Gene Reports 11 (2018) 42–51

Contents lists available at ScienceDirect

Gene Reports

journal homepage: www.elsevier.com/locate/genrep

DNA methyltransferases and tRNA methyltransferase DNMT2 in developing pig brain - expression and promoter methylation

Knud Larsen[a,*], Kaja Kjær Kristensen[a], Henrik Callesen[b]

[a] Department of Molecular Biology and Genetics, Aarhus University, Blichers Allé 20, DK-8830 Tjele, Denmark
[b] Department of Animal Science, Aarhus University, Blichers Allé 20, DK-8830 Tjele, Denmark

ARTICLE INFO

Keywords:
Brain
DNA methyltranferase
Methylation
Pig

ABSTRACT

DNA methylation constitutes an epigenetic mechanism used by cells to control gene expression and chromatin organization. DNA methylation is a biochemical process, catalyzed by DNA methyltransferases, where a methyl group is added to a nucleotide base. In mammals, DNA methylation is essential for normal development. Here we report on the developmental expression of DNA methyltransferases and DNMT2, a tRNA methyltransferase, in various brain tissues in porcine embryos. Expressions of *DNMT1*, *DNMT2*, *DMNT3A* and *DNMT3B* transcripts were determined by qRT-PCR in five different brain tissues at four or five time points in embryo development. Low transcript levels from all these genes were detected as early as 60 days of gestation in all tissues examined. From 60 to 115 days of gestation, a reduction in *DNMT1* and *DMNT3A* and *DNMT3B* mRNA was seen in brain stem, hippocampus, basal ganglia, cortex and cerebellum. No significant changes were seen for *DNMT2* mRNA. In heart, a bimodal expression was observed for all four DNMTs analyzed. Analysis of DNA methylation status of two selected regions in the *DNMT3A* promoter revealed a very low degree of methylation (< 3%). It was concluded that DNA methylation of the examined region of the *DNMT3A* gene is not determining expression of its transcript.

1. Introduction

DNA methylation is a biochemical process where a methyl group is added to a nucleotide base, and the reaction is catalyzed by DNA methyltransferases. DNA methylation and histone modifications represent two key determinants in epigenetic regulation of gene expression in mammalian cells (O'Neill, 2015). DNA methylation is crucial for normal development and is involved in processes such as gene expression and genomic imprinting, X-chromosome inactivation, inhibition of recombination, aging and carcinogenesis (Bird, 2002). In mammals, DNA methylation is essential for normal development and has several functions. In general, methylation within gene regulatory elements suppresses gene expression (Razin and Riggs, 1980; Jones and Takai, 2001). Methylation of gene-deficient regions is vital for maintenance of chromosome structure and integrity (Robertson and Jones, 2000; Friso and Choi, 2002; Choi and Friso, 2009). Among the many functions of DNA methylation, the necessary connection between promoter methylation and gene silencing has yielded the most convincing evidence (Choi and Friso, 2009). DNA methylation changes during development both in gametogenesis and embryogenesis. After fertilization, parental genomes undergo demethylation with the exception of imprints (Stuppia et al., 2015). After implantation, the embryo undergoes *de novo* methylation (He et al., 2011). Imprints are erased and reestablished during gametogenesis. Methylation of DNA occurs at the 5-position of cytosine and is mediated by enzymatic action of DNA methyltransferases (DNMT). In mammals, the family of DNMTs comprises four members: DNMT1, DMNT3A, DNMT3B and DNMT3L. DNMT2 functions as a tRNA methyltransferase.

DNMT1is the dominating enzyme responsible for maintenance of the DNA methylation pattern and the primary enzyme responsible for copying methylation patterns following DNA replication (Moore et al., 2013). DNMT1 maintains the mitotic inheritance of the original methylation pattern in a cell lineage during replication by copying the methylation pattern of the parent DNA strand onto the newly synthesized strand (Gibney and Nolan, 2010; Moore et al., 2013). During replication, DNMT1 completes this task by localizing to the replication fork where newly synthesized hemimethylated DNA is formed and by targeting hemimethylated DNA over unmethylated DNA (Prokhortchouk and Defossez, 2008; Moore et al., 2013). By mimicking the original methylation pattern, DNMT1 catalyzes the transfer of the

Abbreviations: DNMT, DNA methyl transferase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NCBI, National Center for Biotechnology Information; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase PCR

* Corresponding author.
E-mail address: Knud.Larsen@mbg.au.dk (K. Larsen).

https://doi.org/10.1016/j.genrep.2018.02.003
Received 24 November 2017; Accepted 7 February 2018
Available online 08 February 2018

methyl group from *S*-adenosylmethionine when it is bound to the newly synthesized DNA, and DNMT1 also possesses the ability to repair DNA methylation (Moore et al., 2013). DNMT1 is able to discriminate between hemimethylated and unmethylated DNA (Prokhortchouk and Defossez, 2008). DNMT1 is highly expressed in mammalian tissues including the brain (Moore et al., 2013). DNMT1 transcript and protein is also expressed in many human organs and tissues with particularly high expression levels in placenta, testis and lymph node (The Human Protein Atlas). Transcripts encoding DNMT1 are present in preimplantation embryos and contribute to the increasing methylation during early embryo development. The DNMT1 protein is abundantly expressed in cells of the developing embryo, and it is detected in the early post-implantation embryo (Yoder and Bestor, 1998).

