A novel marsupial pri-miRNA transcript has a putative role in gamete maintenance and defines a vertebrate miRNA cluster paralogous to the miR-15a/miR-16-1 cluster

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Abstract

Successful maintenance, survival and maturation of gametes rely on bidirectional communication between the gamete and its supporting cells. Before puberty, factors from the gamete and its supporting cells are necessary for spermatogonial stem cell and primordial follicle oocyte maintenance. Following gametogenesis, gametes rely on factors and nutrients secreted by cells of the reproductive tracts, the epididymis and/or oviduct, to complete maturation. Despite extensive studies on female and male reproduction, many of the molecular mechanisms of germ cell maintenance remain relatively unknown, particularly in marsupial species. We present the first study and characterisation of a novel primary miRNA transcript, *pri-miR-16c*, in the marsupial, the stripe-faced dunnart. Bioinformatic analysis showed that its predicted processed miRNA – *miR-16c* – is present in a wide range of vertebrates, but not eutherians. *In situ* hybridisation revealed dunnart *pri-miR-16c* expression in day 4 (primordial germ cells) and day 7 (oogonia) pouch young, in primary oocytes and follicle cells of primordial follicles but then only in follicle cells of primary, secondary and antral follicles in adult ovaries. In the adult testis, *pri-miR-16c* transcripts were present in the cytoplasm of spermatogonial cells. The oviduct and the epididymis both showed expression, but not any other somatic tissues examined or conceptuses during early embryonic development. This pattern of expression suggests that *pri-miR-16c* function may be associated with gamete maintenance, possibly through mechanisms involving RNA transfer, until the zygote enters the uterus at the pronuclear stage.

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Introduction

Neonate mammals have germ cells that remain quiescent until puberty. The testis contains spermatogonial stem cells that can self-renew indefinitely and differentiate into spermatogonia A and B. Spermatogonia B first enter meiosis at puberty to become spermatozoa (reviews, Bellve *et al.* 1977, Vergouwen *et al.* 1991, Aponte *et al.* 2005). In contrast, the pool of female germ cells is finite, and at birth, oocytes are arrested at prophase I of meiosis in primordial follicles (review, Picton *et al.* 1998).

Successful primordial follicle maintenance involves bidirectional communication between germ cells and the follicle cells, which includes autocrine and paracrine signalling (De La Fuente & Eppig 2001, Matzuk *et al.* 2002, Hutt *et al.* 2006), and gap junctions facilitating the diffusion/transfer of metabolites and other signalling molecules, important for oocyte maintenance and growth (Heller & Schultz 1980, Eppig 1991, Goodenough *et al.* 1996, Wright *et al.* 2001).

Primordial follicle maintenance in eutherians appears to involve anti-Müllerian hormone (AMH; Durlinger *et al.* 1999, 2002), FOXO3 (Castrillon *et al.* 2003, Hosaka *et al.* 2004, John *et al.* 2007) and PTEN (Reddy *et al.* 2008). The lack of any of these factors in mutant mice results in depletion of primordial follicles at an early age (Durlinger *et al.* 1999, Castrillon *et al.* 2003, Hosaka *et al.* 2004, John *et al.* 2007, Reddy *et al.* 2008).

Follicle maintenance, growth and differentiation in later stages of folliculogenesis in eutherians involve members of cytokines and growth factors derived from the follicle cells such as KIT ligand (reviewed by Hutt *et al.* (2006)), leukaemia inhibitory factor (LIF; Nilsson *et al.* 2002) and bone morphogenic proteins (BMP4, BMP9 and BMP15, Lee *et al.* 2001, Nilsson & Skinner 2003, Craig *et al.* 2007, Feary *et al.* 2007, McNatty *et al.* 2007). It also involves oocyte genes such as *KIT* (review, Hutt *et al.* 2006), *SOHLH1* (Pangas *et al.* 2006), LIM homeobox 8 (*Lhx8*; Pangas *et al.* 2006, Choi *et al.* 2008), growth differentiation factor 9 (*GDF9*; McGrath *et al.* 1995, Dong *et al.* 1996, Aaltonen *et al.* 1999,

Hayashi *et al.* 1999, Bodensteiner *et al.* 2000, Feary *et al.* 2007) and *c-mos* (Hashimoto 1994) which maintains the oocyte at metaphase II arrest until fertilisation.

In marsupials, the molecular aspects of follicle maintenance and development are less well understood and limited to *AMH* (Juengel *et al.* 2002), *LIF* (Cui & Selwood 2000), *KIT* (Greenwood *et al.* 1996, Eckery *et al.* 2002a), *GDF9* and *BMP15* (Eckery *et al.* 2002b) in *Trichosurus vulpecula* and *AMH* in *Macropus eugenii* (Pask *et al.* 2004). These genes show similar ovarian expression patterns in marsupials and eutherians, suggestive of conserved roles.

