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Non-coding RNA and the Reproductive System

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Non-coding RNA and the Reproductive System

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Preface

Non-coding RNAs and the Reproductive System

Reproduction is a fundamental feature of all known life and can be classified into asexual and sexual reproduction. For successful sexual reproduction, both the internal and external organs have to develop and function properly to work together for procreation. Faults during development or maintenance of the reproductive system result in diseases that ultimately lead to infertility. In contrast to most other organ systems, it displays clear anatomical, morphological and molecular differences between the two sexes, male and female. These include the testes, epididymis and prostate in males and ovary, uterus and mammary glands in females, as well as the sexual dimorphism of the secondary sexual characteristics such as external genitalia. The development of these organs is tightly regulated by a network of gene expression and function in each of these organs as well as crosstalk between the organs through the production, secretion and reactivity to hormones.

Until recently, the focus of reproductive science was the identification of protein-coding genes that play important roles in the development of the different organs and are mutated in diseases affecting the reproductive system, such as disorders of sex development, endometriosis, male and female infertility, as well as testicular, ovarian, prostate and breast cancer. However, in the last decade, it became obvious that focusing on protein-coding genes will only provide one part of the picture, as the regulatory role of non-coding RNAs became more and more apparent. These RNAs, which have little to no protein-coding potential, have been shown to regulate most if not all physiological processes, including the development and function of the reproductive organs, through transcriptional, post-transcriptional and epigenetic regulation of gene expression.

Non-coding RNAs can be divided into different classes based on their size, their biogenesis and their protein partners. The main categories include microRNAs (miRNAs), small interfering RNAs (siRNAs), PIWI-interacting RNAs (piRNAs) and long non-coding RNAs (lncRNAs). While these molecular regulators have been studied extensively in the last few years, there are still many open questions with respect to their functions in driving biological processes. However, the rapid development of new technologies such as high-throughput sequencing to detect non-coding RNA expression and genome-editing tools such as the TALENs and CRISPR/CAS9 systems to analyse

their in vivo function has accelerated the speed of new discoveries in a way not seen before. In this book, experts in reproductive biology discuss the findings and advances we have made to elucidate the role of these new regulators of gene expression in the development of the reproductive organs and their contribution to disease. While the main focus is on the mammalian system, other model organisms that have been proven useful in the study of non-coding RNAs, such *Drosophila* and *C. elegans*, are also considered.

The editors like to express their gratitude to all authors who have contributed a review in their respective fields and to Springer Publisher, especially Sara Germans and Thijs van Vlijmen, who have worked closely with us to make this book happen.

Parkville, VIC, Australia — Dagmar Wilhelm
Melbourne, VIC, Australia — Pascal Bernard

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1 The Reproductive System

Andrew Pask

Abstract

Correct sexual development is arguably the most important trait in an organism's life history since it is directly related to its genetic fitness. The developing gonad houses the germ cells, the only legacy we pass on to subsequent generations. Given the pivotal importance of correct reproductive function, it is confounding that disorders of sex development (DSDs) are among the most common congenital abnormalities in humans (Lee et al. J Pediatr Urol 8(6):611–615, 2012). Urogenital development is a highly complex process involving coordinated interactions between molecular and hormonal pathways in a tightly regulated order. The controls that regulate some of the key events in this process are beginning to be unraveled. This chapter provides an overview of our understanding of urogenital development from the gonads to the urogenital ducts and external genitalia.

Keywords

Urogenital system • Testis • Ovary • External genitalia • Mammary gland • Sexual differentiation

1.1 Development of the Indifferent Gonadal Ridge

The gonadal ridge first appears as a bulge of intermediate mesoderm on the ventromedial surface of the intermediate embryonic kidney, the mesonephros, at around 10.5 days *post coitum* (dpc) in mouse. At this stage, the gonad is identical in

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structure between males and females and is comprised largely of somatic cells with germ cells migrating in from surrounding tissues. The somatic cells will contribute to the supporting, interstitial and steroid-producing cell lineages, while the germ cells will form the gametes (Merchant-Larios et al. 1993). As development proceeds, the epithelium and underlying mesenchyme of the gonad and mesonephros proliferates, and both organs increase in size. The mesonephros contains the mesonephric and paramesonephric ducts that facilitate fluid movement during kidney development but will later form aspects of the male and female reproductive tracts respectively. Both ducts exist as paired structures that sit adjacent to the gonads (Fig. 1.1). Several homeobox genes including *Lhx1*, *Lhx9* and *Emx2* have been implicated in the early pat-

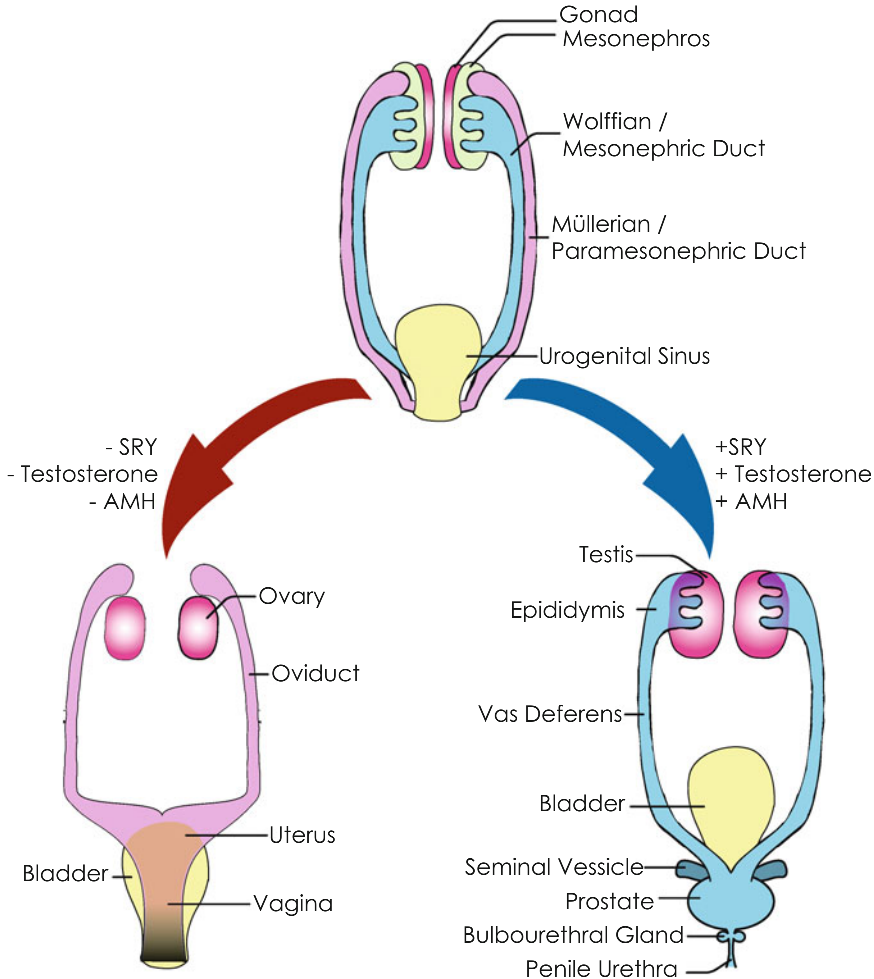

Fig. 1.1 *Sexual differentiation of the urogenital system.* The early embryo has a bipotential urogenital system (*top diagram*) that can proceed towards a female (*left*) or male (*right*) fate. Female urogenital development occurs in the absence of testis development and subsequent absence of AMH and testosterone. Under these conditions the Wolffian/mesonephric duct will fail to proliferate while the Müllerian/paramesonephric duct will develop in to the oviduct, uterus and upper portion of the vagina (*pink*). The lower region of the vagina (*black*) is derived from the urogenital sinus. Male urogenital development occurs in the presence of a testis and the subsequent production of AMH and testosterone. AMH actively drives the regression of the paramesonephric ducts while testosterone promotes the differentiation of the Wolffian ducts to form the epididymis, vas deferens, seminal vesicle, prostate and bulbourethral glands (*blue*). The penile urethra forms from the fusion of tissue from the urogenital sinus and urorectal septum

terning of the gonadal ridge and mesonephros ducts (Svingen and Koopman 2007). Mutations in the Wilms' tumour 1 gene (*Wt1*) cause the gonads to fail to progress past the indifferent stage as well as defects in mesonephric development and kidney tumours (Kreidberg et al. 1993). Interestingly, loss of *Wt1* has been shown to alter the expression of several long noncoding RNAs and their gene targets during tumorigenesis (Hubertus et al. 2011). Mutations in the gene encoding steroidogenic factor 1 (*Sf1*) and *M33* also cause a block in early gonadal development (Luo et al. 1994; Shinoda et al. 1995; Katoh-Fukui et al. 1998).

Following the formation of the sexually indifferent bipotential gonad, molecular factors will initiate its progression towards one of two separate developmental fates; testis or ovary. The developing gonad then produces the hormones and factors necessary to facilitate the coordinated development of the associated ductal systems and development of the appropriate external genitalia and other secondary sexual characteristics. An outline of the key events involved in each of these processes in males and females will be discussed below.

1.2 Sex Determination

In therian mammals, the decision to follow a male or female fate is held largely in the hands of the Y-linked *Sry* gene (Gubbay et al. 1990; Lovell-Badge and Robertson 1990; Koopman et al. 1991; Harry et al. 1995). *Sry* is the master trigger for testis differentiation, initiating Sertoli cell differentiation (Koopman et al. 1990; Rossi et al. 1993). Recently it was shown that *Sry* expression from the Y-chromosome is epigenetically regulated by the autosomal histone H3K9 demethylase JMJD1A (Kuroki et al. 2013). *Sry* expression triggers the direct upregulation of the critical testis gene *Sox9* where it acts as a transcriptional activator of the testis differentiation pathway (Sekido and Lovell-Badge 2008). Interestingly, SOX9 is initially present in the indifferent gonad of both XX and XY foetuses. In males it is rapidly upregulated and translocated to the nucleus, while in females, in the absence of the Y chromosome and *Sry*, *Sox9* transcription is not upregulated, the protein remains cytoplasmic and the gonad will proceed towards an ovarian developmental fate (Malki et al. 2005; Pask et al. 2010). The upregulation of *Sox9* is arguably the most critical step in the initiation of a testis (Qin et al. 2004), since loss of *Sox9* in XY gonads results in ovarian development (Barrionuevo et al. 2006), while ectopic expression of *Sox9* in XX gonads can induce testis formation. Thus, SOX9 is both necessary and sufficient for testicular development (Bishop et al. 2000; Vidal et al. 2001; Qin and Bishop 2005).

1.3 Testicular Development

Following the upregulation of *Sry* and *Sox9* in the bipotential gonad, the first morphological signs of testis development are the appearance of male pattern vasculature, including a prominent coelomic vessel in eutherian mammals, and the formation of testicular cords. SRY also triggers proliferation of the coelomic epithelium, a feature that is required for testis development but not ovarian differentiation (Schmahl and Capel 2003). All these features contribute to a greatly increased abundance of cellular proliferation within the XY gonad, a key hallmark of male development (Schmahl et al. 2000).

The rapid growth of the testis is further facilitated by cell immigration (Capel et al. 1999). *Sry* triggers the movement of mesonephric cells, which invade the developing testis and were believed to contribute to the Leydig, peritubular myoid and endothelial cell lineages (Fig. 1.2) (Martineau et al. 1997). However, recent research has shown that these cells exclusively comprise of migrating endothelial cells, which help to establish testis-specific vasculature, including the development of the characteristic coelomic vessel on the outermost surface of the testis. The formation of side branches from the coelomic vessel delineates the position and division of the developing testis cords (Coveney et al. 2008).

Testis cords are initially composed of three cell types: germ cells which will form spermatogonia; Sertoli cells which enclose the germ cells, and

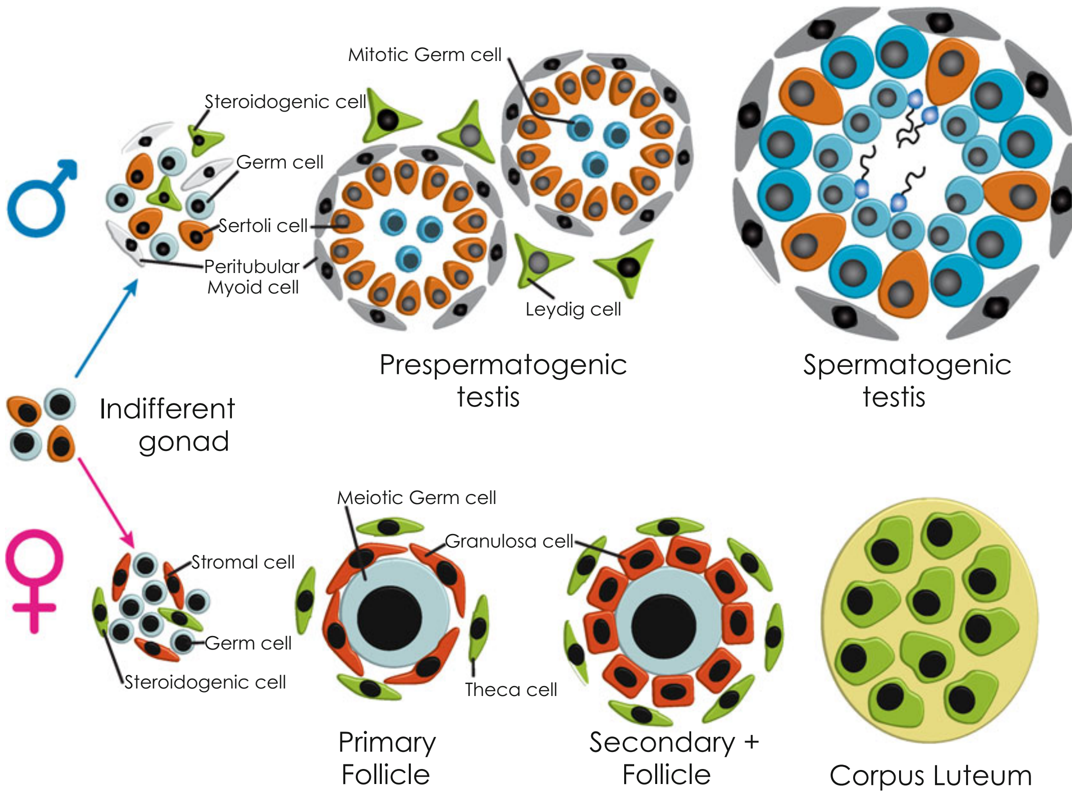

Fig. 1.2 *Cell lineages of the developing gonads.* The indifferent or bipotential gonad (*left*) contains a mixture of somatic (*orange*) and germ (*blue*) cells, with the latter migrating in from surrounding tissues. In males (*top row*), SRY triggers the development of Sertoli cells (*orange*) that organize themselves into cords surrounding groups of germ cells (prespematogenic testis). The cord is surrounded by peritubular myoid cells (*grey*), which together with Sertoli cells provide structural integrity to the cord through the deposition of the basal lamina. Between the cords, the steroidogenic Leydig cells (*green*) secrete testosterone. In the spermatogenic testis, mitotic spermatogonial stem cells, located at the outer edge of the cord (*blue*), provide for continuous spermatogenesis. In females (*bottom row*), the absence of SRY causes the supporting cell lineage (*orange*) to follow a granulosa cell fate. These cells eventually surround individual germ cells (*blue*) to form primordial follicles. The steroidogenic cell lineage (*green*) become theca cells which surround the granulosa cells. Following ovulation, the granulosa and theca cells luteinize to form the steroidogenic corpus luteum

peritubular myoid (PM) cells which surround and contribute to the structural integrity of the testis cords. A basement membrane is deposited around the testis cord forming a distinct barrier. Located between the cords are interstitial cells, which include Leydig and endothelial cells (Fig. 1.2).

