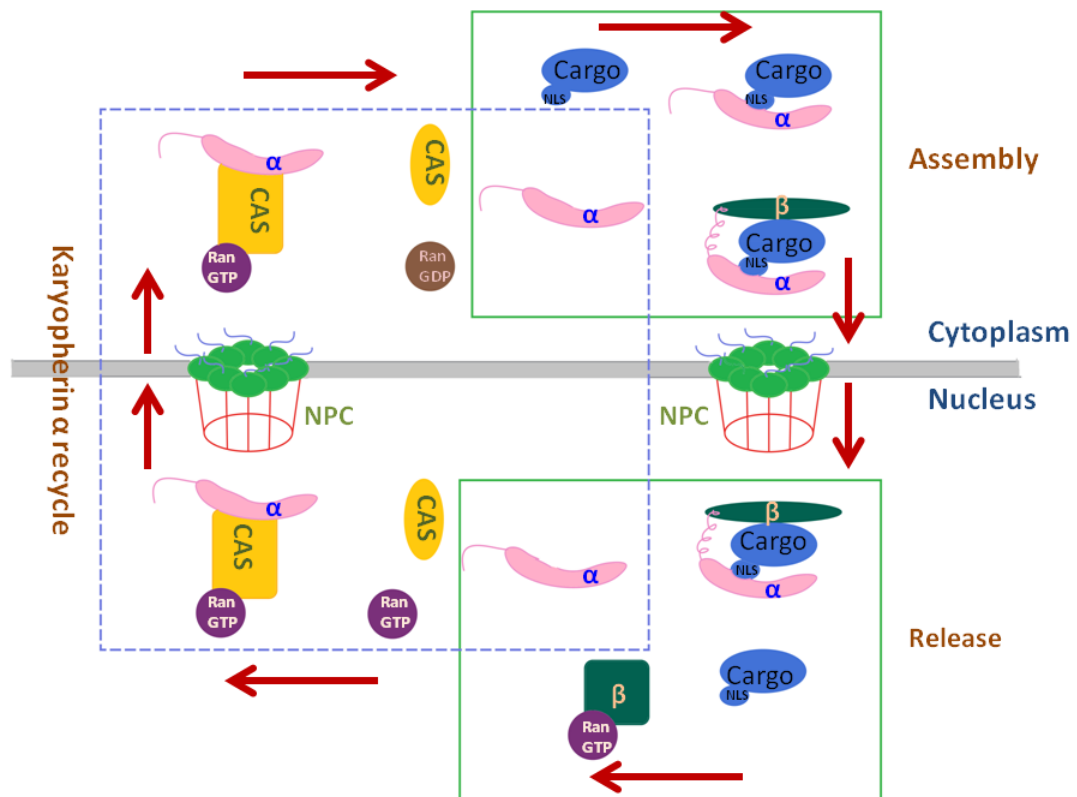
Figure 1.1: Karyopherin α/β mediated import system. In this system, karyopherin α acts as an adaptor protein, which can recognize the NLS within the cargo and bind to it to form a dimeric complex. The dimeric complex interacts with karyopherin β afterwards to form a trimeric complex, which subsequently interacts with NPC structural proteins, and thus translocates the cargo into the nucleus. Once the complex reaches the nucleus, the binding of RanGTP to karyopherin β induces the conformational change of karyopherin β and detaches the cargo from the complex. Following this, karyopherin αs are recycled back to the cytoplasm.

CHAPTER 2 DYNAMIC CHANGES IN NUCLEAR IMPORT OF AN NLS-BEARING SUBSTRATE IN 8-CELL STAGE PORCINE EMBRYOS

Abstract

Coordinated intracellular trafficking is critically important for proper timing of major cellular events during embryogenesis. Nuclear import mediated by the karyopherin α/β (also referred to as importin α/β) heterodimer is perhaps the best characterized nuclear trafficking system in eukaryotic cells. Seven karyopherin α subtypes have been identified in the domestic pig, and while each karyopherin α subtype transports proteins bearing classical nuclear localization signals (NLSs), individual karyopherin α subtypes have been shown to preferentially transport specific cargoes. The aim of this study was to determine the mechanism by which BRN2, a transcription factor previously reported to be transported by the karyopherin α/β heterodimer, gains access to the nucleus in porcine oocytes and embryos. Using a combination of *in vivo* and *in vitro* assays, we tested the hypothesis that nuclear import mediated by discrete karyopherin α subtypes was

dynamically regulated during cleavage development. Our results show that

ectopically expressed BRN2 adopts a nuclear localization in all nuclei through the

4-cell stage of development, while only a subset of blastomeres in 8-cell stage

embryos possess nuclear BRN2. This pattern is unique to BRN2 as another

ectopically expressed NLS-containing protein is able to adopt a nuclear

localization in all blastomeres of 8-cell stage embryos.

Introduction

Precise partitioning of intracellular proteins between the nuclear and cytoplasmic

compartments enables cells to respond promptly to internal and external stimuli.

Several intracellular protein transport systems have been characterized in

eukaryotic cells. One of the best characterized transport systems is the

karyopherin α/β heterodimer (also referred to as the importin α/β heterodimer);

this system mediates the import of proteins bearing classical NLSs into the

nucleus (Görlich and Kutay, 1999). Karyopherin α serves as the NLS receptor

(Adam and Gerace, 1991); NLSs recognized by karyopherin α are characterized

by short stretches of basic amino acids, typically lysines, in the primary amino

acid sequence. These NLSs are exemplified by the monopartite NLS in the SV-40

T-antigen (-**PKKKRKV**-) and the bipartite NLS found in nucleoplasmin

(-**KR**PAATKKAGQA**KKK**-) (Dingwall and Laskey, 1991). Karyopherin β1 interacts with karyopherin α after karyopherin α binds to the NLS-bearing cargo (Görlich *et al.*, 1996). This trimeric complex then contacts components of the nuclear pore complex, travels to the nucleus, and dissociates to release the cargo inside the nucleus (Ribbeck *et al.*, 1998).

Seven karyopherin α subtypes, karyopherin α1-karyopherin α7 (KPNA1 – KPNA7), have been identified in mammals (Tejomurtula et al., 2009; Wang *et al.*, 2012). Although each karyopherin α subtype shares a high degree of amino acid identity and major protein motifs to mediate NLS and karyopherin β1 binding (the Armadillo (ARM) repeats and importin β domain (IBB) domain, respectively; Kamei *et al.*, 1999), evidence suggests that karyopherin α subtypes are not functionally redundant. Firstly, expression patterns for these subtypes vary by tissue and cell type. For example, transcripts encoding the seven karyopherin α subtypes vary in abundance at discrete stages of cleavage development in the porcine embryo. Transcripts encoding KPNA2 and KPNA5 are detectable in porcine oocytes and become undetectable in blastocyst stage embryos (Wang *et al.*, 2012). KPNA7 appears to be expressed only in the oocyte and cleavage stage embryo (Wang *et al.*, 2012; Tejomurtula *et al.*, 2009); KPNA7 transcripts are the most abundant karyopherin α subtype transcripts detectable in the immature

porcine oocyte (Wang *et al*., 2012). Second, although karyopherin α subtypes are able to bind a variety of NLS-bearing cargoes, some karyopherin α subtypes are able to interact with only a subset of NLS-bearing cargoes. For example, Ran GTP/GDP nucleotide exchange factor (RCC1) has been shown to be preferentially imported by KPNA3 (Talcott and Moore, 2000), while KPNA2 and KPNA1 have been shown to have differing affinities *in vitro* for specific NLSs (Köhler *et al*., 1999). In addition, protein:protein interaction screens have revealed different intracellular cargoes are able to interact with discrete karyopherin α subtypes (Ly-Huynh *et al*., 2011; Fukumoto *et al*., 2011).

In addition to carrying specific subsets of NLS-bearing cargoes, ablation and overexpression studies in cell culture and live animals suggest that karyopherin α-mediated nuclear import may serve regulatory roles during differentiation and gamete and embryo development. Mice that carry a homozygous deletion of KPNA6 (also referred to as importin α7) are viable but infertile (Rother *et al*., 2011), while mice that carry a homozygous deletion of KPNA1 (also referred to as importin α5) are viable and fertile (Shmidt *et al*., 2007). RNA interference (RNAi)-based knockdown experiments in bovine (Tejomurtula *et al*., 2009) and porcine (Wang *et al*., 2012) embryos show that KPNA7 appears necessary for cleavage development, although this may not be true for all karyopherin α

subtypes (Cabot and Prather, 2003). Overexpression of *KPNA1* (also referred to as importin α5) and ablation of *KPNA2* (also referred to as importin α1) promote differentiation of murine embryonic stem (ES) cells (Yasuhara *et al*., 2007). In the same study, reductions in *KPNA1* and overexpression of *KPNA2* blocked ES cell differentiation when ES cells were grown in media that promoted differentiation (Yasuhara *et al*., 2007).

Based on these findings, we hypothesized that nuclear import mediated by discrete karyopherin α subtypes was dynamically regulated during cleavage development. The objective of the work presented here was to determine the mechanism by which BRN2, a transcription factor previously shown to be a cargo transported by the karyopherin α/β heterodimer, localized to the nuclei of porcine GV-stage oocytes and cleavage stage embryos. BRN2 was reported to specifically require murine KPNA1 for nuclear import, making BRN2 a marker for KPNA1-mediated nuclear import (Yasuhara *et al*., 2007). Using a combination of *in vivo* nuclear trafficking assays, we find that 8-cell stage porcine embryos undergo a dramatic change in their ability to transport NLS-bearing proteins.

Results

GFP-BRN2 adopts a nuclear localization during porcine embryo development

Ectopically expressed GFP-BRN2 adopted a nuclear localization in GV-stage oocytes (n=33/33) and pronuclear (n=17/17), 2-cell (n=21/21), and 4-cell (n=13/13) stage embryos produced by *in vitro* fertilization. Interestingly, GFP-BRN2 displayed a varying pattern of nuclear localization in blastomeres of 8-cell stage embryos where between 1 and 4 blastomeres possessed nuclear GFP-BRN2 (n=11/11). These data are summarized in Table 2.1; representative images are found in Figure 2.1. As a control, ectopically expressed NLS-DsRed adopted a nuclear localization in all blastomeres in 8-cell stage embryos (n=9/9). Co-microinjection of a mixture of GFP-BRN2 mRNA and NLS-DsRed mRNA revealed that both NLS-DsRed and GFP-BRN2 adopted a nuclear localization in all nuclei in 2-cell (n=11/11) and 4-cell stage (n=7/7) parthenogenetic embryos. In 8-cell stage embryos, NLS-DsRed was detectable in all blastomeres, while GFP-BRN2 was detectable in 2 nuclei of this embryo (n=1/1; Figure 2.2).

In vivo derived embryos arrest before 8-cell stage upon microinjection with GFP-BRN2 mRNA

Microinjection was performed to determine the intracellular localization of exogenous GFP-BRN2 in *in vivo* embryos. No data of the intracellular localization of GFP-BRN2 in 8-cell stage embryo were collected. Briefly, all *in vivo* embryos arrested in development prior to the 8-cell stage in both injection treatment group (N=8; 1-cell stage embryos n=2/8; 2-cell stage embryos n=2/8 and 4-cell stage embryos n=4/8) and noninjection group (N=5; 2-cell stage embryo n=1/5 and 4-cell stage embryo n=4/5). These data are summarized in Table 2.2.

Recombinant BRN2 interacts with multiple karyopherin α subtypes in vitro

A series of *in vitro* binding assays were performed to determine the ability of karyopherin α subtypes to interact with recombinant BRN2. While KPNA1, KPNA2, KPNA3, KPNA5, KPNA6 and KPNA7 interacted with GST-BRN2, KPNA7 exhibited the highest relative binding affinity for recombinant GST-BRN2 *in vitro*. These data are summarized in Figure 2.3.

RNAi-mediated knockdown of KPNA7 does not affect the intracellular localization of GFP-BRN2 in 4-cell stage embryos

An RNA interference approach was used to determine the effect of KPNA7 knockdown on the intracellular localization of GFP-BRN2. No difference in intracellular localization of GFP-BRN2 was detectable between parthenogenetic embryos co-injected with either KPNA7-interfering RNAs or negative control interfering RNAs. At the 4-cell stage of development, GFP-BRN2 adopted a nuclear localization in the KPNA7-RNAi group (n=10/10), as did all embryos in the control RNAi group (n =8/8; Figure 2.4). The effectiveness of KPNA7 transcript knockdown is shown in Figure 2.5.

Discussion

Understanding how transcription factors and enzymes that regulate epigenetic remodeling are partitioned between the nucleus and cytoplasm in cells of the cleavage stage embryos is of critical importance to understanding how transcription is regulated during cleavage development in mammalian embryos. It is well-established that embryos manipulated *in vitro* can display altered patterns of gene expression, perturbed epigenetic modifications, and reduced developmental potential, therefore it is important to understand how factors that

play regulatory roles in these processes gain access to the nucleus to exert their functions. Previous reports indicate that discrete members of the karyopherin α family of nuclear trafficking receptors may serve critical roles during cleavage development (Rother *et al*., 2011; Cabot and Prather, 2003; Tejomurtula *et al*., 2009; Wang *et al*., 2012). The goal of this study was to determine how a known karyopherin α cargo could serve as a reporter to determine if nuclear trafficking mediated by a specific karyopherin α subtype was differentially regulated in cells of the porcine cleavage stage embryo.

Previous data indicated that transport of BRN2, a transcription factor known to play a role in neural differentiation, was mediated by the karyopherin α/β heterodimer (Yasuhara *et al*., 2007). We wanted to determine if BRN2 served as a substrate for import in porcine oocytes and cleavage stage embryos. Results from our ectopic overexpression approach revealed that a GFP-BRN2 fusion protein adopted a nuclear localization in GV stage porcine oocytes and cleavage stage embryos. Although GFP-BRN2 was found in all nuclei in GV-stage oocytes and in all blastomeres of all embryos through the 4-cell stage of development, GFP-BRN2 was only found in a subset of nuclei in 8-cell stage embryos.

In order to compare the intracellular localization of GFP-BRN2 in *in vivo* embryos

with *in vitro* embryos, we also performed microinjection with GFP-BRN2 mRNA on

in vivo embryos. However, the results were inconclusive because all *in vivo*

derived embryos arrested in development prior to the 8-cell stage after allocation

to treatment groups. In the progression of *in vitro* embryo production, all cells

were cultured in established artificial mediums at 39°C and 5% CO_2 in air with 100%

humidity, whereas, *in vivo* derived embryos underwent several times of allocation

along with changes of temperature. It is likely that these *in vitro* manipulations on

embryos reduced the developmental competence of *in vivo* derived embryos.

Another explanation could be that these *in vivo* embryos we harvested were not

true embryos because they were not successfully fertilized yet, and the following

cleavage of the cells could be caused by the environmental changes during the

allocations to treatments. Therefore, we cannot make any determinations about

the intracellular localization of GFP-BNR2 in *in vivo* 8-cell stage embryos.

We wanted to determine if this unexpected pattern of intracellular localization in

8-cell stage porcine embryos was due to a general change in karyopherin

α/β-mediated nuclear import at the 8-cell stage. The finding that the NLS-DsRed

protein was able to adopt a nuclear localization in all nuclei in 8-cell stage porcine

embryos strongly suggests that the atypical localization pattern of GFP-BRN2 at

this stage of development was not an artifact of the microinjection approach we employed. Despite this finding, we cannot completely exclude the possibility that GFP-BRN2 is selectively degraded in specific blastomeres of 8-cell stage porcine embryos. The NLS from the SV40 T-antigen represents a classical cargo, able to be imported by all karyopherin α subtypes except for karyopherin α7 (Kelley *et al*., 2010). It has previously been shown that the NLS from the SV40 T-antigen functions as a nuclear import signal in porcine oocytes and embryos as well (Cabot and Prather, 2003; Park *et al*., 2012). The finding that the NLS-DsRed protein was able to adopt an equivalent nuclear localization in all blastomeres of the 8-cell stage embryo decreases the possibility that the mRNA injected at the pronuclear stage (that the protein translated from this mRNA) was restricted in its ability to adopt a uniform distribution prior to cytokinesis. We hypothesized that the specific karyopherin α subtype or subtypes that control the import of BRN2 undergo a regulatory change that is reflected during the 8-cell stage of development in the porcine embryo.