Following ovulation, the oocyte undergoes further maturation induced by oviduct secretions (reviews, Leese *et al.* 2001, Harris *et al.* 2005). In hamsters (Ortiz *et al.* 1986) and mares (Weber *et al.* 1991), interactions between the oocyte and the oviduct have been noted as fertilised eggs are transported faster than unfertilised eggs. In the oviductal fluid of many eutherian species, oviduct-specific glycoproteins bind to the zona pellucida of oocytes and conceptuses, indicating a role in fertilisation and early development (reviewed by Verhage *et al.* (1997)). In marsupials and some eutherian mammals, a mucoid coat is secreted by the oviduct and deposited around ovulated oocytes (Roberts *et al.* 1994). In marsupials, this mucoid coat plays a role in preventing polyspermy by binding excess sperm (Selwood 1992, 2000) and is also believed to provide nourishment for the conceptus, as supported by ultrastructural observations of endocytosis of mucoid material in *Didelphis virginiana* (Krause & Cutts 1985), *Acrobates pygmaeus* (Ward & Renfree 1988) and *Sminthopsis macroura* (Kress & Selwood 2004). Oviductal transit is very rapid in *S. macroura*, and can take as little as 4 h for oocytes/zygotes to travel the length of the oviduct to the uterus (Menkhurst *et al.* 2007), where cleavage begins.

Similarly, spermatogonial stem cell maintenance requires communication between the germ cells and their supporting Sertoli cells (reviewed by Johnson *et al.* (2008)). Several factors involved in the regulation of spermatogonial stem cell maintenance include Sertoli cell-derived factors such as Ets-related molecule (Chen *et al.* 2005), glial cell line-derived neurotrophic factor (GDNF; Meng *et al.* 2000, Kubota *et al.* 2004, Hofmann *et al.* 2005, Naughton *et al.* 2006), spermatogonial stem cell proteins such as GDNF family receptor α (GFRA; Bugeaw *et al.* 2005) and RET (Jain *et al.* 2004, Naughton *et al.* 2006), which are co-receptors for GDNF, and promyelocytic leukaemia zinc finger (PLZF; Buas *et al.* 2004, Costoya *et al.* 2004). None of these testis factors have been studied in any marsupial species.

Following spermatogenesis in mammals, spermatozoa undergo cytoplasmic maturation and acquire progressive motility (reviewed by Bedford (2008)) during transit through the epididymis. Capacitation occurs in the oviducts and results in the hyperactivity of sperm, reorientation of the sperm head in marsupials

and reorganisation of the sperm plasma membrane for binding to the egg, essential to initiate the acrosome reaction and ultimately achieve fertilisation (reviews, Breed 1994, Bedford 2008). Furthermore, increasing evidence suggests that the epididymis (Fuentes-Prior *et al.* 2002, Ensslin & Shur 2003, Asquith *et al.* 2005, Nixon *et al.* 2005) and the oviduct (King & Killian 1994, Abe *et al.* 1995, Boatman & Magnoni 1995, Buhi *et al.* 2000, McCauley *et al.* 2003) secrete factors that adhere to sperm and egg surfaces and function in sperm–egg adhesion and fertilisation, further confirming the significance of epididymal and oviductal factors on gamete maintenance.

Although recent studies have shed light on germ cell maintenance, the mechanisms remain largely unknown and the understanding of the molecular mechanisms by which the reproductive tract secretory products affect germ cells is generally unclear.

In this study, we identified a putative long non-coding (lnc) RNA corresponding to the primary transcript of a novel microRNA, *miR-16c*, from a marsupial, the stripe-faced dunnart (*S. macroura*), which we named primary miRNA-16c (*pri-miR-16c*). We previously isolated dunnart *pri-miR-16c* as one of the 49 clones that screened positive in a study to search for ovary-specific genes (Au *et al.* 2008). In this study, we have cloned the dunnart *pri-miR-16c* cDNA and analysed its expression by RT-PCR and *in situ* hybridisation to elucidate the potential roles of its processed miRNA in dunnart reproductive and gamete biology. We also show that *pri-miR-16c* is present in a wide range of vertebrates, but not eutherian mammals.

Results

pri-miR-16c sequence analyses

We previously isolated the partial sequence of dunnart *pri-miR-16c* (Au *et al.* 2008). In this study, the full-length sequence of the transcript was determined by 5' and 3' RACE using testis RNA to be 669 bp (Fig. 1A), matching the size of the transcript determined by northern blot analysis (results not shown). Translation of the nucleotide sequence in six reading frames revealed many putative short open reading frames (ORFs), the longest being 130 codons in length. None of the ORFs contained the canonical methionine (ATG) start codon.

BLAST searches of the full-length sequence against the tammar wallaby (*M. eugenii*) whole-genome shotgun (WGS) database revealed homologous sequences within Contig312420 (GenBank accession ABQO010312407) and Contig1027888 (GenBank accession ABQO011027844), which partially overlapped and were able to be joined to form a single contig. This enabled a five-exon structure of the dunnart *pri-miR-16c* transcript to be determined (Fig. 2A). Exons 1, 2, 4 and 5 were also found to be conserved in the opossum (*Monodelphis*

Figure 1 The dunbart *pri-miR-16c* sequence analyses. (A) Dunbart *pri-miR-16c* nucleotide sequence. Nucleotide sequences of all forward (for) and reverse (rev) primers used in 5' and 3' RACE are shown. (B) Predicted secondary structure of the dunbart *pri-miR-16c* nucleotide sequence. The predicted microRNA stem-loop structure of the part of the sequence similar to *miR-16-1* is circled.

domestica) genome (not shown), in which it mapped to chromosome X adjacent to hypoxanthine phosphoribosyltransferase 1 (*HPRT1*; Fig. 2B).

