

# Manipulation of *SMARCA2* and *SMARCA4* transcript levels in porcine embryos differentially alters development and expression of *SMARCA1*, *SOX2*, *NANOG*, and *EIF1*

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## Abstract

Epigenetic reprogramming plays a pivotal role during embryogenesis, including both covalent and non-covalent modifications to chromatin. In this study, we investigated the role of SNF2 chromatin remodeling ATPases (*SMARCA2* (previously known as *BRAHMA*), *SMARCA4* (previously known as *BRG1*), *SMARCA5* (previously known as *SNF2H*), *SMARCA1* (previously known as *SNF2L*), *CHD3*, and *CHD5*) during porcine preimplantation embryonic development. Transcript levels for these ATPases change dynamically throughout development. We also investigated the effect of altering transcript levels of *SMARCA2* and *SMARCA4* via mRNA injection. Overexpression of *SMARCA2* and *SMARCA4* severely impaired embryo development. Results from these experiments show that embryos injected with *SMARCA2* mRNA arrest between the four-cell and blastocyst stages. However, embryos injected with either wild-type *SMARCA4* or a dominant negative variant or *SMARCA4* arrest before zygotic genome activation. No differences in transcript abundance of *SOX2*, *POU5F1*, *NANOG*, and *EIF1* (previously known as *EIF1A*) were detected after injection with *SMARCA2* or its dominant negative variant at 48 h post-injection. Conversely, embryos injected with wild-type *SMARCA4* and its dominant negative variant possessed altered expression of these genes. Examination of SNF2-type ATPase transcript abundance across all treatment groups revealed that only *SMARCA1* was altered following injection with wild-type *SMARCA2* and wild-type and dominant negative *SMARCA4*. We conclude that the arrest in porcine embryo development observed after injection is specific to the ATPase injected. Our data strongly support the hypothesis that *SMARCA2* and *SMARCA4* play different but fundamental roles controlling gene expression during early mammalian embryogenesis.

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## Introduction

Epigenetic reprogramming during cleavage development is a dynamic process. Soon after fertilization, several mammalian embryos (mouse, cattle, and human) undergo global DNA demethylation to erase epigenetic marks previously accumulated during germ cell development (Monk *et al.* 1987, Dean *et al.* 2001, Fulka *et al.* 2004). It is likely that DNA demethylation is necessary for the re-establishment of pluripotency that occurs through the reactivation of *POU5F1*, *NANOG*, and *SOX2* (Reik *et al.* 2001, Bortvin *et al.* 2003, Wuensch *et al.* 2007). Modifications on the histone tails are also important during cleavage development. Methylation on specific arginine residues on histone H3 mark cells in the four-cell-stage mouse embryo that will form the pluripotent inner cell mass (ICM); when histone methylation was ectopically increased on these particular arginine residues, direct up-regulation of *SOX2* and *NANOG* transcripts was observed (Torres-Padilla *et al.* 2007). Covalent modifications

including mono-, di-, trimethylation on the lysine 9 residue of histone H3 are associated only with the maternal pronucleus, and are in part responsible for the epigenetic asymmetry between the two parental genomes in the murine embryos (Liu *et al.* 2004, van der Heijden *et al.* 2005). The disturbance of epigenetic reprogramming is thought to contribute to developmental abnormalities observed in embryos produced by using somatic cell nuclear transfer (Armstrong *et al.* 2006).

Manipulation of the chromatin architecture is referred to as chromatin remodeling. Chromatin remodeling plays a crucial role during epigenetic reprogramming. Histones H3 and H4 and variant H3.3 are translated from maternal mRNA and promptly replace the sperm protamines after fertilization (Wiekowski *et al.* 1997). Histone H3.3 is responsible for maintaining epigenetic memory in *Xenopus* (Ng & Gurdon 2008), and it has been shown that this histone variant is distributed differently between the two parental genomes, with histone H3.3 incorporated preferentially in the male

pronucleus of mouse embryos (Torres-Padilla *et al.* 2006). Interestingly, H3.3 needs the chromatin remodeling factor CHD1 to be incorporated in the male pronuclei in *Drosophila* embryos (Konev *et al.* 2007). Another example of the importance of chromatin remodeling comes from a study in the mouse where the deletion of the chromatin remodeling factor *Smarca4* results in the repression of a large number of genes during zygotic genome activation (ZGA; Bultman *et al.* 2006).

CHD1 and *Smarca4* are members of a large family of enzymes called the SNF2-type ATP-dependent chromatin remodeling enzymes. They generally exist in multi-protein complexes and are able to actively rearrange nucleosomes using the energy obtained from ATP hydrolysis. These chromatin remodeling complexes are built around a core ATPase that possesses a helicase domain and various accessory domains that allow them to interact with specific histone tail modifications (Sif 2004, Choudhary & Varga-Weisz 2007, Gangaraju & Bartholomew 2007). In mammals, these complexes are traditionally divided into three large subfamilies depending on the accessory domains found on the ATPase subunits: SWI/SNF (bromodomain), ISWI (SANT domain), and CHD (chromodomain).

The SWI/SNF subfamily of ATP-dependent chromatin remodeling complexes uses two different ATPases (*SMARCA2* (previously known as BRAHMA) and *SMARCA4* (previously known as BRG1)) depending on the specific complex formed (Kwon & Wagner 2007). SWI/SNF complexes participate in a large number of cellular processes and have been shown to be necessary for embryonic development; deletion of *Smarca4* or other subunits such as *SNF5/ini5*, *BAF60*, *SMARCC1*, and *PBRM1* are all lethal at peri-implantation stages (as reviewed by de la Serna 2006). *SMARCA2* and *Smarca4* share 75% identity at the amino acid level but are not functionally redundant as shown by deletion of *SMARCA2*. Mice that lack functional *SMARCA2* do not present major abnormalities aside from a mild overgrowth phenotype (Bultman *et al.* 2000).