We next wanted to determine the mechanism behind this dramatic change in trafficking of an NLS-bearing protein in the 8-cell stage porcine embryo. Yasuhara and colleagues used an *in vitro* nuclear import assay to show that BRN2 can be imported by KPNA1 (importin α5), and not KPNA2 or KPNA4

(importin α1 or importin α3) (Yasuhara *et al*., 2007). Although an exhaustive screen of all murine karyopherin α subtypes was not reported, BRN2 appeared to be imported by only a subset of karyopherin α subtypes. To determine the karyopherin α subtypes traffic BRN2 to the nuclei of porcine oocytes and embryos, we performed an *in vitro* binding assay to determine the porcine karyopherin α subtypes had the ability to interact with BRN2. Results of our *in vitro* binding assays indicate that while multiple karyopherin α subtypes were able to interact with recombinant BRN2, KPNA7 displayed the highest relative binding to BRN2 *in vitro*. We had intended to use an *in vitro* nuclear import assay on permeabilized cells to measure the *in vivo* transport of BRN2. In this assay, we attempted to build an artificial transport system on permeabilized porcine fetal fibroblast cells with required transport factors and energy regenerators. We could test the requirement of certain karyopherin α for BRN2 transport by selectively add one specific karyopherin α in this transport system followed by determining the intracellular localization of GFP-BRN2 in the permeabilized porcine fetal fibroblast cells. The essential step of this assay is to optimize the permeabilization of porcine fetal fibroblast cells. We first tested the optimization with commercial Hela cell cytosol, which contains both natural transport factors and energy regenerators. However, we failed to build the nuclear import assay system because porcine fetal fibroblast cells we permeabilized were detached and

washed away after three times wash with cold phosphate buffered saline (PBS)

buffer PBS following nuclear import reaction. Cytosol used in nuclear import

assay contains all transport factors involved in nuclear trafficking, but the Hela cell

cytosol we used in this assay also contains 1 mM ethylenediaminetetraacetic acid

(EDTA). It is possible that EDTA detached the cells easily from the petri dish when

the cells were incubated for import reaction. Another possible reason could be

some conditions of the assay, such as temperature for permeabilization and

import reaction or culture time for import reaction.

Based on this finding, and our previously published data that revealed KPNA7 to

be a nuclear protein in GV stage oocytes, 2-cell stage and 4-cell stage embryos,

but not detectable in blastocyst stage embryos (Wang *et al*., 2012), we

hypothesized that KPNA7 may play a key role in targeting BRN2 to the nucleus

prior to the 8-cell stage of development. We attempted an RNAi-mediated

knockdown of KPNA7 using an approach previously reported by our group.

Following co-injection of KPNA7 interfering RNAs and GFP-BRN2 mRNA at the

pronuclear stage, we imaged resultant 4-cell stage embryos to determine the

intracellular localization of BRN2. Results from this study show that knockdown of

KPNA7 does not affect the intracellular localization of GFP-BRN2.

One possible explanation for this observation is that although KPNA7 may be able to bind and import BRN2, other karyopherin α subtypes may also perform this task. For instance, our *in vitro* binding assay revealed multiple karyopherin α subtypes have the ability to interact with recombinant BRN2. Although we have been able to detect transcripts encoding all seven karyopherin α subtypes in porcine oocytes (Wang *et al*., 2012), the relative abundance of these transcripts varies at discrete stages of cleavage development. For instance, KPNA7 transcripts have been shown to be the most abundant of all karyopherin α transcripts in GV-stage oocytes, and while KPNA7 transcripts can be detected in blastocyst stage embryos, KPNA7 protein cannot be detected immunocytochemically at this stage. A second factor that could confound this interpretation of the results of this assay is that the embryos used in this assay were derived from parthenogenetic activation rather than *in vitro* fertilization. Parthenogenetic and bi-parental embryos certainly differ in their developmental capacity and it is possible that there are intrinsic differences in nuclear dynamics between embryos derived from these two methods. Parthenogenetically-derived 4-cell stage embryos were a useful model in our KPNA7 knockdown experiment as we wanted to assess transcript knockdown at a defined time-point and cell stage following oocyte activation. Importantly, our ectopic overexpression studies reported here were performed in embryos produced by *in vitro* fertilization and

parthenogenetic activation with the results of the two experiments yielding similar results (Figures 2.1 and 2.2). In addition, porcine parthenogenetic embryos possess a high degree of synchrony following parthenogenetic activation allowing us to readily obtain a number of embryos to assay by both confocal microcopy and for RT-PCR from each experimental replicate, without the need to pool embryos over multiple days. Therefore, despite their limitations in some developmental assays, there was a clear benefit to use parthenogenetic embryos in this particular experiment.

Secondly, our BRN2 intracellular location assay was not designed to measure differences in import kinetics, the possibility remains that KPNA7 may be a dominant transporter for BRN2 in cleavage stage embryos and that the level of KPNA7 depletion we obtained was not sufficient to prevent BRN2 import. In addition, BRN2 may interact directly with chromatin or chromatin-associated proteins as mitotic cell division occurs, and therefore remain nuclear, rather than being a cargo for nuclear import. Given the previous characterizations of the import dynamics of BRN2 in murine embryonic stem cells and the import of GFP-BRN2 in porcine oocytes, this explanation appears rather unlikely.

Although KPNA2 transcripts have been detected in GV-stage oocytes and not detected in blastocyst stage embryos, we have detected KPNA2 protein in the nuclei of blastocyst stage embryos immunocytochemically (Wang, 2012). We cannot exclude the possibility that KPNA2, or other karyopherin α subtypes, play a role in trafficking BRN2 to the nucleus in porcine oocytes and cleavage stage embryos. Additionally, although we have shown that both KPNA7 transcript and protein can be effectively knocked down 2 days after interfering RNA microinjection (Figure 2.5), we cannot exclude the possibility that the reduced levels of KPNA7 protein may retain sufficient functionality at the 4-cell stage.

Finally, we must be mindful that our results are based on observations derived solely from porcine embryos produced *in vitro.* Although the embryo production protocols we employed (maturing immature oocytes derived from pre-pubertal gilt ovaries, fertilizing them *in vitro*, and culturing embryos in the laboratory) have been used to yield embryos that lead to term development, such embryos are known to have reduced developmental potential as compared to *in vivo* derived embryos. While we do not rule out the possibility that the unusual intracellular localization of BRN2 we report in 8-cell stage embryos may be an artifact of *in vitro* embryo production, we speculate that functional differences in intracellular protein trafficking may reflect differences in embryo quality. Alternatively, because

the blastomeres within 8-cell stage porcine embryos are fundamentally different from blastomeres in earlier stage embryos (e.g., zygotic genome activation during the 4-cell stage), it is possible that the change in intracellular localization of BRN2 observed at the 8-cell stage of development may reflect a more global change in cellular physiology.

Although we hypothesize that a change in karyopherin α/β-mediated nuclear import is responsible for this observation, it is possible that a nuclear export system changes its activity during the 8-cell stage. This export activity may increase from levels observed prior to the 8-cell stage, such that BRN2 begins to maintain a predominantly cytoplasmic localization at steady state due to export activity out competing import activity. The asymmetrical intracellular localization of BRN2 among individual blastomeres at the 8-cell stage may reflect the age of individual blastomeres. As cleavage divisions are not synchronous, blastomeres of slightly different ages are likely present at the time point we examined our injected embryos. This "age" effect may reflect a change in nuclear envelope structure or activity of a given nuclear trafficking pathway.

The atypical intracellular localization of BRN2 at the 8-cell stage is intriguing. Together our data reveal that BRN2, a protein previously shown to be a cargo

selectively trafficked to the nucleus in a karyopherin α subtype-specific manner

exhibits an atypical intracellular localization in 8-cell stage porcine embryos. The

findings reported here clearly show a dramatic change in intracellular localization

of a karyopherin α cargo protein; a change that does not apply to all karyopherin α

cargoes globally. Determining the nature of this change will provide insights into

changes in nuclear trafficking dynamics that can potentially impact embryo

development.

Materials and Methods

Oocyte collection

All chemicals were obtained from Sigma Chemical Company (St. Louis, MO)

unless stated otherwise. Ovaries from prepubertal gilts were obtained from a local

abattoir and transported to the laboratory in an insulated container. Follicular fluid

was manually aspirated from antral follicles; pooled follicular fluid was allowed to

settle by gravity for 20 minutes. Cumulus-oocyte complexes (COCs) were washed

once in Hepes-buffered medium containing 0.01% polyvinyl alcohol (Hepes-PVA);

COCs with multiple layers of well-defined cumulus cells were selected

(Abeydeera *et al*., 1998). For experiments involving GV-stage oocytes, COCs

were vortexed for 5 minutes in 0.1% hyaluronidase in Hepes-PVA to remove

cumulus cells. Oocytes with an intact plasma membrane and evenly granulated

cytoplasm were selected and used for the experiments.

In vitro maturation

Fifty to 75 COCs were placed in 500µl of Tissue Culture Medium 199 (TCM 199;

Gibco BRL, Grand Island, NY) supplement with 0.14% polyvinyl alcohol, 10 ng/ml

epidermal growth factor, 0.57 mM cysteine, 0.5 IU/ml porcine FSH, and 0.5 IU/ml

ovine LH for 42-44 hours at 39°C and 5% CO_2 in air with 100% humidity

(Abeydeera *et al*., 1998). Following maturation, cumulus cells were removed by

vortexing matured oocytes in Hepes-PVA containing 0.1% hyaluronidase for 4

minutes. Denuded oocytes were washed three times in Hepes-PVA.

In vitro fertilization, parthenogenetic activation, and embryo culture

For *in vitro* fertilization, mature denuded oocytes were washed three times in a

modified Tris-buffered medium (mTBM). Cumulus free matured oocytes were

placed in mTBM and fertilized according to an established protocol, using fresh,

extended boar semen (Abeydeera and Day, 1997). Briefly, 30-35 oocytes were

placed in 100 µl of mTBM and incubated with spermatozoa at a concentration of

5×10^5 spermatozoa/ml. Presumptive zygotes were cultured in Porcine Zygote

Medium 3 (PZM3) supplemented with 3 mg/ ml fatty acid-free BSA at 39°C and 5%

CO_2 in air with 100% humidity (Yoshioka *et al.*, 2002). Pronuclear, 2-cell, 4-cell, and 8-cell stage embryos were collected at 18, 30, 48 and 72 hours after gamete mixing, respectively. Only embryos at the indicated developmental stages at the corresponding time points were assayed. For parthenogenetic activation, mature denuded oocytes were placed in activation medium (300 mM mannitol, 0.1 mM $CaCl_2$, 0.1 mM $MgSO_4$, 0.5 mM Hepes, 0.01% BSA) between two platinum electrodes of an Electrocell manipulator (BTX, San Diego, CA) and activated by two DC pulses of 1.2Kv/cm for 30 μsec. Two minutes following the electrical pulse discharge, activated oocytes were placed in PZM3 embryo culture medium containing 3 mg/ml BSA. Parthenogenetic embryos were collected at the following stages: 2-cell stage (30 hours post-activation), 4-cell stage (48 hours post-activation) and 8-cell stage (72 hours post-activation). Only embryos at the indicated developmental stages at the corresponding time points were assayed.

In vivo derived embryos

All procedures were performed according to a protocol approved by institutional animal care and use committee (Purdue Animal Care and Use Committee, 1311000982). Pubertal gilts were checked daily for the onset of estrus; gilts displaying estrus were inseminated with fresh, extended boar semen. Embryos were recovered surgically by mid-ventral laparotomy. Briefly, gilts were

anesthetized with an intravenous injection of ketamine (2 mg/kg) and xylazine (2

mg/kg); anesthesia was maintained by isoflurane inhalation (2-5%). The horns

were flushed with 25 ml of Hepes-buffered medium. The recovered medium was

examined under a dissecting microscope to collect the embryos. Surgery was

performed at 36 hours after the onset of estrus to recover pronuclear stage

embryos. Pronuclear stage embryos were immediately microinjected with

GFP-BRN2 mRNA.

Vector construction

Expression constructs encoding the epitope-tagged versions of porcine KPNA1,

KPNA2, KPNA3, KPNA5, KPNA6, KPNA7, KPNB, and GST-NLS were described

previously (Foust *et al*., 2012; Park *et al.,* 2012). The BRN2 expression constructs

were generated by amplifying the open reading frame of BRN2 by PCR from a

BRN2 expression construct (Yasuhara *et al*., 2007), kindly provided by Dr. Noriko

Yasuhara, Osaka University, and inserting this open reading frame into

pENTR/SD/D-TOPO vector (Invitroge , Carlsbad, CA, USA). Forward and reverse

primers were, 5'-CACCATGGCGACCGCAGCGTCT and 5'-TCACTGGACGGGC

GTCTGCACC, respectively; PCR was performed under the following conditions:

2 minutes at 95°C, followed by 35 cycles of 30 seconds at 95°C, 90 seconds at

63°C, and 70 seconds at 72°C, followed by a final extension at 72°C for 10

minutes. After sequencing to confirm the identity of the insert, BRN2 was

recombined into pDEST53 (Invitrogen) to generate a BRN2 expression construct

with an N-terminal GFP tag; this vector is referred to as GFP-BRN2 throughout

this manuscript. The NLS-DsRed construct was produced as follows: A pair of

oligonucleotide primers that encoded the amino acid sequence of the NLS from

the SV-40 T-antigen (5'-CATGCTAGCCATGGGCCCAAAGAAAAAGCGCAAG

GTGAAGCTTGGG and 5'-CCCAAGCTTCACCTTGCGCTTTTTCTTTGGGCC

CATGGCTAGCATG') was synthesized (IDT DNA Technologies) and annealed;

these duplexed oligos were then digested with *Hin*dIII and *Eco*RI. The

pDsRed2-N1 vector (Clontech) was digested with *Hin*dIII and *Eco*RI; following

digestion the insert encoding the SV-40 NLS was ligated into the digested

pDsRed2-N1 vector using T4 ligase (Promega) following the manufacturer's

protocol. Ligations were used to transfect DH5α cells; resultant plasmid DNA

isolated from selected bacterial tansformants was sequenced to confirm the

identity of the plasmid.

In vitro transcription

The GFP-BRN2 construct was linearized by digestion with *Stu*I and used as a

template for *in vitro* transcription using the mMessage mMachine T7 *in vitro*

transcription kit (Ambion, Austin, TX, USA) following the manufacturer's

instructions. Template NLS-DsRed mRNA was generated by PCR. A forward

oligonucleotide primer (5'-ATTAATACGACTCACTATAGGGGCTAgCCATGGGC

CCA) that contained the T7 promoter and 16 bases of the NLS-DsRed vector

covering the start codon was paired with a reverse primer (5'-ATTGATGAGTTT

GGACAAACCACAACTAG; corresponding to positions 1562-1590 of the

pDsRed2-N1 vector). PCR was performed using these oligonucleotides as

primers and the NLS-DsRed2 vector as template. Conditions for PCR were as

follows: 30 seconds at 98°C, followed by 35 cycles of 10 seconds at 98°C, 30

seconds at 62°C, and 30 seconds at 72°C, followed by a final extension at 72°C

for 10 minutes. Following PCR, products were electrophoresed on a 1% agarose

gel to assess PCR product size; products were purified using the QIAquick PCR

Purification Kit (Qiagen) following the manufacturer's instructions and then used

as a template for *in vitro* transcription using the mMessage mMachine T7 *in vitro*

transcription kit (Ambion, Austin, TX, USA) following the manufacturer's

instructions. Aliquots of mRNA were stored at -80°C until microinjection.

Oocyte and embryo microinjection

Oocyte and embryo microinjection assays were performed using an Eppendorf

Femtojet microinjection system (Eppendorf, Hauppauge, NY) attached to Leica

DM-IRB inverted microscope equipped with a heated stage (Leica, Buffalo Grove,

IL, USA). Pronuclear stage embryos and denuded GV-stage oocytes were placed

in Hepes-buffered medium containing 3 mg/ml BSA for microinjection. Following

injection, GV-stage oocytes were incubated in *in vitro* maturation medium at 39°C

and 5% CO_2 in air; embryos were cultured in embryo culture medium at 39°C and

5% CO_2 in air. Oocytes and embryos that lysed immediately following

microinjection were discarded. For imaging oocytes and embryos expressing GFP

and DsRed fusion proteins, cells were fixed at the appropriate developmental

stage in 3.7% paraformaldehyde at room temperature for 15 minutes, washed

three times in PBS, and stored in PBS at 4°C until further processing (up to 14

days). Fixed cells were stained with Hoechst 33342 (2 µg/ml) for 15 minutes.

Cells were mounted on slides in Vectashield (Vector Laboratories, Inc.,

Burlingame, CA), covered with a glass coverslip, and sealed with nail polish.

Oocytes and embryos were examined using a Leica DM-IRB inverted microscope

equipped with epifluorescence and a Zeiss LSM 710 confocal microscope.

Expression, purification and quantification of GST fusion proteins

BL21 cells were transfected with DNA vectors encoding GST fusion proteins.

Transfected cells were grown in liquid culture medium to an OD600 ranging

between 0.5-0.8 at 37°C; GST-NLS, GST-BRN2, and GST protein expression was

induced with 0.2 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) for 12-16

hours at 18°C, while GST-KPNB expression was induced with 0.2 mM IPTG and 0.2% arabinose for 5 hours at room temperature. Liquid cultures were cooled to 4°C following induction and centrifuged to pellet the bacteria. Bacterial pellets were resuspended in 20 ml PBS containing 0.3% Triton X-100 and protease inhibitors (Complete Protease Inhibitor Cocktail, catalog number 04-683-159-001, Roche, Indianapolis, IN), and lysed by sonication. Bacterial lysates were centrifuged at 12,000 x g for 15 minutes; supernatants were applied to a glutathione-agarose column. Proteins were incubated on the column at 4°C for 2 hours. Following column binding, the column was washed twice at 4°C with cold PBS containing 1% Triton X-100 and washed four times at 4°C with cold PBS. Following the washes, proteins were eluted from the column with 10 mM reduced glutathione; eluted proteins were dialyzed, quantified with Bradford assay and stored at -80°C prior to use in the *in vitro* binding assays.