A region within exon 5 shared identity with sequences from a large range of vertebrate species. Within the mouse genome, the highest identity was found with the miRNA-encoding gene *miR-16-1*, which forms a cluster together with *miR-15a* on chromosome 14 near the gene *TRIM13* (Fig. 2B). Accordingly, the dunbart *pri-miR-16c* transcript was predicted to form a stem-loop structure (Fig. 1B) suggesting that it represents a miRNA precursor. Notably, a region within the predicted intron 4 of the *M. eugenii* contig contained sequence homologous to *miR-15a*, suggesting a miRNA cluster in marsupials homologous to the *Mus musculus* *miR-15a/miR-16-1* cluster.

A thorough search and analysis of sequences homologous to *miR-15a* and *miR-16-1* in vertebrate genome

databases (Fig. 3) revealed that the marsupial *miR-16c*-associated miRNA cluster is not orthologous to the eutherian *miR-15a/miR-16-1* cluster, as marsupial orthologues of *miR-15a* and *miR-16-1* were also identified adjacent to *TRIM13* on chromosome 4 of *M. domestica* (Fig. 2B). We thus named the marsupial homologues *miR-15c* and *miR-16c* respectively. The paralogous relationship between *miR-15c/miR-16c* and *miR-15a/miR-16-1* was evident throughout a wide range of vertebrates: the former cluster invariably flanks orthologues of *HPRT1* while the latter flank tripartite motif-containing 13 (*TRIM13*; Fig. 2B). Notably, eutherians represent the only taxon in which *miR-15c* and *miR-16c* are apparently absent, suggesting that they became extirpated in this lineage.

Cimmino *et al.* (2005) identified a conserved motif within both *miR-15a* and *miR-16-1* complementary to

Figure 2 Schematic diagram showing: (A) alignment of the five-exon structure of dunbart *pri-miR-16c* to *M. eugenii* orthologous genomic sequence. *miR-15c* and *miR-16c* are situated in intron 4 and exon 5 of *pri-miR-16c* respectively. (B) Location of *miR-15c/miR-16c* and *miR-15a/miR-16-1* clusters in the genome of various representative vertebrate species. Note the absence of a *miR-15c/miR-16c* cluster in the *M. musculus* genome.

Figure 3 Sequence alignment of *miR-15c/miR-15a* and *miR-16c/miR-16-1* sequences from various vertebrate species showing strong sequence homology conservation. Solid-bordered boxes enclose a conserved motif complementary to the 3' UTR of amniote *BCL2*. Broken-bordered boxes enclose their complementary sequences within the predicted miRNA hairpin structure that contain mismatches in *miR-15a/miR-15c*, but not in *miR-16-1/miR-16c*.

sequences within the 3' UTR of eutherian B cell lymphoma 2 (*BCL2*), which they negatively regulate. We determined that a core six-nucleotide portion of this sequence is also conserved in both *miR-15a* and *miR-16-h* orthologues from a range of vertebrates (Fig. 3), suggesting that they might also regulate *BCL2*. However, we found that the complementary sequence is conserved in the *BCL2* 3' UTR only in amniotic vertebrates, but not in frog or fish (not shown), suggesting there are other more ancestral target genes of both *miR-15a/miR-16-1* and *miR-15c/miR-16c*.

Transcript analysis by RT-PCR

RT-PCR showed that *pri-miR-16c* transcript was present in *S. macroura* ovary and testis, and not in any somatic tissues examined (Fig. 4A, $n=2$). In secondary follicles, dunnart *pri-miR-16c* was present in follicle cells but not in primary oocytes (Fig. 4B, $n=2$). Dunnart *pri-miR-16c* was present in pouch young (PY) bodies collected at day 4 and day 7 *post partum* (Fig. 4C, $n=2$), containing primordial germ cells and oogonia, respectively, but was not detected in any of the conceptuses examined from zygote to the 64-cell stage (Fig. 4D, $n=3$).

Dunnart *pri-miR-16c* expression in female and male reproductive tracts

Because expression remained in follicle cells but not in oocytes from the primary follicle stage (see *in situ* hybridisation results below), *pri-miR-16c* expression in female and male reproductive tracts was examined ($n=2$). In females, *pri-miR-16c* was expressed in oviducts but not in the uteri (Fig. 4E). In males, expression occurred in the epididymis but not in the vas deferens (Fig. 4E). To determine whether *pri-miR-16c* is continuously expressed in oviducts, we collected and examined expression in oviducts from females that were anoestrous (not breeding) and from females at different stages of their reproductive cycle: day 4 and day 5 of the

follicular phase; day of ovulation; pregnant with zygotes, two-cell conceptuses, four-cell conceptuses, 16-cell conceptuses, unilaminar blastocysts, bilaminar blastocysts or fetuses; and during the non-pregnant luteal phase with one- or four-cell parthenotes. Dunnart *pri-miR-16c* was expressed in oviducts from anoestrous and reproductive females at all stages of oestrus, pregnancy and non-gravid luteal phases examined (Fig. 4F).

In situ hybridisation of dunnart *pri-miR-16c*

In situ hybridisation of *S. macroura* ovaries ($n=3$) revealed that *pri-miR-16c* transcripts were localised to the cytoplasm of primary oocytes and follicle cells of primordial and late primordial follicles (Fig. 5A and C) but were only present in the cytoplasm of follicle cells of all later follicles including primary, secondary, tertiary (Fig. 5D and F) and antral (Fig. 5G).