Sertoli cells differentiate early in testicular development and are critical mediators of testis patterning. While SRY is the primary trigger for Sertoli cell development, it is not necessary. Sertoli cells can develop from XX bearing cells lacking the Y-chromosome (and *Sry*) altogether in mouse XY – XX chimeric gonads (Palmer and Burgoyne 1991). Prostaglandin D_2 (PGD_2), a paracrine signal secreted by Sertoli cells is able to recruit neighbouring supporting cell precursors to a Sertoli fate (Wilhelm et al. 2005). As such, Sertoli cells both direct and help reinforce an overall testis phenotype from the developing gonad.

Once Sertoli cells are specified, the formation of testicular cords involves their aggregation around clusters of immigrant germ cells (Kanai et al. 1991, 1992). Sertoli cells form tight junctions with one another building a continuous barrier around each cord, followed by another

peripheral layer of flattened PM cells (Pelliniemi and Frojdman 2001; Frojdman et al. 1989). A basement membrane, which forms the blood-testis barrier, is deposited on the basal side of the Sertoli cell through interactions with the adjacent PM cells (Richardson et al. 1995; Tung and Fritz 1993). The basement membrane (basal lamina) is an essential component for male fertility and the structural integrity of the testis. Structurally, it is comprised of laminin (Kleinman et al. 1993), collagen (Paulsson 1992) and heparin sulphate proteoglycans (Timpl 1993). Similar structural components are also found in the ovary but are fragmented and do not arrange into continuous basement membranes.

The germ cells, which will later form the sperm, are critical for male fertility but, interestingly, are dispensable for testicular differentiation. In germ cell deficient mice, testicular cords form normally (Merchant 1975). However, the converse does not old true and Sertoli cell development and cord formation are necessary for triggering male germ cell development and spermatogenesis. The decision for a germ cell to follow a male or female fate is not intrinsically determined by its chromosomal complement XX or XY, but instead is determined extrinsically by the identity of the supporting cells which surround it (Palmer and Burgoyne 1991; Burgoyne et al. 1988). In both males and females the gonad is flooded with retinoic acid (RA), which triggers germ cell entry into meiosis, a female developmental fate during foetal life. However, in males, the meiosis-inducing signal from RA is rapidly catabolized to prevent it from reaching the germ cells. This is mediated by the CYP26B1 enzyme, produced in large quantities by the Sertoli cells (Bowles et al. 2006; Koubova et al. 2006). In the absence of an appropriately timed meiotic entry signal from RA, germ cells in a testis instead arrest in mitosis, preserving their proliferative potential in the mature gonad.

Leydig cells are the major steroidogenic cell lineage of the testis that reside between the testicular cords, and are primarily associated with blood vessels. Two different types of Leydig cells have been identified. The foetal Leydig cells are derived in part from mesonephric cells, while adult Leydig cells form much later in development and have different embryological origins (Payne et al. 1996). Both types, however, serve to produce testosterone, responsible for the coordinated masculinization and virilisation of the reproductive system (Payne et al. 1996).

1.4 Male Urogenital Tract Differentiation

The early embryo has both Wolffian (mesonephric) and Müllerian (paramesonephric) ducts contained within the mesonephros (Fig. 1.1). Males must promote the differentiation of the Wolffian duct (Josso 1970a, b), while actively driving the removal of the Müllerian ducts. The maintenance and elaboration of the Wolffian ducts is driven by testosterone produced initially by the fetal Leydig cell population. Under the influence of androgens, the Wolffian duct will undergo regionally restricted differentiation to form the epididymis, vas deferens and seminal vesicles (Fig. 1.1). The differential development of regions of the Wolffian duct is controlled in part by homeobox genes and growth-factors that show restricted domains of expression throughout the tubule, each acting downstream of androgen signalling (Hannema and Hughes 2007).

The removal of the Müllerian ducts is critical for normal testicular descent to occur in males. This is mediated by Müllerian inhibiting substance (MIS), also known as anti-Müllerian hormone (AMH), one of the first proteins produced by the Sertoli cells in the early gonad. AMH actively drives the degeneration of the Müllerian ducts (Behringer et al. 1994; Behringer 1994) through binding to the AMH receptor, *AMHR2*, and inducing apoptosis (Kobayashi et al. 2011). Mutations in either *AMH* or *AMHR2* cause persistent Müllerian duct syndrome (PMDS) in human. If left untreated, PMDS can dramatically affect male fertility and most often requires surgical intervention to retrieve abdominal testes and remove the Müllerian derivatives, which interfere with the development of the vas deferens and epididymis (Ju et al. 2013).

The development of the prostate and male external genitalia is driven by conversion of foetal testosterone to the more potent androgen, dihydrotestosterone (DHT), by the enzyme 5α-reductase (Yamada et al. 2006). The prostate forms from the internal (pelvic) urethral tissue of the urogenital sinus. Androgen stimulation causes prostatic buds to form from the urethra, which then undergo elongation and further branching morphogenesis to form the basis of the adult prostate (Keil et al. 2012). Although the adult mouse and human prostate have dissimilar morphologies, their embryological origin and function are the same (Allgeier et al. 2010). Continued epithelial budding of the urogenital sinus under the influence of androgens also gives rise to the bulbourethral glands, a male accessory organ that produces a viscous fluid to aid in ejaculation, caudal to the prostate and adjacent to the penile urethra (Fig. 1.1) (Allgeier et al. 2010).

Not unlike the indifferent gonad, the external genitalia initially form as bipotential anlagen identical in structure between males and females. This structure, located anterior to the cloaca is known as the genital tubercle (Fig. 1.3). Under the influence of androgens, namely DHT, the genital tubercle shows increased cellular proliferation and outgrowth in males. The urethra becomes internalized in the forming penis by the invasion of the urorectal septum (URS) into the phallus. The URS originates from within the cloaca and grows distally into the phallus, resulting in the urethral meatus terminating at the tip of the penis (Perriton et al. 2002; Cohn 2004). Androgens also drive the fusion of the labioscrotal bulges, which form the male scrotum into which the testes will later descend.

Thus, the development of the male sexual phenotype is a very active process, driven by the rapid development of the testis, which immediately begins to output androgen and AMH, which together act to masculinize the reproductive system. This process is characterized by increased cellular proliferation throughout the male urogenital system, but particularly in the gonads and external genitalia. This is in contrast to several aspects of female urogenital development, which is, at least at the outset, a less active process.

1.5 Ovarian Development

The indifferent gonad will proceed towards an ovarian fate in the absence of the Y-chromosome and/or the upregulation of the *Sry* gene. Unlike the rapid growth and gross morphological remodelling that happens in a very short time window in the early developing testis, ovarian development starts at a more modest pace. The first gross morphological sign of ovarian development is the distinction between the cortex and medulla and the formation of germ cell nests in the outer cortex which will be the site of oogenesis (Wilhelm et al. 2007). In all mammals, this morphological event occurs at a later developmental time point to the formation of cords in the testis.

The ovary is made up of the same cast of cell lineages as the testis albeit arranged differently to support oogenesis (Fig. 1.2). The germ cells are surrounded by granulosa cells, the female equivalent of the Sertoli cells, which will nurture germ cell development. As development progresses each individual germ cell becomes surrounded by its own layer of granulosa cells forming the primordial follicle (Brennan and Capel 2004). The female equivalent of the Leydig cells are the steroidogenic theca cells, which form around the primordial follicles and provide the hormones necessary for follicle growth and oocyte maturation. The theca cells differentiate from the ovarian stroma in response to signals secreted from the growing follicle (Magoffin 2005). Once a follicle has ovulated, the theca and granulosa cells luteinize to form the highly steroidogenic corpus luteum, which produces progesterone to maintain pregnancy should the ovulated oocyte become fertilized (Henkes et al. 2003).

Early vascularization of the ovary is also very different to that seen in the testis. The ovary forms an extensive network of small capillaries that initially surround each of the germ cell nests and later the individual primordial follicles (Bullejos et al. 2002). These vessels are thought to ensure delivery of growth factors to nurture follicular development and, following ovulation, they export hormones out of the corpus luteum.

Female germ cell proliferation only takes place during early development, in contrast to the

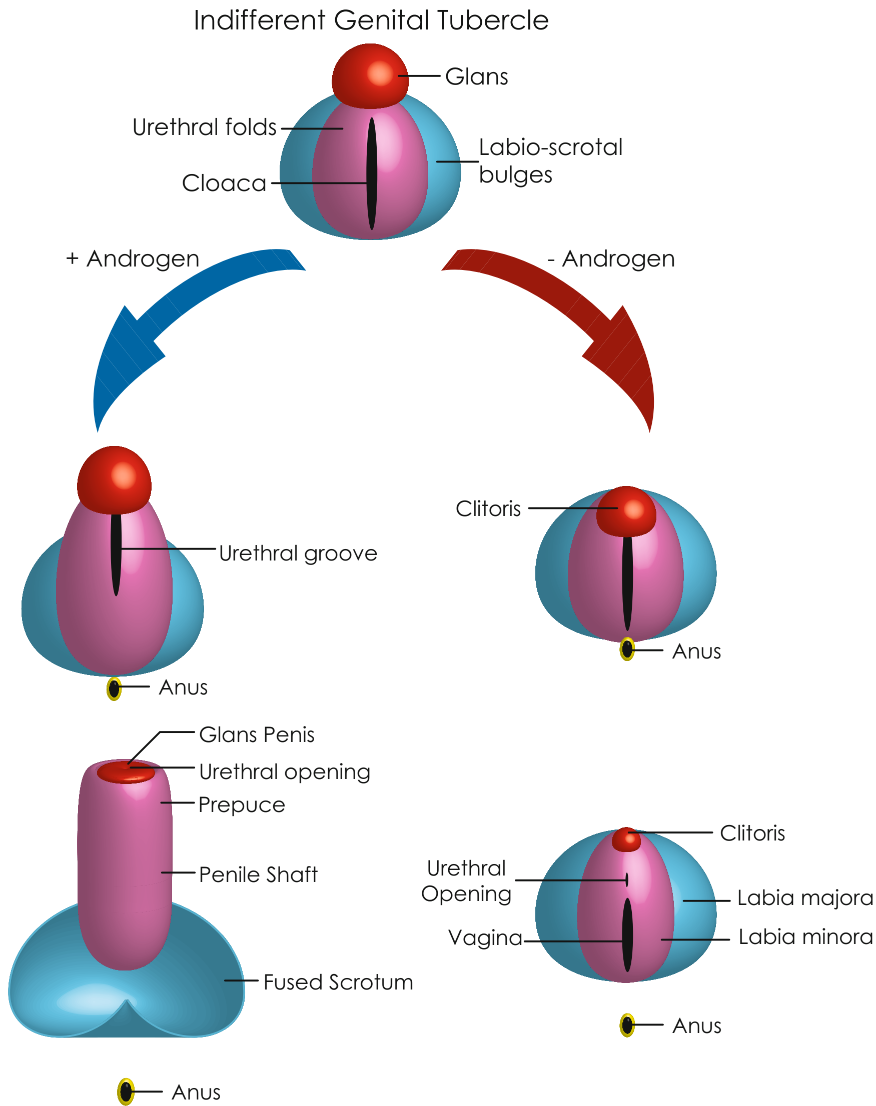

Fig. 1.3 *Sexual differentiation of the external genitalia.* The external genitalia initially form the sexually indifferent genital tubercle. This structure will develop into the male and female external genitalia, dependent on the presence or absence of testosterone respectively. In the presence of androgen (namely testosterone and DHT), the genital tubercle will under go outgrowth and elongation. The glans (*red*) will form the glans penis and the urethral folds (*pink*) will fuse and form the penile shaft and prepuce eventually covering the glans penis. The labio-scrotal bulges (*blue*) will fuse at the midline to form a continuous scrotum into which the testes will descend. The perineum also undergoes active proliferation in males resulting in a distally located anus (*yellow*) which septates from the cloaca early in development. This leads to a greater anogenital distance (distance between the anus and genital opening) in males compared to females. In the absence of androgens, the glans (*red*) forms the clitoris and proliferates only marginally compared to males. The urethral folds (*pink*) and labio-scrotal bulges (*blue*) fail to fuse at the midline and go on to form the labia minora and labia majora respectively. The anus (*yellow*) septates from the cloaca early in development like in males, but fails to migrate as far distally in the absence of androgens, leading to reduced anogenital distance in females

continuous proliferation seen in the male germ line. This results in a finite number of follicles being present in the mature ovary. Interestingly, in addition to their finite numbers, the female germ cells also undergo large amounts of apoptosis in early development resulting in an even more limited number of primordial follicles (Borum 1961). This is thought to be a selection process that results in the retention of only the fittest germ cells (Morita et al. 1999). It is still unknown if this represents those germ cells that are intrinsically more fit and/or physically located in a better position within the developing ovary.

The number of ovarian germ cells becomes finite once the cells have entered meiotic arrest in the early foetus. Entry into meiotic arrest is triggered by RA that floods the developing gonads of both males and females. RA triggers the expression of *Stra8*, which in turn directs germ cell entry into meiosis (Koubova et al. 2006; Bowles et al. 2006).

Interestingly, formation and maintenance of the somatic ovarian environment is dependent, at least in part, on the presence of meiotic germ cells. This is in contrast to the testis where Sertoli cells and testicular cords will form in the complete absence of germ cells (Couse et al. 1999; Hashimoto et al. 1990). In the ovary, the absence of germ cells causes granulosa cell to transdifferentiate into Sertoli-like cells and arrange into cords, reminiscent of a testis-like morphology (Couse et al. 1999). These Sertoli-like cells upregulate key male genes such as *Sox9* as if they were in a testis (Britt et al. 2004). Thus, there appears to be vital communications between the germ and somatic cell lineages in the developing ovary that are critical for its correct patterning and maintenance.

Oestrogen is also a critical factor for maintaining ovarian fate. In the oestrogen receptor deficient or aromatase deficient mice (ERKO and ArKO respectively), early ovarian differentiation appears to proceed normal, however, shortly after birth the germ cells are lost. As seen in the developing germ cell-deficient ovary (described above) the granulosa cells transdifferentiate to Sertoli-like cells, form cords and upregulate *Sox9* (Britt et al. 2002). Upon administration of oestrogen back to ArKO mice, the ovarian histology is restored and *Sox9* is downregulated (Britt et al. 2004). Furthermore, exogenous oestrogen administration to the bipotential gonad in marsupial mammals can drive complete ovarian development from XY gonads (Pask et al. 2010). Together, these findings suggest that ovarian somatic cell fate is malleable and can transdifferentiate towards a Sertoli cell fate in the absence of oestrogen or meiotic germ cells.

