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ABSTRACT

Background: Our understanding of epididymal physiology and function has been transformed over the three decades in which the International Meeting Series on the Epididymis has been hosted. This transformation has occurred along many fronts, but among the most significant advances has been the unexpected discovery of the diversity of small non-protein-coding RNAs (sRNAs) expressed in the epididymal epithelium and differentially accumulated in the luminal population of spermatozoa.

Objectives: Here we survey recent literature pertaining to profiling the sRNA landscape of the mammalian epididymis with an ultimate goal of demonstrating the contribution that these key regulatory elements, and their associated pathways, make to epididymal physiology and sperm maturation.

Results and Discussion: High throughput sequencing strategies have fueled an unprecedented advance in our understanding of RNA biology. In the last decade, such high throughput profiling tools have been increasingly applied to study the mammalian epididymis, presaging the discovery of diverse classes of sRNA expressed along the length of the tract. Among the best studied sRNA classes are the microRNAs (miRNA), a sRNA species shown to act in concert with endocrine signals to fine tune the segmental patterning of epididymal gene expression. In addition to performing this homeostatic role, epithelial cell derived sRNAs also selectively accumulate into the epididymosomes and spermatozoa that occupy the duct lumen. This exciting discovery alludes to a novel form of intracellular communication that contributes to the establishment of the sperm epigenome and its modification under conditions of paternal stress.

Conclusion: Compelling literature has identified sRNAs as a crucial regulatory tier that allows the epididymis to fulfil its combined roles of sperm transport, maturation and storage. Continued research in this emerging field will contribute to our growing understanding of the

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etiology of male factor infertility and potentially allow for the future design of rational therapeutic options for these individuals.

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INTRODUCTION

The production of fertilization competent mammalian spermatozoa encompasses a complex sequence of cytological differentiation and functional maturation events. The latter coincides with passage through the epididymis, during which time the transcriptionally and translationally inert spermatozoa are remodeled in such a way that they gain the potential to display forward progressive motility, complete capacitation and express affinity for the cumulus-oocyte complex (Cornwall, 2009; Dacheux & Dacheux, 2014). Such functional transformation is not an intrinsically encoded program, instead this transformation is driven by the complex array of extrinsic factors that the sperm encounter as these cells transit the epididymis (Aitken*, et al.*, 2007). A defining feature of this microenvironment is that it is continuously modified throughout the epididymal tubule as a result of two opposing cellular mechanisms, namely the secretory and absorptive activity of the surrounding epithelial cells (Dacheux*, et al.*, 2016).

For decades, research has sought to characterize the complex interplay between epididymal secretory / absorptive activity and sperm maturation. A focus of this research has been to document the complement of proteins secreted into the epididymal lumen, and subsequently, to functionally characterize the ability of secreted proteins to influence sperm maturation (Syntin*, et al.*, 1996; Nixon*, et al.*, 2002; Nixon*, et al.*, 2002; Nixon*, et al.*, 2002; Zhou*, et al.*, 2004; Dacheux*, et al.*, 2009; Belleannee*, et al.*, 2011; Belleannee*, et al.*, 2011; Dacheux & Dacheux, 2014; Nixon*, et al.*, 2014; Labas*, et al.*, 2015; Dacheux*, et al.*, 2016; Nixon*, et al.*, 2016; Nixon*, et al.*, 2018). Taken together, this work has highlighted the importance of specialized epididymal segments as playing discrete roles in the post-testicular maturation and storage of spermatozoa. Indeed, high-resolution transcriptional profiling has identified greater than 17,000 transcripts expressed in the epididymis of model species such as the mouse, with 2,168 genes demonstrated differentially expressed (that is, gene expression is either elevated or reduced by more than 4-fold between at least two epididymal segments) along the length of the tract (Johnston*, et al.*, 2005). In the mouse epididymis, such transcriptomic studies have now defined six transcriptionally distinct segments (Johnston*, et*

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al., 2005), a finding that alludes to the involvement of a highly sophisticated regulatory network to allow such strict spatial control over segmental gene expression. While we continue to debate these mechanisms, it is apparent that steroidal hormones, and in particular androgens, are key regulators that tightly control epididymal gene expression (reviewed in (Belleannee*, et al.*, 2012; Sipila & Bjorkgren, 2016)). Despite this knowledge, the balance of evidence indicates that neither androgens, nor the myriad of alternative lumicrine factors synthesized in the testis, are sufficient to fully account for the intricate coordination of gene expression, and hence the creation of distinct segmental microenvironments, along the length of the epididymis (Sipila & Bjorkgren, 2016).

Instead, it has been a long held view that to enable the degree of complexity of gene expression variation along the length of the epididymis, an additional tier of regulation exists. At the forefront of candidates that could direct this degree of flexible control of gene expression are the small non-protein-coding RNAs (sRNAs); a diverse class of master molecular regulators originally identified in the mammalian epididymis by the pioneering work conducted by the Zhang (Zhang*, et al.*, 2010; Ni*, et al.*, 2011; Zhang*, et al.*, 2011; Li*, et al.*, 2012; Ma*, et al.*, 2013; Chu*, et al.*, 2015), Sullivan and Belleannee (Belleannee*, et al.*, 2012; Belleannee*, et al.*, 2012), and Sipila (Bjorkgren*, et al.*, 2012; Bjorkgren*, et al.*, 2015; Bjorkgren & Sipila, 2015) research groups. Small RNAs direct RNA silencing at either the transcriptional or posttranscriptional level to regulate gene expression, and in the mammalian epididymis, a particular emphasis has been placed on the regulatory role directed by the microRNA (miRNA) class of sRNA. Specifically, miRNAs are abundantly expressed, and highly conserved, in the epithelial cells that line each of the three distinct segments of the mammalian epididymis (Anderson*, et al.*, 2015; Nixon*, et al.*, 2015). Building on these initial discoveries, recent advances in sRNA profiling techniques has fueled rapid growth in our appreciation of the tremendous volume and diversity of sRNAs that accumulate in epididymal tissues as well as to advance our understanding of their contribution to controlling gene expression along the length of this tract (Sharma*, et al.*, 2016; Hutcheon*, et al.*, 2017). Furthermore, an emerging body of recent evidence indicates that the role of epididymal sRNAs may extend well beyond that of homeostatic gene expression regulation within the epithelium, to include a more direct influence over the process of sperm maturation (Nixon*, et al.*, 2015; Conine*, et al.*, 2018; Sharma*, et al.*, 2018). In this context, our still emerging understanding points to complex pathways of intracellular communication between the epididymal soma and spermatozoa. Thus, epididymal derived sRNAs have been shown to

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selectively accumulate into extracellular vesicles, termed epididymosomes, culminating in their delivery to the spermatozoa held within the lumen of duct (Belleannee*, et al.*, 2013; Reilly*, et al.*, 2016; Sharma*, et al.*, 2018). This novel form of intercellular communication has, in turn, been implicated in the establishment of the sperm epigenome as well as its modification under conditions of paternal stress. Accordingly, in this review, we survey recent literature pertaining to profiling the sRNA landscape of the mammalian epididymis with an ultimate goal of demonstrating the contribution that these key regulatory elements, and their associated pathways, make to epididymal physiology and sperm maturation.