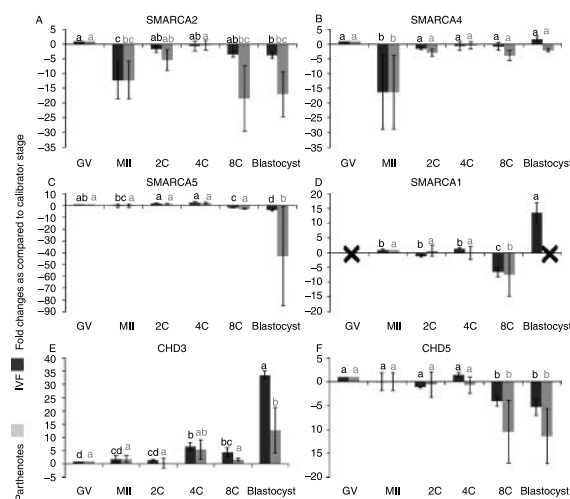
ATP-dependent chromatin remodeling is important for mammalian cleavage development. The objective of the present study was to investigate the pattern of expression of six other core ATPases (*SMARCA4*, *SMARCA2*, *SMARCA5* (previously known as *SNF2H*), *SMARCA1* (previously known as *SNF2L*), *CHD3*, and *CHD5*) belonging to the SNF2 family of chromatin remodelers in the porcine embryo, and to assess the effects of *SMARCA2* and *SMARCA4* misregulation on porcine embryo development. We hypothesized that altering levels of *SMARCA2* and *SMARCA4* will interfere with the regulation of key genes involved in ZGA and the maintenance of pluripotency and also perturb the expression of other SNF2-type chromatin remodeling ATPases.

## Results

### *SNF2-type ATPases are dynamically regulated through porcine cleavage development*

*SMARCA4* and *SMARCA2*, the SWI/SNF ATPase subunits, are differentially expressed through cleavage development in porcine embryos (Fig. 1A and B). *SMARCA4* mRNA levels are at first downregulated in MII oocytes (−16-fold, germinal vesicle (GV) versus MII,  $P < 0.05$ ). *SMARCA4* transcript levels then return to levels similar to those found in GV oocytes at the two-cell stage and do not change afterward (GV versus 2C, 4C, 8C, and blastocyst,  $P > 0.05$ ) in both parthenogenetic and *In vitro*-fertilized (IVF) embryos. Conversely, *SMARCA2* transcript levels are reduced in MII oocytes (−12-fold, GV versus MII,  $P < 0.05$ ) and reach the minimum during the eight-cell and blastocyst stages (18- and 16-fold, GV versus 8C and BL respectively  $P < 0.05$ ) in parthenogenetic embryos. *In vitro*-fertilized embryos possessed a significantly lower amount of *SMARCA2* only at the blastocyst stage when compared with GV-stage oocytes (minus fourfold, GV versus blastocyst,  $P < 0.05$ ).

Transcripts for the ISWI ATPases, *SMARCA5* and *SMARCA1*, were also examined in cleavage-stage embryos (Fig. 1C and D). In embryos obtained by IVF, transcripts for *SMARCA5* were increased at the two- and four-cell stages when compared with MII oocytes (3- and 4-fold, MII versus 2C and 4C respectively,  $P < 0.05$ ) and then reduced at the eight-cell and blastocyst stages (−1.9- and −3.8-fold, GV versus 8C and blastocyst



**Figure 1** Relative abundance of SNF2-type ATPase in porcine oocytes and cleavage embryos. The pattern of expression of SNF2-type ATPases changes throughout cleavage development. Different subscripts represent significant differences across development stages within each treatment after Tukey's multiple comparison post-test ( $P < 0.05$ ). Fold differences were calculated with respect to the GV (or MII for *SMARCA1*)-stage oocyte. The results shown here are the average of three independent experimental replicates. Bars represent s.d. Treatments are labeled as follows: *in vitro*-fertilized embryos (IVF) and parthenogenetic embryos (parthenotes).

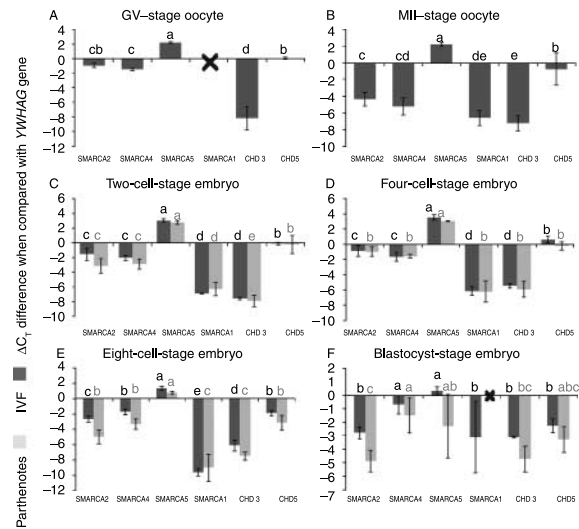
respectively,  $P < 0.05$ ). In parthenogenetic embryos, *SMARCA5* transcripts were dramatically decreased at the blastocyst stage (−42-fold, GV versus BL,  $P < 0.05$ ). Interestingly, *SMARCA1* transcripts were not detectable in either GV-stage oocytes or parthenogenetic blastocyst-stage embryos. *SMARCA1* transcripts were present in low abundance in mature oocytes and further downregulated at the eight-cell stage (minus sevenfold, MII versus 8C,  $P < 0.05$ ). *SMARCA1* was downregulated also at the eight-cell stage in IVF embryos (minus sixfold, MII versus 8C,  $P < 0.05$ ). Surprisingly, we found *SMARCA1* transcripts to be present in higher abundance in IVF-derived blastocyst-stage embryos (13-fold, MII versus blastocyst,  $P < 0.05$ ).

Finally, we examined the expression patterns of *CHD3* and *CHD5*, two ATPases of the CHD family (Fig. 1E and F). Embryos produced by IVF showed increased *CHD3* transcripts at the four-cell stage (6 fold, GV versus 4C,  $P < 0.05$ ) and then at the blastocyst stage (33-fold, GV versus BL,  $P < 0.05$ ). Parthenogenetic embryos had a fivefold increase in *CHD3* transcript levels at the four-cell stage, although not statistically significant, and 13-fold increase at the blastocyst stage (13-fold, GV versus blastocyst,  $P < 0.05$ ). *CHD5* transcripts were in lower abundance in the eight-cell- and blastocyst-stage embryos obtained by IVF (−4 and −5 fold, GV versus 8C and blastocyst respectively,  $P < 0.05$ ). In parthenogenetic embryos, *CHD5* transcripts were found in lower abundance in the eight-cell and blastocyst-stage embryos (−10- and −12-fold, GV versus 8C and blastocyst respectively,  $P < 0.05$ ).