RNA interference

Synthetic, custom Stealth™ RNAi nucleotides (Invitrogen) targeting porcine KPNA7 were used to knockdown levels of endogenous KPNA7 transcripts as previously reported (Wang *et al*., 2012). Briefly, annealed oligonucleotides targeting KPNA7 (5'-CAUGAAUGCUUAACGCCCUUAACAA and 5'-UUGUU AAGGGCGUUAAGCAUUCAUG) and a set of oligonucleotides serving as a

negative control (5'-CAUCGUAAAUUCCGCAUUCAAGCAA-3' and 5'- UUGCUU

GAAUGCGGAAUUUACGAUG-3') were stored at a concentration of 20 µM at

-20°C. Immediately prior to microinjection, Stealth RNAi nucleotides were diluted

to a final concentration of 2 µM in DEPC-treated nuclease-free water and mixed

1:1 with *in vitro* transcribed GFP-BRN2 mRNA.

Fluorescent labeling of BRN2, GST and NLS

GST-NLS, GST-BRN2, and GST proteins were expressed and purified as

described above. Following the three washes with PBS, but before elution from

the beads, GST-BRN2 and GST proteins were labeled with

fluorescein-5-maleimide (Pierce, Rockford, IL) according to the manufacturer's

protocol. GST-NLS was labeled with Alex 594 (Invitrogen) according to the

manufacturer's protocol. Following labeling, the agarose beads were washed

three times with PBS to remove unbound dye. Labeled proteins were eluted from

the beads with a solution of 10mM reduced glutathione in 50 mM Tris-HCl pH 8.0.

Eluted proteins were dialyzed, quantified using a Bradford assay and stored at

-80°C prior to use in the nuclear import assay.

Nuclear import assay in permeabilized porcine fetal fibroblast cells

An *in vitro* nuclear import assay was developed to examine the transport of

GST-NLS *in vivo*. Briefly, Primary porcine fetal fibroblast cells obtained from a

porcine conceptus at day 35 of gestation were seeded on a 10 cm tissue culture

plate (Falcon). When the porcine fetal fibroblast cells reached 50-80% confluence,

cells were permeabilized with 5 µg/ml of digitonin (Roche) for 5 min at room

temperature in transport buffer (TB) (20 mM Hepes, PH 7.4, 110 mM KOAC, 5

mM Na(OAc)$_2$, 2 mM Mg(OAc)$_2$, 2 mM DTT, 1 mM EGTA, 1 µg/ml of leupeptin and

pepstatin). All proteins used in the assay were solved in TB. Following

permeabilization and three times wash with transport buffer, cells were incubated

in 75µl import reaction buffer (2.5 mg/ml Hela cell cytosol (SPEED BioSystems,

LLC), ATP-regenetating system (1 mM ATP, 1 mg/ml CP, 15 U/ml CPK, and 0.1

mM GTP), and 2 µM FITC-labeled NLS) for 30 minutes at 30 °C on a prewared

metal box. Cells were immediately fixed and washed with cold PBS, and then

visualized using a Leica DM-IRB inverted microscope equipped with

epifluorescence.

In vitro binding assay

Methionine-[^{35}S] labeled versions of porcine KPNA1, KPNA2, KPNA3, KPNA5,

KPNA6, and KPNA7 were produced *in vitro* in rabbit reticulocytes using the TNT

Quick Coupled Transcription/Translation System (Promega Corporation, Medison, WI, USA) according to the manufacturer's directions. Binding reactions for each karyopherin α subtype were carried out as described previously (Foust *et al*., 2012). Briefly, 10 µg of GST-BRN2, GST-NLS, GST-KPNB or GST were bound to glutathione agarose beads. After a series of washes, reticulocyte lysates containing the radiolabeled versions of the porcine karyopherin α subtypes were co-incubated with the GST proteins on the column in binding buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM EGTA, and 1% Triton X-100, pH 7.3) for 2 hours at 4°C. Glutathione agarose beads were washed three times with PBS. Glutathione agarose beads were mixed with Laemmli loading buffer, boiled for 5 minutes and resolved on a 10% TGX precast gel (Bio-Rad). Gels were stained with Coomassie blue, dried and exposed to film at -80°C for 24–48 hours. Binding reactions were performed such that each respective *in vitro* transcribed and translated karyopherin α subtype was divided among four binding reactions (GST-BRN2, GST-NLS, GST-KPNB, and GST). The intensities of protein bands were analyzed with Molecular Imaging Software (Carestream Health, Woodbridge, CT). For each karyopherin α subtype, all band intensities from each binding reaction were normalized to the band intensity found in a lane that contained 40% of total reticulolysate input added to each binding reaction. For GST-BRN2, GST-NLS, GST-KPNB, and GST, karyopherin α binding intensities were

normalized to the intensity of the karyopherin α subtype that exhibited the highest

relative binding ability with corresponding GST fusion protein, thereby making this

relative measure a value of 1; binding intensities of all other karyopherin α

subtypes were then calculated to be a fraction relative to this value.

RNA isolation, reverse transcription and quantitative RT-PCR

Activated porcine oocytes were injected with KPNA7-interfering RNAs (or control

RNAs) to assay the level of KPNA7 knock down in 4-cell stage porcine embryos.

Here, activated oocytes were assigned to one of three treatment groups:

injected with KPNA7-RNAi, injected with control-RNAim, or non-injected. All

oocytes were placed in PZM3 embryo culture medium (Yoshioka *et al*., 2002) in

their respective treatment groups until 48 hours post-activation, at which time

4-cell stage embryos were harvested. Messenger RNA was then isolated from 30

intact 4-cell stage embryos from each treatment using Dynabeads reagent

(Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

RT-PCR was performed on each mRNA sample amplify YWHAG and KPNA7

from each sample using methodology previously reported (Wang *et al*., 2012).

Briefly, reverse transcription was carried out using the iScript reagent according to

the manufacturer's protocol (Bio-Rad, Hercules, CA, USA). KPNA7 and YWHAG

were amplified from each cDNA during the same RT-PCR run; each RT-PCR run

also contained a negative control where water was added in place of reverse

transcriptase. Reactions were performed on a MyiQ single-color real-time PCR

detection system (Bio-Rad). Each PCR reaction contained 12.5 µL of 2X

SybrGreen Mastermix (Bio-Rad), 5 µL of 1µM forward primer, 5µL of 1µM reverse

primer and 2.5 µL cDNA. The PCR began with an initial denaturation at 94°C,

followed by 40 cycles of 5 seconds at 94°C, 30 seconds at 60°C and 30 seconds

at 72°C. Real time fluorescence data were collected during the extension time.

KPNA7 and YWHAG were amplified in duplicate across a minimum of three

replicates. The threshold cycle of detection (or CT value) for KPNA7 was

subtracted from the Ct value of YWHAG to obtain a change in CT (ΔCT).

Statistical analysis

For the band intensity assay in Experiment 3, relative values were imported into

SAS (Statistical Analysis Software; Cary, NC, USA) and analyzed with a general

linear model one-way ANOVA procedure. For quantitative RT-PCR, ΔCT values

were analyzed by one-way ANOVA using GLM procedures of SAS. For multiple

comparisons ranking, Tukey's post-test was performed and P<0.05 was

considered significant.

Experiment 1: Intracellular localization of GFP-BRN2 in porcine embryos

Two microinjection assays were performed to determine the intracellular

localization of ectopically expressed GFP-BRN2 in porcine GV-stage oocytes and

cleavage stage embryos. First, denuded GV-stage oocytes were injected with

GFP-BRN2 mRNA and cultured in *in vitro* maturation medium at 39ºC, 5% CO_2 for

8 hours. Injected GV-stage oocytes were fixed and examined using confocal and

epifluorescent microscopy to determine the intracellular localization of GFP-BRN2.

To determine the intracellular localization of GFP-BRN2 in cleavage stage

embryos, *in vitro* matured porcine oocytes were injected with GFP-BRN2 mRNA 5

hours after co-incubation with sperm. Presumptive zygotes were cultured and

subsets of embryos removed at the pronuclear, 2-cell, 4-cell and 8-cell stages and

fixed. Fixed cells were examined using confocal microscopy to determine the

intracellular localization of GFP-BRN2.

Experiment 2: Intracellular localization of GFP-BRN2 and NLS-DsRed in

porcine embryos

To determine if the atypical intracellular localization pattern of GFP-BRN2

observed in 8-cell stage porcine embryos occurred with other NLS-bearing

proteins that were ectopically overexpressed, mRNA encoding NLS-DeRed was

microinjected into parthenogenetically activated porcine oocytes. Microinjected,

parthenogenetically activated oocytes were fixed at the 8-cell stage and examined using epifluorescence microscopy to determine the intracellular localization of NLS-DsRed. In addition, mRNAs encoding GFP-BRN2 and NLS-DeRed were co-microinjected into parthenogenetically activated porcine oocytes. Microinjected, parthenogenetically activated oocytes were fixed at the 2-cell, 4-cell and 8-cell stages and examined using confocal microscopy to determine the intracellular localization of both GFP-BRN2 and NLS-DsRed.

Experiment 3: In vitro binding assay

Methionine-[^{35}S] labeled versions of porcine KPNA1, KPNA2, KPNA3, KPNA5, KPNA6, and KPNA7 were produced *in vitro* in rabbit reticulocytes using the TNT Quick Coupled Transcription/Translation System (Promega Corporation, Medison, WI, USA) according to the manufacturer's directions. Binding reactions for each karyopherin α subtype were carried out as described previously (Foust *et al*., 2012). Briefly, 10µg of GST-BRN2, GST-NLS, GST-KPNB or GST were bound to glutathione agarose beads. After a series of washes, reticulocyte lysates containing the radiolabeled versions of the porcine karyopherin α subtypes were co-incubated with the GST proteins on the column in binding buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM EGTA, and 1% Triton X-100, pH 7.3) for 2 hours at 4°C. Glutathione agarose beads were washed three times with PBS. Glutathione

agarose beads were mixed with Laemmli loading buffer, boiled for 5 minutes and

resolved on a 10% TGX precast gel (Bio-Rad). Gels were stained with Coomassie

blue, dried and exposed to film at -80°C for 24–48 hours. Binding reactions were

performed such that each respective *in vitro* transcribed and translated

karyopherin α subtype was divided among four binding reactions (GST-BRN2,

GST-NLS, GST-KPNB, and GST). The intensities of protein bands were analyzed

with Molecular Imaging Software (Carestream Health, Woodbridge, CT). For each

karyopherin α subtype, all band intensities from each binding reaction were

normalized to the band intensity found in a lane that contained 40% of total

reticulolysate input added to each binding reaction. For GST-BRN2, GST-NLS,

GST-KPNB, and GST, karyopherin α binding intensities were normalized to the

intensity of the karyopherin α subtype that exhibited the highest relative binding

ability with corresponding GST fusion protein, thereby making this relative

measure a value of 1; binding intensities of all other karyopherin α subtypes were

then calculated to be a fraction relative to this value.

Experiment 4: KPNA7 transcript knockdown and GFP-BRN2 intracellular

localization

Synthetic, custom Stealth™ RNAi nucleotides (Invitrogen) targeting porcine

KPNA7 were used to knockdown levels of endogenous KPNA7 transcripts as

previously reported (Wang *et al*., 2012). Briefly, annealed oligonucleotides

targeting KPNA7 (5'-CAUGAAUGCUUAACGCCCUUAACAA and 5'-UUGUUAAG

GGCGUUAAGCAUUCAUG) and a set of oligonucleotides serving as a negative

control (5'-CAUCGUAAAUUCCGCAUUCAAGCAA-3' and 5'- UUGCUUGAAUGC

GGAAUUUACGAUG-3') were stored at a concentration of 20 µM at -20ºC.

Immediately prior to microinjection, Stealth RNAi nucleotides were diluted to a

final concentration of 2 µM in DEPC-treated nuclease-free water and mixed 1:1

with *in vitro* transcribed GFP-BRN2 mRNA. Parthenogenetically activated oocytes

were co-injected with these substrates 5 hours after electroporation, such that

embryos were in one of the three following treatment groups: Control RNAi +

GFP-BRN2, KPNA7 RNAi + GFP-BRN2, or non-injected cells. Microinjected

oocytes were cultured in PZM3 containing 3 mg/ml BSA for 48 hours after

microinjection and assayed at the 4-cell stage to determine the intracellular

localization of GFP-BRN2 using confocal microscopy. We also determined the

level of KPNA7 transcript knockdown in 4 cell–stage embryos following injection

of interfering RNAs. Here, parthenogenetically activated porcine oocytes were

assigned to one of the three following treatment groups: Control RNAi, KPNA7

RNAi, or non-injected cells. Microinjected parthenogenetically activated oocytes

were cultured in PZM3 containing 3 mg/ml BSA for 48 hours after microinjection

and assayed at the 4-cell stage to determine KPNA7 transcript abundance. Briefly,

mRNA was isolated from equal numbers of intact 4-cell stage parthenogenetic embryos from each treatment using Dynabeads; RT-PCR was performed on each mRNA sample using primers to amplify YWHAG and KPNA7 from each sample using methodology previously reported (Wang *et al*., 2012).

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Table 2.1: Distribution of GFP-BRN2 and NLS-DsRed in porcine oocytes and

embryos

Developmental stage	Protein	Intracellular localization	N
Germinal vesical oocytes	GFP-BRN2	BRN2 in the nucleus	33
1-Cell embryos	GFP-BRN2	BRN2 in all nuclei	17
2-Cell embryos	GFP-BRN2	BRN2 in all nuclei	21
4-Cell embryos	GFP-BRN2	BRN2 in all nuclei	13
8-Cell embryos	GFP-BRN2	BRN2 in one of eight nuclei ($n = 3$) BRN2 in two of eight nuclei ($n = 7$) BRN2 in four of eight nuclei ($n = 1$)	11
8-Cell embryos	NLS-DsRed	DsRed in eight of eight nuclei ($n = 9$)	9

Table 2.2: Distribution of arrest points of *in vivo* derived embryos

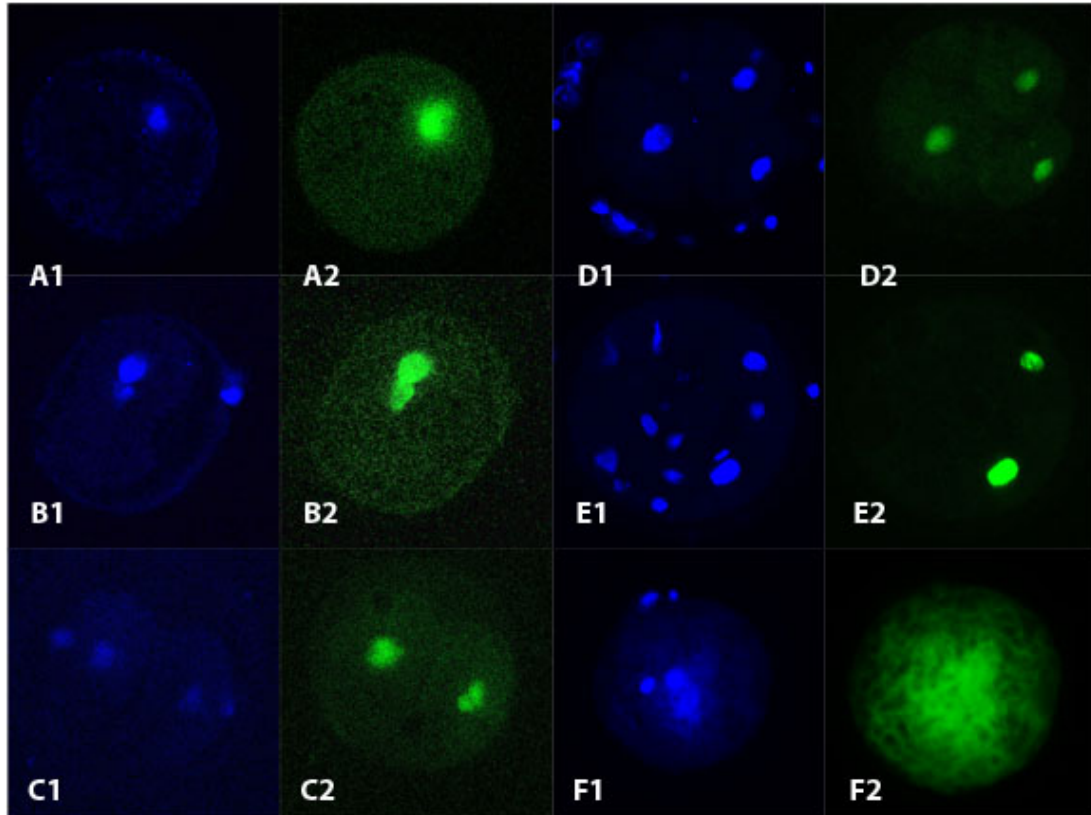| Treatments	Harvest stage	Arrest points	N
Injection with GFP-BRN2 mRNA	1-cell stage (*n*=8)	1-cell stage (*n*=2)	8
		2-cell stage (*n*=2)	
		4-cell stage (*n*=4)	
Noninjection	1-cell stage (*n*=1)	1-cell stage (*n*=1)	5
	2-cell stage (*n*=4)	2-cell stage (*n*=4)	