Overview of small non-protein-coding RNAs

Shortly after the First International Meeting on the Epididymis was convened in 1992, the first miRNA (*lin-4*) was discovered in *Caenorhabditis elegans* where it was shown to act as an endogenous regulator of the expression of genes involved in developmental timing control (Lee*, et al.*, 1993). Five years later, and coinciding with the Second International Meeting of the Epididymis, Andrew Fire and colleagues reported that exogenously applied double-stranded RNA (dsRNA) specifically silenced the activity of genes of high complementarity via a mechanism of gene expression regulation termed RNA interference (RNAi) (Fire*, et al.*, 1998; Montgomery & Fire, 1998). Over the course of the ensuing two decades, the term RNAi has come to encompass an expansive family of interrelated pathways with far reaching practical and applied implications. Indeed, the thousands of studies emanating from these seminal discoveries have transformed our understanding of RNA biology, with one of the most significant knowledge advances stemming from the discovery that small (< 200 nucleotides) non-protein-coding RNAs exert their regulatory function at virtually all genomic levels, including controlling chromatin structure and chromosome segregation, regulating RNA transcription, processing and stability, and influencing amino acid translation efficiency and/or protein stability. Since the functional effects of sRNAs are principally inhibitory (Guo*, et al.*, 2010), the regulatory mechanisms they govern are considered under the broad theme of RNA silencing (Castel & Martienssen, 2013). In this capacity, most sRNA species primarily serve as a specificity factor, responsible for guiding the activity of an effector protein or complex to which the sRNA is bound to direct the targeting of complementary nucleic acids via either sRNA:DNA or sRNA:RNA base-pairing interactions.

Almost invariably, the functional core of the protein effector machinery is formed by a member of the Argonaute (AGO) superfamily of endonucleases (Hutvagner & Simard,

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2008). Although numerous classes of sRNAs have now been documented across evolutionarily diverse eukaryotes, they are broadly classified into the main categories of miRNAs, short-interfering RNAs (siRNAs), Piwi-interacting RNAs (piRNAs), and transfer RNA (tRNA)-derived RNA fragments (tRFs), a classification system based on the genomic origin, the structure of the precursor molecule, and the biological role directed by the AGO-bound sRNA (Leung*, et al.*, 2016). As additional subclasses of sRNAs are continuously being identified, the boundaries defining each sRNA class are becoming increasingly blurred (Kuksa*, et al.*, 2018). However, the most distinguishing feature of each sRNA class is their association with distinct subset(s) of AGO effector proteins. More specifically, miRNAs and siRNAs bind to distinct members of the AGO clade of the AGO protein superfamily (Meister*, et al.*, 2004), whereas piRNA sRNAs bind to members of the PIWI clade of this protein superfamily (Fu & Wang, 2014). Although we do not wish to discount the importance of the regulatory role directed by other sRNA subclasses, for the purpose of this review, we will only discuss the production and action of the miRNA, tRF and piRNA sRNA classes, prior to presenting examples of their proposed functional roles in epididymal physiology.

miRNAs: studies of the biological roles of canonical RNAi pathways have focused largely on the regulation of gene expression in which miRNAs fulfil the role of endogenous specificity guides to direct the regulatory action of the pathway. Indeed, miRNAs have emerged as almost ubiquitous components of gene regulatory circuits, fulfilling an analogous role to that of transcription factors, albeit at the level of post-transcriptional control of biological pathways (Berezikov, 2011). miRNAs are defined as short (approximately 19-22 nucleotides (nt) in length), single-stranded, non-protein-coding molecules of regulatory RNA. miRNAs are initially synthesized by RNA polymerase II to form primary miRNA (pri-miRNA) transcripts that can be several hundred nucleotides long, and upon folding, form a stem-loop structured molecule of imperfectly dsRNA (Bartel, 2004). Sequence motifs that flank the stem-loop region of the pri-miRNA mediate the binding of the nucleus localized dsRNA-binding protein, DiGeorge syndrome critical region 8 (DGCR8), and once bound, DGCR8 ensures accurate positioning and therefore, efficient processing of the stem-loop region of the pri-miRNA by the endonuclease, DROSHA (Ha & Kim, 2014). Liberation of the pri-miRNA stem-loop by the DGCR8/DROSHA functional partnership generates the precursor-miRNA (pre-miRNA), a processing intermediate of shorter length that is exported from the nucleus into the cytoplasm of the cell for a subsequent round of processing. In the

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cytoplasm, the pre-miRNA is bound by a second dsRNA-binding protein, transactivation response element RNA-binding (TRBP), which like DGCR8 in the nucleus, ensures accurate and efficient pre-miRNA processing by its partnering endonuclease, DICER, to generate the miRNA:miRNA* duplex of approximately 22-nt in length (Berezikov, 2011). The miRNA/miRNA* duplex is subsequently loaded into the catalytic core of the miRNA-directed RNA silencing complex (miRISC), wherein Argonaute2 (AGO2) retains one duplex strand while degrading the other duplex strand to form an activated miRISC. Although either strand of the duplex may potentially act as a functional miRNA, one strand is usually preferentially utilized to guide activated miRISC to target mRNA transcripts, that is; mRNAs that harbor complementary binding sequences within their 3' untranslated regions (3' UTRs). Upon target transcript binding, miRISC elicits its posttranscriptional regulatory function, with the fate of the targeted mRNA varying from enhanced degradation or destabilization of the transcript, or repression of the efficiency of translation initiation (O'Brien*, et al.*, 2018).