### Relative transcript abundance of SNF2-type ATPases at different time points in porcine cleavage development

The amount of SNF2 ATPases transcripts is relative to the specific developmental stage (Fig. 2). In the GV-stage oocytes, *SMARCA5* ATPase transcripts were present in the highest abundance (*SMARCA5* versus all others,  $P < 0.05$ ). *SMARCA2* and *SMARCA4* transcripts were present at similar levels, while *CHD3* was in extremely low abundance; *SMARCA1* transcripts were not detectable. This profile is similar to that found in MII oocytes, where *SMARCA5* and *CHD5* were again the most abundant ATPases ( $P < 0.05$ ). *SMARCA2* and *SMARCA4* were present at similar levels; *CHD3* and *SMARCA1* were the least abundant transcripts at this stage.

In IVF-derived embryos at the two-cell stage, we found *SMARCA5* to be in the highest abundance; *CHD5* transcripts were also highly represented while *SMARCA2* and *SMARCA4* transcripts were expressed at similar levels. Also at this stage, *SMARCA1* and *CHD3* transcripts continued to be present at low levels. During the four-cell stage, *SMARCA5* remained present in the highest abundance, but *SMARCA2* and *SMARCA4* transcripts were expressed at levels similar to *CHD5* (*SMARCA2* and *SMARCA4* versus *CHD5*,  $P > 0.05$ ); *SMARCA1* and



**Figure 2** Relative abundance of SNF2-type ATPase at a defined stage of porcine development. The pattern of expression of SNF2-type ATPases is defined at each stage of cleavage development. Different subscripts represent significant differences across SNF2-type ATPase transcript levels within each treatment after Tukey's multiple comparison post-test ( $P < 0.05$ ).  $\Delta C_T$  values were calculated with respect to the *YWHAG* transcript levels. The results shown here are the average of three independent experimental replicates. Bars represent s.d. Treatments are labeled as follows: *in vitro*-fertilized embryos (IVF) and parthenogenetic embryos (parthenotes).

*CHD3* were again in the lowest abundance. At the eight-cell stage, *SMARCA5* was in the highest abundance, followed by *SMARCA4* and *CHD5*. Also at the eight-cell stage, *SMARCA2* transcripts were detected at an intermediate level and *SMARCA1* and *CHD3* transcripts were present at low levels. In the blastocyst-stage embryos, we found *SMARCA4* and *SMARCA5* transcripts to be present in the highest abundance. All the other ATPases were found to be present at similar levels.

We found that embryos obtained by parthenogenesis possessed a pattern of expression which closely resembled that observed in IVF embryos. Exceptions to this were found at the blastocyst stage. Parthenogenetic embryos had a lower amount of *SMARCA5* and did not possess transcripts for *SMARCA1*.

### Ectopic expression of SMARCA2 and SMARCA4 lead to arrested development in porcine parthenogenetic embryos

No significant differences were detected in the proportion of embryos that progressed to the four-cell stage across treatment groups (injected with *SMARCA2*-WT, *SMARCA2*-DN, GFP, and non-injected; data not shown). When we ectopically expressed wild-type *SMARCA4* or dominant negative *SMARCA4* in parthenogenetic embryos, we observed a more dramatic phenotype (Table 1). The dominant negative mutations consist of a single amino acid mutation that inactivates the ATPase

**Table 1** *In vitro* developmental potential of porcine parthenogenetic embryos injected with *in vitro*-synthesized SMARCA4 mRNA.

Treatment	% Blastocyst	% Cleaved	% Four-cell stage	Average nuclei number	N
SMARCA4-WT	1.8 <sup>a</sup>	10.6 <sup>a</sup>	6.9 <sup>a</sup>	1.4 ± 4.9 <sup>a</sup>	234
SMARCA4-DN	4.1 <sup>a</sup>	28.6 <sup>b</sup>	18.3 <sup>b</sup>	2.9 ± 6.5 <sup>b</sup>	308
GFP	28.6 <sup>b</sup>	53.3 <sup>c</sup>	67.2 <sup>c</sup>	10.9 ± 13.3 <sup>c</sup>	382
Non-injected	32.8 <sup>b</sup>	52.9 <sup>c</sup>	67.7 <sup>c</sup>	10.5 ± 11.5 <sup>c</sup>	424

All embryos were included in the analysis presented here; degenerated embryos were considered as embryos with zero cells for the purposes of determining cell number. Different superscripts indicate differences at  $P < 0.001$ . Treatments are labeled as follows: wild-type SMARCA4 (SMARCA4-WT), dominant negative SMARCA4 (SMARCA4-DN), GFP (GFP), and non-injected control (non-injected).

activity without altering complex formation (Muchardt & Yaniv 1993, Dunaief *et al.* 1994). Embryos injected with wild-type SMARCA4 tended to cleave at a lower rate and reach the four-cell stage with a significantly lower frequency when compared with embryos injected with dominant negative SMARCA4 (10.6% and 6.9% vs 28.6% and 18.3%, SMARCA4-WT and SMARCA4-DN versus GFP and non-injected respectively,  $P < 0.05$ ). Embryos injected with SMARCA4-WT and SMARCA4-DN had a significantly lower developmental potential as shown by blastocyst formation and nuclei number (1.8% and 1.4; 4.1% and 2.9 vs 28.6% and 10.9; 32.8% and 10.5%; SMARCA4-WT and SMARCA4-DN versus GFP and non-injected respectively,  $P < 0.05$ ).