In situ hybridisation of *S. macroura* testis ($n=2$) showed that *pri-miR-16c* transcripts were only localised to the cytoplasm of spermatogonial cells (spermatogonial stem cells and differentiating spermatogonia A and B, Fig. 6A and D, black arrows) and were not expressed in the supporting Sertoli cells (Fig. 6A and D, arrow heads) or later stages of spermatogenesis.

pri-miR-16c transcripts in the *S. macroura* epididymis ($n=2$) were present in the cytoplasm of epithelial cells (principal cells, basal cells, narrow, apical and clear cells; Fig. 6G, white arrows). It was not possible to distinguish between the clear cells and the principal cells by the current staining methods as these cell types are of equivalent size, but expression was likely to reside at least in principal and/or basal cells as they make up the majority of cells in the epididymal epithelium of marsupials (Cummins *et al.* 1986). The specificity of the *pri-miR-16c* antisense DIG-labeled riboprobes was confirmed by a lack of hybridisation in any cell type using sense riboprobes (Figs 5B, E, H and 6B, E, H). Dunnart *pri-miR-16c* localisation in ovaries, testes and epididymides did not vary between animals.

Figure 4 The presence of *pri-miR-16c* transcripts in *S. macroura* tissues, oocytes, follicle cells and conceptuses as determined by RT-PCR. (A) Transcript is present in adult gonadal and somatic tissues, $n=2$ (refers to the number of repeat experiments). o, ovary; b, brain; t, testis; h, heart; li, liver; k, kidney; lu, lung; s, spleen; hg, hindgut. (B) Transcript is present in secondary follicles separated into primary oocytes and follicle cells, $n=2$. (C) Transcript is present in day 4 and day 7 pouch young (PY) bodies, $n=2$. (D) Dunnart *pri-miR-16c* expression analysis in early embryonic development from zygote to 64-cell unilaminar conceptuses, $n=3$. (E) Transcript is present in female and male reproductive tracts: oviducts, uteri, epididymis and vas deferens ($n=2$). (F) Transcript is present in oviducts ($n=2$) collected from 1. Anestrous females, females at specific stages of her reproductive cycle: 2. day 4 follicular phase, 3. day 5 follicular phase, 4. day of ovulation, 5. pregnant with zygotes, 6. pregnant with two-cell stages, 7. pregnant with four-cell conceptuses, 8. pregnant with 16-cell conceptuses, 9. pregnant with unilaminar blastocysts, 10. pregnant with bilaminar blastocysts, 11. pregnant with fetuses, 12. non-gravid luteal phase with one-cell parthenotes and 13. non-gravid luteal phase with four-cell parthenotes. 4E, early four-cell conceptuses; 4A, arrested four-cell conceptuses; 4L, late four-cell conceptuses stages. Negative controls (-ve) contained the gene-specific primers but no templates. Positive controls (+ve) contained dunnart *pri-miR-16c* primers and dunnart *pri-miR-16c* plasmid cDNA.

Discussion

In this study, the entire 669 bp dunnart *pri-miR-16c* transcript was cloned. Secondary structure prediction of the dunnart *pri-miR-16c* nucleotide sequence showed the possibility of a miRNA duplex formation. In mammalian cells, primary miRNA transcripts containing the fold-back stem-loop RNA structure are processed

by DROSHA and DICER or AGO2 to produce 21- to 23-nucleotide regulatory RNAs that control gene expression (reviews, Winter *et al.* 2009, Yi & Fuchs 2011). The strong conservation of *miR-15c* and *miR-16c* sequences among vertebrates, and their clear homology with the well-characterised miRNAs *miR-15a* and *miR-16-1*, respectively, provide compelling evidence that the former also function as miRNAs. Dunnart *pri-miR-16c* thus appears to represent a primary transcript (pri-miRNA) for processing to *miR-16c* that becomes incorporated in the RNA interference pathway to regulate the expression of target gene(s).

miR-15a and *miR-16-1* were the first miRNAs to be implicated in cancer progression, being deleted or down-regulated in cases of chronic lymphocytic leukaemia (Calin *et al.* 2002, Hanlon *et al.* 2009), pituitary adenomas (Bottoni *et al.* 2005) and ovarian cancer (Bhattacharya *et al.* 2009). Recently, deletion of *miR-15a* and *miR-16-1* has been shown to accelerate proliferation in B cells by regulating the expression of multiple oncogenes including *BCL2* (Cimmino *et al.* 2005) and genes controlling cell cycle progression and differentiation (reviewed by Aqeilan *et al.* (2010)). Consistent with this, the regulation of *BCL2* expression in mouse ovary (Guillemin *et al.* 2009) and testis (Furuchi *et al.* 1996) is important in the maintenance of the germ cell.

The sequence similarity between the *miR-15a*/*miR-16-1* and *miR-15c*/*miR-16c* clusters suggests that they may regulate some similar genes but this requires further analysis. Conversely, the conservation of both clusters throughout most vertebrates suggests that they have at least partially distinct roles, and that the roles of *miR-15c* and *miR-16c* have become redundant in eutherian mammals for unknown reasons. Thus, examining the function of *miR-15c* and *miR-16c* may be relevant to studies of cancer as well as to understanding features that make eutherians distinct from other mammals. Some protein-coding genes have been characterised that are present in marsupials and/or other vertebrates, but absent in eutherians. These include a novel class of T-cell receptors (Parra *et al.* 2007), *POU2* – a paralogue of the well-characterised pluripotency regulator *POU5F1* (Frankenberg *et al.* 2010) – and DPAT (a marsupial-specific PAT protein (Au *et al.* 2010b)). Devor *et al.* (2009) identified 14 miRNAs that are unique to marsupials, however to our knowledge, this study represents the first characterisation of a putative miRNA conserved throughout vertebrates, including marsupials, but specifically absent in eutherians.