The mechanism of silencing directed by a RISC-loaded sRNA is influenced by the complementarity of the sRNA:target gene hybrid, and by the catalytic activity of the AGO protein at the functional core of RISC (Bartel, 2009). Herein lies an important distinction between the miRNAs and the siRNA subclass of sRNA, that is; due to low levels of miRNA:mRNA complementarity, each miRNA can potentially regulate the expression of many functionally unrelated target genes. By contrast, endo-siRNAs only direct expression repression of target genes that harbor target sites of perfect complementarity. Thus, each endo-siRNA only regulates the expression of a single target gene. Furthermore, the mechanism of endo-siRNA target gene expression repression, RISC-catalyzed target transcript cleavage, is also distinct to that of the predominant mechanism of silencing directed by miRISC, translational repression (Claycomb, 2014). Whilst sRNA-directed RISC activity can be readily linked to homeostatic regulation of epididymal structure and function (Bjorkgren & Sipila, 2015), sRNA-directed RNA silencing would seem of little biological relevance for the transcriptionally and translationally inert spermatozoa residing within the lumen of the duct. Nevertheless, and as discussed below, the sRNA signature of epididymal sperm displays considerable plasticity (Nixon*, et al.*, 2015; Hutcheon*, et al.*, 2017), with such changes potentially influencing the downstream trajectory of early embryo development and ultimately, the health of offspring (Yuan*, et al.*, 2016; Conine*, et al.*, 2018).

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tRFs: tRNA-derived fragment biogenesis is initiated in the nucleus via the transcription of precursor tRNA (pre-tRNA) transcripts from tRNA loci by RNA polymerase III. Following transcription, the pre-tRNA undergoes extensive modification, with the 5' and 3'-trailer sequences being catalytically cleaved off the precursor via the action of RNase P and RNase Z, respectively. A triplet CCA motif is next added to the 3'-terminus of the pre-tRNA to eventually serve as the site of amino acid attachment, and the mature tRNA so formed is then transported across the nuclear envelope. Post export to the cytoplasm, mature tRNAs fulfil their primary function as a molecular linker during protein translation on the ribosomes by providing the specificity that links the decoded genetic sequence of the transcript template to the amino acid chain being translated (Kumar*, et al.*, 2016). However, aside from this essential role, a considerable portion of the total pre-tRNA pool transcribed, also experiences significant, yet highly specific fragmentation, leading to the production of the tRNA-derived fragment (tRF) class of sRNA (Kumar*, et al.*, 2016). tRF sRNAs are single-stranded RNAs, 14 to 32-nt in length, and are distinct from the stress-induced tRNA fragments that are generated via cleavage of the anti-codon loop of a mature tRNA (Lee*, et al.*, 2009). Different tRF structural subtypes originate from the extreme 5' (tRF-5) or 3' ends (tRF-3) of mature tRNAs, or alternatively, from the 3' trailer sequence of each pre-tRNA (tRF-1) (Kumar*, et al.*, 2016). Such cleavage events are mediated by a number of endonucleases, including angiogenin, DICER, RNase Z and RNase P, and since they are derived from mature tRNAs of highly conserved sequence, the sequence of most tRF-5 and tRF-3 sRNAs are highly similar across species (Keam & Hutvagner, 2015).

It has been estimated that in human and mouse cells, the total tRF population is composed of more than 150 tRF sRNAs of distinct sequence composition, with some tRFs accumulating to frequencies of more than 20 reads per million sequenced reads; a level of abundance that is significantly higher than the read numbers recorded for most miRNAs detected in the same tissues. Different tRF subtypes have a diverse spectrum of functional activities, having been shown to be loaded into AGO-catalyzed RISCs to direct expression repression by guiding RISC activity to either the promotor region, or the 5' UTR of a targeted gene/target gene transcript (Kumar*, et al.*, 2016). Alternatively, tRFs can contribute to the formation of stress granules, or can displace mRNAs already bound by regulatory RNA-binding proteins (Ivanov*, et al.*, 2011). As discussed below, tRFs have risen to prominence in the field of male reproductive health owing to their abundant accumulation to become the dominant sRNA species harbored by mature sperm residing in the distal caudal segment of the epididymis (Sharma*, et al.*, 2016; Hutcheon*, et al.*, 2017). Of further interest, the sperm-

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borne tRF profile also exhibits significant alteration in response to a variety of paternal stressors (Chen*, et al.*, 2016; Sharma*, et al.*, 2016).

piRNAs: P-element induced wimpy testes (PIWI)-interacting RNAs are among the most highly expressed sRNA class within the mammalian testis where they exert influence over multiple stages of germline development, encompassing *de novo* DNA methylation, meiosis and spermiogenesis (Fu & Wang, 2014). Such function forms a central component of an elaborate defensive mechanism that ensures genomic integrity of the male germline via repressing the activity of the many numerous different types of mobile genetic element, including suppression of transposable element activity (Siomi*, et al.*, 2011). This is achieved via piRNA sRNA association with canonical PIWI proteins leading to the formation of piRNA-induced silencing complexes (piRISC) (Hutvagner & Simard, 2008). In contrast to miRNAs, which only number in the hundreds to thousands, piRNA analysis has revealed the existence of hundreds of thousands of individual piRNA sequences, few of which are conserved among different species (Siomi*, et al.*, 2011). Furthermore, with the exception of a strong bias for uridine at sRNA nt position 1, piRNAs generally share few other distinguishing sequence motifs. Nevertheless, despite their exceptional diversity, most piRNAs originate from a relatively small number of genomic regions, which are referred to as piRNA clusters (Siomi*, et al.*, 2011). piRNA clusters extend from several to more than 200 kilobases in length and comprise multiple sequences capable of encoding piRNA sRNAs. Whilst the precise mechanisms responsible for primary processing of piRNA clusters remains to be fully resolved, it is known that these genomic loci are initially transcribed as long (i.e., tens to hundreds of kilobases) single stranded transcripts. These nascent piRNA precursor transcripts are then subjected to 5′ capping and 3′ polyadenylation before being processed into shorter piRNA intermediates and loading into a corresponding PIWI protein (Siomi*, et al.*, 2011).