### Ectopic expression of SMARCA2 and SMARCA4 specifically alters gene expression

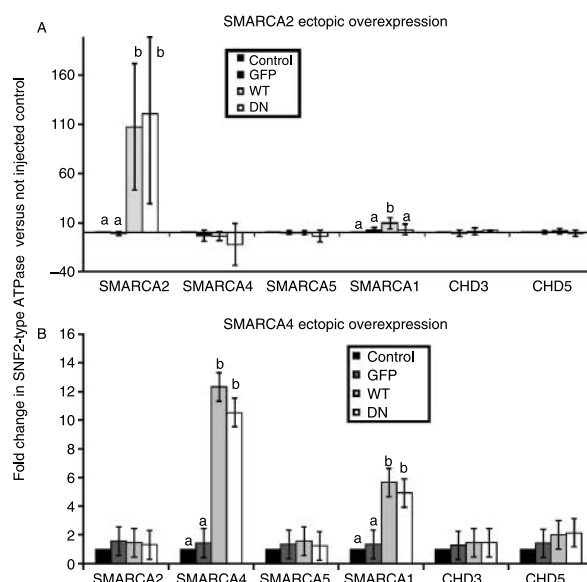
While ectopic overexpression of SMARCA2 did not have any significant effect 48 h post-injection on the expression of POU5F1, NANOG, and eukaryotic elongation initiation factor 1 (EIF1; previously known as eIF1A), the dominant negative form of SMARCA2 led to an increased amount of SOX2 transcripts (4-fold, DN versus WT,  $P < 0.05$ ; Figs 3A and 4A). Furthermore, embryos injected with the wild-type form of SMARCA2 showed an increased amount of SMARCA1 (10-fold, WT versus non-injected control,  $P < 0.05$ ).

Embryos injected with SMARCA4 or its dominant negative variant had perturbed gene expression 48 h post-injection. Transcriptional levels of SOX2 were statistically lower in abundance in embryos injected with wild-type SMARCA4 when compared with the other treatment groups (–10-fold, WT versus all other treatments,  $P < 0.05$ ). While POU5F1 expression was not perturbed, we could not detect NANOG transcripts in embryos injected with wild-type SMARCA4. Injection with dominant negative SMARCA4 resulted in a statistically lower amount of NANOG (–5-fold, DN versus GFP and non-injected control,  $P < 0.05$ ). EIF1, a marker gene for ZGA, was also misregulated: injection of either wild-type or dominant negative SMARCA4 resulted in lower transcript levels for EIF1 (–7- and –11-fold, SMARCA4-WT and SMARCA4-DN versus GFP and non-injected control respectively,  $P < 0.05$ ). We were able to detect an increased amount in SMARCA4 transcript both in the

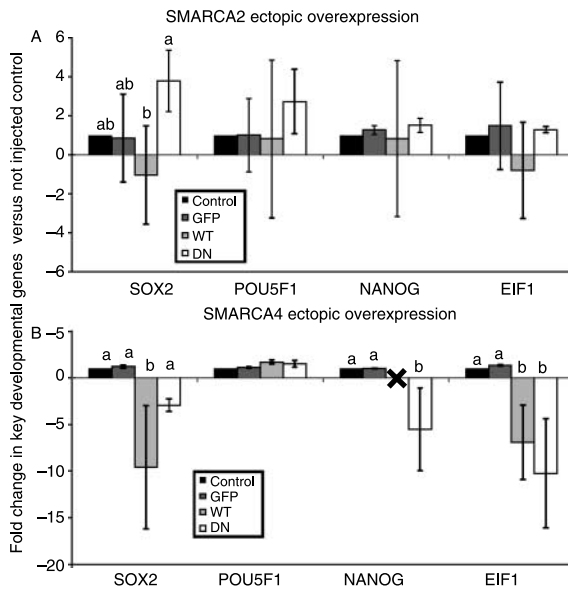
wild-type and in the dominant negative treatments (13- and 11-fold, SMARCA4-WT and SMARCA4-DN versus GFP and control respectively,  $P < 0.05$ ). We also found a 5-fold increase in SMARCA1 transcript in both SMARCA4-WT and SMARCA4-DN treatments (5-fold, SMARCA4-WT and SMARCA4-DN versus GFP and non-injected control,  $P < 0.05$ ).

### Discussion

Non-covalent modifications of chromatin may have a significant impact on the embryonic transcription profile and influence embryo development. Alterations of the chromatin structure often accompany transcriptional regulation and require the activity of complexes able to alter the nucleosome array. Few studies have addressed the dynamics of chromatin remodeling during



**Figure 3** Embryos injected with wild-type or dominant negative SMARCA2 or SMARCA4 have altered expression of SNF2-type ATPases. Embryos injected with the SWI/SNF ATPases SMARCA2 or SMARCA4 possess altered levels of other SNF2-type ATPases 48 h post-injection. Different subscripts represent significant differences in transcript levels across treatments within each treatment after the LSD multiple comparison post-test ( $P < 0.05$ ). Fold differences were calculated using non-injected control as the calibrator. The results shown here are the average of three independent experimental replicates. Bars represent s.d. Treatments are labeled as follows: wild-type ATPase (WT), dominant negative ATPase (DN), GFP (GFP), and non-injected control (control).



**Figure 4** Embryos injected with wild-type or dominant negative *SMARCA2* or *SMARCA4* have altered gene expression. Embryos injected with the SWI/SNF ATPases *SMARCA2* or *SMARCA4* possess altered levels of key developmental genes 48 h post-injection. Different subscripts represent significant differences in transcript levels across treatments within each treatment after the LSD multiple comparison post-test ( $P < 0.05$ ). Fold differences were calculated using a non-injected control as the calibrator. The results shown here are the average of three independent experimental replicates. Bars represent s.d. Treatments are labeled as follows: wild-type ATPase (WT), dominant negative ATPase (DN), GFP (GFP), and non-injected control (control).

mammalian embryogenesis. Here, we show that misregulation of the catalytic ATPases of the SWI/SNF family results in perturbed embryonic development possibly via misregulation of other key developmental genes.

In regard to the relative abundance of SNF2 ATPase transcripts in mammalian embryos, our data demonstrate that a species-specific difference exists between the murine model and other mammals. In mouse embryos, *Smarca2* transcripts are downregulated after the two-cell stage and become almost undetectable from the four-cell stage until the blastocyst stage, whereas *Smarca2* transcripts become more abundant (LeGouy *et al.* 1998, Bultman *et al.* 2000). LeGouy noted that *SMARCA2* protein becomes restricted to the ICM at the blastocyst stage. Interestingly, they also found that ES cells (which are derived from the ICM) did not possess *SMARCA2*, but they express *Smarca2* upon differentiation (LeGouy *et al.* 1998). On the other hand, *Smarca4* transcripts are in relatively high abundance during mouse cleavage development (LeGouy *et al.* 1998). We showed that in parthenogenetic porcine embryos and those produced by IVF, *SMARCA2* is expressed at a comparable level with *SMARCA4* during most of cleavage development. *SMARCA2* is then downregulated at the blastocyst stage (Figs 1 and 2). This profile is similar to the one observed in primate embryos

(Zheng *et al.* 2004), suggesting that the pig embryo may represent a better model for human cleavage-stage embryo development in studies involving chromatin remodeling when compared with the mouse embryo.