The expression of dunnart *pri-miR-16c* in primordial germ cells and oogonia, in early germ cells (spermatogonial cells and oocytes of primordial follicles) and subsequently gamete-supporting cells (follicle, oviductal or epididymal cells), is suggestive of a potential role in gamete maintenance by regulating the expression of genes required for gamete cell cycle progression and differentiation to retain germline fate and potency.

Figure 5 Localisation of *pri-miR-16c* in adult *S. macroura* ovary sections by *in situ* hybridisation, $n=3$ (refers to the number of repeat experiments). Hybridisation with antisense riboprobes (A, D and G); or sense riboprobes (B, E and H). (A and B) Primordial and late primordial follicles. (D and E) Primary, late primary, secondary and tertiary follicles. (G and H) Antral stage follicle. (C) Enlarged view of primordial follicles observed in inset in section A. Specific staining in follicle cells of all follicles but also in the cytoplasm of oocytes. (F) Enlarged view of secondary and tertiary follicles observed in inset in section D. Specific staining in follicle cells only. Pd, primordial follicle; LPd, late primordial follicle; P, primary follicle; LP, late primary follicle; S, secondary follicle; T, tertiary follicle; An, antral follicle. Scale bar: 100 μm .

Evidence of miRNAs involved in the maintenance of pluripotency has been demonstrated in numerous studies (reviewed by Martinez & Gregory (2010)). The miR-290 cluster, expressed extensively in primordial germ cells and spermatogonia (Hayashi *et al.* 2008), has recently been implicated in the maintenance of germline potency (Blakaj & Lin 2008). Moreover, mouse gametes that lack *Dicer* (the enzyme required for miRNA biogenesis) also showed deficiency in spermatogenesis (Hayashi *et al.* 2008) and chromosomal defects during

meiotic maturation in primary oocytes (Mattiske *et al.* 2009), suggesting an important function for miRNAs in gamete biology. The first miRNAs identified, *lin-4* (Lee *et al.* 1993) and *let-7* (Reinhart *et al.* 2000) in *Caenorhabditis elegans*, regulate epithelial cell differentiation further confirming the roles of miRNAs in the maintenance of cell identity. After initial production in the gametes, dunnart *pri-miR-16c* is likely produced by their supporting cells in the ovary, oviduct and epididymus, perhaps due to transcription limitations in

Figure 6 Localisation of *pri-miR-16c* in adult *S. macroura* testis and epididymis sections by *in situ* hybridisation, $n=2$ (refers to the number of repeat experiments). (A and D) Testis tubules hybridised with antisense dunnart *pri-miR-16c* riboprobes. Inset in D shows enlarged view of specific staining in spermatogonia (arrows). Arrowheads show lack of staining in Sertoli cells (B and E) testis tubules hybridised with sense dunnart *pri-miR-16c* riboprobes. (G) Epididymis tubule sections hybridised with antisense dunnart *pri-miR-16c* riboprobes. Inset shows enlarged view of specific staining in epididymal tubule cell cytoplasm (white arrows). (H) Epididymis tubule sections hybridised with sense dunnart *pri-miR-16c* riboprobes. Hematoxylin stained testes (C and F) and epididymis (I). Inset in F shows spermatogonia (arrows) and Sertoli cells (arrowheads). Inset in I shows epididymal tubule cells (white arrows). Scale bar: 100 μm .

gametes (Schultz 1993, Latham 1999, Latham & Schultz 2001), but possibly functions primarily on gametes by providing developmental cues necessary for gamete maintenance.

During and following dunnart cleavage, *pri-miR-16c* is no longer required, as suggested by the lack of expression in the uteri and in cleavage-stage conceptuses. While eutherian conceptuses begin cleavage during oviduct transit, in marsupials, this does not occur until uterine entry (Selwood & Hickford 1999). This difference in the location of cleavage division may explain the redundancy of *pri-miR-16c* in eutherian species allowing cleavage to proceed in the oviducts.

In the male, *pri-miR-16c* appears to be required in early germ cells but not differentiated sperm cells or Sertoli cells. We speculate that down-regulation of dunnart *pri-miR-16c* expression in differentiated sperm cells may be a mechanism to allow for the removal of deficient sperm cells during spermatogenesis (reviewed by Eddy (2002)).

In contrast, dunnart *pri-miR-16c* expression in the ovary and oviducts suggests its requirement during every stage of gametogenesis, folliculogenesis and during oviductal transit. Such differences may reflect a higher requirement to preserve the finite population of oocytes that cannot be replaced or self-renew (reviewed by Picton *et al.* (1998)). Since dunnart *pri-miR-16c* function is not limited to oocytes, the continuous expression in oviducts also may be related to the necessity to maintain sperm which can be stored up to 12 days in the oviducts (Menkhurst *et al.* 2007) and during asynchronous ovulation and oviduct transit which lasts 7.5 (Menkhurst *et al.* 2007) to 12 h (Selwood & Woolley 1991). Fertilisation occurs in the ampulla and zygotes enter the uterus at the pronuclear stage (Selwood & Hickford 1999).