1.6 Female Urogenital Tract Differentiation

Without the formation of a testis there is no early production of testosterone or AMH. Testosterone and its more potent metabolite DHT are required for the persistence and elaboration of the Wolffian (mesonephric) ducts, and in their absence in females, the ducts passively regress. Conversely, in the absence of AMH, the Müllerian (paramesonephric) ducts persist and elaborate to form the fallopian tubes, uterus, cervix and upper portion of the vagina (Fig. 1.1). Because development of the female urogenital system happens in the absence of both testosterone and AMH it is often referred to as the passive or default pathway. However, development of the Müllerian ducts is still an active process that requires precise regional development that is controlled, in part, by *Hox* genes (Du and Taylor 2004).

Similarly, in the absence of testosterone and DHT in the circulation of early female embryos, the external genitalia become feminized. Rather than this being an active process, the feminization results from failure of the external genitalia to become masculinized. In the absence of testosterone the embryonic phallus elongates only marginally and becomes the clitoris. The urethral folds and genital swellings remain separated (unlike in males where these fuse to form the penis and scrotum) and become the labia minora and labia majora respectively (Fig. 1.3). These structures proliferate as the embryo grows and eventually surround the clitoris to form the

female external genitalia. The urogenital sinus remains open and contributes to the formation of the lower vagina (Yamada 2005).

Another sexually dimorphic feature and a defining characteristic of mammals is the development of the mammary glands. The underlying mammary line (milk duct) forms around the time when gonads first appear in the early embryo. The mammary placodes form along the mammary lines and bud inwards from an invagination of the epithelium. These mammary buds then undergo branching morphogenesis in association with the mammary fat pad and will eventually give rise to the nipples. As development progresses oestrogen production from the ovary triggers the mammary ducts to continue to expand through branching morphogenesis to invade the underlying mammary fat pad (Hens and Wysolmerski 2005). During pregnancy and partuition the mammary ducts undergo further dynamic rounds of cell division and differentiation to prepare for milk production and let-down. Luminal cells within mammary aveoli formed from the branching ducts are the sites of milk production. Milk is channelled through the ductal system to the nipple through myoepithelial contractions (Daniel and Smith 1999). Following weaning of the young the mammary gland undergoes significant rounds of apoptosis (involution) to remodel to the pre-pregnancy state. A large number of dynamically expressed non-coding RNAs have been identified during specific stages of mammary development and involution, however their precise roles in these processes are yet to be defined (Hennighausen and Robinson 2005; Siegel and Muller 2010).

Interestingly, several genes subject to genomic imprinting are expressed in the mammary gland that are either lncRNAs themselves or regulated in part by lncRNAs (Adriaenssens et al. 1999; Stringer et al. 2012b). Genomic imprinting is hypothesized to have evolved to help regulate nutrient provisioning between mother and offspring. As a result, many imprinted genes are expressed in the invasive placenta of eutherian mammals (Angiolini et al. 2006). In contrast, marsupial mammals have a relatively short gestation, with a substantially less invasive placenta that appears to be less reliant on imprinted gene expression (Renfree et al. 2008). However, the short gestation is traded for a long and sophisticated period of lactation during which the bulk of maternal provision to the young occurs (Tyndale-Biscoe and Renfree 1987). Thus, in marsupials, the primary site of maternal-young nutrient exchange occurs through the mammary gland (Renfree et al. 2013). Consistent with a role for imprinted genes in nutrient provision, several imprinted genes known to be regulated by long non-coding RNAs, have been identified in the marsupial mammary gland (Stringer et al. 2012a, b). Thus it appears that long non-coding RNAs may play a highly conserved and fundamental role in the regulation of this sexually dimorphic organ.

1.7 Summary

Development of the gonad and associated urogenital and secondary sexual characteristics is a highly dynamic, tightly regulated and critical for species fitness. However, despite its importance, the process is also highly fallible. The following chapters will describe recent advances in our understanding of the control of the molecular pathways underpinning reproductive development and the role that non-coding RNAs play in this complex and critical process.

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Non-coding RNAs: An Introduction

2

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Abstract

For many years the main role of RNA, it addition to the housekeeping functions of for example tRNAs and rRNAs, was believed to be a messenger between the genes encoded on the DNA and the functional units of the cell, the proteins. This changed drastically with the identification of the first small non-coding RNA, termed microRNA, some 20 years ago. This discovery opened the field of regulatory RNAs with no or little protein-coding potential. Since then many new classes of regulatory non-coding RNAs, including endogenous small interfering RNAs (endo-siRNAs), PIWI-associated RNAs (piRNAs), and long non-coding RNAs, have been identified and we have made amazing progress in elucidating their expression, biogenesis, mechanisms and mode of action, and function in many, if not all, biological processes. In this chapter we provide an introduction about the current knowledge of the main classes of non-coding RNAs, what is know about their biogenesis and mechanism of function.

Keywords

MicroRNAs • Endo-siRNAs • Long non-coding RNAs • Gene regulation • piRNAs • snoRNAs

2.1 Introduction

After the discovery of nucleic acids by Friedrich Miecher in 1868 (Dahm 2005) it took more than 50 years to identify the chemical and biological differences between DNA and RNA (Allen 1941). During the late 1950s, the concept of messenger RNA emerged, from which Francis Crick, the English scientist who discovered the structure of DNA, developed the "central dogma of Molecular Biology" to describe the flow of genetic information in all cells: DNA is transcribed into RNA and

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RNA is translated into protein (Crick 1970). Information can flow between DNA and RNA, but not from protein to nucleic acid.

For decades, the only known biological catalysts were proteins. RNA was assumed to be the essential bridge between DNA and protein, acting as a template for protein synthesis (Kozak 1983). However, in the 1980s, Thomas Cech and Sidney Altman showed that also certain RNAs can function as enzymes, so-called ribozymes, which are involved in the processing of other RNA molecules such as the biogenesis of transfer RNA or splicing of nuclear pre-messenger RNA (Doudna and Cech 2002; Guerrier-Takada et al. 1983; Zaug and Cech 1980). Nevertheless, scientists around the world focussed their research on the properties and biological functions of protein-coding RNAs or messenger RNAs (mRNAs), even though only 1–2 % of the human genome is protein-coding and the remaining 98 % was considered to be "junk" DNA (Lander et al. 2001; Venter et al. 2001). Interestingly, it became apparent that most of this junk DNA is actually transcribed into RNA (Carninci et al. 2005; Denoeud et al. 2007; Kapranov et al. 2007a, b; Okazaki et al. 2002), with many of these RNAs having no or little coding potential, raising the hypothesis that they are non-functional. However, a rapidly increasing number of specific non-coding RNAs (ncRNAs) has been identified as key regulators of many biological processes, including regulation of gene expression, cell cycle control, apoptosis, cell identity decisions, chromatin remodelling, and epigenetic modifications (Bartel 2004; Mercer et al. 2009; Taft et al. 2007).

Additionally, the increasing number of functional ncRNA in complex organisms may be the answer to the inconsistent correlation between the complexity of an organism, its cellular DNA content and the number of protein-coding genes (Mattick 2007, 2009). Comparison between the nematode *C. elegans* and humans revealed a similar number of protein-coding genes, around 20,000, in the two organisms, however, there is an expansion of complexity with respect to RNA processing and an increase in the number of ncRNAs from worm to human. Thus, it is clear that the complexity of organisms cannot be explained based on the number of proteins but rather, at least partly, on the complexity of RNA.

ncRNAs include "house-keeping" RNAs such as ribosomal RNA (rRNA) and transfer RNA (tRNA), as well as regulatory RNAs. Regulatory RNAs are categorised, rather arbitrarily, according to their transcript length into small ncRNAs that are shorter than 200 nucleotides (nt) and long ncRNAs (>200 nt). Members of small regulatory ncRNAs include microRNAs (miRNAs), PIWI-interacting RNAs (piRNAs) and endogenous small interfering RNAs (endo-siRNAs) (Carninci 2009). They are characterized by their biogenesis pathways, their length and their interaction with different proteins of the Argonaute family (Carthew and Sontheimer 2009; Kim et al. 2009). In contrast, long ncRNAs are a diverse class of mRNA-like, non-coding transcripts that participate in a variety of biological processes through numerous mechanisms including chromatin modification, regulation of the activity or localization of proteins, organizational and structural frameworks, and as precursors for small ncRNAs (for review see: Fatica and Bozzoni 2014; Ponting et al. 2009; Wilusz et al. 2009; Zhang et al. 2014a). In this chapter we will discuss more details for each class of ncRNAs with the exception of piRNAs for which we refer to Chap. 4.

2.1.1 MicroRNAs

The first miRNA, lin-4, was reported in 1993 in *C. elegans* (Lee et al. 1993; Wightman et al. 1993). Lin-4 was found to negatively regulate the mRNA of the protein-coding gene *lin-14*, ensuring the proper transitions between *C. elegans* larval stages (Lee et al. 1993; Wightman et al. 1993). Lin-4 binds complementary to elements within the 3′ untranslated region (3′UTR) of the *lin-14* mRNA leading to its degradation (Reinhart et al. 2000). The discovery did not capture much attention as lin-4 was found to be specific to worms. The second known miRNA, let-7, was also discovered in *C.elegans*. Similar to lin-4, let-7 also controls cell differentiation and proliferation (Reinhart et al. 2000). However in contrast to lin-

4, let-7 is not species-specific, but is evolutionary conserved (Reinhart et al. 2000), indicating that miRNAs have essential functions in many organisms. Since then the miRNA field has exploded with more than 35,000 mature miRNAs listed in miRBase (release 21; Griffiths-Jones 2004).

Biogenesis of miRNAs

Within the genome, miRNAs are encoded at various locations as either independent or clustered transcriptional units (Bartel 2004; Carthew and Sontheimer 2009). miRNAs are typically transcribed by RNA polymerase II into a precursor transcripts called primary miRNA (pri-miRNA). Pri-miRNAs have a characteristic hairpin structure with an imperfectly paired stem of approximately 33 bp, a terminal loop and flanking sequences of which the 5′end is capped and the 3′end polyadenylated (Bartel 2004; Carthew and Sontheimer 2009). In the nucleus, a microprocessor complex that contains the RNase III type endonuclease DROSHA and its cofactor, the double stranded RNA-binding protein DGCR8 in mammals and PASHA in flies, cleaves pri-miRNAs (Fig. 2.1). DGCR8 binds the pri-miRNA stem and interacts with DROSHA, which cleaves the double strand stem region off the pri-miRNA to generate a 60–80 nt pre-miRNA, a hairpin structure with a distinctive two-nucleotide overhang at the 3′end, a 5′ phosphate and a 3′ hydroxyl group (Denli et al. 2004; Gregory et al. 2004; Lee et al. 2003). A second pathway uses the splicing machinery to generate pre-miRNAs from introns (Okamura et al. 2007; Ruby et al. 2007). Lariat structures are formed by the spliced introns and interact with the lariat-debranching enzyme LDBR, which leads to the formation of the pre-miRNA hairpin structure. These so-called mirtrons are not very common and enter the miRNA-processing pathway downstream of DROSHA/DGCR8. Pre-miRNAs are shuttled out of the nucleus by exportin 5, which recognises the 2 nt-3′ overhang and export the pre-miRNAs into the cytoplasm through Ran-GTPase (Bohnsack et al. 2004; Lund et al. 2004; Yi et al. 2005). In the cytoplasm, pre-miRNAs are further processed by another RNase III enzyme, DICER, and its partner RNA-binding protein TRBP (Grishok et al. 2001; Han et al. 2004; Hutvagner et al. 2001; Ketting et al. 2001). The PAZ domain of DICER allows it to bind to the 3′ overhang of pre-miRNAs in order to cleave off the terminal loop to generate an approximately 22 nt double-stranded miRNA duplex. The miRNA duplex associates with an AGO protein that rapidly unwinds the duplex; one strand, called guide strand, is retained, whereas the other strand, the passenger or star (miRNA*) strand, is lost (Kawamata et al. 2009). In *Drosophila* and *C. elegans*, the choice of the guide strand depends on the thermodynamic stability of the duplex's ends, whereby the strand that is more stably base-paired at its 5′end is degraded (Khvorova et al. 2003; Schwarz et al. 2003). In contrast, the mechanism of strand selection in mammals is still unknown. At this stage, the mature miRNA associates with an AGO protein and additional accessory proteins such as GEMIN3, GEMIN4, MOV10, IMP8 and GW182 (Bartel 2004; Liu et al. 2005a; Meister et al. 2005; Till et al. 2007; Weinmann et al. 2009) to regulate mRNA expression. Of these accessory proteins, GW182 has been shown to be necessary and sufficient for miRNA-bound AGO to repress gene expression (Eulalio et al. 2008b; Jakymiw et al. 2005). This complex is referred to as miRISC, microRNA-induced silencing complex (Fig. 2.1).

Similar to the processing of mirtrons that depends on DICER but not on DROSHA/DGCR8, another alternative pathway has been described that uses other ncRNAs such as tRNAs (Lee et al. 2009), small nuleolar RNAs or snoRNAs (Ender et al. 2008), or small nuclear RNA-like viral RNAs (Cazalla et al. 2011) as miRNA precursors. In addition, an unusual case of miRNA processing has been described for one miRNA, miR-451, which is DROSHA-dependent, but DICER-independent. DROSHA-mediated processing produces a pre-miRNA that is too short to be loaded onto DICER, instead *pre-mir-451* is directly bound by AGO2, which slices it in the middle of its 3′strand generating a 30-nucleotide RNA that is further trimmed into the mature miR-451 by the ribonuclease PARN (Cheloufi et al. 2010; Cifuentes et al. 2010; Yang and Lai 2010; Yang et al. 2010; Yoda et al. 2013).