Similarly to the SWI/SNF ATPases (*SMARCA4* and *SMARCA2*), the ISWI ATPases (*SMARCA5* and *SMARCA1*) are 75% identical at the nucleotide level and share 81% of their amino acid sequence. Studies in the mouse have shown that *SMARCA5* deletion leads to post-implantation lethality (Stopka & Skoultschi 2003). The expression pattern of *SMARCA5* has not been extensively investigated in cleavage-stage embryos. *SMARCA5* was present in high abundance as maternal mRNA and then degraded at the eight-cell stage in rhesus monkey embryos (Stopka & Skoultschi 2003). Interestingly, *SMARCA1* mRNA was barely detectable throughout cleavage development in the rhesus monkey (Zheng *et al.* 2004), similar to our findings that *SMARCA1* transcripts are not detectable in the GV-stage porcine oocytes and parthenogenetic blastocyst-stage porcine embryos and are in extremely low abundance at all other stages of development examined (Figs 1 and 2).

The third family of SNF2-type chromatin remodeling complexes is represented by the CHD complexes (also known as Nurd or Mi-2 in human; Bowen *et al.* 2004, Hall & Georgel 2007). Relatively less is known about the function of the CHD complexes and no studies have been conducted to investigate their expression pattern in mammalian embryos, although it has been shown that *CHD2* is essential for embryo development (Marfella *et al.* 2006). We found that *CHD3* and *CHD5* were expressed at differing levels until the blastocyst stage, where they are present in similar abundance. Our data, taken collectively, show that chromatin remodeling may be regarded as a fine-tuned process in cleaving embryos, with many ATPases that can form several alternative complexes in order to interact with specific loci at specific times of development. More experiments are needed to address the protein localization of such ATPases during embryo development.

We expected to observe a detrimental effect following *SMARCA4* dominant negative injection, since it may outcompete the endogenous maternal *SMARCA4*, mimicking a knock-out phenotype. Functional maternal *SMARCA4* is necessary for ZGA in mouse embryos; ablation of *SMARCA4* resulted in the downregulation of ~30% of  $\alpha$ -amanitin-sensitive genes (Bultman *et al.* 2006). This explains why embryos injected with dominant negative *SMARCA4* tend to arrest at the four-cell stage (Table 1), at the same stage when ZGA occurs in the pig (Jarrell *et al.* 1991). This is also consistent with the downregulation of *EIF1* observed following the injection of dominant negative *SMARCA4*. Transcripts for *EIF1* are a good marker for ZGA in many species (Latham *et al.* 1992, De Sousa *et al.* 1998). Our results support the hypothesis that *SMARCA4* is important for the onset of ZGA in pig embryos.

Conversely, we did not expect the dramatic developmental arrest observed in embryos injected with the wild-type *SMARCA4*. The dynamics of the arrest, with the majority of embryos blocking before the two-cell stage, led us to hypothesize that excess *SMARCA4* may have interfered with the first cleavage division. *SMARCA4* has been linked extensively to the control of the cell cycle (as reviewed in Muchardt & Yaniv 2001). It is possible that the arrest comes from the antiproliferative properties of *SMARCA4* in association with the tumor suppressor protein retinoblastoma (pRb; Dunaief *et al.* 1994). The pRb protein is one of the major cell cycle regulators that control the G1/S transition as well as progression through S phase. pRb and *SMARCA4* form a repressor complex (Dunaief *et al.* 1994). Co-expression of *SMARCA4* and pRb in human C33a cells (a cell line that does not possess *SMARCA4*) led to cell cycle arrest (Zhang *et al.* 2000). *SMARCA4* overexpression led to pRb-mediated growth arrest also in *SMARCA4*/*SMARCA2* lacking human SW13 cells (Dunaief *et al.* 1994, Shanahan *et al.* 1999, Strobeck *et al.* 2000). Interestingly, forced ectopic expression of *SMARCA4* in U2OS cells (cells that do possess *SMARCA4*) led to growth arrest as well (Zhang *et al.* 2000). pRb protein is present at the two-cell stage during zygotic gene activation in the mouse (Xie *et al.* 2005). The *SMARCA4*-pRb block is mediated by cyclin A and E degradation. It will be interesting to analyze the expression pattern of these two cyclin molecules in embryos injected with wild-type and dominant negative variants of *SMARCA4*. The point mutation in the dominant negative variant of *SMARCA4* is outside the loop involved in pRb-*SMARCA4* contact, so we assume that dominant negative *SMARCA4* can still form the SWI/SNF complex. Our data nonetheless suggest that functional *SMARCA4* is required to arrest the cell cycle, since embryos injected with dominant negative *SMARCA4* have a significantly different phenotype (Table 1).

It is important to note that the data shown in Table 1 include all injected embryos that did not lyse immediately after microinjection. Degenerated and fragmented embryos scored for cell number at 144 h post-activation, the time when *in vitro*-produced porcine embryos will have reached the blastocyst stage of development, were considered as embryos with zero cells. By scoring embryo development in this manner, we include all embryos in our analysis, but greatly increase the range of values for the average cell number in each treatment group. It is important to note that the group injected with wild-type *SMARCA4* had a significantly lower average number of nuclei per embryo even when compared with the group injected with dominant negative *SMARCA4*. This was largely due to arrest of embryos injected with *SMARCA4* at an early stage of cleavage, thereby lowering the number of embryos that progressed through development.

Retinoblastoma protein pRb is able to form a complex with *SMARCA2* as well. Interestingly, pRb, *SMARCA2*, and HDAC1 are responsible for the formation of the repressor protein HP1 $\beta$  heterochromatin foci in aging human melanocyte cells (Bandyopadhyay *et al.* 2007). *SMARCA2*, like *SMARCA4*, is able to arrest growth in *SMARCA2*- and *SMARCA4*-deficient cells in a pRb-dependent fashion (Strober *et al.* 1996, Trouche *et al.* 1997, Reisman *et al.* 2002). Curiously, we did not observe the same dramatic arrest of embryo development as for *SMARCA4* overexpression. This suggests that *SMARCA2* and *SMARCA4* have distinct roles in cell cycle control during early embryogenesis. *SMARCA2* may be less important during the first cleavage divisions, as demonstrated by mouse knock-out models (Bultman *et al.* 2000). Given that at 48 h post-injection, we were able to detect a 10-fold increase in *SMARCA2* mRNAs, but only a tenfold increase in *SMARCA4* transcripts, lends support to this hypothesis. These two genes may be differentially translated, since similar concentrations of each mRNA were injected in all treatments groups. Thus, mammalian embryos may rely more heavily on *SMARCA4* than *SMARCA2* during cleavage development.