The specific expression of dunnart *pri-miR-16c* in spermatogonial cells suggests that it may represent a novel specific genetic marker for spermatogonial cells. One current challenge is to be able to maintain spermatogonial stem cells indefinitely in culture followed by differentiation *in vitro* or *in vivo* when transplanted to recipient testes (van Pelt *et al.* 2002). Further studies of *pri-miR-16c* may provide better culture systems. The expression pattern of *pri-miR-16c* in both sexes in the gametes and the reproductive tract suggests a requirement for it at all stages of oogenesis and oocyte oviductal transit, during spermatogonial stages in the testis and maturation of the spermatozoa in the epididymis and in the fertilised zygote until it enters the uterus at the pronuclear stage. This could involve exchange of *pri-miR-16c* between the tract and the gametes. Recently, the transfer of proteins and RNA molecules (including immature and/or mature miRNAs) packaged in microvesicles has been demonstrated in mouse (Yuan *et al.* 2009) and human (Collino *et al.* 2010) stem cells, with implications in cellular reprogramming

and maintenance (reviewed by Camussi *et al.* (2010)). Collino *et al.* (2010) demonstrated the transfer of miRNAs from microvesicles of human bone marrow-derived mesenchymal stem cells (MSCs) to murine tubular epithelial cells (mTECs) and also showed that the expression of *miR-16-1* in these microvesicles can induce down-regulation of *BCL2* in target mTECs under the presence of a transcription inhibitor, α -amanitin. In addition, Hunter *et al.* (2008) detected *miR-16-1* in human plasma microvesicles, suggesting that they are transported in the bloodstream to regulate target cells. In MSC-derived microvesicles, the presence of AGO2 (Collino *et al.* 2010), an Argonaute protein which has recently been demonstrated to be involved in miRNA biogenesis (Cheloufi *et al.* 2010, Cifuentes *et al.* 2010), suggests that immature miRNA transcripts are also packaged for subsequent miRNA processing whether in the microvesicles and/or in destination cells. The sequence similarity between the *miR-15a/miR-16-1* and *miR-15c/miR-16c* clusters presented in this study and the high accumulation of membrane-bound vesicles of unknown composition in dunnart oocytes previously demonstrated by Kress *et al.* (2001) suggest that a similar mechanism could also be operating here.

We present here a putative non-coding RNA gamete maintenance factor, *pri-miR-16c*, and for the first time a gene with specific expression in gonads and reproductive tracts of both female and male. Further reverse genetic studies of *pri-miR-16c* will confirm its role in marsupial gamete maintenance and may shed light onto a novel signalling mechanism of miRNA-directed gene regulation between gametes and supporting cells.

Materials and Methods

Animals

The *S. macroura* used in this study were from a laboratory colony maintained by Lynne Selwood at the University of Melbourne, Department of Zoology, following Australian National Health and Medical Research Council Guidelines for the Care and Use of Animals for Scientific Purposes and held under permits issued by the Victorian Department of Sustainability and Environment.

RNA extraction

RNA was extracted from 50 to 100 mg of homogenised adult tissues (ovary, brain, testis, heart, liver, kidney, lungs, spleen, hindgut, oviducts, epididymis or vas deferens), or day 4 or day 7 PY bodies (heads removed) by two different methods sequentially, an acid phenol method (Trizol, Invitrogen) followed by column purification (RNAeasy Mini Kit, Qiagen) according to the manufacturers' protocols. PY bodies contain early stages in gametogenesis: day 4 PY, primordial germ cells and day 7, female PY oogonia (Kress *et al.* 2001). RNA was analysed on a 1.5% (w/v) agarose/

1 × MOPS/3% formaldehyde gel against 2.0 µg RNA markers (Promega) and its concentration was determined spectrophotometrically.

Cloning the full-length sequence of dunnart *pri-miR-16c* using 5' and 3' RACE

5' and 3' RACE were performed to obtain the 5' and 3' ends of the dunnart *pri-miR-16c* transcript, using the FirstChoice RLM-RACE kit (Ambion, Melbourne, VIC, Australia).

5' RACE: 15 µg of testis total RNA was treated with calf intestinal alkaline phosphatase to remove free 5'-phosphates from molecules such as rRNA, tRNA, any fragmented RNA and contaminating genomic DNA if present. This was followed by phenol/chloroform (v/v, 1:1) extraction and tobacco acid pyrophosphatase treatment to remove the 5' cap structure (^{7m}G-P-P-P) from full-length mRNA and ligation of processed full-length mRNA to 5' RACE adapters according to the manufacturer's protocols. Processed and ligated RNA was reverse transcribed using random decamers according to the manufacturer's protocols (FirstChoice RLM-RACE kit, Ambion). This first-strand cDNA was the template in PCR, using GoTaq Master Mix (Promega) according to the manufacturer's instructions, with 5' RACE adapter outer primer complementary to the 5' RACE adapter and 5' RACE *pri-miR-16c* outer primer (Fig. 1A) at 55 °C annealing temperature, 1 min extension time for 35 cycles. A nested PCR was performed by 2 µl of PCR product from the first PCR, 5' RACE adapter inner primer and 5' RACE *pri-miR-16c* inner primer (Fig. 1A) at 55 °C annealing temperature, 1 min extension time for 35 cycles. PCR products were analysed on a 1.5% agarose (w/v) gel in 1 × TAE buffer.