Figure 2.1: GFP-BRN2 adopts a nuclear localization in porcine oocytes and cleavage stage embryos. Representative images are shown of germinal vesicle stage oocytes (A), pronuclear stage (B), 2- (C), 4- (D) and 8-cell (E) stage embryos injected with GFP-BRN2 mRNA. (F) Representative images of a pronuclear stage embryo injected with green fluorescent protein (GFP) mRNA. In all cases, panels beginning with the same letter are derived from the same representative oocyte or embryo. (A–E) Images were captured as an optical section using confocal microscopy; (F) image was captured using epifluorescent microscopy. (A1–F1) DNA; (A2–F2) GFP.
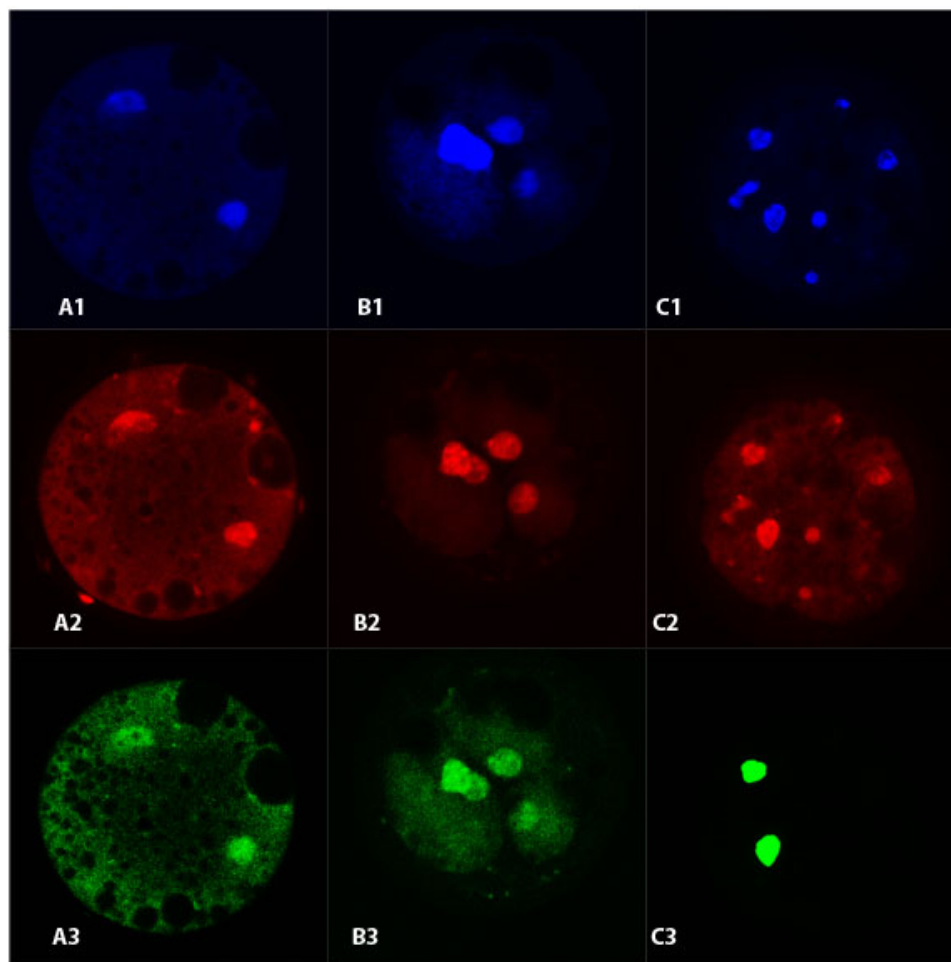
Figure 2.2: NLS-DsRed and GFP-BRN2 adopt a nuclear localization in 2-, 4- and 8-cell stage parthenogenetic embryos. Representative images of 2- (A), 4- (B) and 8-cell (C) stage embryos coinjected with both *NLS-DsRed* and *GFP-BRN2* mRNA are shown. Panels beginning with the same letter are derived from the same representative embryo. Images were captured as an optical section using confocal microscopy. (A1–C1) DNA; (A2–C2) DsRed; (A3–C3) GFP.
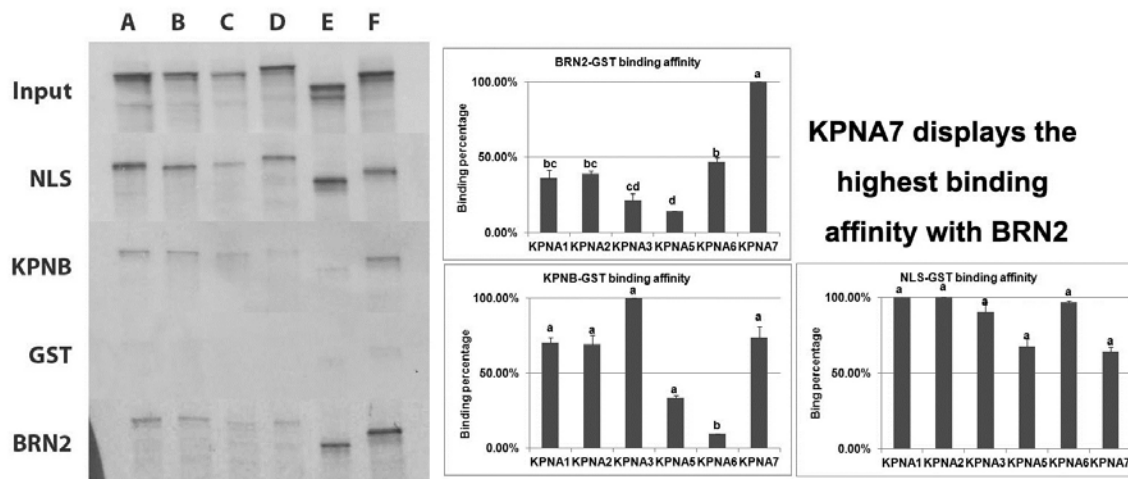
Figure 2.3: Recombinant BRN2 binds to multiple karyopherin α subtypes.

Representative autoradiographs derived from the BRN2-karyopherin α binding

assay reveal karyopherin α7 (KPNA7) to have the highest relative binding affinity

for BRN2 compared with the other karyopherin α subtypes analysed. The

autoradiographs show the amount of radiolabelled karyopherin α subtype that

was added to each binding reaction (Input), the amount of radiolabelled

karyopherin α subtype recovered upon incubation with GST-NLS (NLS),

karyopherin β1 (KPNB), the GST tag alone (GST) and GST-BRN2 (BRN2). Lanes

A–F indicate KPNA1–KPNA7, respectively. The bar graphs on the right reveal the

relative binding intensity of BRN2, NLS, and KPNB with each karyopherin α

subtype, respectively. Data are the mean ± s.e.m. Columns with different letters

differ significantly ($P < 0.05$).
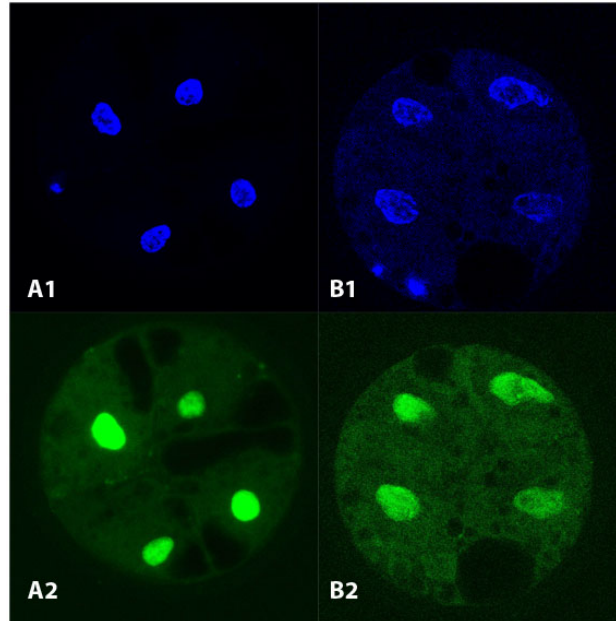
Figure 2.4: Knockdown of karyopherin α7 (*KPNA7*) does not affect the

intracellular localization of GFP-BRN2 in 4-cell stage embryos. (A) Representative

images of an embryo injected with control RNAs previously shown to have no

effect on *KPNA7* transcript abundance and mRNA encoding GFP-BRN2. (B)

Representative images of an embryo injected with *KPNA7* interfering RNA and

mRNA encoding GFP-BRN2.
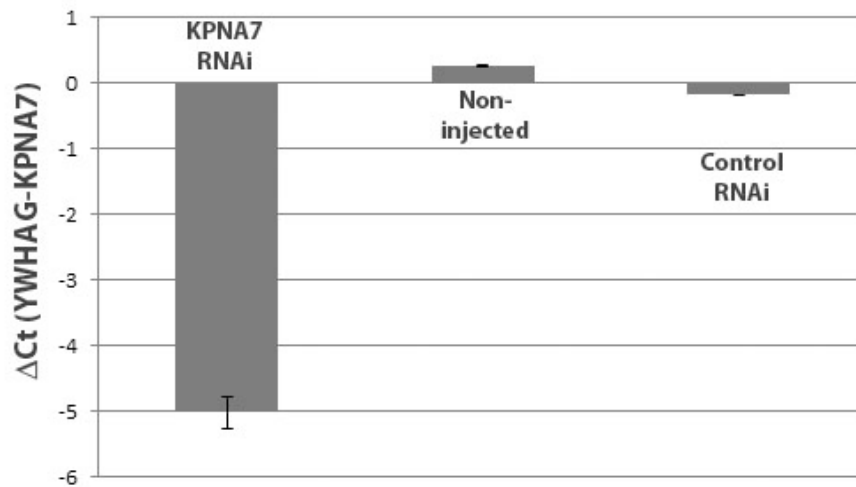
Figure 2.5: Validation of RNA interference (i)-mediated knockdown of karyopherin

α7 (*KPNA7*) transcripts in 4-cell stage porcine embryos. A quantitative reverse

transcription–polymerase chain reaction assay was used to determine the level of

reduction in *KPNA7* transcript abundance in 4-cell stage porcine embryos 48 h

after injection of interfering RNAs targeting *KPNA7*. Data show ΔCT values,

obtained from subtracting the CT value for *KPNA7* from the CT value for the

housekeeping gene *YWHAG*. Data are the mean ± s.e.m.

CHAPTER 3 REDUCTION IN KARYOPHERIN α1 IS ASSOCIATED WITH DEVELOPMENTAL ARREST AND IMPAIRED NUCLEAR IMPORT IN PORCINE CLEAVAGE STAGE EMBRYOS

Abstract

Intracellular communication between the nucleus and cytoplasm is central to coordinate cellular events during cleavage development. The karyopherin α/β heterodimer is an intracellular nuclear trafficking system that mediates nuclear import of proteins that bear classical nuclear localization signals (NLSs). Seven karyopherin α subtypes have been identified in mammals. Although each of these karyopherin α subtypes is able to bind to an NLS, individual karyopherin α subtypes have been shown to transport specific NLS-bearing proteins. The aim of this study was to determine the developmental requirements of karyopherin α1 (KPNA1) during cleavage development in the porcine embryo. Our results suggest that karyopherin α1 is required for cleavage development, and that changes in KPNA1 levels result in reduced import of NLS bearing proteins.

Introduction

Cleavage stage embryos undergo a profound amount of chromatin remodeling during the first few cell cycles after fertilization. Aberrant epigenetic modifications can lead to perturbed patterns of gene expression associated with reduced embryo developmental competence and developmental failure. Transcription factors and chromatin remodeling factors must be transported across the nuclear membrane to the nucleus to remodel chromatin and direct embryo development.

A series of transport pathways are known to regulate the movement of factors between the nuclear and cytoplasmic compartments in eukaryotic cells. Perhaps the best characterized nuclear transport system is nuclear import mediated by the karyopherin α/β heterodimer (Görlich and Kutay, 1999). In this system, karyopherin α binds to intracellular proteins that possess a classical NLS. The NLS recognized by karyopherin α typically consists of short stretches of basic amino acids, primarily lysine residues. These NLSs are found in one of two canonical forms: monopartite signals typified by the NLS found with the SV40-T antigen (-**PKKKRKV**-) or a bipartite signal consisting of two clusters of basic amino acids separated by a short spacer, which resembles the NLS from nucleoplasmin (-**KR**PAATKKAGQA**KKK**-) (Reichelt *et al.*, 1990). After

karyopherin α interacts with the NLS-bearing cargo, cargo-bound karyopherin α

binds to karyopherin β (Görlich *et al.*, 1996) and this trimeric complex then

interacts with the structural proteins of the nuclear pore complex and moves the

NLS-bearing cargo into the nucleus (Ribbeck *et al.*, 1998).

Seven karyopherin α subtypes have been identified in mammals. The individual

karyopherin α subtypes can be identified by their standardized nomenclature and

referred to as karyopherins α1-α7 (KPNA1-KPNA7) (Wang *et al.*, 2012;

Tejomurtula *et al.*, 2009). Karyopherin α subtypes share many general structural

motifs, including an N-terminal IBB domain, which is responsible for the

interaction with karyopherin β and multiple repeated ARM motifs that contain an

NLS-binding pocket.

Previous studies have shown that although karyopherin α subtypes share a high

degree of sequence identity and are able to bind to and transport multiple

NLS-bearing substrates, certain NLS-bearing substrates are preferentially

imported by specific karyopherin α subtypes. For example, murine importin α5

(KPNA1) is able to bind with several transcription factors *in vitro* such as Anp32a,

TRβ1, and FUBP1, while Arip3, Hop2, and Chrp can interact with murine importin

α2 (KPNA2) (Fukumoto *et al.*, 2011; Ly-Huynh *et al.*, 2011). SP17 and RREB are

able to interact with multiple porcine karyopherin α subtypes (Park *et al*., 2012).

In addition, RanBP3 and RCC1 have been shown to be preferentially imported by

KPNA3 (Talcott and Moore, 2000; Yeung *et al*. 2006; Welch *et a*l., 1999), and

KPNA6 and KPNA7 have been shown to be carriers of STAT3 (Cao and Ma,

2005), while NF-κB is transported into nucleus by KPNA3 and KPNA4 (Fagerlund

et al., 2005).

Evidence also suggests that individual karyopherin α subtypes are not functionally

redundant and individual karyopherin α subtypes may serve regulatory roles

during embryo development. Mice that lack KPNA6 (also referred to as importin

α7) are viable but infertile (Rother *et al*., 2011). Karyopherin α7 appears to be

required for cleavage development in both bovine (Tejomurtula *et al*., 2009) and

porcine (Wang *et al*., 2012) embryos, although this may not be true for all

karyopherin α subtypes (Cabot and Prather, 2003). These observations enable us

to hypothesize that distinct karyopherin α subtypes differentially direct

development by transporting discrete intracellular cargoes.

In murine stem cells, the transcription factor BRN2 (which has been reported to

serve regulatory roles in the neural differentiation pathway) has been reported to

require KPNA1 for nuclear import (Yasuhara *et al.,* 2007). Our group has

previously shown that the NLS in this transcription factor interacts with both KPNA1 and KPNA7 in the domestic pig. While the NLS found in BRN2 directs a nuclear localization until the 4-cell stage of development in porcine embryos, a varied pattern of nuclear location is observed at the 8-cell stage of development in the pig (Li *et al.,* 2013). This observation indicates that intracellular partitioning of NLS-bearing cargoes mediated by members of the karyopherin α family may serve a role in regulating access to chromatin surrounding the time of zygotic genome activation and the early events associated with blastocoele formation.

In this study, we report that RNAi-mediated knockdown of *KPNA1* leads to reduced cell numbers in porcine cleavage stage embryos. We also show that knockdown of *KPNA1* significantly reduces the ability of BRN2 to accumulate in the nuclei of 4-cell stage porcine embryos. Our data suggest that KPNA1 is required for porcine embryonic development, and indicate that KPNA1 plays an important role in the nuclear import of proteins that possess NLSs similar to those found in BRN2.

Results

RNA interference mediated knockdown of KPNA1 leads to embryo developmental arrest

Injection of *KPNA1* interfering RNAs resulted in a reduced proportion of embryos with the capacity to develop to the 8-cell stage and beyond (13.5% vs 48.2% or 40% for control interfering RNAs and non-injected control embryos, respectively; $p<0.05$). Embryos injected with *KPNA1* interfering RNAs possessed significantly fewer nuclei following six days of embryo culture than embryos injected with control interfering RNA or non-injected control embryos (5.6 nuclei per embryo vs 9.1 or 11.5 nuclei per embryo, respectively; $p<0.05$). These data are summarized in Table 3.1. The level of *KPNA1* transcript knockdown is shown in Figure 3.1.

RNAi-mediated knockdown of KPNA1 affects the intracellular localization of GFP-BRN2 in 4-cell stage embryos

An RNAi approach was used to determine the effect of *KPNA1* knockdown on the intracellular localization of GFP-BRN2. At the 4-cell stage of development, GFP-BRN2 adopted a nuclear localization in parthenogenetic embryos injected with *KPNA1* interfering RNAs (n = 5/5) and control interfering RNAs (n = 5/5, Figure 3.2A-3.2C). However, the ratio of fluorescence intensity between nuclear

and cytoplasmic compartments in embryos injected with *KPNA1* interfering RNAs was significantly reduced as compared to embryos injected with control interfering RNAs (17.7% vs 21.2%; *p*<0.05, Figure 3.3).