Ectopic *SMARCA4* may also be integrated in other complexes with repression functions. *SMARCA4* has been physically associated with six different complexes, including the mSin3A/HDAC co-repressor complex (Sif *et al.* 2001, Kuzmichev *et al.* 2002, Pal *et al.* 2003) and the N-CoR complex (Underhill *et al.* 2000). Both these complexes possess histone deacetylases (HDAC1 and HDAC2 in mSin3A, HDAC3 in nCoR). Histone deacetylation has been linked extensively with repressed chromatin (reviewed in Hildmann *et al.* 2007). Histone deacetylase transcripts (HDAC2) have been detected in pig embryos as early as the four-cell stage (earlier stages were not assessed; Kumar *et al.* 2007). Bovine and mouse embryos possess all three HDACs as maternal transcripts in the GV- and metaphase II-stage oocytes and two-cell-stage embryos (McGraw *et al.* 2003, Inoue *et al.* 2006). Transcripts for the three deacetylases were also detected in rhesus monkey embryos, with HDAC2 being predominant. The bromodomain in *SMARCA4* can interact with acetylated lysine residues on histone proteins (reviewed in Mujtaba *et al.* 2007). It is possible that exogenous *SMARCA4* targets mSin3A/HDAC and nCoR complexes onto specific targets and repress transcription in an ATPase-dependent way.

The transcriptional repression of NANOG after *SMARCA4* injection further suggests that histone deacetylation may be involved. After retinoic acid induced differentiation of mouse embryonic stem (ES) cells, NANOG expression is shut down by p53 through mSin3A deacetylase activity (Lin *et al.* 2005). Moreover, Hattori *et al.* (2007) showed that NANOG is repressed in trophectoderm (TE) cells and expressed in ES cells. Histones H3 and H4 were hypoacetylated in the NANOG promoter region in TE cells, correlating with its

repressive state, while the same region in ES cells contained hyperacetylated histones. This suggests a mechanism by which wild-type SMARCA4 represses NANOG expression at 48 h post-injection. NANOG is typically activated during this period in porcine embryos (Magnani & Cabot 2008). SMARCA4 may recruit the mSin3A/HDAC complex onto the NANOG promoter and actively repress its transcription.

SOX2 transcripts were also repressed, although at a lower extent, in embryos injected with wild-type SMARCA4. There is evidence that histone acetylation on the SOX2 promoter is responsible for its inducible activation or silencing (Ruau *et al.* 2008, Sikorska *et al.* 2008). Moreover, the injection of a dominant negative variant of SMARCA4 or a SMARCA4 morpholino in *Xenopus* embryos led to increased cell proliferation and expansion of SOX2-positive cells (Seo *et al.* 2005), suggesting that SMARCA4 is required for SOX2 control and cell proliferation.

Ectopic expression of SMARCA2 in porcine embryos leads to a reduction in blastocyst formation; however, these embryos developed to the four-cell stage equally to the non-injected control (data not shown). Interestingly, at 48 h post-injection, we were able to detect a significant increase in SOX2 expression in the group injected with the dominant negative variant of SMARCA2. This indicates that functional SMARCA2 may be necessary to control SOX2 expression. However, this seems to be in contrast with the observation that functional SMARCA2 is required to activate SOX2 in rat neural cells (Kondo & Raff 2004). It is also possible that SOX2 transcription is differently regulated in the cleavage embryo when compared with neural cells.

Injection of wild-type SMARCA2 resulted in a tenfold increase in SMARCA1. SMARCA1 transcripts are present at extremely low concentrations during development, and further decrease around the eight-cell stage. Interestingly, parthenogenetic porcine embryos do not possess detectable SMARCA1 transcripts at the blastocyst stage. SMARCA1 knock-out mice have not been reported. The increase in SMARCA1 may be detrimental at this stage of development. It would be interesting to monitor the amount of SMARCA1 at later stages following the injection of wild-type SMARCA2, as well as to directly overexpress SMARCA1. It is interesting to note that SMARCA1 is associated with differentiation in multiple cell lines (LeGouy *et al.* 1998, Lazzaro & Picketts 2001, Lazzaro *et al.* 2006, Itoh *et al.* 2008). Thus, it is intriguing to speculate that a connection exists between SWI/SNF and ISWI ATPases may then exist in relation to the differentiation process (SMARCA4/ SMARCA5 to SMARCA2/ SMARCA1). SMARCA4 and SMARCA5 appear to be present at the same actively transcribed foci in early mouse embryos at the time of ZGA (Torres-Padilla & Zernicka-Goetz 2006).

Human SMARCA2 and SMARCA4 have been shown to be involved in splicing processes. Interestingly, SMARCA2, but not SMARCA4 has been shown to be necessary for

splicing of SWI/SNF target genes such as CD44 and e-cadherin in an ATPase-independent fashion (Batsché *et al.* 2006). SMARCA4 and SMARCA2 were shown to be directly linked to the methylation status at the same loci, suggesting that SWI/SNF complexes may exert activation/repression functions in one of several ways (Banine *et al.* 2005). SMARCA2, and possibly SMARCA4, also controls splicing variants for the telomerase reverse transcriptase gene via interaction with p54 (Ito *et al.* 2008). At this point, we cannot exclude that the phenotype observed in our experiments is a direct effect of mis-splicing events of essential transcripts.

The results from our studies presented here were obtained using porcine embryos derived from parthenogenetic activation and IVF. Porcine embryos produced *in vitro* are known to possess reduced developmental competence, when compared with embryos produced *in vivo*. Although polyspermy is one major challenge in producing porcine embryo by using IVF, the protocols used by our laboratory have been reported to lead to term development (Abeydeera & Day 1997, Abeydeera *et al.* 1998). In addition, although parthenogenetic embryos cannot develop to term, they represent a robust model for molecular events occurring during preimplantation development. Our group has recently shown that the regulation of fundamental genes such as *POU5F1*, *NANOG*, and *SOX2* is closely recapitulated in embryos produced by parthenogenetic activation or IVF when compared with *in vivo*-derived embryos (Magnani & Cabot 2008).