Distinct from either miRNA or siRNA precursors, the regions flanking the sequences processed into mature piRNAs, lack the propensity to form dsRNA structures, and therefore, piRNA production is a DICER-independent process. The PIWI-loaded piRNA intermediates subsequently undergo 3' to 5' exonucleolytic trimming and 2'-O-methylation at their 3' terminus to lead to the formation of mature primary piRNAs. Of interest, piRNAs associating with different members of the PIWI protein family are of distinct size. In mice for example, 26, 28 and 30-nt piRNAs preferentially associate with PIWIL2 (MILI), PIWIL4 (MIWI2) and

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PIWIL1 (MIWI), respectively, a finding that strongly suggests that the PIWI proteins assist in the determination of the size of the mature piRNA produced, and further; piRNA size sorting may occur to direct a different mechanism of target sequence suppression, or to distinguish between the sequences targeted for suppression (Siomi*, et al.*, 2011).

Of the many thousands of piRNAs produced in a cell, only a small fraction of these will be used by the cell to trigger the formation of a secondary population of piRNAs, a production pathway known as the ping-pong cycle. This amplification loop is initiated once a PIWI protein loaded with a mature primary piRNA binds to its targeted transposon-derived transcript to catalyze PIWI-mediated cleavage at the 5' end of the targeted non-protein-coding transcript. Post cleavage, this new piRNA molecule undergoes further maturation as per the primary piRNA production pathway, to produce a mature secondary piRNA (Iwasaki*, et al.*, 2015). Aside from their surveillance over germline transcription, recent studies in mice have shown that piRNAs generated by the primary processing pathway, disproportionately accumulate in the mature sperm harbored in the distal cauda segment of the epididymis; a dramatic contrast to their baseline abundance in their immature counterparts that enter the caput epididymis (Hutcheon*, et al.*, 2017). The impetus to investigate the epididymal contribution to this phenomenon is given credence by the fact that the piRNA sRNA class can also constitute as much as 17% of the complex sRNA profile of ejaculated spermatozoa of normozoospermic individuals (Krawetz*, et al.*, 2011) and moreover, that this profile of sperm-borne piRNAs is dramatically altered in response to pathologies such as obesity (Donkin*, et al.*, 2016).

Endogenous sRNA pathways in epididymal tissue

The pseudostratified epithelium of the epididymal tubule is dominated by principal cells, with this cell type constituting as much as 80% of the peritubular interstitium (Abe*, et al.*, 1983). Within the proximal caput and corpus segments of the epididymis, principal cells are characterized by their exceptional capacity for protein synthesis and secretion (Hermo & Robaire, 2002; Dacheux*, et al.*, 2009). By contrast, in the more distal cauda segment, principal cells act in tandem with the clear cell population to mediate the uptake and recycling of various luminal components (Hermo & Robaire, 2002). This gradient of differing cellular activity, combined with the distribution of a small number of additional cell types (including narrow cells, apical cells, basal cells as well as those involved in immune

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functions), contributes to the creation of successive, regionally distinct luminal microenvironments (Wong, *et al.*, 2002). Indeed, since the majority of testicular fluid is reabsorbed prior to entering the proximal segments of the epididymis, the luminal composition of downstream segments is dictated by the combined secretory and absorptive activity of the epithelial cells that line the duct (Dacheux, *et al.*, 2016). Accordingly, comprehensive transcriptomic analysis has identified the expression of more than 17,000 genes in the epididymal epithelium of model species such as the mouse (Johnston, *et al.*, 2005). Amongst the expressed genes, ~13% are characterized by changes in expression of at least 4-fold between any two segments; a number that increases to ~36% if the criterion is relaxed to include transcripts that vary in abundance by at least 2-fold across two epididymal segments (Johnston, *et al.*, 2005). Exactly how this precise regulation is imposed remains unclear, but it is notable that similar profiles of epididymal gene and protein expression have been reported in many species, including domestic ungulates and humans (Dacheux, *et al.*, 2009; Guyonnet, *et al.*, 2011; Browne, *et al.*, 2016). One possible explanation is the expression of a myriad of sRNAs within the epididymal epithelial cells (Figure 1).

To date, the focus for most sRNA investigations has been the miRNA class. Thus, the application of microarray and high throughput sequencing methodologies has led to the identification of complex profiles of several hundred miRNAs in the epididymis of species such as the human (Zhang, *et al.*, 2010; Belleannee, *et al.*, 2012; Li, *et al.*, 2012), rat (Zhang, *et al.*, 2011), mouse (Nixon, *et al.*, 2015) and bovine (Belleannee, *et al.*, 2013); with several of these miRNAs being significantly enriched or unique to the epididymis. With the caveat that most of these studies have not sought to demarcate the epididymis beyond the broad segments of the caput, corpus and cauda, they have nevertheless shown that a large portion of epididymal miRNAs are equivalently expressed along the length of the tract (i.e., ~75% of miRNAs in the mouse epididymis), and are also conserved between different species (e.g., ~31% of miRNAs identified in the mouse and human epididymis are conserved) (Nixon, *et al.*, 2015). Such findings raise the prospect that miRNAs may fulfil key regulatory roles in the maintenance of epididymal homeostasis. By contrast, as many as 15% of miRNAs identified in the mouse epididymis appear to accumulate differentially with greater than 2-fold changes in abundance documented across different epididymal segments (Nixon, *et al.*, 2015). In some instances, these segmental changes in miRNA abundance are highly pronounced with key examples being the *miR-204-5p* and *miR-196b-5p* sRNAs, which have reduced and elevated fold changes in abundance of ~39- and ~45-fold respectively, between

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the caput and caudal segments of the mouse epididymis (Nixon, *et al.*, 2015).