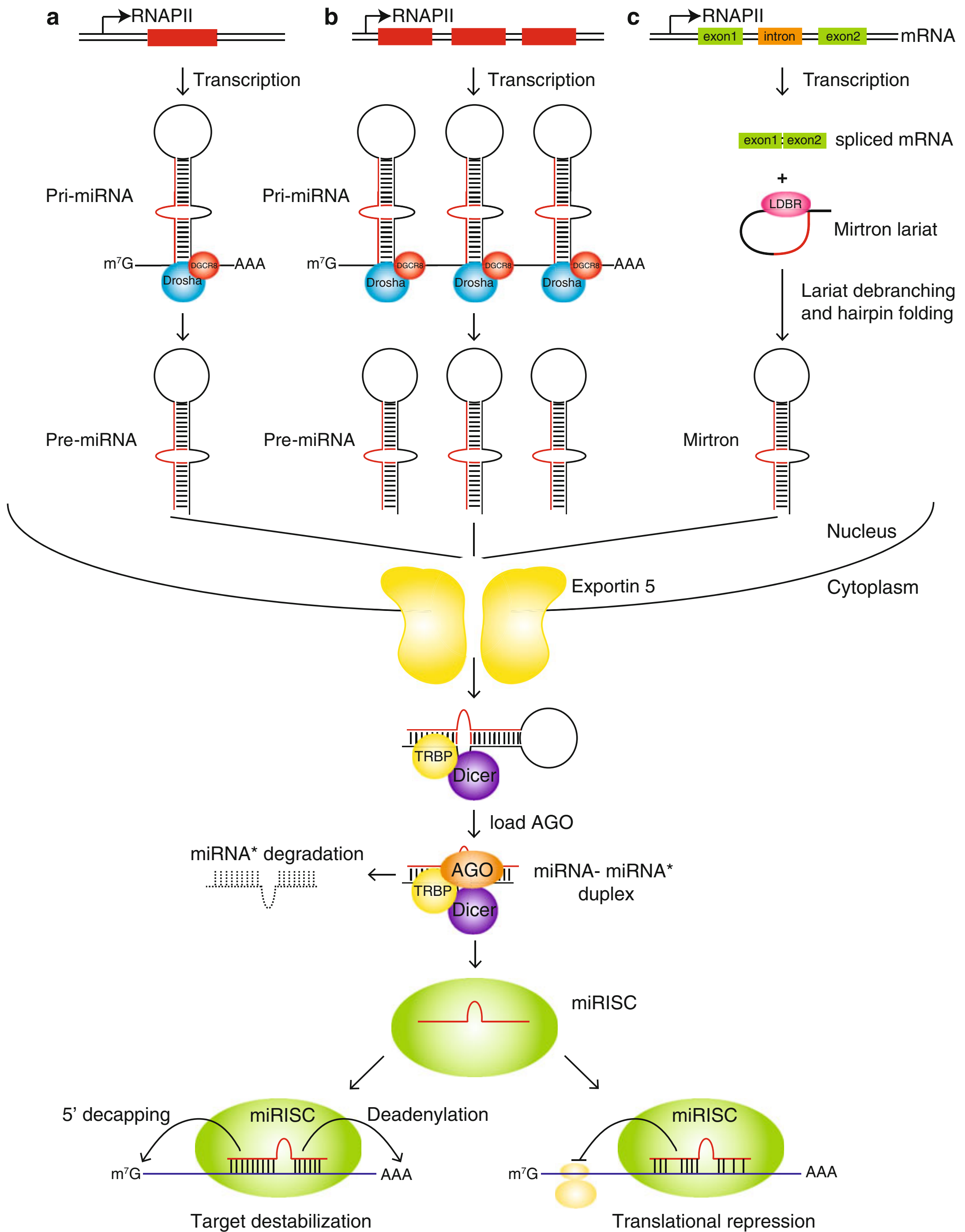

Fig. 2.1 Biogenesis of miRNAs exist as independent (**a**) or clustered (**b**) transcriptional units. First primary transcripts (pri-miRNAs) are transcribed by RNA polymerase II and are characterised by a 5′cap structure, a polyadenylated 3′end and a hairpin with a terminal loop. Pri-miRNAs are processed by endonuclease DROSHA and its cofactor DGCR8 to generate pre-miRNA transcripts. Alternatively miRNAs can derive from introns, termed mirtrons (**c**). Here, the pre-miRNAs are formed in a DROSHA-independent manner in which short introns with hairpin potential are linearized by the lariat debranching enzyme LDBR. Pre-miRNAs derived from A, B and C are exported by Exportin 5 into the cytosol and are further processed by DICER and its binding partner TRBP resulting in the formation of mature miRNA/miRNA* duplexes. After unwinding of these duplexes by AGO, the passenger strand (miRNA*) is degraded, whereas the guide strand (miRNA) binds to AGO and additional accessory proteins to form the microRNA-induced silencing complex (miRISC). miRISC binds to mRNAs and inhibits gene expression either by destabilizing the mRNA or by repressing translation

Regulation of miRNA Biogenesis

To ensure correct miRNA expression, the miRNA biogenesis process is regulated at the transcriptional, DROSHA, DICER and RISC level. As mentioned above, most miRNA genes are transcribed by RNA polymerase II and are therefore regulated at this level in the same way as protein-coding genes, i.e. their transcription is controlled by RNA Pol II-associated transcription factors and epigenetic regulators (Cai et al. 2004; Lee et al. 2004).

The generation of pre-miRNAs from pri-miRNAs by DROSHA/DGCR8 appears to be regulated by numerous proteins. This includes proteins that bind to DROSHA as well as proteins that interact with the terminal loop structure of the pri- and pre-miRNA. While the microprocessor complex containing only DROSHA and DGCR8 is sufficient for miRNA processing, these proteins have been shown to exist in a larger complex, which also includes the DEAD-box RNA helicases p68 (DDX5) and p72 (DDX17) (Gregory et al. 2004). Endogenous p68 and p72 not only promote DROSHA processing of certain miRNAs (Fukuda et al. 2007), but also act as interacting platforms for other proteins that influence DROSHA activity. One example is the association of p68 with SMAD, which is one of the downstream effector of TGFβ signalling, resulting in the increased processing of *pri-mir-21* (Davis et al. 2008). Similarly, the interaction of p68 with the tumour suppressor p53 promoted the processing of various pri-miRNAs in response to DNA damage (Suzuki et al. 2009).

In addition to the association with p68/p72, a number of proteins regulate DROSHA processing through their interaction with the terminal loop of the pri- and/or pre-miRNA. Probably one of the best-known mechanisms is the regulation of *let-7* processing by LIN28. Let-7 was one of the first two miRNAs identified in *C. elegans* (Reinhart et al. 2000) and is highly conserved across animal species. While the *let-7* family consist of multiple copies within the genome, the number of copies varies between different organisms (Roush and Slack 2008). The RNA-binding protein LIN28 interacts with the terminal loops of *let-7* family members via a conserved sequence and blocks the processing of *pri-* and *pre-let-7* (Newman et al. 2008; Piskounova et al. 2008; Rybak et al. 2008; Viswanathan et al. 2008). In addition, LIN28 interacts with the terminal uridylyl transferase 4 (TUT4, also known as ZCCHC11) and 7 (TUT7, ZCCHC6), resulting in the oligo-uridylation of *pre-let-7* at the 3′end in the cytoplasm, which leads to inhibition of processing by DICER and degradation of the pre-miRNA (Hagan et al. 2009; Heo et al. 2008; Lehrbach et al. 2009; Thornton et al. 2012). The negative regulation of *pre-let-7* by TUT4/7 switches off in the absence of LIN28. In cells lacking LIN28, TUT4/7 and a third terminal uridylyl transferase, TUT2, redundantly mono-uridylate *pre-let-7*, which in turn promotes DICER processing (Heo et al. 2008).

Another example of regulation of miRNA processing is the interaction of the terminal loop with the RNA-binding protein heterogenous nuclear ribonuleoprotein A1 (hnRNPA1), which facilitates the processing of miR-18a (Guil and Caceres 2007). Interestingly, miR-18a is the only member of the *mir-17–92* cluster whose processing is regulated by hnRNPA1, explaining, at least in part, different expression levels of individual mature miRNA members in this cluster (Lu et al. 2007; Yu et al. 2006). Moreover, another activator of both DROSHA and DICER processing is the KH-type splicing regulatory protein (KSRP), which promotes the biogenesis of certain miRNAs including let-7a, miR-21 and miR-16. After binding to DROSHA and the terminal loop of the target pri-miRNA in the nucleus, KSRP is still associated to the precursor miRNA during nucleo-cytoplasmic transit and becomes part of the DICER-miRNA complex in the cytosol (Trabucchi et al. 2009).

In addition to these interacting factors, the activity of both the microprocessing complex DROSHA/DGCR8 and DICER is also regulated by post-translational modifications. The nuclear localization of DROSHA depends on its phosphorylation by glycogen synthase kinase 3β (GSK3β; Tang et al. 2010, 2011). Similarly, DGCR8 is phosphorylated, in this case by mitogen-activated protein kinase (MAPK), which stabilizes the protein (Herbert et al. 2013).

MAPK also phosphorylates TRBP, which interacts with DICER in the cytoplasm, leading to the specific up-regulation of growth-promoting miRNAs (Paroo et al. 2009). In addition, DGCR8 is deacetylated by HDAC1, resulting in a higher affinity for pri-miRNAs (Wada et al. 2012). Moreover, AGO2 is regulated by phosphorylation of Tyr393 by the epidermal growth factor receptor (EGFR) under hypoxic stress, leading to a reduced binding affinity of AGO2 to DICER and a reduced rate of processing for some tumour-suppressor-like miRNAs (Shen et al. 2013).

Similarly, the miRNA itself can be modified to affect its processing. For example RNA editing, which is the conversion of adenosine to inosine by ADARs, has been observed in some pri-miRNAs, which lowers their affinity for DROSHA (Yang et al. 2006) or DICER (Kawahara et al. 2007a, b). Some pre-miRNAs such as *pre-mir-145* and *pre-mir-23b* can also be methylated by the RNA methyl transferase BCDIN3D, leading to reduced processing by DICER (Xhemalce et al. 2012). Furthermore, a recent study showed that the processing of *pri-mir-195* is regulated by a long ncRNA, *Uc.283+A* (Liz et al. 2014). In this case, *Uc283+A* hybridises through an ultraconserved region to the lower stem of the pri-miRNA and inhibits processing by the microprocessor complex (Liz et al. 2014); a feasible mechanism that might also occur for other miRNAs.

Finally, the level of miRNAs in any given cell is not only determined by its biogenesis but also by its degradation, for which there are still many open questions on how it is regulated. Most miRNAs appeared to be very stable for days or even weeks (Bail et al. 2010; Gantier et al. 2011; Papaioannou et al. 2009). However, there are also examples for short-lived miRNAs such as miR-29b in HeLa cells (Hwang et al. 2007), neuronal-enriched miR-9, miR-125b and miR-146a (Sethi and Lukiw 2009), and retinal-enriched miR-183/-96/-128, miR-204 and miR-211 (Krol et al. 2010). For different miRNAs different intrinsic sequence motifs have been identified that lead to destabilization of these specific miRNAs. However, these sequences do not result in decay if introduced to a random context. In addition, these motifs appear to not have a preference with respect to the region within the miRNA sequence. In miR-503, two motifs are necessary for its decay, one in the seed sequence and the other within the 3′end (Rissland et al. 2011). In miR-382, the degradation motif encompasses seven nucleotides, GGAUUCG, at the 3′end (Bail et al. 2010), and in miR-29b it is determined by three Us at position 9–11 (Zhang et al. 2011). In addition, it was shown that the *Herpes virus saimiri* destabilizes two host-miRNAs, miR-27a and b, in infected T cells using two of its seven non-coding RNAs named *H. saimiri* U-rich RNA 1 and 2 or *Hsur1* and *2*. These non-coding RNAs bind sequence-specifically miR-27 and direct its degradation (Cazalla et al. 2010).

In addition to these intrinsic motifs, several miRNA degrading enzymes have been identified. To date, all identified miRNA-ribonucleases are exoribonucleases. These include XRN1 and XRN2 in *C. elegans* (Chatterjee et al. 2011; Chatterjee and Grosshans 2009), XRN1, but not XRN2, in human embryonic kidney cells (HEK293T) leading to miR-378 and miR-382 degradation (Bail et al. 2010), and the human interferon-inducible, exoribonuclease polyribonucleotide nucleotide-transferase 1, PNPT1, that causes degradation of miR-221, miR-222 and miR-106b (Das et al. 2010). Apart from exoribonucleases, exosomes seemed to play a role as well. Knockdown of the exosomal subunit RRP41 resulted in an increase of miR-278 and miR-382 (Bail et al. 2010), suggesting that exosomes are required for lowering the levels of specific miRNAs.

Interestingly, the interaction of a miRNA with its target, not only results in the inhibition of target gene expression, but also can influence the stability of the miRNA itself. It appears that in *Drosophila*, mouse and human cells miRNAs are destabilized if they have a high degree of complementary to their target mRNAs (Ameres et al. 2010; Baccarini et al. 2011; Xie et al. 2012). During this process the miRNA seems to be “marked” for degradation by the addition of uridines or adenosines, so-called tailing, and/or shorter, trimmed version of the miRNA (Ameres et al. 2010; Baccarini et al. 2011; Xie et al. 2012).

Functions of miRNAs

The best-known function for miRNAs is the repression of gene expression by either mRNA destabilization and/or inhibition of translation (Fig. 2.1, and Eulalio et al. 2008a; Filipowicz et al. 2008). The miRNA sequence confers specificity to recognize particular mRNAs. In most cases, the 3′UTR of the target mRNAs is bound by the miRNA through imperfect complementary base pairing. The key feature for recognition is Watson-Crick base pairing of the miRNA seed sequence, which encompasses nucleotides 2–8, to the mRNA (Grimson et al. 2007; Hibio et al. 2012; Lewis et al. 2005; Lim et al. 2005). In addition, complementary sites at the 3′end might also contribute to the binding (Grimson et al. 2007). Gene silencing can happen through mRNA degradation induced by deadenylation from the 3′end and/or 5′ decapping. Alternatively or in combination, the AGO protein interacts with several translation factors, causing inhibition of translation at the initiation and/or elongation step (for review see Filipowicz et al. 2008). Translationally inactive mRNAs accumulate in discrete cytoplasmic foci, called P bodies, that function as mRNA storage and degradation sites (Bhattacharyya et al. 2006; Brengues et al. 2005; Eulalio et al. 2007; Parker and Sheth 2007) and are enriched in AGO proteins, miRNAs and mRNAs repressed by miRNAs (Jakymiw et al. 2005; Leung et al. 2006; Liu et al. 2005a, b; Pillai et al. 2005), demonstrating compartmentalization of miRNA repression. However, there are reports that miRNAs not only repress but also up-regulate gene expression (Mortensen et al. 2011; Vasudevan et al. 2007). This process is rare and very selective, based on RNA sequence context, interacting proteins, and occurs in response to cellular circumstances such as G0 arrest (Orom et al. 2008; Vasudevan et al. 2007).

In addition to these well-studied cytoplasmic functions, recent research has uncovered a likely nuclear role for miRNAs. Both miRNAs and their protein co-factors such as AGOs and DICER have been detected in mammalian cell nuclei (Chen et al. 2012; Jeffries et al. 2011; Khudayberdiev et al. 2013; Liao et al. 2010; Nishi et al. 2013; Ohrt and Schwille 2008; Rudel et al. 2008; Till et al. 2007; Weinmann et al. 2009). They are believed to directly regulate gene expression by targeting the promoters of various genes. This regulation can be positive, i.e. RNA activation (RNAa), or negative, depending on the miRNA and the cell type. For example, miR-373 activates the E-cadherin and *Csdc2* promoters (Place et al. 2008), whereas miR-744, miR-1186 and miR-466d-3p induce cyclin B1 expression in mouse cell lines through association of the RNA-bound AGO protein with the *Ccnb1* promoter (Huang et al. 2012) and miR-483 regulates *Igf2* expression (Liu et al. 2013). In contrast, miR-423-5p represses transcription from the progesterone receptor promoter (Younger and Corey 2011). In most cases, however, it is not clear if the miRNAs bind directly to the promoter DNA, forming a triplex, or if they interact indirectly through RNA transcripts that are produced within promoters and enhancers (Carninci et al. 2005; Taft et al. 2009c). The latter has been shown to occur for the positive regulation of the *Cox2* promoter by miR-589 (Matsui et al. 2013). Either way, it seems that for this nuclear mechanism of gene regulation the binding of the miRNA by AGO and the complementarity of the seed sequence to the promoter is important (Place et al. 2008; Younger and Corey 2011), whereas the slicing activity of AGO is not required (Matsui et al. 2013).