Our data taken collectively show that chromatin remodeling may be regarded as a fine-tuned process in the cleavage-stage mammalian embryos, with many ATPases that can form several alternative complexes in order to interact with specific loci at specific times of development. Our data show that overexpression of SMARCA2 and SMARCA4, the two highly similar ATPases of the SWI/SNF complex, results in different phenotypes suggesting different roles for the two proteins during porcine cleavage development. We demonstrated that alterations in the expression of SMARCA2 and SMARCA4 perturb developmentally important genes and result in the disruption of embryo development. In conclusion, our data reinforce the idea that chromatin remodeling has a central role during embryonic development via regulation of gene expression.

## Materials and Methods

### Oocyte collection

All chemicals were obtained from Sigma Chemical Company, unless stated otherwise. Porcine (*Sus scrofa*) ovaries from prepubertal gilts were collected at a local slaughterhouse and transported to the laboratory in an insulated container at 37 °C. Antral follicles between 3 and 6 mm in diameter were aspirated manually with a disposable 10 cc syringe and an 18 gauge

needle. Follicular fluid was pooled and allowed to settle by gravity. Cumulus–oocyte complexes (COCs) were resuspended in HEPES-buffered medium containing 0.01% polyvinyl alcohol (PVA; Abeydeera *et al.* 1998). Under a dissecting microscope, COCs with multiple layers of intact cumulus cells were selected for the experiments. For GV-stage oocytes used in PCR studies, COCs were vortexed in 0.1% hyaluronidase in HEPES-buffered medium for 6 min to remove the cumulus cells.

### **In vitro maturation**

50–75 COCs were cultured in 500 µl tissue culture medium 199 (TCM-199; Gibco BRL) containing 0.14% PVA, 10 ng/ml epidermal growth factor, 0.57 mM cysteine, 0.5 IU/ml porcine FSH, and 0.5 IU/ml ovine LH. COCs were matured for 42–44 h at 39 °C, 5% CO<sub>2</sub> in air, and 100% humidity (Abeydeera *et al.* 1998). Matured COCs were then vortexed in 0.1% hyaluronidase in HEPES-buffered medium containing 0.01% PVA for 4 min to remove the cumulus cells.

### **Parthenogenetic activation**

Mature oocytes were artificially activated as described previously (Magnani & Cabot 2007), by placing mature oocytes in activation medium (300 mM mannitol, 0.1 mM CaCl<sub>2</sub>, 0.1 mM MgSO<sub>4</sub>, 0.5 mM HEPES, 0.01% BSA) between two platinum electrodes in an Electrocell chamber and given two DC pulses of 1.2 kV/cm for 30 µs provided by a BTX Electro-Cell Manipulator 200 (BTX, San Diego, CA, USA).

### **IVF**

Mature denuded oocytes were placed in a modified Tris-buffered medium (mTBM) and fertilized according to an established protocol (Abeydeera & Day 1997), using fresh extended boar semen. Briefly, boar semen was extended in Modena Boar Semen Extender (Swine Genetics International, Cambridge, IA, USA) and kept at 17.5 °C for up to 3 days. Before fertilization, 1 ml extended semen was mixed with 10 ml Dulbecco's phosphate-buffered saline (DPBS) and centrifuged at 1000 *g*, 25 °C, for 4 min. Sperm were washed thrice with DPBS. The final concentration of sperm used in the fertilization experiments was 5 × 10<sup>5</sup> cells/ml. Groups of 30–35 oocytes were placed in 100 µl mTBM; gametes were co-incubated for 5 h at 39 °C and 5% CO<sub>2</sub>.

### **Embryo culture and collection**

Porcine zygote medium 3 (PZM3) containing 3 mg/ml fatty acid-free BSA was used to culture all embryos (Yoshioka *et al.* 2002). The embryos were maintained at 39 °C, 5% CO<sub>2</sub>, and 100% humidity until the appropriate cell stage. IVF embryos were collected as follows: two-cell (24 h post-IVF), four-cell (48 h post-IVF), eight-cell (72 h post-IVF), and blastocyst stages (day 6 post-IVF). Parthenogenetic embryos (PA) were collected as follows: two-cell (24 h post-activation), four-cell (48 h post-activation), eight-cell (72 h post-activation), and blastocyst stages (day 6 post-activation). Only embryos with the correct morphology at the above-indicated time points were used.

### **Microinjection**

The sequence of human *SMARCA2*, *SMARCA4*, and their dominant negative variants were a generous gift from Dr Anthony Imbalzano (University of Massachusetts Medical School, Worcester, MA, USA; de La Serna *et al.* 2000). Messenger RNA was prepared as described previously (Magnani & Cabot 2007). *In vitro*-produced mRNA was run on a denaturing agarose gel to verify size and integrity. Prior to microinjection, activated oocytes were divided randomly into four groups and placed in HEPES-buffered medium supplemented with 3 mg/ml BSA. For *SMARCA2* studies, the four groups were as follows: non-injected controls, *GFP* mRNA injected, wild-type *SMARCA2* mRNA and *GFP* mRNA injected (*SMARCA2*-WT), and dominant negative *SMARCA2* mRNA and *GFP* mRNA injected (*SMARCA2*-DN). For *SMARCA4* studies, the four groups were as follows: non-injected controls, *GFP* mRNA injected, wild-type *SMARCA4* mRNA and *GFP* mRNA injected (*SMARCA4*-WT), and dominant negative *SMARCA4* mRNA and *GFP* mRNA injected (*SMARCA4*-DN). Messenger RNA concentrations were µg/µl; equal amount of ATPase RNAs and GFP were mixed before injection. *GFP* mRNA co-injected to verify the integrity of injected mRNA translation of GFP demonstrated the quality of the mRNA sample injected. *In vitro*-synthesized mRNA was loaded into a sterile injection capillary attached to an Eppendorf FemtoJet microinjector (Westbury, NY, USA); ~8 pl of each respective mRNA was injected into the activated oocytes. Observance of movement of the cytoplasm was used as a reference for successful injection. Embryos that lysed immediately following microinjection were discarded and not included in the analysis. After injection, the embryos were transferred into PZM3 (Yoshioka *et al.* 2002) embryo culture medium and cultured for 6 days (to evaluate embryo development) or cultured for 48 h for mRNA isolation. On day 6, the embryos were stained with Hoechst 33342 and nuclei were counted under an epifluorescent microscope.