DNMT3 enzymes are responsible for establishing *de novo* methylation because they can introduce methylation into naked DNA and hence control meiotic inheritance and because *de novo* DNMTs are capable of methylating both native and synthetic DNA without the preference for hemimethylated DNA (He et al., 2011; Moore et al., 2013). DNMT3A is expressed ubiquitously while DNMT3B is mainly expressed in the thyroid, testes and bone marrow (Moore et al., 2013). DNMT3B is expressed as early as at the 8-cell stage in bovine embryos (Graf et al., 2014). The third member of the DNMT3 family, DNMT3L, has no catalytic activity but acts as a regulator factor of DNMT3A and DNMT3B for *de novo* methylation (Deplus et al., 2002; Moore et al., 2013). DNMT2, also named TRDMT1, methylates RNA instead of DNA; more specifically it methylates cytosine 38 in the anticodon loop of aspartic acid transfer RNA (Goll et al., 2006). DNMT2 is probably also involved in recognition of DNA damage, DNA recombination and mutation repair (Hermann et al., 2003).

During aging a global loss of methylation can be observed both in human and mice (Fraga and Esteller, 2007; Jones et al., 2015; Wang et al., 2017). A comparison of global methylation in newborns and centenarians revealed more hypomethylated DNA sequences in the advanced age group (Heyn et al., 2012).

Our study has examined the spatial expression of DNMT1, DNMT3A, DNMT3B and DNMT2 transcripts in various organs and tissues from adult pig. Furthermore we have investigated the developmental expression of the same genes in five different brain tissues from pig embryos at different stages. Here, we also present an analysis of the methylation status of the DNMT3A promoter in seven different pig brain tissues sampled at day 60 to day 115 of gestation.

2. Materials and methods

2.1. Ethics statement

Housing of pigs and approval of experimental procedures have been described elsewhere (Henriksen et al., 2013). The pigs were sacrificed by an intravenous injection with 30 mg/kg Pentobarbital.

2.2. Biological subjects

Nine different brain regions were included in this study: hippocampus, brain stem, basal ganglia, frontal cortex, cerebellum, occipital cortex, parietal cortex, hypothalamus and mesencephalon. Brain samples were collected from Danish Landrace developing embryos at 60, 80, 100 and 115 days of gestation. Samples from heart and liver were also selected at 40, 60, 80, 100 and 115 days of gestation. Biological samples for determination of the spatial expression of DNMTs were collected from two one-year old Danish Landrace pigs (adult) weighing 125–150 kg.

2.3. Bioinformatic analyses

Sequence analysis was performed using online software NCBI (http://ncbi.nlm.gov) and Expasy (http://expasy.org). The putative amino acid sequence was deduced using the Expasy translate tool (http://expasy.org/translate/). Homologues of DNMTs were retrieved from NCBI using blastx. ClustalW (http://www.genome.jp/tools/clustalw/) was used for sequence alignment. MethPrimer (http://www.urogene.org/methprimer/) was used to predict CpG islands in the *DNMT3A* promoter sequence and to design bisulfite-conversion-based methylation PCR primers.

2.4. Extraction of nucleic acids and cDNA synthesis

RNA was isolated from pig organs and tissues as previously described (Larsen et al., 2014). DNA was isolated from biological samples according to standard purification protocols (Green and Sambrook, 2012). Synthesis of the cDNA used for cloning was performed as previously described (Henriksen et al., 2009). cDNAs used for expression analysis were synthesized from RNA isolated from various adult porcine organs and tissues, and from fetal brain tissues sampled at 60, 80, 100 and 115 days of gestation using random hexamer primers (Roche) and the manufacturer's protocol.

2.5. Expression analysis

Porcine *DNMTs* mRNA expression was determined by RT-PCR analysis. The following porcine organs and tissues were included in the analysis: liver, prostate, lung, testis, spleen, tongue, cerebellum, occipital cortex, parietal cortex, temporal lobe, musculus longissimus dorsii, frontal cortex, heart and jaw muscle. The temporal expression analysis included brain tissues, heart and lung samples at 40, 60, 80, 100 and 115 days of gestation. Heart and lung was also sampled at day 40 of gestation. Three individual biological samples of each type of brain tissue and time in gestation were included. Organ and tissue samples for the spatial expression analysis were collected from three one-year old Danish Landrace pigs weighing 125–150 kg. Based on earlier observations (Madsen et al., 2007) we used *GAPDH* as a reference gene in determination of *DNMT* mRNA expression. DNMT-specific primers were designed to span neighbor exon junctions using the EXIQON Human Probe Library. Sequences of primers for the various DNMTs as well as GAPDH-F and GAPDH-R used in the expression analysis are shown in Table 1.

Probes used were identified from the human probe library and were designed using either the Probe Finder web tool (www.roche-applied-science.com) or the Primer Express software program (Applied Biosystems). Probes for the individual DNMT transcripts are listed in Table 1. The PCR primers and oligonucleotide probe, labeled with the fluorescent reporter SYBR Green or VIC, were designed with the Primer Express software program (Applied Biosystems) and the Probe finder web tool at default settings (www.probelibrary.com). Each reaction was performed in technical and biological triplicates. Quantitative RT-PCR was performed as previously described (Madsen et al., 2007). Ethidium bromide-staining after real-time PCR confirmed specific amplification of the relevant PCR products (data not shown). Expression analysis data were analyzed using the analysis of variance (ANOVA) procedure of the Statistical Analysis Software (version 8.2; SAS Institute Inc. Cary, NC). The equality of *NURR1* expression levels between different gestation times with different embryonic brain tissues was tested for statistical significance using the Relative Expression Software Tool (REST) (Madsen et al., 2007). The level of probability was set at $P < 0.03$ as statistically significant and 50,000 randomization steps were implemented in each comparison (Madsen et al., 2007). Data were analyzed using the analysis of variance (ANOVA) procedure of the Statistical Analysis Software (version 8.2; SAS Institute Inc. Cary, NC).

2.6. Methylation analysis of the DNMT3A promoter

The methylation status of *the DNMT3A promoter* was performed by bisulfite sequencing. Genomic DNA from different brain tissues of