3' RACE: first-strand cDNA was synthesised from 1 µg ovary total RNA using 3' RACE adapter following the manufacturer's instructions. This cDNA was subjected to PCR, but using 3' RACE adapter outer primer complementary to the 3' RACE adapter and 3' RACE *pri-miR-16c* outer primer (Fig. 1A) at 55 °C annealing temperature, 1 min extension time for 35 cycles. A nested PCR was performed by 1 µl of PCR product from the first PCR, 3' RACE adapter inner primer and 3' RACE *pri-miR-16c* inner primer (Fig. 1A) at 55 °C annealing temperature, 1 min extension time for 35 cycles. PCR products were analysed on a 1.5% agarose (w/v) gel in 1 × TAE buffer.

5' and 3' RACE PCR products from the nested PCR were subcloned into pGEM-T Easy Vector (Promega) before transforming JM109 High Efficiency Competent Cells (Promega) according to the manufacturer's instructions. Colonies were grown overnight in 4 ml Luria-Bertani broth containing 400 µg ampicillin, before plasmid DNA preparation (QIAprep Spin Miniprep kit, Qiagen) following the manufacturer's instructions. Plasmid DNA was analysed by PCR using 5' RACE adapter inner primer and 5' RACE *pri-miR-16c* inner primer or 3' RACE adapter inner primer and 3' RACE *pri-miR-16c* inner primer, at 55 °C annealing temperature, 1 min extension time for 35 cycles, to identify clones containing the insert. PCR products were analysed on a 1.5% agarose (w/v) gel in 1 × TAE buffer to allow selection of positively transformed clones before sequencing using a commercial service.

Dunnart *pri-miR-16c* sequence analysis

The dunnart *pri-miR-16c* cDNA sequence was submitted to GenBank (accession number GQ844868). A nucleotide BLAST (NCBI) of the dunnart *pri-miR-16c* sequence was performed against the nucleotide collection, WGS and expressed sequence tag (EST) databases using the 'somewhat similar sequences' search setting. The dunnart *pri-miR-16c* nucleotide sequence was translated using the Translate tool programme (ExpASY) in six reading frames. Prediction of secondary structure was performed by a RNA secondary structure prediction programme (GeneBee).

pri-miR-16c expression analysis of dunnart gonadal, reproductive and somatic tissues by RT-PCR

Two micrograms of total RNA from adult *S. macroura* ovary, testis, brain, heart, liver, kidney, lungs, spleen, hindgut, oviducts, epididymis or vas deferens, or day 4 PY (primordial germ cells) or day 7 female PY (oogonia) were reverse transcribed in a 25 µl reaction volume containing: M-MLV RT 1 × Reaction Buffer (final concentration: 50 mM Tris-HCl (pH 8.5 at 25 °C), 75 mM KCl, 3 mM MgCl₂, 10 mM dithiothreitol), 40 nM dNTPs, 0.5 µg Oligo dT and 200 Units of M-MLV RT (M-MLV Reverse Transcriptase/RNase H Minus/Point Mutant, Promega). First-strand cDNA was used as template for PCR with dunnart *pri-miR-16c* forward (5'-GCTAGGCCCAAGTCAATCAAG-3') and reverse (5'-AAGCAGCAAGGCAATACTGG-3') primers. The 50 µl PCR contained: 5 µl RT reaction as template, 1 × PCR Buffer (final concentration: 10 mM Tris-HCl (pH 9.0 at 25 °C), 50 mM KCl and 0.1% Triton X-100), 0.2 µM dNTPs, 2 mM MgCl₂, 5 U Taq DNA Polymerase and 0.2 µM of each forward and reverse primer. First-strand cDNA templates were denatured at 94 °C for an initial 2 min, followed by 35 cycles (94 °C 30 s; at annealing temperature of 58 °C 30 s; 72 °C 30 s); followed by a final extension at 72 °C for 5 min. *GAPDH* was included as a positive control for loading of first-strand cDNA template in each RT-PCR using 0.2 µM (final concentration) of each *GAPDH* forward (5'-ACCCACGGCAAGTTCACCG-3') and reverse (5'-GCATGTCAGATCCACAACGG-3') primer for 35 cycles at an annealing temperature of 60 °C.

Collection of oocytes and follicle cells from secondary follicles

To determine localisation of ovarian expression, follicles were dissected free using two insulin syringe needles according to Merry *et al.* (1995) in warmed DMEM with L-glutamine (Invitrogen), on a 35 °C plate, under a dissecting microscope using transmitted light (Zeiss, North Ryde, NSW, Australia). Follicle diameters were measured on the longest axis, and the orthogonal axis, using a calibrated ocular micrometre. Follicles containing two layers of cuboidal follicle cells between 228–285 µm in diameter on the long axis and 217–277 µm in diameter on the short axis were identified as secondary follicles, and were within the expected range (Kress *et al.* 2001). Secondary follicles were digested in 0.05% trypsin (Sigma-Aldrich)/0.02% EDTA with vigorous pipetting using a 10 µl tip until the connections between follicle cells and oocyte

appeared loose. The addition of half volume of 1% BSA/PBS (Sigma–Aldrich) stopped digestion. Finally, the oocyte and follicle cells were separated mechanically using two insulin syringe needles, then washed in warm calcium- and magnesium-free PBS at 35 °C, collected in a minimal volume of PBS and stored at –80 °C.