Regulation of miRNA Activity

Similar to the regulation of miRNA biogenesis their function is also controlled, emphasising the importance of the correct miRNA activity level in cells of an organism. Regulation of miRNA action occurs through the control of miRNA access to their target mRNAs. Three mechanisms have been described that result in a decreased binding of miRNAs to their targets. First, the 3′UTR length of target genes varies depending on the cell cycle and embryonic development (Ji et al. 2009; Sandberg et al. 2008). In proliferating cells, 3′UTRs of many genes are shorter, thereby omitting miRNA target sites, resulting in higher protein expression as compared to the same genes with full-length 3′UTRs (Ji et al. 2009; Sandberg et al. 2008). Secondly, miRNAs can be sequestered

by mRNAs, transcribed pseudogenes, long ncRNAs and circular RNAs that contain miRNA binding sites and thereby function as a sponge (Hansen et al. 2013; Memczak et al. 2013; Poliseno et al. 2010; Sumazin et al. 2011; Tay et al. 2011). Finally, RNA-binding proteins can inhibit the access of miRNAs to their target sites. For example, the RNA-binding protein dead end 1 (DND1) has been shown to bind to 3′UTRs at miRNA recognition sites and thereby preventing the association of the miRNA-RISC complex with the target mRNA (Kedde et al. 2007).

2.1.2 Small Interfering RNAs

While miRNAs are processed from long, single-stranded transcripts to form a short hairpin structure, small interfering RNAs (siRNAs) are generated from long, perfectly complementary, double-stranded RNAs and function as an additional level of gene regulation and as a cellular defence mechanism against foreign and deleterious nucleic acids such as virus RNA and transposable elements. siRNAs are well characterized in *C. elegans*, which uses an RNA-dependent RNA polymerases (RDRPs) to transcribe single stranded RNAs in double-stranded RNA precursor for siRNA production (Sijen et al. 2001; Smardon et al. 2000). In contrast, mammals do not have any RDRPs. In addition, long, double-stranded RNA induce the interferon response and activation of protein kinase R, resulting in a global inhibition of protein translation (Clemens et al. 1975; Lee et al. 1993; Levin and London 1978). Therefore, it was assumed that mammals do not possess endogenous siRNAs (endo-siRNAs). However, high-throughput sequencing analysis demonstrated that also mammalian cells produce endo-siRNAs (Tam et al. 2008; Watanabe et al. 2006, 2008). These endo-siRNAs are synthesised from long, double-stranded RNAs generated by the transcription of palindromic sequences, transposable elements and pseudogenes. In mammals, endo-siRNAs are mainly found in oocytes, as long dsRNA does not activate the interferon response in these cells (Stein et al. 2005; Svoboda et al. 2000).

Endo-siRNAs are generated by DICER in a complex with TRBP and AGO2 (Gregory et al. 2005; Maniataki and Mourelatos 2005). This RISC-loading complex binds and slices the dsRNA precursor, releases the passenger strand and loads the siRNA onto AGO2 to form the functional siRISC (MacRae et al. 2008). The siRNA then guides RISC to either perfectly complementary RNA targets, which will be sliced and degraded (Fig. 2.2), or imperfectly complementary targets that will be silenced by the same mechanisms as through a miRNA. In addition, siRNAs can also induce heterochromatin formation as a way of transcriptional silencing (Grewal and Elgin 2007; Moazed 2009).

2.2 Long Non-coding RNAs

Long ncRNAs (lncRNAs) are a group of diverse heterogeneous ncRNAs that are longer than 200 nucleotides. Within the mammalian genome, lncRNAs are found in widespread loci with an expected number of tens of thousands, which likely make lncRNAs the largest portion of the mammalian non-coding transcriptome (Mercer et al. 2009). Based on their position in the genome, lncRNAs were categorized in three classes, intronic, natural antisense transcripts (NATs) and intergenic (large, intergenic ncRNAs or lincRNAs). However, this classification does not hold true when it comes to their functions. LncRNAs have a wide range of functions (Bernstein and Allis 2005; Mercer et al. 2009), including the regulation of gene expression in *cis* and in *trans*, the regulation of epigenetic chromatin modification, post-transcriptional processes as well as structural functions.

2.2.1 Long ncRNA Biogenesis

In general, lncRNAs are transcribed by RNA polymerase II and display the hallmarks of protein-coding genes. This includes the conservation and chromatin structure of their promoters, their regulation of expression by transcription factors and morphogens, their range of half-lives,

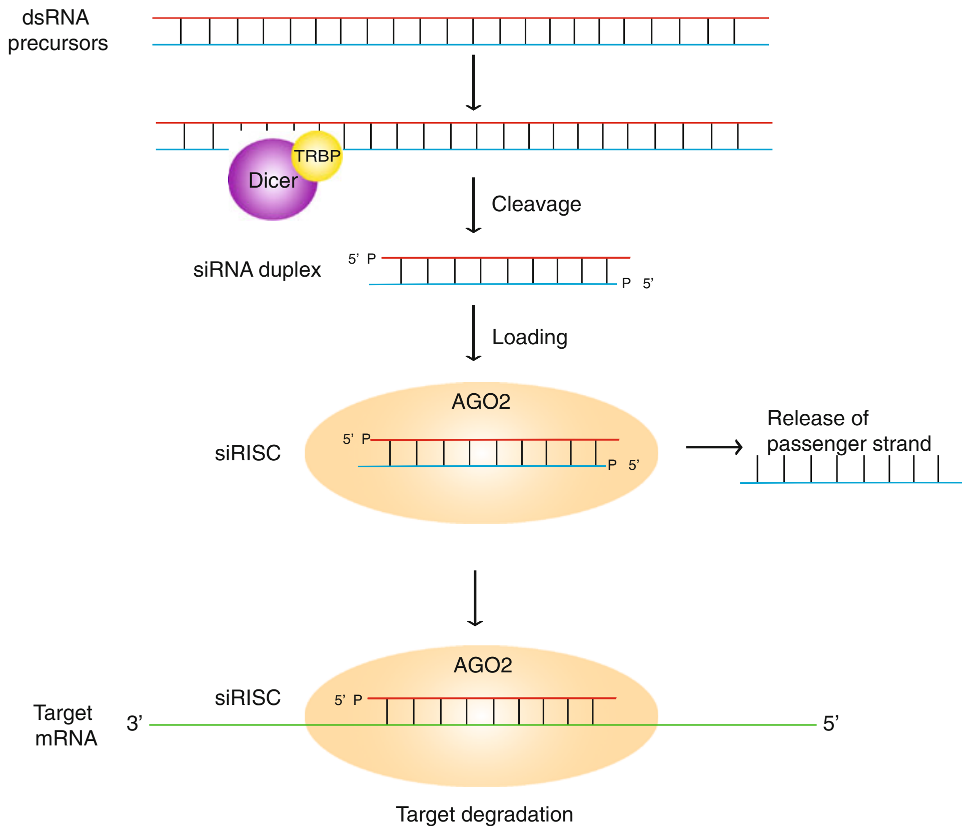

Fig. 2.2 Biogenesis of endo-siRNAs. Long double-stranded RNAs, derived from transcription of palindromic sequences, transposable elements or pseudogenes, can function as precursors for endo-siRNAs. In a first step the endonuclease DICER and its cofactor TRBP form the RISC-loading complex, which bind the precursor double stranded RNA to generate a 19–22 bp long siRNA duplex. The passenger strand of the siRNA duplex is released and the guide strand is loaded onto AGO2, thereby generating the functional siRISC complex in which the siRNA guides RISC to target RNAs. Perfectly complementary siRNA:mRNA combinations result in mRNA degradation

tissue-specific expression, and splicing, including alternative splice variants (Carninci et al. 2006; Cawley et al. 2004; Clark et al. 2012; Ravasi et al. 2006; Rinn et al. 2007).

In addition, many lncRNAs show the same characteristics as protein-coding mRNAs, i.e. they are spliced, 5′end capped and 3′end polyadenylated (Djebali et al. 2012; Martens et al. 2004; Tupy et al. 2005). However, more recently, through the development of more sophisticated approaches, alternative lncRNA structures have been identified. Instead of endonucleolytic cleavage by CPSF73 and subsequent polyadenylation, which is necessary for transcript stability, some lncRNAs possess a triple-helical structure at their 3′end that protects them from degradation (Brown et al. 2012; Wilusz et al. 2012). This triple helical structure is formed by cleavage through RNaseP, which removes a conserved tRNA-structure from the 3′end, leaving a short A-rich tract that can form stable UAA triple helical structure (Brown et al. 2012; Wilusz et al. 2012). Some well-studied examples for lncRNAs with a triple helical 3′end include *MALAT1* (metastasis-associated lung adenocarcinoma transcript), *NEAT1*, also called *MENε/β* (multiple endocrine neoplasia) and RNAs from various viruses (Mitton-Fry et al. 2010; Tycowski et al. 2012).

A second alternative structure, which also results in stable transcripts, is utilized by some lncRNAs derived from excised introns (Louro et al. 2008; Nakaya et al. 2007). Small, nucleolar

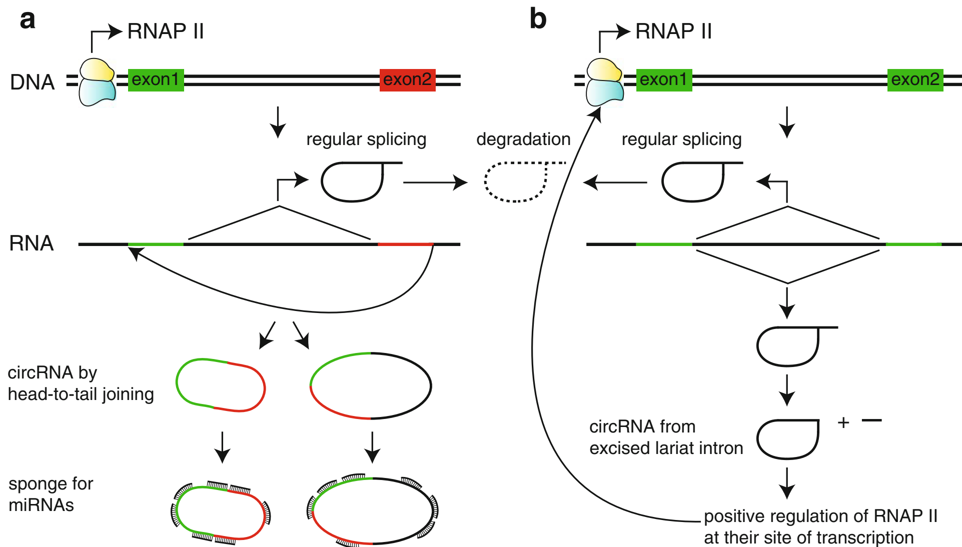

Fig. 2.3 Biogenesis and putative functions of circular RNAs. (**a**) The first type of circular RNA is produced from back-spliced exons by which the 5′end acceptor site from one exon is spliced to a downstream 3′end donor site of another exon. This process results in the generation of either circular exon-exon transcripts or combined exon-intron circRNAs. A number of these circRNAs exhibit several binding sites for miRNAs and are suggested to function as a sponge for miRNAs thereby inhibiting miRNA-dependent gene regulation. (**b**) The second type of circRNA is derived from spliced lariat introns by which a specific consensus sequence near the branch point site protects the lariat intron from debranching and degradation. circRNAs from excised introns interact with RNA polymerase II at their site of transcription thereby increasing the transcription rate

RNAs (snoRNAs) are encoded within introns and play a role in modification of small, nuclear and ribosomal RNAs. They are approximately 70–200 nt long and therefore are generally not classified as lncRNAs. However, many introns contain two snoRNA-like sequences at their ends. Processing of these ends by the snoRNP machinery after splicing, without removal of the sequences in between, results in intronic lncRNAs with snoRNA-like ends that are widely expressed within the human genome (Yin et al. 2012).

A third alternative mechanism of stabilization is circularization. Circular RNAs (circRNAs) can be formed by two different mechanisms (Fig. 2.3). CircRNAs are formed by either head-to-tail joining through back-splicing, by which the 5′end acceptor site is spliced to a downstream 3′end donor site (Hansen et al. 2013; Jeck et al. 2013; Memczak et al. 2013; Salzman et al. 2012) or are derived from excised lariat introns (Zhang et al. 2013, 2014b). Thousands of circRNAs have been identified in human cells (Jeck et al. 2013; Zhang et al. 2013) and some of these, especially those formed through back-splicing, display an enrichment for miRNA binding sites and have been proposed to function as miRNAs sponges, thereby indirectly regulating gene expression (Hansen et al. 2013; Memczak et al. 2013). In contrast, some intron-derived circRNAs accumulate at their site of transcription and positively regulate RNA polymerase II transcription at the elongation step (Zhang et al. 2013). However, it remains to be seen if all circRNA function through these mechanisms or if they also play roles through different mechanism similar to linear lncRNAs.

2.2.2 Mechanisms of Long ncRNA Function

LncRNAs play a role in many different processes, both at the molecular level such as splicing, transcriptional and epigenetic regulation of gene

expression, and at the cellular level, for example in the regulation of proliferation, differentiation and apoptosis. Different groupings have been suggested for these functions, such as repressive, activating, and structural, or functioning in *cis*, in *trans* and as a decoy, or a nuclear vs. cytoplasmic role. However, the more we learn about the different mode of actions of specific lncRNAs the more the boundaries of these categories do not hold true anymore. For example, some lncRNAs function as activators as well as repressors depending on the cellular context. For example, the RNA component, *SRA*, of the steroid receptor coactivator complex, acts as co-activator for the transcription factors MyoD and VDR (Lanz et al. 1999; Leygue 2007), but also as a repressor in association with CTCF (Yao et al. 2010). For some lncRNAs it appears that not the RNA itself plays the important role, but the process of transcription of the RNA (Keller et al. 2013; Uhler et al. 2007). Many lncRNAs function as link or scaffold to bring together specific regions in DNA and/or RNA and proteins, for example the binding and recruitment of the polycomb repressor complex 2 (PRC2) to specific loci by lncRNAs including the *asOct4-pseudogene 5* (Hawkins and Morris 2010), *HEIH* (Yang et al. 2011) and *HOTAIR* (Gupta et al. 2010; Kogo et al. 2011; Tsai et al. 2010). Other lnRNAs, e.g. *MALAT1*, interact with SR splicing factors to regulate alternative splicing (Bernard et al. 2010; Tripathi et al. 2010), or associate with TDF43 to control mRNA decay like the lncRNA *Gadd7* (Liu et al. 2012). In addition, several lncRNAs have been described to be important structural components, especially within the nucleus, such as *Gomafu* (Sone et al. 2007), which constitutes a novel nuclear domain within certain neurons, and *NEAT1*, which is essential for the formation and maintenance of paraspeckles (Chen and Carmichael 2009; Clemson et al. 2009). For more extensive descriptions of the mechanism of function for specific lncRNAs please refer to recent reviews (e.g. Morris and Mattick 2014; Zhang et al. 2014a).

The expression of some lncRNAs is associated with diseases such as cancer and neurodegeneration. A significant number of lncRNAs was linked to prostate, renal, breast and ovarian cancer (e.g. Brito et al. 2008; Perez et al. 2008; Reis et al. 2004). One of the best-studied lncRNAs in cancer is *MALAT1*, which is a known biomarker for lung cancer metastasis and regulates cell migration and invasion of cancer cells (Gutschner et al. 2013; Ji et al. 2014; Schmidt et al. 2011; Tano and Akimitsu 2012). In neurodegeneration the lncRNAs *17A* and *BACE1-AS* are both associated with Alzheimer's disease by regulating either the stability or inducing nonfunctional alternative splice isoforms of their target mRNA (Faghihi et al. 2008; Massone et al. 2011; Modarresi et al. 2011). An overall list, showing an update of all lncRNA and their associated diseases, is given by the database LncRNADisease (http://cmbi.bjmu.edu.cn/lncrnadisease (Chen et al. 2013)).