The biological implications of such differences in miRNA abundance are highlighted by the fact that, owing to the tolerance of relatively low levels of miRNA/target transcript sequence complementarity, each miRNA can potentially regulate the expression of many multiple, functionally unrelated target genes (Bartel, 2009). Illustrative of this, target prediction algorithms suggest that an estimated 8,000 target gene transcripts are putatively under sRNA-directed expression regulation by the pool of miRNAs that differentially accumulate along the length of the mouse epididymis (Wong & Wang, 2015). Therefore, differing spatial profiles of miRNA abundance may impose a formidable level of regulatory control over the unique segmental environments in the epididymis. Adding to this complexity, recent studies have also documented substantial spatio-temporal variation in the global sRNA landscape of epididymal epithelial cells (Hutcheon, *et al.*, 2017) (Figure 1). For instance, the relative abundance of the miRNA component of the global sRNA population is reduced from ~38% in caput epithelial cells, to ~27% in the epithelial cells isolated from the cauda segment. This dynamic reduction in miRNA accumulation occurs commensurate with a reciprocal increase in the proportion of the tRF sRNA class across equivalent epididymal segments, that is; tRFs account for 14% and 27% of the global sRNA population of mouse caput and cauda epithelial cells, respectively (Hutcheon, *et al.*, 2017). Notwithstanding these changes, the proportion of sRNAs belonging to the piRNA, small nucleolar RNA (snoRNAs) and small nuclear RNA (snRNA) classes show little variation along the length of the epididymal tract, together accounting for less than 5% of the global sRNA population determined to accumulate in any one segment (Hutcheon, *et al.*, 2017) (Figure 1).

The notion that miRNAs may be critical for the regulation of epididymal physiology and hence sperm maturation, is supported by both targeted and global miRNA manipulation strategies. In the former, the injection of a synthetic miRNA analog (agomir) directly into the cauda epididymis of adult rats has been shown to precipitate a cascade of molecular changes that are of sufficient biological significance to compromise the fidelity of sperm maturation. More specifically, *mil-HongrES2* microinjection led to a marked reduction in both gene and protein expression of the epididymal carboxylesterase CES7, resulting in defects in sperm capacitation (Ni, *et al.*, 2011). Building on these observations, the extent of miRNA contribution to sperm maturation has been demonstrated through conditional proximal epididymal knock-out of DICER1; a key enzyme responsible for the maturation of miRNAs. This strategy elicited a rapid dedifferentiation of the epididymal epithelium, perturbation of

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steriod signaling, altered lipid homestatis, and a concomitant loss of fertility (Bjorkgren*, et al.*, 2012; Bjorkgren*, et al.*, 2015). However, the prospect that at least some of these responses may be attributed to miRNA-independent mechanisms has been raised by recent work featuring the selective disruption of *Dicer1* in the principal cells of the distal caput epididymis (Tang*, et al.*, 2018). Specifically, this study implicated DICER1 in the direct transcriptional control of a subset of beta-defensin genes; genes that encode key elements of the epididymal antibacterial defense system. Accordingly, the epididymal antibacterial activity of *Dicer1* -/- males was significantly compromised, precipitating age-associated fertility defects analogous to those of the human epididymitis disease (Tang*, et al.*, 2018). Whilst such findings encourage caution in the interpretation of *Dicer1* knockout phenotypes, they do not discount mounting evidence for both indirect and direct roles of sRNAs in promoting sperm maturation.

The sRNA profile of epididymal sperm

It is well established that, despite their transcriptionally inert state, mature spermatozoa are endowed with a substantial and diverse cargo of sRNAs, including miRNAs, endo-siRNAs, tRFs and piRNAs, as well as numerous additional sRNA molecules derived from snRNAs, snoRNAs and ribosomal RNAs (rRNAs) (Miller & Ostermeier, 2006; Krawetz*, et al.*, 2011; Jodar*, et al.*, 2013; Chu*, et al.*, 2017). While the foundations of this sRNA landscape are undoubtedly established during the testicular phases of sperm development, we now appreciate that the detected sRNAs are not simply static remnants of the spermatogenic cycle with limited consequences for fertilization, or early embryonic development. Rather, this early view has been superseded by evidence that sRNAs are capable of being delivered to the oocyte, along with the paternal genome, at the time of fertilization (Jodar*, et al.*, 2013). Thereafter, the sperm-borne sRNAs have been implicated in controlling the stability and translational efficiency of maternal transcripts before the zygotic genome is activated (Conine*, et al.*, 2018). Such findings are of significance since each of these contributions can contribute to sperm dysfunction. Accordingly, it has been shown that spermatozoa exhibiting lesions in their morphology and/or motility, possess different miRNA profiles to those of their phenotypically normal counterparts (Jodar*, et al.*, 2012; Abu-Halima*, et al.*, 2013; Abu-Halima*, et al.*, 2014; Jodar*, et al.*, 2015). Moreover, aberrant miRNA accumulation has been reported in blastocysts derived from patients with male factor infertility, suggestive of a

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contribution from sub-fertile sperm that adversely affected the phenotype of the resulting embryo (McCallie*, et al.*, 2010). Coupled with evidence for the transmission of paternal traits acquired by environmental conditions to the offspring, there is mounting interest in the potential of sperm-borne sRNAs to act as information carriers responsible for relaying non-genetic information between generations (Perez & Lehner, 2019). This potential has in turn stimulated interest in whether the prolonged phases of epididymal transit and storage presents a window for remodeling of the sperm sRNA landscape under normal physiological conditions and/or in response to various pathologies and environmental stressors.

In keeping with this model, a number of studies have begun to report a considerable level of plasticity in the overall sRNA landscape of sperm, the sRNAome. Indeed, interrogation of our own sRNA sequencing datasets has revealed pronounced changes in both the relative abundance, and the overall proportion, of the different sRNA classes harbored by mature mouse spermatozoa versus that of the immature cells sampled from the proximal epididymis (Nixon*, et al.*, 2015; Hutcheon*, et al.*, 2017) (Figure 1). Illustrative of this phenomenon, miRNAs were identified as being highly enriched in caput spermatozoa where they accounted for ~50% of the global sRNA population. However, miRNAs were dramatically reduced in their abundance, to the point where they represented only ~16% of the sRNA transcripts in caudal spermatozoa (Hutcheon*, et al.*, 2017). A number of other trends were also readily evident in the sRNA profiles of spermatozoa, including an accumulation of the tRF component of the total sRNA population of spermatozoa, increasing from 24% in caput spermatozoa, to 65% in spermatozoa sampled from the cauda segment (Figure 1). Such findings have been independently validated (Sharma*, et al.*, 2016), and of considerable interest, is the parallel demonstration of highly similar trends in miRNA and tRF abundance within the epididymal soma (Nixon*, et al.*, 2015). Whilst these data provide correlative evidence of a link between the levels of epididymal epithelial miRNA and tRF sRNAs to those of the sperm within the lumen of the duct, the piRNA class of sRNA does not exhibit a similar trend in changing abundance (Hutcheon*, et al.*, 2017). Indeed, despite a substantial enrichment of piRNAs in cauda sperm (a sRNA class that was essentially absent in immature caput spermatozoa, before representing ~5% of the total sRNA population of mature cauda spermatozoa), no such change was detected in the epithelial cells that line the cauda segment of the epididymis (Hutcheon*, et al.*, 2017).