### **Hoechst staining and nuclei count**

Blastocysts were collected at 144 h post-activation and after injection and placed in HEPES-buffered medium containing 2 µg/ml Hoechst 33342. Embryos were stained for 15 min and mounted on microscope slides. Nuclei were visualized on an epifluorescent microscope. To obtain a developmental score, the nuclei were counted (degenerate embryos counted as 0 nuclei) and averages were obtained for each treatment.

### **Primers for real-time PCR**

Primers were designed on porcine sequences highly similar to human transcripts. Sequences were submitted to GenBank with the following accession numbers: *SMARCA4* (EU780789), *SMARCA2* (EU780788), *SMARCA5* (EU780790), *SMARCA1* (EU780791), *CHD3* (EU780792), and *CHD5* (EU780793). Primers for *YWHAG*, *POU5F1*, *NANOG*, *SOX2*, and all the SNF2-type ATPases were described previously (Magnani & Cabot 2008, Magnani *et al.* 2008); *EIF1* primers were as follows: forward, gggtttcaagaagatgggcaagag and reverse,

tttcctctgatgtgacataacttc. Transcript levels of the gene *YWHAG* were used to normalize transcript abundance in our assay (Magnani & Cabot 2007).

### Isolation of mRNA and RT

For the expression pattern of SNF2-type ATPases, polyadenylated mRNA was extracted from pools of 50–100 GV and MII oocytes using Dynabeads reagent (Invitrogen). Polyadenylated mRNA extracted from pools of oocytes collected on a single day was used as experimental replicate. Polyadenylated mRNA was extracted from pools of 50–100 embryos at the following stages: two-cell, four-cell, eight-cell, and blastocyst. Each mRNA extraction was replicated at least three times for each experiment. Oocytes and embryos were washed three times with PVA-PBS (nuclease free), then lysed in 100  $\mu$ l lysis buffer (Invitrogen), and stored at  $-80^{\circ}\text{C}$  until mRNA isolation. Reverse transcription was performed using the iScript kit, according to the manufacturer's instructions (Bio-Rad). For each experimental replicate, cDNA from one pool of oocytes or embryos was divided to amplify all genes during the same PCR run. For microinjected embryos, polyadenylated mRNA was extracted from 15 to 50 intact embryos 48 h post-activation. Each mRNA extraction was replicated at least three times for each experiment. For each experimental replicate, cDNA from one pool of oocytes or embryos was divided to amplify the six SNF2-ATPases or *SOX2*, *POU5F1*, *NANOG* and *EIF1* genes.

### Quantitative real-time PCR

A PCR Master Mix was prepared for each gene as follows: 12.5  $\mu$ l of  $2\times$  SYBR Green Master Mix (Bio-Rad), 5  $\mu$ l of 1  $\mu$ M forward primer, 5  $\mu$ l of 1  $\mu$ M reverse primer, and 2.5  $\mu$ l of cDNA. Reactions were carried out in duplicate. For each replicate, cDNA from a single pool of oocytes or embryos was subdivided to amplify all genes during the same PCR assay. Templates used as negative controls in the PCR assay included an RT reaction lacking reverse transcriptase and  $\text{H}_2\text{O}$ . PCR was run on an MyiQ single color real-time thermal cycler (Bio-Rad). The following program was used: initial denaturation at  $94^{\circ}\text{C}$  for 5 min followed by 40 cycles of 5 s at  $94^{\circ}\text{C}$ , 30 s at  $60^{\circ}\text{C}$ , and 30 s at  $72^{\circ}\text{C}$ . Real-time fluorescence data were collected during the extension time. A melting curve was produced to verify individual PCR amplicons. Products were further confirmed on an agarose gel. Products from the first replicate were sequenced to verify identity.

### Quantification of transcripts levels

For the expression pattern of SNF2-type ATPases, transcript levels of all genes were quantified using the relative quantification method based on comparative threshold cycles values ( $C_T$ ). The abundance of each single gene was determined relative to the abundance of the housekeeping gene *YWHAG*. *YWHAG* was chosen as normalizer gene since it had been previously shown to maintain a constant level of expression throughout cleavage development in porcine embryos (Whitworth *et al.* 2005). The  $C_T$  value, or the cycle

number during the log-linear phase of the PCR at which time the amount of detected PCR product rises above background levels, was determined for each reaction. Briefly, the  $C_T$  value for each single gene was subtracted from the  $C_T$  value for *YWHAG* to obtain a change in  $C_T$  ( $\Delta C_T$ ). To analyze the relative amount of SNF2-type ATPases in the same-stage embryos, we compare  $\Delta C_T$ . To analyze the relative amount of *SMARCA2*, *SMARCA4*, *SMARCA5*, *CDH3*, and *CHD5* during embryo development, we compared fold differences relative to the GV stage (GV stage as calibrator); for analysis of the relative amount of *SMARCA1*, comparisons were made relative to the MII-stage oocytes (MII stage as calibrator). Fold differences were calculated assuming an amplification efficiency of 100% and using the equation  $2^{-(\Delta\Delta C_T)}$ . To analyze the relative abundance of all genes following injection, we compared fold difference using the non-injected control as calibrator.

### Statistical analysis

For quantitative PCR,  $2^{-(\Delta\Delta C_T)}$  values were imported into SAS (SAS Institute Inc., Cary, NC, USA) and analyzed with a GLM one-way ANOVA procedure. The model incorporated stage and replicate as the main factors. For the expression pattern of SNF2-type ATPases, a multiple comparisons ranking using Tukey's post-test was performed and  $P < 0.05$  was considered significant. For the microinjection experiment, a multiple comparison ranking using the LSD post-test was performed and  $P < 0.05$  was considered significant. Analysis of the proportion of embryos developing to the two-cell, four-cell, and blastocyst stages were performed using a logistic regression model and was analyzed with PROC GENMODE in SAS. For nuclei count analysis, data were transformed with a log transformation and analyzed with a two-way ANOVA with replicate and treatments as the main factors.

### Declaration of interest

The authors declare no conflicts of interest that would prejudice the impartiality of the research reported here.

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