2.3 Other Non-coding RNAs

In addition to these well-studied small and long regulatory RNAs, other classes of ncRNAs have been described in the literature (for review see Farazi et al. 2008), but extensive characterization and elucidation of their function is still awaiting exploration.

Clusters of short, 22–200 nt-long RNAs were detected at the 5′ and 3′end of human and mouse genes, which were called promoter-associated short RNAs, PASRs, and termini-associated short RNAs, TASRs. Almost half of the genes that are expressed in a given cell are associated with PASR and/or TASRs, whereas only about 20 % of silenced genes have PASRs and/or TASRs (Kapranov et al. 2007a). However, no function has been described for these classes of RNAs yet. Similarly, we have described in an independent study ncRNAs that appeared to be processed from the 3′UTRs of protein-coding genes. We named these ncRNAs uaRNAs, for 3′UTR-associated ncRNAs. The size of these ncRNAs ranges between 50 and 200 nt; they are in sense direction to the protein-coding gene, and show stage-, sex- and subcellular-specific expression (Mercer et al. 2011). These RNAs could also function as decoy or sponges similar to other

ncRNAs and sequester away RNA-binding proteins and/or miRNAs that mainly regulate gene expression through the binding to the 3′UTRs of genes.

Several groups have identified a class of small ncRNAs that are derived from tRNA precursors (Haussecker et al. 2010; Kawaji et al. 2008; Lee et al. 2009). These were named tRNA-derived RNA fragments (tRF) or tRNA-derived small RNAs (tsRNAs). Some tsRNAs appeared to be processed by Dicer, while others are Dicer-independent and are generated through RNaseZ, leaving a 5′phosphate end, and transcription termination by RNA polymerase III forming their 3′end (Haussecker et al. 2010). These small RNAs are able to associate with AGO3 and AGO4 (Haussecker et al. 2010), and for one, *tRF-1001*, it has been shown that is highly expressed in a range of cancer cell lines, but had low expression in normal tissues (Lee et al. 2009). Furthermore, knockdown of *tRF-1001* by siRNAs resulted in impaired proliferation, suggesting a specific biological role for these small RNAs (Lee et al. 2009). However, it remains to be seen if this role in cell proliferation is mediated through AGO association and inhibition of target gene expression.

Similar to tRNAs, small nucleolar RNAs (snoRNA), have also been shown to be processed into small, miRNA-like molecules called sno-derived RNAs or sdRNAs (Ender et al. 2008; Saraiya and Wang 2008; Taft et al. 2009b). SnoRNAs play a role in guiding enzymes to target RNAs for modification (Matera et al. 2007). There are two classes of snoRNAs, C/D and H/ACA box, of which the small RNAs processed from the first, C/D box snoRNAs, are derived from the 5′end, whereas small RNAs from the H/ACA class are generated from the 3′end of the snoRNAs (Taft et al. 2009b). In contrast to tsRNAs, their biogenesis appears to be DICER-dependent (Taft et al. 2009b). Further research is required to determine if sdRNAs also associate with AGO proteins to regulate the expression of target genes.

Possibly the smallest RNA class identified to date, tiny RNAs or transcription initiation RNAs (tiRNAs), are predominantly 18 nt in length and map to the transcription start site of highly expressed transcripts and sites of RNA polymerase II binding. They are conserved from fly to human, and are mostly found at GC-rich promoters (Taft et al. 2009a). Similar to some of the other classes of ncRNAs, the function of tiRNAs is currently unknown. They may be a product of RNA polymerase II backtracking (Pal et al. 2001), or they could have specific roles such as the recruitment of chromatin modifying enzymes comparable to other ncRNAs.

2.4 Conclusions

The identification and rapid expansion of the roles of regulatory ncRNAs has caused a paradigm shift from RNAs mainly function as messengers between DNA and proteins to taking a central role in all processes at the molecular, cellular and whole organism level. Future development and refinement of techniques to detect ncRNAs and to analyse their function is likely to further change our picture of the processes that drive gene regulation, cell differentiation and the development of organs and whole organisms.

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3

How Many Non-coding RNAs Does It Take to Compensate Male/Female Genetic Imbalance?

Jean-François Ouimette and Claire Rougeulle

Abstract

Genetic sex determination in mammals relies on dimorphic sex chromosomes that confer phenotypic/physiologic differences between males and females. In this heterogametic system, X and Y chromosomes diverged from an ancestral pair of autosomes, creating a genetic disequilibrium between XX females and XY males. Dosage compensation mechanisms alleviate intrinsic gene dosage imbalance, leading to equal expression levels of most X-linked genes in the two sexes. In therian mammals, this is achieved through inactivation of one of the two X chromosomes in females. Failure to undergo X-chromosome inactivation (XCI) results in developmental arrest and death. Although fundamental for survival, a surprising loose conservation in the mechanisms to achieve XCI during development in therian lineage has been, and continues, to be uncovered. XCI involves the concerted action of non-coding RNAs (ncRNAs), including the well-known *Xist* RNA, and has thus become a classical paradigm to study the mode of action of this particular class of transcripts. In this chapter, we will describe the processes coping with sex chromosome genetic imbalance and how ncRNAs underlie dosage compensation mechanisms and influence male-female differences in mammals. Moreover, we will discuss how ncRNAs have been tinkered with during therian evolution to adapt XCI mechanistic to species-specific constraints.

Keywords

Dosage compensation • X-chromosome inactivation • Xist • Tsix • Sex chromosomes

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3.1 Foundations of X-Chromosome Inactivation

The X and Y chromosomes in therians have significantly diverged from one another during evolution. Indeed, while the human X chromosome contains around 1300 genes with a full range of function, the Y chromosome gene content is rather small, with around a 100 genes, most of them specialized in male-determining and/or advantageous functions, like *Sry* (Graves 2010). Furthermore, the Y chromosome has evidently undergone extensive remodelling during mammalian evolution while the X chromosome displays a much more stable evolutionary history, having preserved synteny and gene content. These different evolutionary trajectories presumably take their roots in the suppression of recombination between the X and Y during male meiosis that appeared early in mammalian evolution. Absence of recombination has rendered the Y chromosome susceptible to deleterious genomic events, such as rearrangements, insertions and deletions that have created extensive genetic imbalance between both sex chromosomes. The presence of a single copy of X-linked genes in males versus the diploid set in females is compensated for in mammals by inactivating an entire X chromosome in females and by upregulating the active X in both males and females to achieve levels of expression equivalent to that of the diploid sets of autosomes (Deng et al. 2011). The latter process being deployed in both sexes, we focus here on the process of X-chromosome inactivation (XCI) and its impact on male/female differences.

XCI was first conceptualized by Mary Lyon, thanks to various observations on sex chromatin characteristics (Barr and Bertram 1949; Ohno et al. 1959) and on the effect of X-linked gene mutations on the observed mosaicism of the coat colour of female mice (Lyon 1961). The hypothesis laid in the seminal paper published in 1961, which has become to be known as the Lyon law, is that one X chromosome, which can be either of maternal or paternal origin, is genetically inactivated in female cells. This inactive state, once established, is stably maintained and transmitted clonally over cell divisions. Considerable efforts have been invested since then to decipher the whys and wherefores of X-chromosome inactivation.

We know now that initiation of XCI in female starts early in embryonic development, with the expression of *Xist*, a ncRNA specifically expressed from the inactive X (Brockdorff et al. 1991; Brown et al. 1991a; Borsani et al. 1991). This ncRNA is conserved in eutherians and has been associated with XCI in all species of this lineage investigated so far (Chureau et al. 2002; Duret et al. 2006). Once upregulated from a single X chromosome, *Xist* accumulates and coats this chromosome in *cis* (Clemson et al. 1996; Brown et al. 1992). Through the direct and indirect targeting actions of the *Xist* RNA, gene silencing begins and features of heterochromatin accumulate on the Xi (de Napoles et al. 2004; Heard et al. 2001; Peters et al. 2002; Plath et al. 2003; Rasmussen et al. 2000). A combination of epigenetic modifications, including histone and DNA methylation, are believed to act in concert to secure the maintenance and clonal transmission of the Xi.

Some genes, known as escapers, bypass this chromosome-wide silencing, even though expression from the Xi is frequently lower than from the Xa (reviewed in Berletch et al. 2010). The degree of escape varies in a tissue-specific and loci-dependent manner. Escaping can result from immediate resistance to XCI or from a progressive relaxation due to an inability to maintain XCI. Escaping genes without homologues on the Y chromosome likely establish in females a unique signature that underlies some male/female differences.

3.2 Relating Sex-Chromosome Complement to Phenotypic Male/Female Differences

As illustrated with escaping genes, XX and XY sex-chromosome complement induces intrinsic differences that XCI cannot prevent. This is further supported by recent studies taking use of the

four core genotypes in mice (XY^{sry-} and XX females or XY^{sry-};Sry^{tg} and XX;Sry^{tg} males), which have revealed both developmental and adult phenotypes that are related to the gonosome complement and not to the sex. These include differences in brain differentiation that affect behavioural traits and metabolic differences in liver and in adipose tissue that contribute to obesity (De Vries et al. 2002; Chen et al. 2013). These studies clearly highlighted that, although dosage compensation (DC) mechanisms coping with genetic imbalance between male and female are used, differences still arise in mammals from sex chromosomes dimorphism.

The timing of XCI may influence in the long-term male/female development. There is indeed a time window, prior to the initiation of XCI, where females harbour two active X chromosomes. This temporal window of dosage imbalance is likely to impact on the development of female embryos. Observed delay in the developmental progression of female embryos compared to males, seen prior to gonadal differentiation in most mammalian species, may be an effect of this imbalance (Burgoyne et al. 1995). Male/female differences acquired during early development may be consequential of X-linked genes' influence on autosomal genes. Many chromatin modifiers and readers are X-linked and serve to establish transcriptional regulation, not only on X-linked loci, but genome-wide. Investigation of sexual dimorphism before gonadal development in the embryonic brain, an organ known for displaying morphological and phenotypical sex-related differences, has uncovered genes with sex-biased expression, many of which are autosomal genes (Dewing et al. 2003).

Apart from escaping genes and XCI timing, the process of XCI in itself generates sexually dimorphic gene expression. Mosaicism in female, a direct consequence of random XCI, influences in particular female susceptibility to X-linked diseases and makes females less susceptible than males to the consequences of X-linked mutations. In addition, the penetrance of a phenotype of a recessive mutation will vary between female individuals bearing the same mutation if skewing in the XCI process has favoured inactivation of either the wild type or mutant allele. Finally, Y-linked genes also contribute to male-female differences. Clearly, XCI cannot circumvent all the consequences of sex chromosome dimorphism. Nevertheless, gene dosage compensation through XCI has been selected for to buffer sex chromosomes divergences and equalizes X-linked gene product output.

3.3 Various Roads Lead to XCI: Developmental Regulation of the Process

X-chromosome inactivation in therians can be achieved through imprinted (iXCI) and/or random (rXCI) mechanisms (Fig. 3.1). In both cases, the inactivated X chromosome acquires features of heterochromatin that lead to its stable silencing. The two modes of inactivation were identified in mice as successive developmental waves of XCI, but this pattern is not conserved in the therian lineage. Similarities have only been found in rat and bovine species (Dindot et al. 2004; Wake et al. 1976), although the extent to which they have been investigated has so far remained limited. Other eutherian species appear to only undergo rXCI, as evidenced in human, equine, porcine and rabbit (reviewed in Dupont and Gribnau 2013), while marsupials display only iXCI, leading to silencing of the paternal X chromosome.

For practical reasons, mouse XCI dynamics have been the most characterized among mammals. As in marsupials, iXCI in mouse leads to silencing of the paternal X-chromosome (Xp). This takes place prior to implantation, around the 4–8-cell stage, through an imprint of unknown nature. Imprinted X-chromosome inactivation is maintained in extra-embryonic tissues (Takagi and Sasaki 1975), which makes this lineage sensitive to extra copies of Xm (as only Xp can be silenced through iXCI), contributing to the failure of mouse parthenogenote to specify trophectoderm derivatives (McGrath and Solter 1984; Barton et al. 1984; Shao and Takagi 1990).

In the inner cell mass (ICM) of the early mouse blastocyst embryos, the imprint is

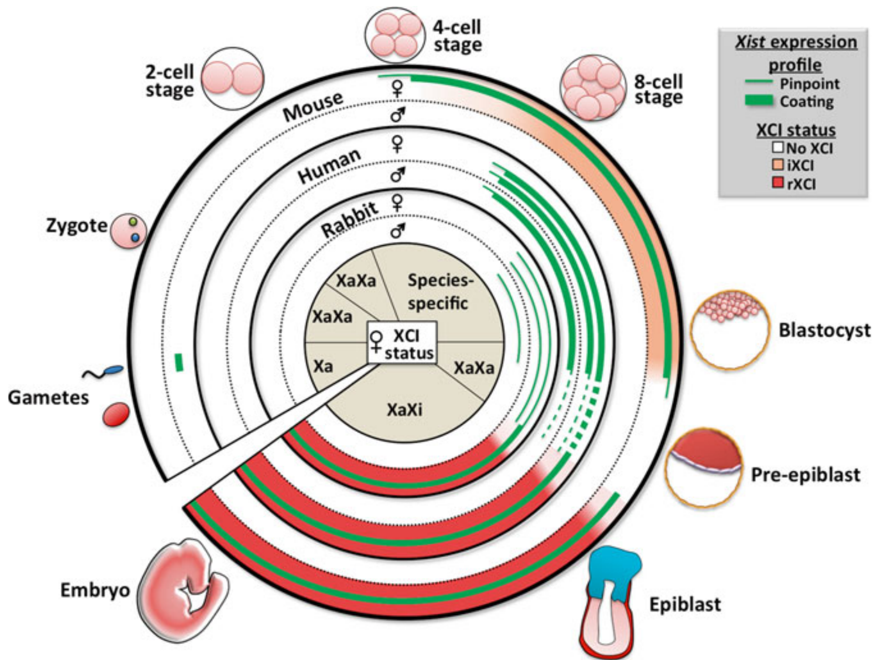


Fig. 3.1 Comparative dynamics of X-chromosome inactivation in eutherian species. Kinetics of XCI and *Xist* expression during mouse, human, and rabbit embryonic development; for each species, males and females are represented. The corresponding developmental stages are depicted on the outside of the circles. The fillings delineate time windows for imprinted (iXCI, in *orange*) and random (rXCI, in *red*) phases of X-inactivation, which occur in females only. *Xist* expression profile is schematized by *curved lines* (punctuate expression) and *boxes* (coating). *Two parallel lines/boxes* indicate bi-allelic expression. *Dashed lines* and *boxes* represent extrapolated profiles

somehow erased and XCI is reversed (Fig. 3.1). This allows for complete reactivation of the paternal X chromosome (Okamoto et al. 2004; Mak et al. 2004). The resulting XaXa status is however transient in vivo and is rapidly resolved through random XCI (Rastan 1982; Takagi et al. 1982), based on a stochastic choice between the maternal and paternal X chromosome. Random XCI is first detected in epiblast cells, but in vivo studies have shown that the acquisition of a Xi is not a synchronous event. Analysis of X-linked endogenous or transgenic markers to monitor chromosome silencing has rather demonstrated slow and tissue-specific kinetics, rXCI being completed well after gastrulation takes place (Tan et al. 1993). As such, it is unlikely that all epiblast cells have undergone complete XCI and the stable XaXi status displayed by the recently derived epiblast stem cells (EpiSC) is probably acquired, stabilized or selected for during derivation (Brons et al. 2007). In contrast, mouse embryonic stem (ES) cells can stably maintain, in their undifferentiated state, the XaXa status characterizing the ICM from which they are derived, but random XCI is achieved upon differentiation, a property extensively exploited to decipher the mechanisms underlying this process.