Discussion

As stated in the introduction above, import of proteins bearing classical NLSs is mediated by the karyopherin α/β heterodimer. Several karyopherin α subtypes have been reported to be required for embryonic development. For instance, RNAi-mediated knockdown of *KPNA7* has been shown to lead to reduced cell numbers in bovine and porcine embryos (Tejomurtula *et al*., 2009; Wang *et al..* 2012). In addition, porcine embryos appear to have differing developmental requirements for KPNA2 and KPNA3 (Cabot *et al*., 2003). It has also been reported that KPNA6 is required for murine embryos to develop beyond the 2-cell stage of development (Rother *et al*., 2011). Based on these data, we wanted to determine the developmental requirements of karyopherin α1 (KPNA1) during cleavage development in porcine embryos. Our results showed KPNA1 transcript levels were significantly reduced 24 hours after injection of interfering RNAs; we also found that a significantly lower proportion of embryos injected with *KPNA1* interfering RNAs developed beyond the 8-cell stage as compared to control embryos.

Our group previously showed that *KPNA1* transcripts were detectable in porcine oocytes and cleavage stage embryos, with no statistically significant change in *KPNA1* transcript abundance from immature GV-stage oocyte to blastocyst stage embryo (Wang *et al*., 2012; Li *et al*., 2013). Our group found that BRN2, a transcription factor that possesses a classical NLS, was able to bind with multiple porcine karyopherin α subtypes *in vitro*. While BRN2 could interact with all karyopherin α subtypes in this assay, BRN2 bound most robustly with KPNA7 (Li *et al*., 2013). An *in vivo* nuclear import assay revealed that although KPNA7 could bind robustly to BRN2 *in vitro*, KPNA7 itself was not solely responsible for BRN2 import in porcine embryos (Li *et al*., 2013). A related series of experiments using murine cells showed that KPNA1 was the only karyopherin α subtype able to mediate import of BRN2 using an *in vitro* import assay (Yasuhara *et al*., 2007).

Because our RNA interference assay indicated that *KPNA1* was required for cleavage development, we next aimed to determine the nature of this developmental arrest. To determine if the disruption in development following knockdown of *KPNA1* could be attributed to a change in nuclear trafficking dynamics, we assayed the import dynamics of fluorescently labeled BRN2 in control and *KPNA1*-depleted embryos. Our results revealed a significant reduction in the proportion of labeled protein that accumulated in the nuclei of 4-cell stage porcine embryos upon *KPNA1* depletion. Our results that show a significant reduction in embryo development potential upon KPNA1 knockdown in the pig differs from the phenotypes of two independent KPNA1-knockout mice. In

2007, Shmidt *et al.* reported that KPNA1-null mice (also referred to as importin α5-null mice) generated by a gene-trap approach exhibited no overt reproductive phenotype (in that null animals were viable and fertile) and possessed no discernable neural phenotype (Shmidt *et al*., 2007). In contrast, Moriyama reported that another murine KPNA1-null (generated using a Cre-lox system) was viable, but possessed a striking reproductive phenotype characterized by ovarian and uterine hypoplasia (Moriyama *et al*., 2011). While the differences in phenotypes reported for these two animals may be rooted in the systems used to generate these two knock-outs, the viability of both of these murine KPNA1-null animals differs from the clear developmental arrest we observe during cleavage development in the porcine embryo. These differences could be due to requirements for KPNA1-mediated trafficking in the porcine embryo that are unique from those in the murine system. In support of this argument, it should be noted that knockout mice that lack KPNA7 are viable (Hu *et al.,* 2010), while RNAi-mediated knockdown of KPNA7 in both bovine and porcine embryos leads to dramatic disruption of cleavage development (Tejomurtula *et al*., 2009; Wang *et al.,* 2012).

Zygotic genome activation (ZGA) occurs during the 4-cell stage in porcine embryos (Norberg, 1970). Because the majority of KPNA1-depleted porcine embryos fail to develop beyond the 8-cell stage, it is possible that KPNA1 may serve a critical role of partitioning intracellular cargoes that are directly involved in ZGA, or that direct development immediately after ZGA. Several transcription

factors involved in pluripotency maintenance and stem cell self-renewal, such as OCT4 and SOX2 are transported via the karyopherin α/β heterodimer (Oestrup *et al.,* 2009; Yasuhara *et al.,* 2007). Ordered partitioning of intracellular proteins mediated by multiple transport receptors may be of critical importance during ZGA. For instance, the null mutation in KPNA6 in mice also results in developmental arrest at the time of ZGA (Rother *et al.*, 2011).

While our findings indicate that KPNA1 can function in BRN2 import (as evidenced by the reduction in GFP-BRN2 accumulation in the nuclear of KPNA1-depleted embryos), we cannot rule out the possibility that BRN2 is trafficked by additional karyopherin α subtypes (or other transport pathways) during this developmental timeframe. It has been reported that multiple karyopherin α subtypes can mediate trafficking of discrete intracellular proteins. For example, STAT3 has been shown to be transported by both KPNA1 and KPNA6 (Ma and Cao, 2006) and SOX2 has been shown to be imported by both KPNA3 and KPNA5 (Yasuhara *et al.,* 2007). It is also possible that residual KPNA1 remaining after RNAi-mediated knockdown is sufficient to allow embryos to proceed in development to the 4-cell stage, and a complete depletion at an earlier stage in development may result in an earlier developmental arrest (i.e., prior to ZGA). In addition, because BRN2 is a transcription factor, which can regulate gene transcription by binding and interacting with DNA, we cannot exclude the possibility that the interaction between BRN2 and chromatin could

cause BRN2 to remain in the nucleus as mitotic cell division occurs from 2-cell stage to 4-cell stage embryo.

Lastly, it is important to consider the source of the materials used in our study. The embryos used in our BRN2 import assay were derived from parthenogenetic activation. The use of parthenogenetic embryos allowed us to generate cohorts of embryos that maintained a high degree of developmental synchrony, whereby a high percentage of embryos reached the 4-cell stage within 48 hours of activation. However we must be mindful that the embryos produced by perthenogenetic activation differ from embryos produce by fertilization. It is possible that parthenogenetic and biparental embryos differ in nuclear dynamics in the early stage development, and thus not fully replicate the trafficking dynamics observed in biparental embryos, although many aspects of early cleavage are modeled nicely in parthenogenetic embryos (e.g., timing of ZGA, timing of initial cleavage divisions) (Paffoni *et al.,* 2008). At the same time, we must keep in mind that all the embryos observed in this study were produced *in vitro*. Despite the fact that the embryo production protocols used in our study have been used to yield embryos that lead to term development, embryos produced *in vitro* are known to have reduced developmental potential as compared to embryos produced *in vivo*.

In summary, the data discussed above support the hypothesis that karyopherin α1 serves a critical role in early cleavage stages embryonic development. Our data also suggest that karyopherin α1 is involved in the transport of transcription factor

BRN2 in 4-cell stage embryos. Together, our results once again indicate that discrete classes of NLS-bearing nuclear proteins may be preferentially imported by individual karyopherin α subtypes. This level of regulation may be critical to ensure proper timing of major cellular events during cleavage development, including ZGA.

Materials and Methods

Oocyte collection

Unless stated otherwise, all chemicals were obtained from Sigma Chemical (St. Louis, MO, USA). Porcine ovaries from prepubertal gilts were obtained from a local abattoir and transported to the laboratory in an insulated container. Follicular fluid was aspirated from antral follicles 3-6 mm in diameter using a 20-gauge needle and 10 ml syringe. Follicular fluid was allowed to settle by gravity for 20 minutes. After removal of follicular fluid, cumulus–oocyte complexes (COCs) were washed once in HEPES-buffered medium containing 0.01% polyvinyl alcohol (HEPES-PVA; Abeydeera *et al*., 1998). Using a stereomicroscope, COCs with multiple layers of well-defined cumulus cells were selected for experiments. For experiments involving germinal vesicle stage (GV–stage) oocytes, COCs were vortexed for 5 minutes in HEPES-PVA containing 0.1% hyaluronidase to remove

cumulus cells. Denuded GV-stage oocytes with an intact plasma membrane and evenly granulated cytoplasm were selected and used for experiments.

In vitro maturation

COCs were matured in a chemically defined maturation medium. Briefly, 50-75 COCs were placed in tissue culture medium 199 (TCM199; GIBCO BRL, Grand Island, NY, USA) supplemented with 0.14% PVA, 10 ng mL^{-1} epidermal growth factor, 0.57 mM cysteine, 0.5 IU mL^{-1} porcine FSH, and 0.5 IU mL^{-1} ovine LH for 42–44 hours at 39°C and 5% CO_2 in air with 100% humidity (Abeydeera *et al*., 1998). Following maturation, COCs were vortexed in HEPES-PVA containing 0.1% hyaluronidase for 4 minutes to remove cumulus cells. Denuded oocytes were washed three times in HEPES-PVA. Mature, denuded oocytes with an intact plasma membrane were then assigned to studies involving *in vitro* fertilization or parthenogenetic activation (described below).

In vitro fertilization, parthenogenetic activation, and embryo culture

For *in vitro* fertilization (IVF), mature denuded oocytes were washed three times in a modified Tris-buffered medium (mTBM); mTBM consisted of 113.1 mM NaCl, 3.0 mM KCl, 7.5 mM $CaCl_2{\times}2H_2O$, 20 mM Tris (crystallized free base), 11 mM glucose, 5 mM sodium pyruvate, 0.1% bovine serum albumin（BSA）, and 1 mM

caffeine (Abeydeera *et al.,* 1998). Denuded, mature oocytes were placed in mTBM

and fertilized according to an established protocol, using fresh extended boar

semen (Abeydeera and Day, 1997). Briefly, 30–35 oocytes were placed in 100 μL

mTBM and incubated with spermatozoa at a concentration of 5×10^5 spermatozoa

mL^{-1} for five hours at 39°C and 5% CO_2 in air with 100% humidity. Presumptive

zygotes were cultured in porcine zygote medium 3 (PZM3) containing 3 mg mL^{-1}

BSA (Yoshioka *et al*., 2002); presumptive zygotes were cultured at 39°C and 5%

CO_2 in air with 100% humidity. Blastocyst stage embryos were collected six days

after gamete mixing. For parthenogenetic activation, denuded, mature oocytes

were placed in activation medium (300 mM mannitol, 0.1 mM $CaCl_2$, 0.1 mM

$MgSO_4$, 0.5 mM HEPES, 0.01% BSA) between two platinum electrodes of an

Electrocell manipulator (Magnani and Cabot, 2008) (BTX, San Diego, CA, USA)

and activated by two DC pulses of 1.2 kV cm^{-1} for 30 μs. Activated oocytes were

placed in PZM3 embryo culture medium containing 3 mg mL^{-1} BSA and cultured

for 48 hours, at which point 4-cell stage embryos were collected.

Vector construction and in vitro transcription

The vector referred to as GFP-BRN2 was used as a template to produce

GFP-BRN2 mRNA as described previously (Li *et al.,* 2013). Briefly, the GFP-BRN2

construct was linearized by digestion with *Stu*I and used as a template for *in vitro*

transcription using the mMessage mMachine T7 *in vitro* transcription kit (Ambion,

Austin, TX, USA) according to the manufacturer's instructions (Li *et al.,* 2013).

Oocyte and embryo microinjection

Oocyte and embryo microinjection assays were performed using an Eppendorf

Femtojet microinjection system (Eppendorf, Hauppauge, NY, USA) attached to

Leica DM-IRB inverted microscope equipped with a heated stage (Leica, Buffalo

Grove, IL, USA). Oocytes and pronuclear stage embryos were placed in 50 μL

HEPES-buffered medium containing 3 mg mL^{-1} BSA covered in mineral oil

immediately prior to microinjection. Following injection, oocytes were cultured in

TCM199 and embryos were cultured in PZM3 containing 3mg mL^{-1} BSA. Both

oocyte and embryos were cultured in their respective media at 39°C and 5% CO_2

in air with 100% humidity. Oocytes and embryos that lysed immediately following

microinjection were discarded. For imaging embryos, cells were fixed at the

appropriate developmental stages in 3.7% paraformaldehyde at room temperature

for 15 minutes, washed three times in phosphate-buffered saline (PBS) and stored

in PBS at 4°C until further processing (up to 10 days). Fixed cells were stained with

Hoechst 33342 (2 μg mL^{-1}) for 15 minutes. Cells were mounted on glass slides in

Vectashield (Vector Laboratories, Burlingame, CA, USA), covered with a glass

coverslip and sealed with nail polish. Embryos were examined using a Leica

DM-IRB inverted microscope equipped with epifluorescence and a Nikon A1R_MP confocal microscope (Nikon Instruments Inc.).

RNA isolation and transcript quantification

Porcine GV stage oocytes were injected with *KPNA1*-interfering RNAs (or control RNAs) to assay the level of *KPNA1* knockdown in porcine oocytes. Oocytes were assigned to one of three treatment groups: injected with *KPNA1*-RNAi, injected with control-RNAi, or non-injected. All oocytes were cultured for 24 hours in TCM199 at 39°C and 5% CO_2 in air with 100% humidity in their respective treatment groups. The mRNA was then isolated from 55 GV stage oocytes from each treatment group using Dynabeads reagent (Invitrogen) according to the manufacturer's instructions. Reverse transcription–polymerase chain reaction (RT-PCR) was performed on each mRNA sample to amplify *YWHAG* and *KPNA1* from each sample using methodology reported previously (Wang *et al.*, 2012). Briefly, reverse transcription was performed using the iScript reagent (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. *KPNA1* and *YWHAG* were amplified from each cDNA during the same PCR run; each PCR run also contained a negative control, in which water was added in place of the template. Reactions were performed on a MyiQ single-color real-time PCR detection system (Bio-Rad). Each PCR reaction contained 12.5 µL of 2×

SybrGreen Mastermix (Bio-Rad), 5 µL of 1 µM forward primer, 5 µL of 1 µM

reverse primer and 2.5 µL cDNA. The PCR began with an initial denaturation at

94°C followed by 40 cycles of 5 seconds at 94°C, 30 seconds at 60°C and 30

seconds at 72°C. Real-time fluorescence data were collected during the extension

time. *KPNA1* and *YWHAG* were amplified in duplicate; the threshold cycle of

detection (or CT value) for *KPNA1* was subtracted from the CT value of *YWHAG* to

obtain a change in CT (ΔCT).

Measurement and quantification of intracellular localization of GFP-BRN2

Embryos were examined with a Nikon A1R_MP confocal microscope (Nikon

Instruments Inc.). Briefly, a series of 24 optical sections were imaged on

whole-mount porcine embryos. A compiled image was generated by merging the

Z-stack series of images for each embryo. NIS-Elements AR Analysis software

(Nikon Instruments Inc.) was used to quantify the fluorescence detected from GFP

in each composite image. For each embryo, the amount of fluorescence detected

in the whole embryo and the amount of fluorescence in nuclear compartments

were measured and a ratio of fluorescence intensity between nuclear and

cytoplasmic regions within an embryo was calculated and analysed.

Statistical analysis

For the GFP fluorescence assay, relative values were imported into SAS (Statistical Analysis Software, Cary, NC, USA) and analysed with a *paired-t* test procedure. For experiments of *KPNA1* RNA interference, data were analysed with a general linear model way ANOVA procedure. For multiple comparison ranking, Tukey's post test was performed. *P*<0.05 was considered significant.

Experiment 1: validation of RNA interference mediated knockdown of KPNA1 transcripts in GV porcine stage oocytes

Synthetic, custom Stealth RNAi nucleotides (Invitrogen) were used to knockdown levels of endogenous *KPNA1* transcripts. Custom designed Stealth RNA molecules targeting *KPNA1* (5'-GAGGCCCAGAUUAAUAACAUGGAAA-3' and 5'-UUUCCAUGUUAUUAAUCUGGGCCUC-3') were obtained from Invitrogen along with a set of custom negative control RNAs (5'-GAGACCUAGAAUAAUUACGGG CAAA-3' and 5'- UUUGCCCGUAAUUAUUCUAGGUCUC-3'). Targeting RNAs were directed to positions 225-249 of Genbank accession number NM_001163405. These RNAs were annealed and stored at concentration of 20 μM at –20°C. Immediately before microinjection, interfering RNAs were diluted to a final concentration of 2 μM in diethylpyrocarbonate (DEPC)-treated nuclease-free water. Denuded oocytes were assigned into three following treatment groups: (1)

control RNAi; (2) *KPNA1* RNAi or (3) non-injected oocytes. Microinjected oocytes were cultured in TCM199 for 24 hours at 39°C and 5% CO_2 in air with 100% humidity. Following culture, mRNA was isolated from 55 injected oocytes using Dynabeads; RT-PCR was performed on each mRNA sample using primers to amplify *YWHAG* and *KPNA1* from each sample using methodology reported previously (Wang *et al.*, 2012; Li *et al.,* 2013).

Experiment 2: effect of KPNA1 knockdown on in vitro embryo developmental competence

An RNA interference approach was performed to determine the effect of *KPNA1* knockdown on *in vitro* embryo developmental competence. Fertilized porcine oocytes were assigned to one of three following treatment groups 5 hours after gamete mixing: (1) control RNAi; (2) *KPNA1* RNAi or (3) non-injected oocytes. Immediately prior to microinjection, presumptive zygotes were vortexed in HEPES-PVA containing 0.1% hyaluronidase for one minute to remove excess spermatozoa from the zona pellucida. After microinjection, presumptive embryos were transferred into PZM3 for embryo culture. Embryos were fixed and stained with Hoechst 33342 six days after fertilization to determine the number of nuclei present in each embryo.