While various models for their origin are under consideration (see below), the diversity and abundance of sRNAs within mature cauda spermatozoa, relative to their immature

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counterparts, raises a number of additional questions regarding the consequences of this dynamic remodeling process. We have previously speculated that modification of the sperm miRNA signature may not be intimately tied to the functional maturation of these cells (i.e., miRNAs with altered abundance are not essential for zygote formation or embryo development) (Nixon*, et al.*, 2015) owing to decades of research demonstrating that successful fertilization, and apparently normal embryo development, can be readily achieved via intracytoplasmic injection (ICSI) of spermatozoa aspirated from the testes and proximal epididymal regions (Schoysman*, et al.*, 1993; Schoysman*, et al.*, 1993). Rather, we postulated that sRNAs may possess alternative roles in conditioning of the peri-conceptual environment in the female reproductive tract, as vectors for the transmission of *trans*-generational patterns of inheritance that subtlety alter the developmental trajectory of the offspring, and/or serve as an additional tier of innate defense to protect the mature sperm population (e.g. protection against the horizontal transfer of mobile genetic elements via the exosomes they encounter during/after ejaculation) (Nixon*, et al.*, 2015). Whilst we cannot discount these possibilities, recent work by Conine and colleagues has raised the exciting prospect that epididymal-driven changes to the sRNA population of sperm may be integral for the support of early embryo development (Conine*, et al.*, 2018). Specifically, embryos generated using ICSI of immature caput sperm displayed signatures of aberrant pre-implantation signaling, and as a consequence, failed to implant normally. By contrast, no such defects were observed in embryos generated using cauda epididymal spermatozoa. Furthermore, and of considerable interest, was the authors demonstration that these phenotypic aberrations were ameliorated via the co-injection of embryos with the sRNA cargo extracted from cauda epididymosomes (Conine*, et al.*, 2018). Taken together, these data reinforce the importance of the epididymis in terms of shaping the functional sRNA profile of sperm.

Mechanisms responsible for remodeling of the sRNA profile of epididymal spermatozoa

Whilst we still have much to learn about the physiological importance of the sRNA cargo of sperm, the precise mechanisms responsible for remodeling the sRNA landscape of these cells as they traverse the epididymis, also remains to be elucidated. A plausible explanation for the apparent reduction in individual sRNAs is that these transcripts are encapsulated within the cytoplasmic droplet; a remnant of the germ cell cytoplasm that is

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gradually discarded as spermatozoa undergo epididymal maturation (Hermo*, et al.*, 2010). Thus, in species such as the mouse, only approximately half the spermatozoa possess cytoplasmic droplets upon reaching the cauda epididymis. Less certain however, are the mechanism(s) responsible for sRNA acquisition during epididymal transit, particularly given that spermatozoa are transcriptionally quiescent and therefore incapable of the *de novo* production of sRNA precursor molecules. These cells must therefore rely on an alternate extrinsic mechanism, with potentially important contributions being made by the epididymal luminal environment in which the sperm are bathed. One possible route for sperm sRNA acquisition is delivery via RNA binding proteins, as has been documented for other tissues (Wang*, et al.*, 2010; Arroyo*, et al.*, 2011). Indeed, RNA binding proteins have previously been assigned numerous roles in spermatogenesis (Sutherland*, et al.*, 2015). Currently however, little is known about the presence and/or abundance of equivalent proteins in the epididymal lumen, or their potential for association with maturing spermatozoa. Rather, an alternative route for the bulk delivery of sRNA, involving their encapsulation in extracellular vesicles known as epididymosomes, has attracted the balance of attention (Belleannee*, et al.*, 2013; Sullivan, 2015).

Epididymosomes are broadly defined as small membrane bound extracellular vesicles, which are produced within the principal cells of the epididymal epithelium (Sullivan, 2015). Notwithstanding some degree of heterogeneity, both in terms of their abundance of exosomal markers and capacity to interact with target cells (Frenette*, et al.*, 2006), epididymosomes are delivered to the epididymal lumen via an apocrine secretory pathway (Hermo & Jacks, 2002), which appears to be conserved in the reproductive tract of the hamster (Yanagimachi*, et al.*, 1985), bull (Frenette & Sullivan, 2001), mouse (Rejraji*, et al.*, 2006; Nixon*, et al.*, 2018), ram (Ecroyd*, et al.*, 2004; Ecroyd*, et al.*, 2004), cat (Rowlison*, et al.*, 2018), and human (Thimon*, et al.*, 2008). After their release, epididymosomes interact with neighboring epithelial cells to effect a form of paracrine regulation and thereby exert indirect influence over sperm maturation (Belleannee*, et al.*, 2013). Alternatively, epididymosomes also exert a more direct influence on sperm maturation via delivery of a complex proteomic and lipidomic payload to maturing spermatozoa (Frenette*, et al.*, 2002; Rejraji*, et al.*, 2006). Such interactions appear to involve selective adhesion and transient fusion mechanisms, which culminate in significant compositional modifications of the sperm membrane architecture and cytosolic domains (Griffiths*, et al.*, 2008; Schwarz*, et al.*, 2013; Nixon*, et al.*, 2018). These properties, combined with an ability to encapsulate their ferried cargo within a protective membrane, identify epididymosomes as a highly attractive vehicle for the trafficking of sRNA cargo to

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transiting epididymal sperm (Reilly*, et al.*, 2016) (Figure 2). Consistent with this model, profiling of the sRNA cargo of epididymosomes confirmed the presence of a number of sRNA species, including the miRNAs and tRFs as well as sRNAs derived from snRNAs, snoRNAs and rRNAs (Reilly*, et al.*, 2016; Hutcheon*, et al.*, 2017) (Figure 1). Notably, the profile of sRNAs encapsulated in epididymosomes is distinct from that of the parent cells from which they have originated (Belleannee*, et al.*, 2013; Hutcheon*, et al.*, 2017); a finding that indicates there is likely selective packaging of the sRNA fraction loaded into epididymosomes. Interestingly, similar selective packaging of the sRNA content loaded into exosomes has also been reported for non-reproductive tissues (Guduric-Fuchs*, et al.*, 2012). Also consistent with the intercellular communication pathways proposed for somatic exosomes, the *in vitro* co-culture of epididymosomes and spermatozoa has been shown to effect the delivery of targeted miRNAs (Reilly*, et al.*, 2016), as well as an overall global increase in sRNA abundance, to the recipient sperm cells (Sharma*, et al.*, 2018).