Once established, rXCI is stably propagated during cell divisions and maintained throughout the life. One notable exception is the female germline, in which reversion of XCI is an obligate step to allow for equal transmission of both X chromosomes to the next generation. These cells are specified during gastrulation, migrate from the mesentery to the gonadal ridges and differentiate to form oocytes in the ovary. Emergence of these cells is progressive and, accordingly, they display very heterogeneous XCI dynamics. In female mouse embryos, the onset of XCI seems concomitant with their entry in the gonadal ridges from E9, a rather late timing in comparison to neighbouring cells (Tan

et al. 1993; Tam et al. 1994). Xi reactivation, which precedes the onset of meiosis, is asynchronous in this population, as indicated by the reactivation of X-linked transgenes and enzymes taking place between E11.5 and E17.5 (Tam et al. 1994; Sugimoto and Abe 2007; Chuva de Sousa Lopes et al. 2008).

Variation in X chromosome activity is also observed during male gametogenesis, with inactivation of both X and Y chromosome occurring during meiosis through the formation of the sex body (Solari 1974). Meiotic sex chromosome inactivation (MSCI) is reverted post-meiotically (Hendriksen et al. 1995; Mueller et al. 2008), although it might persist to some extent and be potentially transmitted through fertilization. The inactive gonosomes found in spermatocytes and the inactive X chromosome present in female somatic cells share common features in particular in their chromatin structure (Turner 2007), but the underlying mechanisms are likely different. *Xist* in particular is expressed in testes and found associated with the sex body, yet it is dispensable for spermatogenesis (Marahrens et al. 1997), sex body formation and MSCI (Turner et al. 2002).

While the dynamics of XCI throughout the life cycle has been pretty well deciphered in the mouse, much less is known about its kinetics and regulation in other species. Nevertheless, what is now emerging is that mice might not have been the best model system regarding XCI; it appears from a few pioneer studies that different strategies have evolved in parallel in the different mammalian clades to achieve the same goal: inactivate one X chromosome in females (Fig. 3.1). The existence of iXCI in human has been long debated, but elegant work performed in human embryos obtained from in vitro fertilization clearly demonstrate that human XCI is achieved in a random manner only (Okamoto et al. 2011); in contrast to the mouse, *XIST* can be expressed from both the maternal and the paternal X in human pre-implantation embryos (Ray et al. 1997). Random XCI has also been reported in human extraembryonic tissues (Moreira de Mello et al. 2010). Rabbits, which are closely related to rodents on the evolutionary scale, also proceed without iXCI, suggesting that iXCI in eutherians might be a recent evolutionary event. In addition, in both rabbits and humans XCI is initiated later than in the mouse, and no reactivation takes place at the blastocyst stage.

Differences in XCI patterns are also observed in ES cells of various origins. In particular XCI has already taken place in most human ES cells prior to differentiation, but the inactive status is highly unstable at this stage (Makhlouf and Rougeulle 2011). Whether these differences are related to the variety in XCI kinetics during in vivo development remains to be understood.

Finally marsupials, which have diverged from the common eutherian ancestor some 150 million years ago, have taken a quite different route to achieve XCI. Imprinted XCI, with systematic inactivation of the paternal X, is the rule in marsupials. This inactivation is less stable than in placental mammals: many genes escape inactivation in what appears to be a stochastic manner (Al Nadaf et al. 2010). Frequent reactivation could be linked to the fact XCI in marsupials does not involve DNA methylation. Some of the heterochromatin features of the inactive X, at the level of histone modification, are however conserved between marsupials and eutherians (Koina et al. 2009). More importantly, XCI in marsupials relies on a different set of factors; *Xist* in particular is absent from these species.

In summary, while XCI is an obligate route for mammalian female development, it is surprising to see how flexible this process can be. The reason for such diversity is still unknown, but it is likely to be linked to the important variation between mammals in the dynamics of their early development. This includes in particular the timing of zygotic gene activation, which is much earlier in mice than in other species.

3.4 NcRNAs at the Centre of XCI

The locus controlling XCI has been physically mapped in various species to a single region on the X-chromosome named the X-inactivation center (*Xic* in mouse, *XIC* in human, Fig. 3.2). This mapping took advantage of X-autosome translocations and X chromosome rearrange-

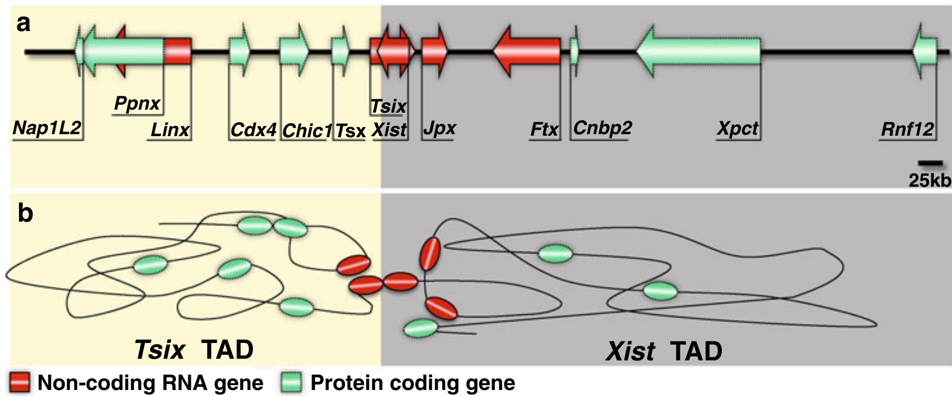


Fig. 3.2 Physical and tri-dimensional organization of the X-inactivation centre. (**a**) Linear representation of the *Xic*, with protein coding genes schematized by *green arrows* and non-coding genes by *red* ones. (**b**) High-order organization of the region based on chromosome conformation capture carbon-copy analysis (Nora et al. 2012). The *Xic* is partitioned into two distinct topologically associated domains (TADs). The boundary is located between *Xist* and *Tsix* promoters so that one TAD contains *Xist* and the other *Tsix*. Within TADs, genes are co-regulated

ments and deletions that recapitulates or hampers XCI. The *XIC* has been mapped to Xq13 in humans and to XD in the mouse. It spans around 1 Mb in the human and 700 kb in the mouse, although the precise borders have been hard to delineate (Rastan 1983; Rastan and Brown 1990; Brown et al. 1991b). Current delineations of this region rely on functional studies of the different genes nearby the physically mapped region that have been implicated in XCI (reviewed in Augui et al. 2011) and on the study of the high-order organization of chromatin and its dynamics to define domains and boundaries (Nora et al. 2012). A striking feature of the *XIC* is the abundance of gene producing long ncRNAs. The murine *Xic* indeed contains five long ncRNA genes, (*Xist*, *Tsix*, *Ftx*, *Jpx* and *Linx*), most of which have been shown, or are believed to participate to the XCI process. Six protein-coding genes (*Nap1L2*, *Cdx4*, *Chic1*, *Zcchc13*, *Slc16a2* and *Rnf12*) and a presumably bi-functional RNA gene (*Tsx*) that could acts both as a protein and as a ncRNA (Simmler et al. 1996; Anguera et al. 2011) are also present within the *Xic*. Among those, only *Rnf12* has been shown clearly to participate to the control of XCI (Barakat et al. 2011; Jonkers et al. 2009; Shin et al. 2010). In addition, the *Xpr* locus, which overlaps with *Slc16a2*, has been proposed to serve as a pairing platform that, upon physical engagement of the two X, would trigger the mechanisms of XCI, although this role is still debated (Augui et al. 2007; Sun et al. 2011). Intriguingly, the *Xic* is partitioned into two distinct topologically associated domains (TAD) which are transcriptionally co-regulated: a XCI-repressive unit containing *Linx/Pplnx, Xite*, *Tsx* and *Tsix* and a XCI-activating unit including *Xist*, *Jpx*, *Ftx* and *Rnf12* (Nora et al. 2012) (Fig. 3.2). These domains, characterized by a high propensity for internal contact and isolated from one another by a binding site for the insulator protein CTCF (Spencer et al. 2011), are essential to coordinate the timely expression of genes inside the respective domains and ultimately of *Xist* and *Tsix*. These results suggest that, apart from the elements present inside the *XIC*, proper organization of the region might be essential to impose functionality; the ncRNA present inside the different TADs could function in this conformational regulation. In line with this hypothesis, deletion of *Ftx* in male ES cells was shown to perturb the transcription of neighbouring genes in a gradient-like fashion (Chureau et al. 2010).

In summary, the X-inactivation centre possesses an essential chromosome-silencing activity mediated by the *Xist* ncRNA. Many of the additional elements of the *Xic*, including the other ncRNAs, also participate to the XCI pro-

cess by facilitating or preventing this silencing activity, either directly through the regulation of *Xist*, or indirectly.

3.5 The Essentials of *Xist*: Coating, Silencing and Regulation

Xist was originally identified as being transcribed solely from the Xi of somatic cells, producing a 17 kb spliced, capped, polyadenylated and nuclear retained RNA devoid of protein-coding activity (Brown et al. 1992; Brockdorff et al. 1992). *Xist* was later demonstrated to be essential for XCI, as X chromosomes carrying *Xist*-null mutation cannot undergo XCI (Penny et al. 1996). The central role of *Xist* in XCI relies on two fundamental properties: the ability of the *Xist* RNA to coat in *cis* the chromosome from which it is expressed, and its capacity to trigger transcriptional silencing at a chromosome-wide level.

A transgenic approach, in which the activity of various truncated and deleted version of *Xist* was measured, demonstrated that coating and silencing functions are mostly independent from each other and mediated by different regions of the *Xist* RNA (Wutz et al. 2002), which are organized as tandem repeats of monomeric units of various lengths. Several elements appear to be responsible for *Xist* RNA chromosomal painting-like distribution. One prevalent domain is the C-repeat, containing 14 tandem repeats with high inter-repeat homology. Although deletion of the C-repeat from the *Xist* transgene does not impact its capacity to trigger XCI (Wutz et al. 2002), competition with antisense peptide nucleic acid and locked nucleic acids leads to loss of *Xist* RNA from Xi (Beletskii et al. 2001; Sarma et al. 2010). The matrix protein hnRNP U interacts with *Xist* through the C-repeat and depletion of this protein during differentiation of female ES cells impair XCI due to mislocalization of the *Xist* RNA. Similar interference with hnRNP U in differentiated cells causes release of *Xist* from the Xi (Hasegawa et al. 2010). Recruitment of this protein on the Xi occurs however after initial *Xist* coating (Pullirsch et al. 2010), raising the question of its importance in the initiation of the process. The transcription factor Yin Yang 1 (YY1) has also been proposed to nucleate *Xist* RNA in *cis* to its site of transcription through simultaneous binding to a *Xist* genomic element and to *Xist* RNA binding sites near the F-repeat (Jeon and Lee 2011). Another interesting recent hypothesis, raised from the identification of the genomic regions interacting with *Xist* RNA, is that *Xist* exploits pre-existing three-dimensional architecture of the chromosome to contact initial target sites (Engreitz et al. 2013).

Xist silencing activity depends on a single region in the 5′ part of the RNA, the A-repeat. Deletion of this element, while not affecting the ability of the RNA to coat the chromosome in *cis*, blocks its silencing activity (Wutz et al. 2002). The A-repeat also appears to be important for spreading of the *Xist* RNA from the initial target sites to the entire chromosome, including gene dense regions (Engreitz et al. 2013). Notably, overexpression of A-repeat mutant *Xist* RNA leads to the recruitment of chromatin remodelers, such as PRC2 and the subsequent heterochromatic marks, indicating that the accumulation of these marks is not causally involved in gene silencing. The first event that follows *Xist* accumulation is eviction of the transcription machinery from the *Xist* RNA compartment, which occurs independently of A-repeat. Relocation of genes to this repressive compartment however depends on the A-repeat (Chaumeil et al. 2006). How the A-repeat, and more generally *Xist*, causes silencing is still poorly understood.

Xist expression per se is not sufficient to trigger XCI in the mouse, and the time-window and cellular contexts in which *Xist* is able to silence have been shown to be rather restricted. Induction of *Xist* up-regulation in differentiated cells or past the first 3 days following the onset of ES cell differentiation does not lead to stable X-chromosome inactivation (Wutz and Jaenisch 2000). Detailed in vivo analysis is missing, but it was shown that *Xist* is able to trigger XCI in only specific contexts including early embryogenesis, pro-B cells, and lymphomas (Savarese et al. 2006; Agrelo et al. 2009). These observations have spurred interest in the identification of com-

petency factors that would mediate *Xist* silencing activity. The chromatin organizer SATB1 was proposed to be an obligate factor in XCI, by triggering the silencing-potency of *Xist* (Agrelo et al. 2009), but this conclusion has been challenged by the observation that SATB1, and the related protein SATB2, are dispensable for XCI in vivo (Nechanitzky et al. 2012).

In parallel to investigating *Xist* silencing function, many studies over the years aimed at deciphering the regulatory network controlling the transcription of this gene, mostly in the mouse system. *Xist* regulation, although not yet fully understood, involves a combination of *cis* and *trans* acting elements of RNA and protein nature. While ncRNAs controlling *Xist* expression will be discussed in the following sections, we will focus here on the involvement of trans-acting proteins. Early observations had highlighted the coupling between XCI and cellular differentiation (Monk and Harper 1979). This paved the way for further analysis investigating the molecular coupling of XCI and *Xist* expression with pluripotency. The pluripotent state in the mouse is indeed well correlated with the repression of *Xist*, whether in vivo (ICM) or in vitro (ES and iPS cells) (reviewed in Makhlouf and Rougeulle 2011). Exit from pluripotency is accompanied, in the case of XX cells, by *Xist* activation and subsequent XCI. Pluripotency factors such as OCT4, SOX2, NANOG, and REX1 have been proposed to exert a repressive effect on *Xist* (Navarro et al. 2008) and an activating function on the *Xist* antagonist *Tsix* (Navarro et al. 2010), even though it is still matter of debate how direct some of these effects are on *Xist* (Minkovsky et al. 2013). REX1 was shown to play part to both events: it binds to *Tsix* regulatory elements and facilitates its expression in ES cells (Navarro et al. 2010). REX1 also appears to directly associate to *Xist* promoter elements to block its up-regulation (Gontan et al. 2012). In vivo, REX1 expression decreases rapidly as ICM cells progress to epiblast state (Rogers et al. 1991; Pelton et al. 2002) and is rapidly lost upon differentiation in vitro (Hosler et al. 1989). This timely down-regulation of REX1 is probably mediated in part by the ubiquitin degradation pathway, as REX1 is the target of the X-linked E3 ubiquitin ligase RNF12/RLIM, also shown to be essential in XCI (Gontan et al. 2012).

While several trans-acting elements have been described to repress *Xist* directly or indirectly, much less is known regarding the factors involved in its transcriptional up-regulation. We have recently obtained evidence that YY1 plays a prominent and conserved role in *Xist* activation, both during the initiation and the maintenance phases of XCI (Makhlouf et al. in preparation).