Experiment 3: KPNA1 transcript knockdown and GFP-BRN2 intracellular

localization

Prior to microinjection, interfering RNAs were diluted to a final concentration of 2

µM in diethylpyrocarbonate (DEPC)-treated nuclease-free water and mixed in a

1:1 ratio with *in vitro*-transcribed GFP-BRN2 mRNA. Parthenogenetically activated

oocytes were injected with these substrates 5 hours after electroporation, such

that embryos were in one of the three following treatment groups: (1) control RNAi

+ GFP-BRN2; (2) *KPNA1* RNAi + GFP-BRN2; or (3) non-injected cells. Injected

oocytes were cultured in PZM3 containing 3 mg mL^{-1} BSA for 48 hours after

microinjection and assayed at the 4-cell stage to determine the intracellular

localization of GFP-BRN2 using confocal microscopy. For each treatment group,

GFP fluorescence was captured from whole mount embryos; the ratio of

fluorescence intensity between nuclear and cytoplasmic regions within an embryo

was calculated using NIS-Elements AR Analysis software (Nikon Instruments

Inc.).

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Table 3.1: Knockdown of KPNA1 in porcine embryos leads to reduced embryo developmental competence *in vitro*.

Treatment	Morphological 8-cell stage embryos	Morphological blastocyst stage embryos	Average nuclei number	N
KPNA1 siRNA	21 (13.5%)[a]	13 (8.4%)[a]	5.6[a]	155
Control siRNA	96 (48.2%)[b]	24 (12.1%)[b]	9.1[b]	199
Non-injected	104 (40%)[b]	54 (20.8%)[b]	11.5[b]	260

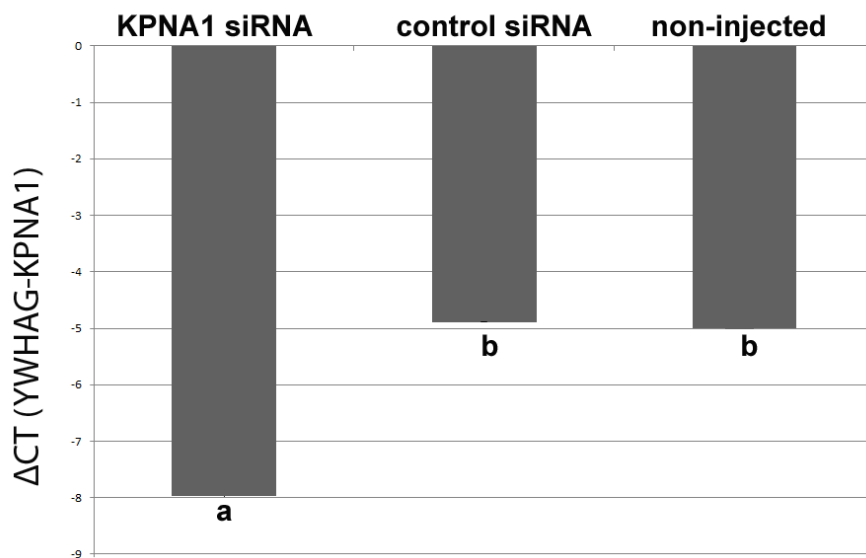Figure 3.1: Validation of KPNA1 transcript knockdown in GV-stage porcine oocytes. A quantitative RT-PCR assay was used to determine the level of reduction in KPNA1 transcript abundance in porcine GV stage oocytes 24 hours after injection of interfering RNAs targeting KPNA1. Data shown are ΔCT values, obtained from subtracting the CT value for KPNA1 from the CT value for the housekeeping gene YWHAG.
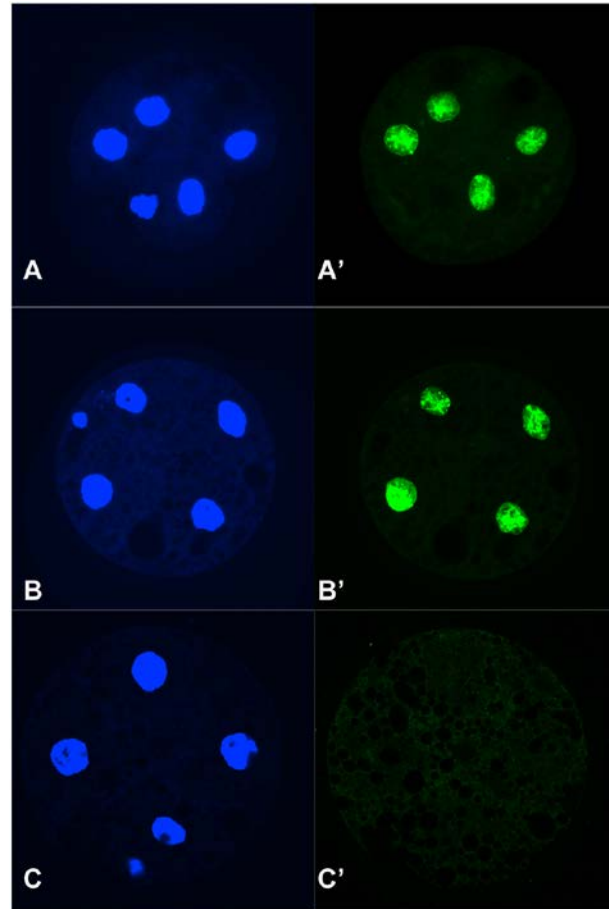
Figure 3.2: Representative images of 4-cell stage embryos expressing GFP-BRN2 under *KPNA1* knockdown and control conditions. Panels A and A' contain representative images of an embryo injected with *KPNA1* interfering RNAs and mRNA encoding GFP-BRN2 (panel A depicts Hoechst staining; panel A' depicts localization of GFP-BRN2). Panels B and B' contain representative images of an embryo injected with control RNAs and mRNA encoding GFP-BRN2 (panel B depicts Hoechst staining; panel B' depicts localization of GFP-BRN2). Panels C and C' contain representative images of a non-injected control embryo (panel C depicts Hoechst staining; panel C' depicts no signal of GFP-BRN2).
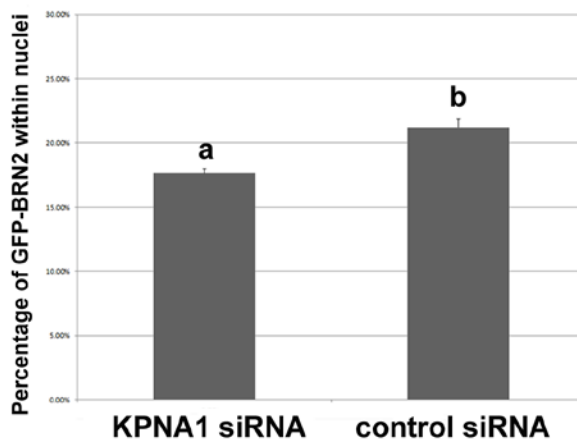
Figure 3.3: Knockdown of KPNA1 alters the intracellular localization of

GFP-BRN2 in 4-cell stage embryos. Percentages indicated on the y-axis reflect

the percentage of fluorescence that is located within the nuclear compartments of

individual embryos. Data are the mean ± s.e.m. Columns with different letters

differ significantly (P < 0.05).

CHAPTER 4 A NUCLEAR PROTEIN IN CLEAVAGE STAGE EMBRYOS INTERACTS WITH MULTIPLE KARYOPHERIN α SUBTYPES IN PORCINE

Abstract

Molecular communication between nuclear and cytoplasmic compartments ensures vital cellular events occur in a proper spatial and temporal manner during embryogenesis. Perhaps one of the best-characterized nuclear transport systems responsible for macromolecule transport is the karyopherin α/β heterodimer-mediated system. Seven karyopherin α subtypes have been identified in mammals, which are referred to as karyopherins α1-α7. The aim of this study was to determine the mechanism by which OCT4 (octamer-binding transcription factor 4), a transcription factor previously reported to be transported by the karyopherin α/β heterodimer, gains access to the nucleus in porcine oocytes and cleavage stage embryos. We hypothesized that discrete karyopherin α subtypes are responsible for the nuclear import of OCT4 in porcine oocytes and cleavage stage embryos. Our results show that ectopically expressed OCT4

adopts a nuclear localization in all nuclei through the 2-cell stage of development, while it is not found in nuclei in 4-cell stage embryos. *In vitro* binding assays indicate that all the tested porcine karyopherin α subtypes are potential transport factors of OCT4.

Introduction

Embryo development is a complex process that involves timely and quantitative control of gene transcription. Nuclear proteins such as transcription factors and chromatin remodeling enzymes play important roles in the initiation of transcription in early stage embryos before embryonic genome activation. Many such factors are required to re-establish the totipotency during embryonic development (Svoboda, *et al*., 2001). The nuclear transport mechanisms for these proteins are highly regulated process. The karyopherin α/β heterodimer-mediated import pathway perhaps is the best characterized nuclear transport system (Görlich and Kutay, 1999). In this system, karyopherin α acts as an adaptor protein that binds to intracellular proteins that possess a classical nuclear localization signal (NLS). The NLS that is recognized by karyopherin α typically consists of short stretches of basic amino acids, primarily lysine residues. These NLSs are found in one of two forms, either monopartite signals typified by the NLS

found with the SV40-T antigen (-**PKKKRKV**-) or a bipartite signal consisting of

two clusters of basic amino acids separated by a short spacer, which resembles

the NLS from nucleoplasmin (-**KR**PAATKKAGQA**KKK**-) (Reichelt *et al.*, 1990).

After karyopherin α interacts with the NLS-bearing cargo, karyopherin α binds to

karyopherin β (Görlich *et al.*, 1996) and this trimeric complex interacts with the

structural proteins of the nuclear pore complex to translocate the NLS-bearing

cargo into the nucleus. Once in the nucleus, the trimeric complex disassociates

upon karyopherin β binding to the GTP-bound form of the GTPase, Ran (Ribbeck

et al., 1998)

Seven karyopherin α subtypes have been identified in mammals. The individual

karyopherin α subtypes were identified by their standardized nomenclature and

referred to as karyopherins α1-α7 (KPNA1, KPNA2, KPNA3, KPNA4, KPNA5,

KPNA6, and KPNA7) (Cabot and Prather, 2003). They are grouped into three

subfamilies based on sequence identity. The first subfamily includes KPNA2 and

KPNA7 (Cuomo *et al.*, 1994; Weis *et al.*, 1995). The second subfamily consists of

KPNA3 and KPNA4 (Kohler *et al.*, 1997; Seki *et al.*, 1997; Nachury *et al.*, 1998),

and the third subfamily includes KPNA1, KPNA5, and KPNA6 (Kohler *et al.*, 1997;

Cortes *et al.*, 1994; Kohler *et al.*, 1999). Members of different subfamilies have

about 50% sequence identity. Within one subfamily, the members share about 85%

sequence identity (Tejomurtula *et al*, 2009). Karyopherin α subtypes are not

expressed in equal amounts in all tissues. All karyopherin α subtypes share the

same general structural motifs, namely, an N-terminal importin β binding (IBB)

domain, which is responsible for the interaction with karyopherin β, and multiple

repeated armadillo (ARM) motifs of roughly 42 amino acids, which are responsible

for the NLS-binding.

OCT4 is a POU-domain-containing transcription factor encoded by *Pou5f1*. The

expression pattern of OCT4 during early embryogenesis varies across

mammalian species. For instance, in mice, OCT4 protein is present in oocytes but

degraded at the end of 2-cell stage (Palmieri *et al*., 1994); in bovine, the amount

of OCT4 appears to stay constant throughout the procession of early stage

embryo development (van Eijik *et al*., 2007); however, in porcine, OCT4

transcripts are in highest abundance at the 2-cell stage as compared with other

stage cleavage (Magnani and Cabot, 2008). Gene targeting experiments in mice

showed that OCT4-deficient embryos survived through the morula stage, but

could not form inner cell mass (ICM) and also failed to give rise to embryonic stem

(ES) cell colonies *in vitro* (Nichols *et al*., 1998). Additionally, increasing the

expression of OCT4 above the endogenous levels in ES cells leads to

differentiation toward the extra embryonic endoderm lineage. It has been shown

that the genetic program of somatic cells can be induced to acquire pluripotency by overexpression of specific transcription factors including OCT4 (Takahashi *et al.*, 2007; Takahashi and Yamanaka, 2006).

Previous studies have shown that although each karyopherin α subtype can transport NLS-bearing substrates, some NLSs are preferentially imported by only specific karyopherin α subtypes. In particular, RanBP3, RAC3 and RCC1 are preferentially imported by KPNA3 (Talcott and Moore, 2000; Yeung *et al.*, 2006; Welch *et al.*, 1999), and KPNA6 and KPNA7 have been shown to be the carriers for STAT3 (Cao and Ma, 2005), while NF-κB is transported into nucleus by KPNA3 and KPNA4 (Fagerlund *et al.*, 2005). Bovine NPM2 showed a much stronger binding affinity with KPNA7 than KPNA2, KPNA4, KPNA5 and KPNA6 (Tejomurtula *et al.*, 2009). It has been reported that OCT4 can be transported by KPNA1, KPNA2 and KPNA4 in mice (Yasuhara *et al.*, 2007). In addition, ablation and over-expression studies provide strong evidence that individual karypopherin α subtypes transport separate classes of intracellular proteins in murine ES cells, which can either induce or block differentiation of ES cells. Specifically, overexpression of KPNA2 and ablation of KPNA1 both block differentiation of ES cells when cells are held in media that promote differentiation (Yahusura *et al.*, 2007).

Based on these findings, we hypothesized that nuclear import of OCT4 mediated by discrete karyopherin α subtypes was dynamically regulated during cleavage development. The aim of this study was to determine the mechanism by which OCT4, a transcription factor previously reported to be a cargo transported by the karyopherin α/β heterodimer，gains access to the nucleus in porcine oocytes and embryos.

Results

GFP-OCT4 adopts a nuclear localization before the 4-cell stage during porcine embryo development

Ectopically expressed GFP-OCT4 adopted a nuclear localization in GV-stage oocytes (n=22/22), and pronuclear (n=32/32) and 2-cell (n=17/17) stage embryos produced by *in vitro* fertilization, while GFP-OCT4 was not found in nuclei in 4-cell stage embryos (n=7/7). These data are summarized in Table 4.1; representative images are found in Figure 4.1.

***Recombinant OCT4 interacts with all tested porcine karyopherin α subtypes
in vitro***

A series of *in vitro* binding assays was performed to determine the ability of
karyopherin α subtypes to interact with recombinant OCT4. KPNA1, KPNA2,
KPNA3, KPNA5, KPNA6 and KPNA7 did not exhibit any significant difference in
the relative binding affinity for recombinant GST-OCT4 *in vitro*. These data are
summarized in Figure 4.2.

Discussion

It is well established that embryos produced *in vitro* have a reduced
developmental potential as compared to embryos produced *in vivo*. Embryos
cultured in the laboratory often display perturbed gene expression, altered
epigenetic states, reduced developmental potential and aberrant patterns of fetal
growth (Reichenbach *et al*., 1992; Boni, *et al*., 1999; Rizos *et al*., 2002). Early
stage embryonic development is a complex process precisely regulated by
nuclear proteins.

It is critical to understand how these proteins gain access to the nucleus to exert
their functions. OCT4 was reported as one of the four transcription factors, whose

transfection together with SOX2 (sex determining region Y-box 2), c-Myc, and Kif4 (Kruppel-like factor 4) into mouse embryonic fibroblasts (MEF) is able to induce a state of induced pluripotency (Takahashi and Yamanaka, 2006). These induced pluripotent stem (IPS) cells have the ability to differentiate into cells of all three germ layers (Evans and Kaufman, 1981). The finding that OCT4 serves a critical in this process indicates that the transport of OCT4 must be strictly controlled in cells.

Previous work in our lab showed transcription factor OCT4 underwent dynamic changes in transcript abundance during cleavage development (Magnani and Cabot, 2008), which indicates the transcription factor OCT4 might be dynamically required and transported during porcine cleavage stage embryo development. In addition, Yasuhara *et al.* reported that OCT4 can be transported by multiple karyopherin α subtypes (Yasuhara *et al*., 2007). Based on these data, we wanted to use OCT4 as a cargo reporter to determine whether and how nuclear transport factors dynamically change during porcine early stage embryo development.

Firstly, we wanted to determine if OCT4 served as a substrate for import in porcine oocytes and discrete cleavage stage embryos by performing a series of microinjection assays. Results from our ectopic overexpression approach

revealed that GFP-OCT4 fusion protein adopted a nuclear localization in GV

stage porcine oocytes and all blastomeres of all embryos through the 2-cell stage

of development, but it was not found in nuclei in 4-cell stage embryos.