Recent studies have also led to an extrapolation of this role through suggestion that epididymosomes and their encapsulated sRNA cargo are potential mediators of *trans*-generational inheritance of environmental stress. Indeed, successive research has revealed that exposure to a number of environmental stressors can influence the sRNA profile of sperm and potentially of epididymosomes (Chen*, et al.*, 2016; Perez & Lehner, 2019). Among the first studies to examine this phenomenon, an increased accumulation of a subset of miRNAs, and reciprocal reduction to the abundance of the piRNA class of sRNA, was reported in mouse spermatozoa exposed to early life trauma (Gapp*, et al.*, 2014). The resulting alterations in the sperm sRNA profile were linked to behavioral phenotypes displayed by the resulting offspring, with causality of these responses being demonstrated by microinjection of total RNA from the sperm of traumatized male mice into fertilized oocytes; a strategy that recapitulate the offspring phenotype (Gapp*, et al.*, 2014). Subsequent examination of this paradigm has confirmed that a diversity of paternal stressors [including: dietary perturbation (Chen*, et al.*, 2016; de Castro Barbosa*, et al.*, 2016; Fullston*, et al.*, 2016; McPherson*, et al.*, 2016; Sharma*, et al.*, 2016), psychological stress (Rodgers*, et al.*, 2013; Saavedra-Rodriguez & Feig, 2013; Pang*, et al.*, 2017; Dickson*, et al.*, 2018), ethanol consumption (Rompala*, et al.*, 2018), environmental toxicants (Metzler-Guillemain*, et al.*, 2015; Paris*, et al.*, 2015) and cigarette smoke exposure (Marczylo*, et al.*, 2012), and even exercise (Short*, et al.*, 2017; Ingerslev*, et al.*, 2018)], can each differentially alter the sRNA profile of effected males as well as to contribute to overt phenotypic disturbances in their

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offspring. Among these studies, a handful have specifically sought to examine the mechanisms by which the sperm sRNA profile is altered, and provided additional evidence of the pivotal role played by epididymosomes. By way of illustration, the tRF cargo of both sperm and epididymosomes has been shown to be equivalently altered in response to paternal insults of either chronic ethanol consumption (Rompala*, et al.*, 2018). If this paradigm were to hold across other forms of stress, it would imply that the physiology of the epididymal epithelial cells are likely being altered, and that this change in the cellular environment is likely influencing the selective packaging of sRNA species that differ from those packaged under normal physiological conditions. While not widely studied, this possibility has been considered in the context of chronic stress, wherein exposure to elevated corticosterone elicits a cascade of responses including aberrant expression of the glucocorticoid receptor and epididymal epithelial cell signaling, putatively resulting in equivalent changes to the sperm sRNA profile (Pang*, et al.*, 2017).

Clearly, a greater understanding of the biochemical mechanisms that result in the differential loading and packaging of sRNAs into epididymosomes in a stressed epididymis represents an exciting avenue for future research. An important caveat however, is that the epididymosome contribution towards establishing sperm sRNA profiles during epididymal transit as well as its possible modification under conditions of stress, may differ for each class of sRNA. In this context, our own work on mouse epididymosomes indicates that they do not harbor substantial amounts of the piRNA class of sRNA (Hutcheon*, et al.*, 2017) (Figure 1), and accordingly, the co-incubation of epididymosomes and spermatozoa *in vitro* does not result in substantial piRNA transfer (Sharma*, et al.*, 2018). Thus, it is unlikely that epididymosomes account for the elevated accumulation of this sRNA class documented in mature cauda spermatozoa. Rather, the identification of proteins involved in piRNA synthesis in cauda spermatozoa invites speculation that sperm may possess precursor piRNA transcripts that can be used as substrates by pathway machinery proteins for piRNA production (Hutcheon*, et al.*, 2017).

Conclusions

Numerous studies have begun to document the diverse sRNA landscape of the epididymis with the majority of these studies focusing on the spatial and temporal accumulation profiles present across the three distinct segments of the tissue. To date, and for the miRNA class of

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sRNA, such studies have revealed a rich and complex landscape that, in turn, has been implicated in; (1) regulating the androgen-dependent development and homeostasis of the organ (Zhang*, et al.*, 2010; Ma*, et al.*, 2012; Ma*, et al.*, 2013); (2) indirectly contributing the secretory and absorptive activities of the epithelium, and hence sperm maturation/storage, via the control of segmental patterns of gene expression (Belleannee*, et al.*, 2012; Li*, et al.*, 2012), and; (3) contributing to the reproductive pathologies such as age-dependent decline in male fertility (Zhang*, et al.*, 2010). Furthermore, additional studies have demonstrated the dynamic nature of the global sRNA population of the epididymis under differing cellular conditions, namely alterations to the abundance of each sRNA species that accumulates in epididymal tissues post paternal exposure to stress (Chen*, et al.*, 2016; Perez & Lehner, 2019). Of particular interest is the demonstration that alteration to the sRNA landscape of the epididymis epithelium is mirrored in the spermatozoa harbored in the tract, and further, that epididymosomes are the likely vectors for the transfer of an altered sRNA payload to the sperm. Continued research into this emerging field may aid in the development of a greater understanding of the etiology of male factor infertility and potentially allow for the design of rational therapeutic options for individuals experiencing male factor infertility in the future.

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Author's contributions

All authors made substantial contributions to the conception of this review and the critical appraisal of the literature summarized herein. All authors approved the final version and submission of this article.

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FIGURE LEGENDS

Figure 1. Profile of mouse epididymal sRNA. Pie charts illustrating the percentage of the six prominent small non-protein-coding RNA (sRNA) classes [i.e. small nucleolar RNA (snoRNAs), small nuclear RNA (snRNA), ribosomal RNA-derived fragments (rRNA), microRNAs (miRNA), transfer RNA-derived fragments (tRFs) and PIWI interacting RNAs (piRNA)] that contribute to the overall sRNA population of the epididymal epithelium and luminal epididymosomes and spermatozoa sampled from the caput and cauda segments of the adult mouse epididymis. Figure adapted from (Hutcheon, *et al.*, 2017).