Embryo collection

Animals were killed by inhalation of Halothane (Rhone Merieux, Melbourne, VIC, Australia), followed by cervical dislocation. The oestrous period of about 7 days is characterised by the presence of cornified epithelial cells in high numbers in the urine, and a rise and then fall in weight on the day of ovulation (Selwood & Woolley 1991). To collect stage-specific embryos, females were killed late on the day of ovulation and on subsequent days of gestation. Early cleavage-stage conceptuses (zygote to 64-cell unilaminar blastocyst stage) were collected between day 0.5 and day 3.5 of gestation, following the most recent *S. macroura* embryonic development timetable (Selwood & Hickford 1999). Some cleavage stages were collected from females induced to ovulate using equine serum gonadotrophin (Au *et al.* 2010a). Collection of these conceptuses occurred between day 7 and day 11 post-initial injection (days 1–4 of gestation), following an induced ovulation timetable (Au *et al.* 2010a). Female reproductive tracts were removed and washed in PBS (35 °C) and examined under a dissecting microscope. Ovaries were examined to determine oestrous cycle stage by the presence of antral follicles or corpora lutea, which were counted to give an estimate of the number of embryos/partenotes in the uteri. The transparent oviducts were examined under a transmitted light dissecting microscope to detect oocytes. Each uterus was transferred to warmed culture medium (DMEM with L-glutamine, Sigma–Aldrich), slit longitudinally along the midline and carefully everted to roll conceptuses into the medium. Embryos were analysed to assess for evidence of fertilisation, stage and normal development, washed in PBS[–] and collected in a minimal volume of PBS and stored at –80 °C.

pri-miR-16c expression analysis of dunnart oocyte, follicle cells and conceptuses by RT-PCR

Secondary follicles separated into primary oocytes and follicle cells, and conceptuses were lysed using 10 µl lysis buffer for every two oocytes or conceptuses and 50 µl per set of follicle cells from each follicle before DNase treatment and 10 µl of lysate reverse transcribed using random decamers (cells-to-cDNA kit, Ambion) following the manufacturer's instructions. First-strand cDNA (2.5 µl) was used as template for a 50 µl PCR with dunnart *pri-miR-16c* forward (5'-GCTAGGCCCAAGTCAATCAAG-3') and reverse (5'-AAG-CAGCAAGGCAATACTGG-3') primers, using GoTaq Master Mix (Promega), using the same conditions as previously described and according to the manufacturer's instructions.

Although *GAPDH* is commonly used as a loading control for RT-PCR, it is inactivated during the meiotic and post-meiotic phase due to inactive glycolysis in germ cells and conceptuses

(Mori *et al.* 1992, Welch *et al.* 1992, Eddy *et al.* 1994, Aswal *et al.* 2008). Thus, *18S* (ribosomal subunit 18S gene) was the control used in RT-PCRs for oocytes, follicle cells and conceptuses. Each loading control RT-PCR contained 0.2 µM (final concentration) of each *18S* forward (5'-GATCCATTG-GAGGGCAAGTCT-3') and reverse (5'-CCAAGATCCAACCTAC-GAGCTTTT-3') primer for 35 cycles at an annealing temperature of 55 °C.

In situ hybridisation

In situ hybridisations were performed by ovaries (from three different adult females all of which were at the same stage of the oestrous cycle), and testes and epididymides from two adult males. *S. macroura* ovaries, testes and epididymides were fixed in 4% paraformaldehyde in 1× PBS overnight at 4 °C before paraffin-embedding. One microgram plasmid (pGem-T Easy vectors, Promega) containing dunnart *pri-miR-16c* cDNA was used to synthesise sense and antisense DIG-labeled riboprobes by *in vitro* transcription using a DIG RNA-labeling kit (SP6/T7) (Roche Diagnostics) according to the manufacturer's instructions. DIG-labeled riboprobes synthesised by SP6 and T7 were 523 and 519 bp respectively. Probes were purified using QIAquick PCR Purification Kit (Qiagen) following the manufacturer's instructions, eluted in 30 µl of DNase/RNase-free water and stored in single use aliquots at –80 °C.

DIG-labeled sense or antisense riboprobes (400 ng/ml) in 50–100 µl hybridisation buffer (50% deionised formamide, 5× sodium chloride–sodium citrate buffer (SSC), 5× denhardtts, 10 µg/ml yeast tRNA (Roche Diagnostics), 0.1% Tween, 10 µg/ml denatured salmon sperm) were added to tissue sections before hybridisation at 55 °C for 16–20 h. Following two immersions in 2× SSC for 10 min at RT, sections were subjected to a final stringent wash (1× SSC for 15 min at 55 °C). Tissues were blocked in 1× PBST (1× PBS/1% Tween)/0.1% sheep serum for 60 min at RT before incubating with anti-DIG-FAB antibody (Roche Diagnostics, 1:200 dilution) for 16–20 h at 4 °C. Colour detection was achieved using 20 µl/ml NBT/BCIP (Roche Diagnostics) in staining buffer (0.1 M Tris pH 9.5, 0.1 M NaCl, 50 mM MgCl₂, 0.1% Tween, 5 mM tetramisole) for up to 60 min at RT. Sections were counterstained in 0.2% nuclear fast green for 5 min.

Follicles were determined to be primordial, primary, secondary, tertiary or antral predominantly based on size but also on morphology for the antral follicle stage. Oocytes were measured along the longest axis, and the axis perpendicular in relation to the scale bar. The measurements were within the expected range for the identified follicles (Kress *et al.* 2001, Nation & Selwood 2009).

All experiments were replicated ($n=2-4$) using samples obtained from different animals or PY.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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