One possible explanation for the change in the intracellular localization of OCT4

in 4-cell stage embryos is that although there are a subset of karyopherin α

protein that may be able to bind and import OCT4 in porcine embryos, the specific

karyopherin α subtype (or subtypes) that perform this task in 4-cell stage embryos

was (or were) not sufficiently expressed for OCT4 import. Previous data obtained

in our lab showed that the relative abundance of transcripts encoding seven

karyopherin α subtypes changes during progression from GV stage oocytes to

blastocyst stage embryos. For instance, transcripts encoding KPNA5 were both

detectable in GV stage and MII stage oocytes, while it became undetectable in

4-cell stage embryos. In addition, the level of KPNA7 transcripts became lower in

4-cell stage embryos as compared with GV or MII stage oocytes (Wang *et al*.,

2012). Additionally, since the transport of NLS-bearing cargoes between nucleus

and cytoplasm is a dynamic process, it is possible that the export speed of OCT4

is faster than the import speed, which leads to the accumulation of GFP-OCT4 in

cytoplasm rather than in nuclei in 4-cell stage embryos. The weak fluorescence

signal in the cytoplasmic compartment of 4-cell stage embryos might indicate that

either the mRNA encoding GFP-OCT4 or the GFP-OCT4 protein itself, may have degraded prior to 4-cell stage.

We next wanted to determine the mechanism by which OCT4 was transported in porcine cleavage stage embryos. Yasuhara and colleagues showed that OCT4 can be imported by KPNA1 (importin α5), KPNA2 (importin α1) and KPNA4 (importin α3) via an *in vitro* nuclear import assay (Yasuhara *et al*., 2007). In order to determine the potential subset of karyopherin α subtypes that transport OCT4 during porcine embryonic development, an *in vitro* binding assay approach was undertaken to determine the porcine karyopherin α subtypes had the ability to interact with OCT4. The data from our *in vitro* binding assay showed that recombinant OCT4 is able to interact with all tested porcine karyopherin α subtypes KPNA1, KPNA2, KPNA3, KPNA5, KPNA6, and KPNA7.

Although it was showed that KPNA1 (importin α5), KPNA2 (importin α1) and KPNA4 (importin α3) are all able to transport OCT4, OCT4 import is regulated by selective switching of karyopherin α subtypes expression during cell fate determination. For instance, OCT4 is transported by KPNA2 (importin α1) in undifferentiated ES cells, while it is transported by KPNA1 (importin α5) in neural differentiated cells (Yasuhara *et al*., 2007). Therefore, it is likely that the

requirement of karyopherin α subtypes for OCT4 import dynamically changes at discrete cleavage stage embryo development.

To further explore the requirement changes of karyopherin α subtypes for OCT4 import, we also planned to perform a series of co-microinjection assay to test the impact of overexpression of karyopherin α7 or karyopherin α5 on the intracellular localization of GFP-OCT4. Specifically, we planned to co-inject the oocytes with the mixture of GFP-OCT4 mRNA and KPNA7 mRNA, and then test the intracellular localization of GFP-OCT4 in injected embryos at 4-cell stage development. We have obtained the correct-sized DNA template for producing KPNA7 mRNA, but we failed to yield KPNA7 mRNA. One possibility could be that mutation happens in the procession of PCR amplification, which results in the malfunction of T7 promoter. Malfunction of promoter consequently causes the failure of transcription from DNA to RNA. Another explanation could be that RNA is too susceptible to degradation. It is highly possible that mRNA is degraded in the procession of transcription *in vitro*.

The current study described here is just a beginning step toward exploring the requirement of karyopherin α subtypes for OCT4 import in porcine embryos. To identify OCT4 as a specific substrate for a certain subset of karyopherin α

subtypes, a series of experimental approaches could be employed in future. First, we could test the intracellular localization of exogenous OCT4 in the *in vivo derived embryos* thus to determine the difference which could be caused by *in vitro* manipulation on embryos. Secondly, we can conduct a series of nuclear import assay to test the function of karyopherin α subtypes for OCT4 import individually. Results from these further experiments could provide more clues to better understand the preference of karyopherin α subtypes in binding and transporting NLS-bearing cargoes, and subsequently provide more insights to better investigate the requirements of karyopherin α subtypes during embryogenesis.

In conclusion, we showed that transcription factor OCT4 is a nuclear protein in porcine GV-stage oocytes, and pronuclear and 2-cell stage embryos. This study again provided evidence that individual karyopherin α subtypes may preferentially bind and interact with discrete classes of NLS-bearing nuclear proteins.

Materials and Methods

Oocyte collection

All chemicals were obtained from Sigma Chemical Company (St. Louis, MO) unless stated otherwise. Ovaries from prepubertal gilts were obtained from a local abattoir and transported to the laboratory in an insulated container.   Follicular fluid was manually aspirated from antral follicles; pooled follicular fluid was allowed to settle by gravity for 20 minutes. Cumulus-oocyte complexes (COCs) were washed once in Hepes-buffered medium containing 0.01% polyvinyl alcohol (Hepes-PVA); COCs with multiple layers of well-defined cumulus cells were selected (Abeydeera *et al*., 1998).   For experiments involving GV-stage oocytes, COCs were vortexed for 5 minutes in 0.1% hyaluronidase in Hepes-PVA to remove cumulus cells. Oocytes with an intact plasma membrane and evenly granulated cytoplasm were selected and used for the experiments.

In vitro maturation

Fifty to 75 COCs were placed in 500µl of Tissue Culture Medium 199 (TCM 199; Gibco BRL, Grand Island, NY) supplement with 0.14% polyvinyl alcohol, 10 ng/ml epidermal growth factor, 0.57 mM cysteine, 0.5 IU/ml porcine FSH, and 0.5 IU/ml ovine LH for 42-44 hours at 39°C and 5% CO_2 in air with 100% humidity

(Abeydeera *et al*., 1998).   Following maturation, cumulus cells were removed by vortexing matured oocytes in Hepes-PVA containing 0.1% hyaluronidase for 4 minutes. Denuded oocytes were washed three times in Hepes-PVA.

In vitro fertilization and embryo culture

For *in vitro* fertilization, mature denuded oocytes were washed three times in a modified Tris-buffered medium (mTBM). Cumulus free matured oocytes were placed in mTBM and fertilized according to an established protocol, using fresh, extended boar semen (Abeydeera and Day, 1997).   Briefly, 30-35 oocytes were placed in 100 μl of mTBM and incubated with spermatozoa at a concentration of 5×10^5 spermatozoa/ml. Presumptive zygotes were cultured in Porcine Zygote Medium 3 (PZM3) supplemented with 3 mg/ml fatty acid-free BSA at 39°C and 5% CO_2 in air with 100% humidity (Yoshioka *et al*., 2002).   Pronuclear, 2-cell, 4-cell, and 8-cell stage embryos were collected at 18, 30, 48 and 72 hours after gamete mixing, respectively. Embryos at the indicated developmental stages at the corresponding time points were assayed.

Vector constructions

Expression constructs encoding the epitope-tagged versions of porcine *KPNA1, KPNA2, KPNA3, KPNA5, KPNA6, KPNA7, KPNB*, and *GST-NLS* (NLS derived

from the SV40T-ANTIGEN; GST-NLS) were described previously (Foust *et al.*,

2012; Park *et al.*, 2012). The OCT4 expression constructs were generated by

amplifying the open reading frame (ORF) of *OCT4* by polymerase chain reaction

(PCR) from a OCT4 expression construct (Yasuhara *et al.*, 2007), kindly provided

by Dr. Noriko Yasuhara, Osaka University, and inserting this open reading frame

into pENTR/SD/D-TOPO vector (Invitrogen, Carlsbad, CA, USA). Forward and

reverse primers were 5'-CACCATGGCTGGACACCTGGCT -3' and 5'-TCAGTT

TGAATGCATGGGAGAGC-3' respectively; PCR was performed under the

following conditions: 2 minutes at 95°C, followed by 35 cycles of 30 seconds at

95°C, 90 seconds at 63°C, and 70 seconds at 72°C, followed by a final extension

at 72°C for 10 minutes. After sequencing to confirm the identity of the insert,

OCT4 was recombined into pDEST53 (Invitrogen, Carlsbad, CA, USA) to

generate an OCT4 expression construct with an N-terminal GFP tag; this vector is

referred to as GFP-OCT4 throughout this manuscript.

In vitro transcription

Templates of GFP-OCT4 mRNA and KPNA7 mRNA were both generated by PCR.

For GFP-OCT4, a forward oligonucleotide primer (5'CACTGCTTACTGGCTTATC

GA-3') that contained the T7 promoter was paired with a reverse primer (5'-TCA

GTTTGAATGCATGGGAGAGC-3'). For KPNA7, a forward oligonucleotide primer

(5'ATTAATACGACTCACTATAGGGCATGGATATGCCGATTTTAGAAGCTCC3')

that also contained the T7 promoter was paired with a revers primer (5'TTTAGG

TTTTTGTTAAGGGCGTTAAGC3'). PCR was performed using these

oligonucleotides as primers and the GFP-OCT4 vector and KPNA7-pENTR vector

as templates. Conditions for PCR were as follows: 30 seconds at 98°C, followed

by 35 cycles of 10 seconds at 98°C, 30 seconds at 62°C, and 30 seconds at 72°C,

followed by a final extension at 72°C for 10 minutes. Following PCR, products

were electrophoresed on a 1% agarose gel to assess PCR product size; products

were purified using the QIAquick PCR Purification Kit (Qiagen, Germantown, MD,

USA) following the manufacturer's instructions and then used as a template for *in*

vitro transcription using the mMessage mMachine T7 *in vitro* transcription kit

(Ambion, Austin, TX, USA) following the manufacturer's instructions. 2 μl aliquots

of mRNA were stored at -80°C until microinjection.

Oocyte and embryo microinjection

Oocyte and embryo microinjection assays were performed using an Eppendorf

Femtojet microinjection system (Eppendorf, Hauppauge, NY) attached to Leica

DM-IRB inverted microscope equipped with a heated stage (Leica, Buffalo Grove,

IL, USA). Pronuclear stage embryos and denuded GV-stage oocytes were placed

in Hepes-buffered medium containing 3 mg/ml BSA for microinjection. Following

injection, GV-stage oocytes were incubated in *in vitro* maturation medium at 39°C

and 5% CO_2 in air; embryos were cultured in embryo culture medium at 39°C and

5% CO_2 in air. Oocytes and embryos that lysed immediately following

microinjection were discarded. For imaging oocytes and embryos expressing GFP

fusion protein, cells were fixed at the appropriate developmental stage in 3.7%

paraformaldehyde at room temperature for 15 minutes, washed three times in

PBS, and stored in PBS at 4°C until further processing (up to 14 days). Fixed cells

were stained with Hoechst 33342 (2 µg/ml) for 15 minutes. Cells were mounted

on slides in Vectashield (Vector Laboratories, Inc., Burlingame, CA, USA),

covered with a glass coverslip, and sealed with nail polish. Oocytes and embryos

were examined using a Zeiss LSM 710 confocal microscope (Carl Zeiss

Microscopy, Thomwood, NY, USA).

Expression, purification and quantification of glutathione S-transferase (GST) fusion proteins

BL21 cells were transfected with DNA vectors encoding GST fusion proteins.

Transfected cells were grown in liquid culture medium to an OD600 ranging

between 0.5-0.8 at 37°C; GST-NLS, GST-OCT4, and GST protein expression was

induced with 0.2mM IPTG for 12-16 hours at 18°C, while GST-KPNB expression

was induced with 0.2mM IPTG and 0.2% arabinose for 5 hours at room

temperature. Liquid cultures were cooled to 4°C following induction and centrifuged at 10,000 × g at 4 °C to pellet the bacteria. Bacterial pellets were resuspended in 20 ml PBS containing 0.3% Triton X-100 and protease inhibitors (Complete Protease Inhibitor Cocktail, catalog number 04-683-159-001, Roche, Indianapolis, IN, USA), and lysed by sonication. Bacterial lysates were centrifuged at 12,000 x g for 15 minutes; supernatants were applied to a glutathione-agarose column. Proteins were incubated on the column at 4°C for 2 hours. Following column binding, the column was washed twice at 4°C with cold PBS containing 1% Triton X-100 and washed four times at 4°C with cold PBS. Following the washes, proteins were eluted from the column with 10mM reduced glutathione; eluted proteins were dialyzed, quantified with Bradford assay and stored at -80°C prior to use in the *in vitro* binding assays.

Statistical analysis

For the band intensity assay in Experiment 3, relative values were imported into SAS (Statistical Analysis Software; Cary, NC, USA) and analyzed with a general linear model one-way ANOVA procedure. For quantitative RT-PCR, ΔCT values were analyzed by one-way ANOVA using GLM procedures of SAS. For multiple comparisons ranking, Tukey's post-test was performed and $P<0.05$ was considered significant.

Experiment 1: Intracellular localization of GFP-OCT4 in porcine embryos

Two microinjection assays were performed to determine the intracellular

localization of ectopically expressed GFP-OCT4 in porcine GV-stage oocytes and

cleavage stage embryos. First, denuded GV-stage oocytes were injected with

GFP-OCT4 mRNA and cultured in *in vitro* maturation medium at 39oC, 5% CO2

for 8 hours. Injected GV-stage oocytes were fixed and examined using confocal to

determine the intracellular localization of GFP-OCT4. To determine the

intracellular localization of GFP-OCT4 in cleavage stage embryos, *in vitro*

matured porcine oocytes were injected with GFP-OCT4 mRNA 5 hours after

co-incubation with sperm. Presumptive zygotes were cultured and subsets of

embryos removed at the pronuclear, 2-cell and 4-cell stages and fixed. Fixed cells

were examined using confocal microscopy to determine the intracellular

localization of GFP-OCT4.

Experiment 2: In vitro binding assay

Methionine-[35S] labeled versions of porcine KPNA1, KPNA2, KPNA3, KPNA5,

KPNA6, and KPNA7 were produced *in vitro* in rabbit reticulocytes using the TNT

Quick Coupled Transcription/Translation System (Promega Corporation, Madison,

WI, USA) according to the manufacturer's directions. Binding reactions for each

karyopherin α subtype were carried out as described previously (Foust *et al*.,

2012). Briefly, 10µg of GST-OCT4, GST-NLS, GST-KPNB or GST were bound to glutathione agarose beads. After a series of washes, reticulocyte lysates containing the radiolabeled versions of the porcine karyopherin α subtypes were co-incubated with the GST proteins on the column in binding buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM EGTA, and 1% Triton X-100, pH 7.3) for 2 hours at 4°C. Glutathione agarose beads were washed three times with PBS. Glutathione agarose beads were mixed with Laemmli loading buffer, boiled for 5 minutes and resolved on a 10% TGX precast gel (Bio-Rad). Gels were stained with Coomassie blue, dried and exposed to film at -80°C for 24–48 hours. Binding reactions were performed such that each respective *in vitro* transcribed and translated karyopherin α subtype was divided among four binding reactions (GST-OT4, GST-NLS, GST-KPNB, and GST). The intensities of protein bands were analyzed with Molecular Imaging Software (Carestream Health, Woodbridge, CT). For each karyopherin α subtype, all band intensities from each binding reaction were normalized to the band intensity found in a lane that contained 40% of total reticulolysate input added to each binding reaction. For GST-OCT4, GST-NLS, GST-KPNB, and GST, karyopherin α binding intensities were normalized to the intensity of the karyopherin α subtype that exhibited the highest relative binding ability with corresponding GST fusion protein, thereby making this

relative measure a value of 1; binding intensities of all other karyopherin α

subtypes were then calculated to be a fraction relative to this value.

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Table 4.1: Distribution of fusion protein consisting of GFP and OCT4 (GFP-OCT4) in porcine oocytes and embryos.

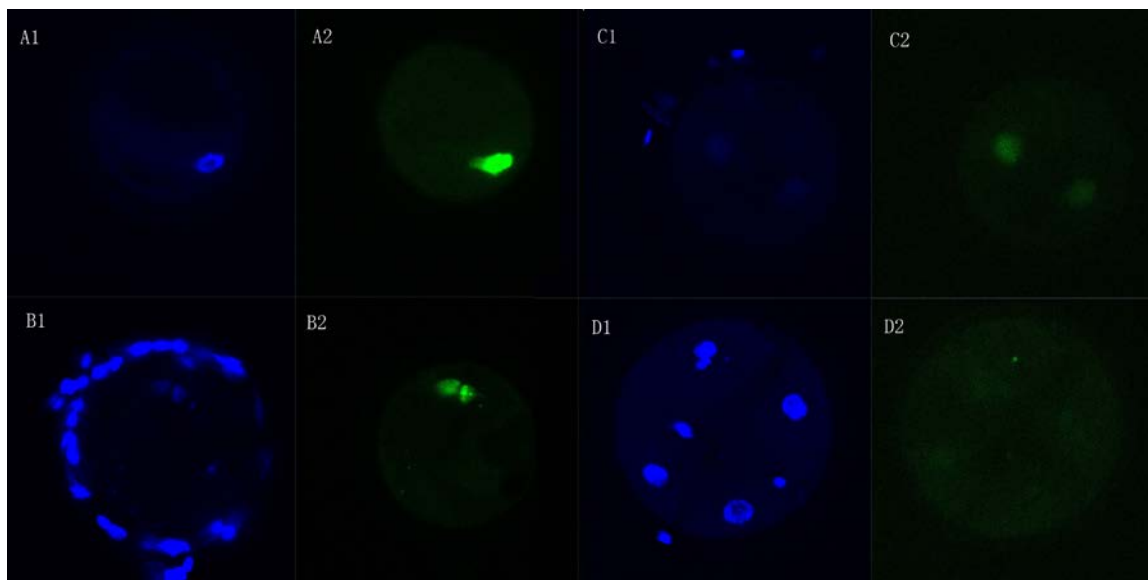| Developmental stage	Protein	Intracellular localization	N
GV-stage oocytes	GFP-OCT4	OCT4 in the nucleus	22
1-cell stage embryos	GFP-OCT4	OCT4 in all nuclei	32
2-cell stage embryos	GFP-OCT4	OCT4 in all nuclei	17
4-cell stage embryos	GFP-OCT4	OCT4 excluded from all nuclei	7

Figure 4.1: A fusion protein consisting of GFP and OCT4 (GFP-OCT4) adopts a

nuclear localization in porcine GV stage oocytes, pronuclear stage, and 2-cell

stage embryos, but was not found in nuclei in 4-cell stage embryos.