Figure 2. Putative origin and roles of epididymal sperm sRNA. Mature spermatozoa harbor a substantial and diverse cargo of sRNAs, the foundations of which are established during spermatogenesis before being dynamically modified as the cells transit the epididymis. One putative mechanism for the delivery of sRNA is via their encapsulation in membrane bound extracellular vesicles known as epididymosomes, which are released from the principal cells of the epididymal epithelium After release, epididymosomes are targeted to downstream epithelial cells and maturing spermatozoa. The resultant modifications of the sperm sRNA landscape may hold functional significance in terms of modulating the peri-conceptual environment of the female reproductive tract, and/or as epigenetic information capable of influencing the stability and translational efficiency of maternal transcripts within the oocyte, and hence the developmental trajectory of the embryo and offspring.

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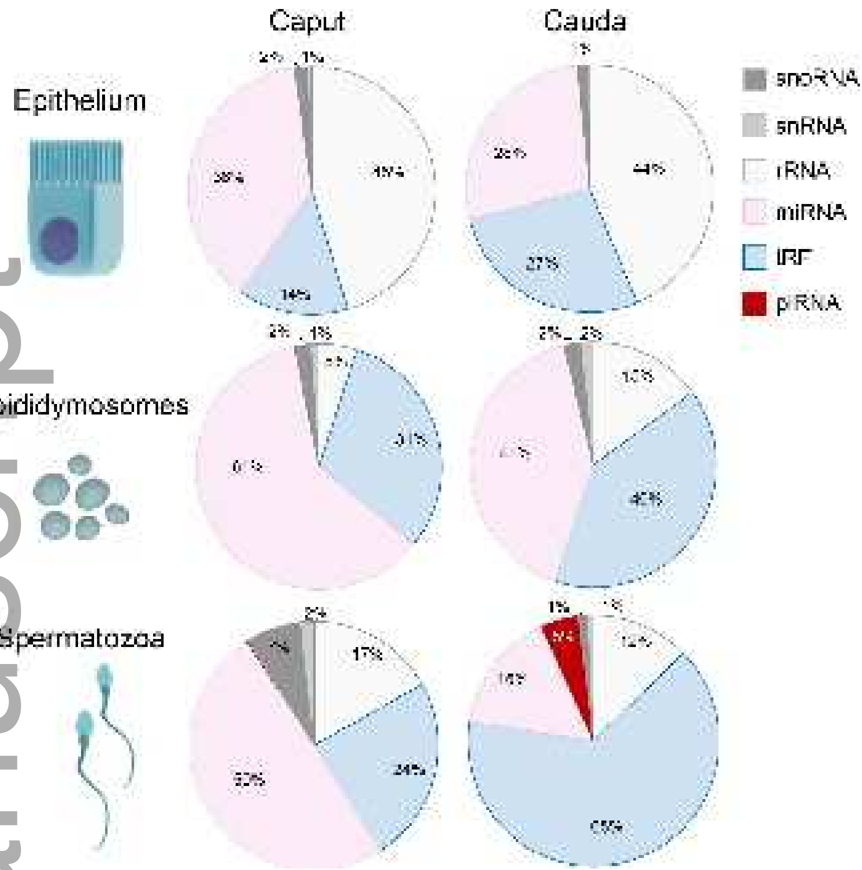

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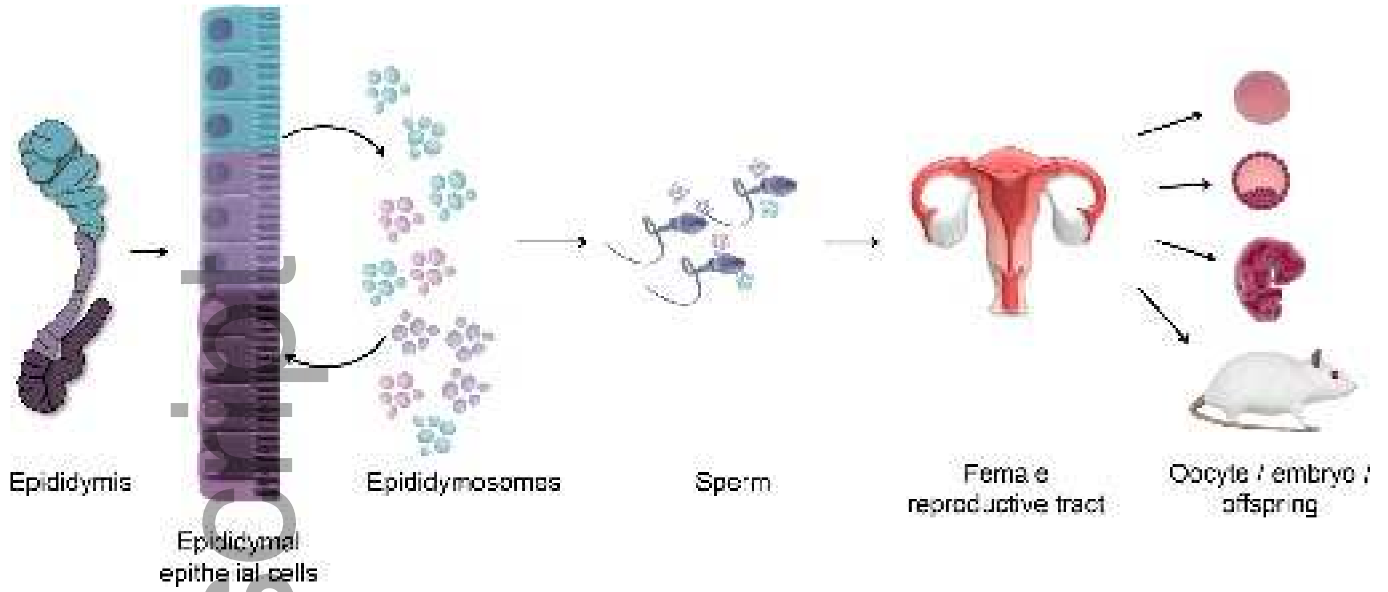

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