Representative images of GV-stage oocytes, pronuclear stage, 2-cell, and 4-cell

stage embryos injected with GFP-OCT4 mRNA are shown in panels A, B, C, and

D, respectively.   In all cases panels beginning with the same letter are derived

from the same representative oocyte or embryo.   Images were captured as an

optical section using confocal microscopy. DNA is shown in panels A1-D1; GFP is
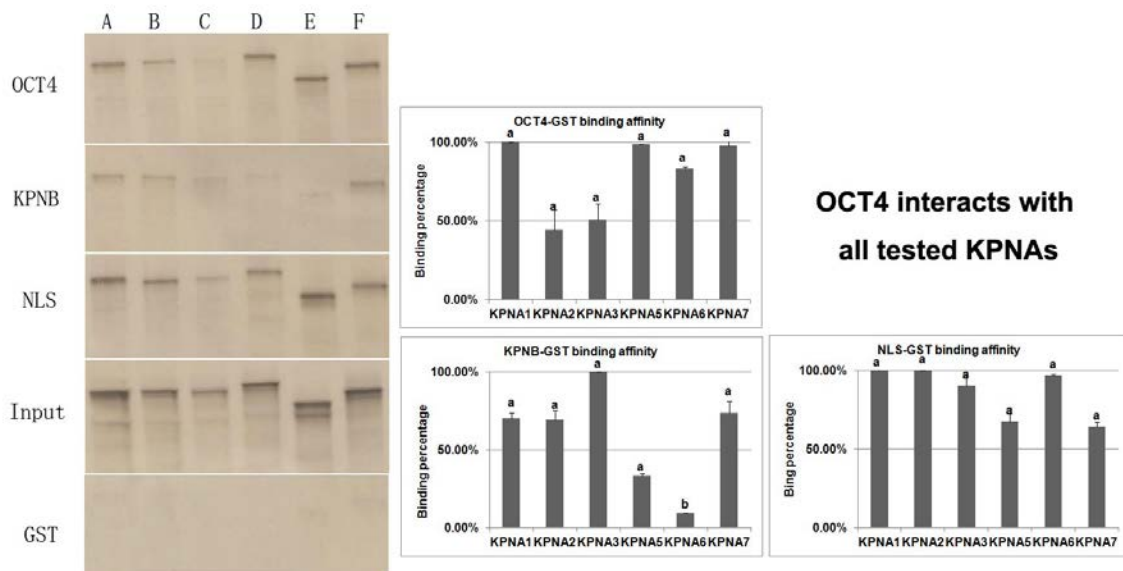
shown in A2-D2.

Figure 4.2: Recombinant OCT4 binds to all tested porcine karyopherin α subtypes.
Representative autoradiographs derived from the OCT4-karyopherin α binding
assay reveal all tested karyopherin α subtypes exhibit no significant difference in
binding affinity for recombinant OCT4. The autoradiographs show the amount of
radiolabeled karyopherin α subtype that was added to each binding reaction
(Input), the amount of radiolabelled karyopherin α subtype recovered upon
incubation with GST-NLS (NLS), karyopherin β1 (KPNB), the GST tag alone (GST)
and GST-OCT4 (OCTS). Column A-F indicate KPNA1-KPNA7 respectively. The
bar graphs on the right reveal the relative binding intensity of OCT4, KPNB, and
NLS with each karyopherin α subtype respectively. Data are the mean ± s.e.m.
Columns with different letters indicate statistical differences (p<0.05).

CHAPTER 5 CONCLUSION AND FUTURE PLAN

Protocols have been well developed over the past decades that enable us to produce embryos in the laboratory. Despite the fact that some *in vitro* produced embryos are capable of developing into healthy and live-born offspring, it is well known that embryos produced *in vitro* often show a reduced ability to undergo successful development, as compared to embryos that are not housed in the laboratory. During porcine oocyte maturation and early stage embryo development, cells undergo a profound amount of gene activation and chromatin remodeling modification. A number of nuclear proteins, including various transcription factors and chromatin remodeling enzymes involved in these crucial processes, need to be imported into the nucleus to exert their regulatory functions. It is possible that some of the manipulations done on embryos produced *in vitro* disrupt the function of these nuclear proteins, which consequently results in poor embryo development with abnormal gene expression and perturbed epigenetic modifications. Understanding how these nuclear proteins gain access to the nucleus during embryo development is central to exploring the regulatory

mechanisms of porcine embryogenesis and improving porcine reproductive efficiency.

Transcription factors BRN2 and OCT4 were reported to be transported into nucleus by the karyopherin α/β heterodimer. In Chapter 2, data from a microinjection assay revealed that BRN2 adopts a nuclear localization at all stages of development from GV stage oocyte to the 4-cell stage embryo. At the 8-cell stage, BRN2 was only detectable in a subset of nuclei.

We chose 4-cell stage as a proper time point to test the requirements of karyopherin α subtypes for BRN2 import because GFP-BRN2 adopted a nuclear localization in 4-cell stage, and the duration of MII stage to 4-cell stage allows the siRNA to knockdown the targeting gene with high efficiency Although all tested KPNAs were capable of binding to recombinant BRN2 *in vitro*, we were interested in KPNA1 and KPNA7 based on the facts that murine KPNA1 was demonstrated to be required for BRN2 trafficking and KPNA7 exhibited the highest relative binding affinity for GST-BRN2.

In Chapters 2 and 3, we examined the impact of KPNA7 and KPNA1 knockdown on the intracellular localization of recombinant BRN2 in 4-cell stage porcine embryos. KPNA1 was implicated in trafficking BRN2 in 4-cell stage porcine embryos in our study. Because we used an RNA interference approach in this investigation, we cannot exclude the possibility that residual target protein

continued to function in nuclear import. It is possible that residual KPNA1 or KPNA7 remained after RNAi-mediated knockdown was sufficient to traffic BRN2 in porcine embryos. Another possible explanation for this observation is that in addition to KPNA1 and KPNA7, other karyopherin α subtypes may also be able to bind and import BRN2. It would be helpful to perform a nuclear import assay in porcine fetal fibroblast cells, which could be an approach to model the nuclear trafficking events mediated by the karyopherin α/β heterodimer. We only knocked down one specific KPNA in one experimental design, and therefore cannot exclude the possibility that other karyopherin α subtypes compensate for the lack of one karyopherin α, however, we could test this by *in vitro* nuclear import assay.

Additionally, it has been indicated that a switching mechanism of karyopherin α subtypes expression might exist in cells. For instance, KPNA1 has been proven to be upregulated in mice with a knockdown of KPNA2 (Shmidt, et al., 2007). More interesting evidence could be provided if we could test the transcript abundance changes of the other six karyopherin α subtypes in the KPNA1 or KPNA7 knockdown embryos. If it comes out that the transcript abundance of any other karyopherin α subtype (or subtypes) is found to be increased in KPNA1 or KPNA7 knockdown embryos,the model that karyopherin α subtypes could compensate for one another would be supported.

The atypical pattern of the intracellular localization of recombinant BRN2 in 8-cell stage embryos in Chapter 2 led us to hypothesize that the differences in

intracellular protein trafficking might be caused by the differences in developmental competence observed between embryos produced *in vivo* and *in vitro*. This finding would be more substantiated if we could determine the intracellular localization of BRN2 in porcine embryos produced *in vivo*. Additionally, BRN2 is a transcription factor which functions by binding with chromatin and regulating transcription. We must keep in mind that the BRN2 studied in our experiments was ectopically expressed and this level of overexpression may in part contribute to the atypical pattern of BRN2 localization.

Data in Chapter 3 showed that loss of KPNA1 in porcine embryos resulted in a reduced proportion of embryos developing beyond the 8-cell stage, as compared to control groups. These findings provided evidence that KPNA1 was required for porcine embryo cleavage development. The evidence that nuclear trafficking of BRN2 was affected by the depletion of KPNA1 in Chapter 3 provided a good explanation to the nature of this developmental arrest of KPNA1-depleted embryos. The fact that the majority of embryos arrest at the 4-cell stage upon KPNA1 depletion, as well as the observation of the significant reduction in the proportion of labeled protein accumulated in the nuclei of KPNA1-depleted embryos at the 4-cell stage, enabled us to speculate that KPNA1 might play a critical role of partitioning intracellular proteins. These proteins may be involved in ZGA or in the later development events right after ZGA. KPNA2, KPNA6, and KPNA7 have all been shown to be required for embryo development (reviewed in Chapter 1).

In Chapter 4 we determined the intracellular localization of recombinant, labeled OCT4. Overexpression studies revealed that recombinant Oct4 adopted a nuclear localization prior to the 2-cell stage of development, while it was not detectable in the nuclei of 4-cell stage embryos. All tested porcine orthologs of the karyopherin α subtypes exhibited *in vitro* binding affinities for OCT4 that did not differ in a statistically significant level. This finding provided us another cargo we could also use to monitor the developmental requirements of karyopherin α subtypes during cleavage development. For example, we could conduct a series of experiments to test the intracellular localization changes of GFP-OCT4 in 4-cell stage embryos upon overexpression of individual karyopherin α subtypes, from which we could also gain some insights into investigating the developmental requirements of karyopherin α subtypes in porcine embryo cleavage development.

A greatly growing amount of evidence has been revealed over the past decades to support the hypothesis that nuclear import mediated by the karyopherin α/β heterodimer plays a crucial regulatory role in cellular differentiation and development, as well as animal reproduction. This dissertation has focused on the developmental requirements of karyopherin α by determining how NLS-bearing nuclear proteins vary in transport efficiency during porcine embryo cleavage development. The data collected from this study provided evidence that certain NLS-bearing nuclear proteins might be preferentially trafficked by individual karyopherin α subtypes. We demonstrated that both BRN2 and OCT4 are nuclear proteins in discrete cleavage stages embryos in porcine. Moreover, we concluded

that KPNA1 is involved in the trafficking of transcription factor BRN2 in 4-cell stage embryos. We also demonstrated the role of KPNA1 serves in early cleavage stages embryonic development. Together the role of KPNA1 in the transport of BRN2, we postulated a potential mechanism that KPNA1 might undergo to perform its function. Taken together, the data collected from this study support the hypothesis that karyopherin α subtypes serve a crucial role during the porcine embryo development by collectively partitioning the discrete NLS-bearing nuclear proteins between the nuclear and cytoplasmic compartments.

Based on these data, we proposed a possible pathway that karyopherin α subtypes might be involved in the context of embryo development. During embryo development progress, genes are turned on and off at precise time points under the control of specific transcription factors and chromatin remodeling enzymes. Karyopherin α subtypes might affect embryonic development through regulating the trafficking of these crucial nuclear proteins. The schematic of the speculated pathway is shown in Figure 5.1.

To further the study of the role that karyopherin α subtypes play in the context of porcine embryo development we proposed to perform *in vivo* assays. We could conduct the microinjection assay in *in vivo* derived embryos and determine the intracellular localizations of BRN2 and OCT4 in discrete stage cleavage embryos. We could compare the trafficking function of karyopherin α subtypes by comparing the intracellular localization of these NLS-bearing cargoes in *in vitro* derived embryos and *in vivo* derived embryos. The information from the

comparison could deepen our understanding about the possible reasons why the developmental competence of *in vitro* derived embryos differs from *in vivo* derived embryos.

The absence of reliable markers for identifying embryos with good developmental competence for transfer at the early cleavage stage may contribute to the low implantation rates of *in vitro* derived embryos. The intriguing finding about the intracellular localization of GFP-BRN2 in 8-cell stage embryos inspires us to explore if BRN2 could be a marker for developmental competence during porcine embryonic development in future. We could culture 8-cell stage embryos injected with GFP-BRN2 mRNA to develop to blastocyst stage embryos and determine the phenotypes of the embryos, such as the blastomere number, size, shape, and presence or absence of extracellular fragments. We also could conduct Q-RT-PCR assay to test the transcript abundance of transforming growth factor β (TGFβ); TGFβ serves as a marker of developmental competence (Schemid *et al*., 1994). By comparing the relative abundance of TGFβ in GFP-BRN2 positive and negative blastomeres, we could determine if BRN2 could be a marker for embryonic developmental competence.

A compensatory mechanism was accepted in the functions of proteins with super families. It is important to investigate the expression pattern of karyopherin α subtypes in porcine embryos in future. The transcripts and proteins levels of other karyopherin α subtypes should be verified in certain karyopherin α-depleted

embryos: Which karyopherin α subtypes are upregulated or downregulated upon the depletion of certain karyopherin α? At which stages do the upregulation or downregulation occur in the progression of embryo development? Data from these experiments could provide us some supports to model the expression pattern of karyopherin α subtypes during porcine embryogenesis. Also, we could compare the phenotypes of porcine embryos upon the depletion of different karyopherin α subtypes, from which, we could learn more about the requirements of karyopherin α subtypes in the progression of embryo development. The information about the expression pattern and developmental requirements of karyopherin α subtypes could provide us some support to improve the quality of embryos produced in the laboratory in future.

In addition to KPNA1 and KPNA7, roles of other karyopherin α subtypes also should be explored. It would be helpful to generate KPNA knockout domestic pigs to assess the role of certain karyopherin α in regulating embryogenesis: What are the phenotypes of the knockout embryos at discrete cleavage stages? What are the rates of successful pregnancy upon knockout embryos transfer? What are the lethality rates of the knockout embryos?

Our current study is just the early step towards exploring the roles of nuclear trafficking serves during embryogenesis. Once the expression pattern and the developmental requirements for specific karyopherin α subtypes during cleavage development are determined, it would be possible to determine the network of

important genes involved in embryogenesis that are regulated by specific nuclear trafficking pathway. We also could determine mechanisms by which specific chromatin modifying enzymes such as DNA methyltransferases, histone methyltransferases and other epigenetic remodeling enzymes access the nucleus in cleavage stage embryos. In addition, we could determine mechanisms behind which epigenetic programming and nuclear reprogramming establish during embryogenesis. All of these findings will provide us some insights to develop improved methods for culturing and manipulating porcine embryos in future.
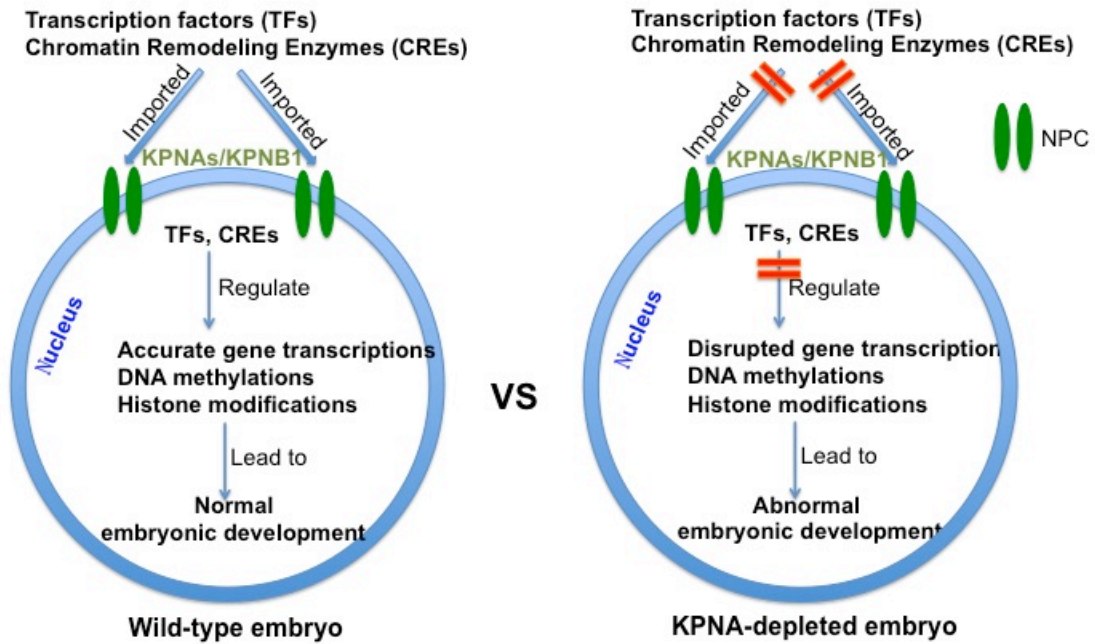
Figure 5.1: The schematic of speculated pathway that karyopherin α subtypes regulate the embryonic development in wild-type embryo versus KPNA-depleted embryo.

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VITA

VITA

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