Xist regulation also takes place at the chromatin level, and differential histone modification and DNA methylation profiles characterize the active and inactive *Xist* promoter region. Loss of de novo and maintenance DNA methyltransferases DNMT1, 3a and 3b results in aberrant activation of *Xist* ex vivo in differentiated cells but not in ES cells (Beard et al. 1995; Panning and Jaenisch 1996; Sado et al. 2004). Similarly, *Xist* repression is compromised in the absence of the methylated DNA binding protein MBD2 (Barr et al. 2007). Normal *Xist* expression is nevertheless observed in the majority of *Dnmt3a* and *Dnmt3b* mutant XX cells in vivo (Sado et al. 2004), indicating that mechanisms other than DNA methylation are involved in setting up the differential expression of *Xist* on the two X chromosomes. Interestingly, links have been proposed between *Tsix* and DNA methylation (Ohhata et al. 2008; Navarro et al. 2005) and *Tsix* RNA could function through association with DNMTs by depositing methylation marks on *Xist* promoter elements. We discuss the various modes of action of *Tsix* in the next section.

3.6 *Tsix*, an Antisense Regulator of *Xist*

Tsix is a non-coding transcript antisense to *Xist* (Fig. 3.2) and was the first element shown to be involved in controlling the latter, both during imprinted and random XCI (Lee and Lu 1999; Lee 2000; Sado et al. 2001, 2002; Luikenhuis et al. 2001). Preventing *Tsix* expression during random XCI through various mutations leads to systematic accumulation of *Xist* on the mutated

chromosome. Conversely, forcing the expression of *Tsix* precludes *Xist* up-regulation in *cis*. These observations demonstrate that *Tsix* acts by blocking *Xist* accumulation in *cis*, participating as such to the choice of the chromosome to be inactivated at the onset of XCI. *Tsix* is however not responsible for the repression of *Xist* in ES cells as this repression is maintained in *Tsix* mutant contexts (Navarro et al. 2005). *Tsix* is expressed from both X chromosomes in wild type female ES cells, but the two alleles are asynchronously silenced during differentiation (Lee et al. 1999), a process that has been correlated to X-chromosomes pairing (Masui et al. 2011). This asymmetric expression of *Tsix* is believed to contribute to the monoallelic activation of *Xist*.

Tsix is also involved in iXCI by protecting the maternal X from inactivation. Maternal inherited *Tsix* deletion leads to ectopic *Xist* expression and aberrant XCI of the Xm resulting in early embryonic lethality, while disruption of *Tsix* on the paternal allele has very little effect. *Tsix* protective effect is however not likely to be the determinant of imprinted XCI as *Tsix* transcription on the maternal X chromosome starts at the morula stage, after paternal-specific *Xist* expression begins in the early embryo (Sado et al. 2001).

Tsix prevalent regulatory function seems to be associated with its transcription per se rather than with its RNA product: genetic manipulations preventing *Tsix* transcription fails to repress *Xist* and replacement with a cDNA does not rescue this phenotype (Shibata and Lee 2004). *Tsix* likely functions through several chromatin-remodelling activities (Navarro et al. 2005, 2006, 2010; Ohhata et al. 2006; Sun et al. 2006), acting at its own locus, at the *Xist* promoter region and more distally, over a large hotspot of H3K9 and H3K27 tri-methylation located 5′ to *Xist* (Rougeulle et al. 2004). In particular, *Tsix* deposits heterochromatin-like marks at the *Xist* promoter. Asymmetric *Tsix* down-regulation at the onset of random XCI therefore creates a differential chromatin structure at this region, which likely contributes to activation of a single *Xist* allele. In addition, *Tsix* remodelling activities were also proposed to act at the transition from imprinted to random XCI. Biallelic expression of *Tsix* at this stage would reset the *Xist* promoter chromatin landscape and erase marks associated with imprinted XCI, thus rendering both *Xist* alleles epigenetically equivalent and equally competent for up-regulation (Navarro et al. 2005).

3.7 Xist/Tsix Neighboring Regulators Include Other ncRNAs

Xist and *Tsix* play a pivotal role in controlling the onset of XCI and the regulation of this pair of sense/antisense transcripts has been linked to trans-acting protein components and in particular to pluripotency factors in the mouse as described above. Non-coding RNAs and genomic elements of the *XIC* are also involved their transcriptional regulation. These are physically and functionally segregated as preventing or facilitating XCI, through their action on *Tsix* or *Xist* respectively (Fig. 3.2).

Tsix expression is enhanced by *Xite*, an upstream transcribed non-coding region (Stavropoulos et al. 2005) and *DxPas34*, a 34mer repeat element within *Tsix* that binds several transcription factors (Navarro et al. 2010; Vigneau et al. 2006; Cohen et al. 2007). *Linx* is another nuclear retained, unspliced transcript, located more than a 100 kb upstream of the major *Tsix* promoter and transcribed in the opposite direction (Nora et al. 2012). *Linx* is co-expressed with *Tsix* in mouse ES cells and in the ICM of the blastocyst. Its expression in female ES cells is frequently monoallelic prior to *Xist* up-regulation. The *Linx* locus shows specific chromosomal interaction with the *Tsix* locus and this could provide the asymmetry-breaking signal to designate the future active X chromosome through *cis* up-regulation of *Tsix*. The implication of the *Linx* RNA in that process is however unclear and remains to be investigated. Indeed, activating mechanisms of *Xite*, *DxPas34* and *Linx* might not directly necessitate RNA products but they could rather acts as classical transcriptional enhancer by facilitating transcription through DNA interactions.

One proposed actor favouring XCI is *Jpx*, a partly spliced ncRNA lying just upstream of *Xist*

and transcribed in the opposite direction (Chureau et al. 2002; Johnston et al. 2002; Tian et al. 2010). *Jpx* expression is not sex-specific, as evidenced by similar induction of *Jpx* RNA in differentiating male and female ES cells (Tian et al. 2010). However, while deleting *Jpx* in male ES cells does not hamper their differentiation, heterozygous deletion in female ES cells prevent *Xist* up-regulation upon differentiation and XCI (Tian et al. 2010), leading to cell death. Rescue experiments indicated that *Jpx* RNA acts in *trans* and further studies have proposed that it functions in a dose-dependent manner to remove a repressor from *Xist* regulatory regions (Sun et al. 2013). In this setting, CTCF would work as a *Xist*-repressing factor, through binding to an exonic region of *Xist*. To relieve the block on *Xist*, *Jpx* RNA would dislodge CTCF mono-allelically. This hypothesis is however in contradiction with previous studies which have demonstrated binding of CTCF at this site on the *Xist*-expressing allele (Calabrese et al. 2012).

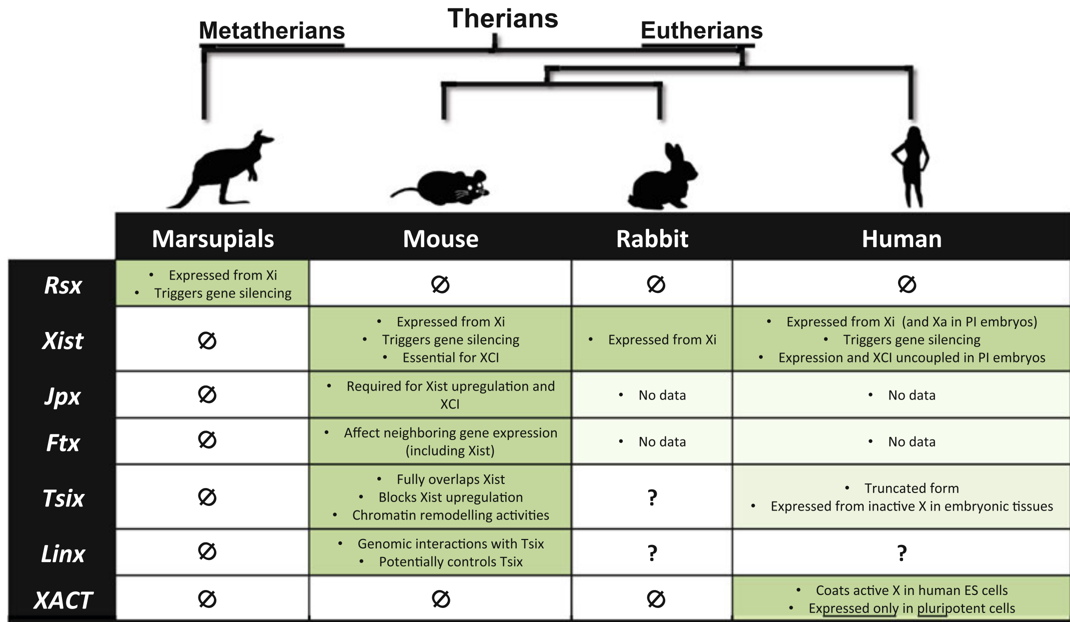
Ftx is another ncRNA proposed to be involved in the positive regulation of *Xist*. It lies further upstream of *Xist* and generates multiple nuclear isoforms through alternative promoter usage and splicing. *Ftx* is up-regulated during female ES cells differentiation, in a profile resembling that of *Xist*. Deletion of *Ftx* promoters in male ES cells abrogates *Ftx* transcription and causes a general decrease in the expression of its neighbouring genes including *Xist* (Chureau et al. 2010). Similar effects have been observed in mutated female ES cells (Romito and Rougeulle personal observation). It however remains to be investigated whether and how *Ftx* impacts on XCI.

3.8 Tinkering with ncRNAs to Adapt to Species-Specific Constraints

Despite many still unanswered questions, the mechanisms and actors of XCI have now been well deciphered in the mouse, revealing a myriad of ncRNAs, not only *Xist* and *Tsix* but also several neighbouring partners, that appear to act in concert to ensure sex-specific and developmental control of this essential process. However, as differences in the XCI strategy used in various mammalian species are now being confirmed, it becomes essential to address the functional conservation of the known actors and the existence of species-specific elements, in particular ncRNAs (Fig. 3.3). Several recent data are indeed illustrating how XCI evolutionary plasticity might be connected with species-specific ncRNAs.

The lack of a *Xist* homologous gene in marsupials has fuelled efforts to identify mechanisms that might account for dosage compensation in this therian branch, especially since the marsupial Xi shares many features with the eutherian Xi (Mahadevaiah et al. 2009). Recently, the long non-coding gene *Rsx* was identified on the marsupial X chromosome (Grant et al. 2012). *Rsx* produce a large RNA that is specifically expressed from the Xi in females and exhibits *cis* coating. It shares important features with *Xist* such as enrichment in tandem repeats in its 5′ part, a high GC-rich content and the ability to induce, at least partially, gene silencing when introduced on mouse autosomes. These properties make *Rsx* a likely candidate for exerting X-chromosome silencing function in marsupial's dosage compensation. Sequence conservation analysis revealed no substantial homology with *Xist* and it seems likely that *Rsx* arose independently in marsupials, making it a fascinating example of convergent evolution through co-option of ncRNAs for dosage compensation.

In eutherians, considering the evolutionary history of the region that controls X-inactivation may help understand the tinkering that has led to species-specific tricks to achieve XCI. A region syntenic to the human *XIC*, with similar gene content and organization, is found in all eutherians including elephant, the most distantly related species of this lineage (Duret et al. 2006). In non-eutherian species, including marsupials, an *XIC* homologous region (*XIC* HR) exists but is devoid of ncRNA genes. The ncRNAs found in the eutherian *XIC* in fact arose through pseudogenization of protein-coding genes found in the *XIC* HR (Duret et al. 2006). Lack of specific con-



	Marsupials	Mouse	Rabbit	Human
Rsx	• Expressed from Xi • Triggers gene silencing	∅	∅	∅
Xist	∅	• Expressed from Xi • Triggers gene silencing • Essential for XCI	• Expressed from Xi	• Expressed from Xi (and Xa in PI embryos) • Triggers gene silencing • Expression and XCI uncoupled in PI embryos
Jpx	∅	• Required for Xist upregulation and XCI	• No data	• No data
Ftx	∅	• Affect neighboring gene expression (including Xist)	• No data	• No data
Tsix	∅	• Fully overlaps Xist • Blocks Xist upregulation • Chromatin remodelling activities	?	• Truncated form • Expressed from inactive X in embryonic tissues
Linx	∅	• Genomic interactions with Tsix • Potentially controls Tsix	?	?
XACT	∅	∅	∅	• Coats active X in human ES cells • Expressed only in pluripotent cells

Fig. 3.3 Evolutionary perspective of the non-coding RNAs involved in X-chromosome inactivation in the therian lineage. The ncRNAs known to be involved in XCI are listed on the *left*. The table indicates their presence, main characteristics or absence (*crossed circle*) for each species. *Question marks* signify that the existence of the ncRNA is not known. *Light filling* indicates that the gene is present but the function is unknown or not conserved

straints on pseudogenes in addition to insertion of genomic repeated sequences presumably from transposable elements have contributed to drive ncRNA evolution both prior to and within the eutherian lineage (Elisaphenko et al. 2008).

The *Xist*/*Tsix* gene pair provides an example of how genomic and functional variation in ncRNAs may contribute to the diversity of XCI mechanisms and kinetics in eutherians. Extensive studies in the mouse have revealed *Tsix* effects on the control of *Xist* expression during iXCI and at the transition with rXCI, more specifically in the choice of the X to be inactivated. Such important control would be expected to be under high selective pressure. However, the genomic architecture and expression patterns of *Tsix* has drastically evolved in the eutherian lineage; although *Xist*-antisense transcription is present in several species, it is found in various truncated forms that do not completely overlap with *Xist*, a feature shown to be essential for its regulatory role (Migeon et al. 2001). Poor conservation of *Tsix* might be related to limited use of imprinted X-chromosome inactivation in mammalian species, indicating that *Tsix* peculiar features might not be ancestrally derived but a rather novel acquisition, specific to the rodent lineage.

In what might also be related to the absence of *Tsix*, profiling of *Xist* in other eutherians has uncovered unexpected behaviours, especially during pre-implantation development (Okamoto et al. 2011). For example, in early rabbit female embryos, both X chromosomes can be transiently coated by *Xist* and display some levels of inactivation. Further investigation in humans revealed another unexpected situation, with *XIST* being expressed from and accumulating on every X chromosomes in both males and females, starting at the morula stage. However, *XIST* coating at these early embryonic stages does not translate into chromosome inactivation and biallelic gene expression is detected in female cells. These unusual situations are eventually resolved around the blastocyst stage, with *XIST* coating a single X in females only, and correlating with proper XCI. These observations strongly suggest that

different *Xist* regulatory mechanisms, involving not only *Tsix* but also likely other factors, are operating in these species.

Variation also exists at the level of the *Xist* sequence itself: while *Xist* is present in the X-inactivation centre of all placental mammals, sequence comparison across species did not reveal extensive homology throughout the transcript. Instead, conserved elements are located at the 5′ end of the transcript, where a series of repeat motifs, A to F were identified, yet with variation in their sizes and sequences (Yen et al. 2007). The existence of *Xist* orthologs in all eutherians led to the assumption that *Xist* plays a conserved and central role in XCI across species. Although likely, it is important to note that in most cases, this hypothesis has not been formally proven.

NcRNAs located outside of the *XIC* may provide additional species-specificity in X-inactivation, as exemplified by our recent discovery of *XACT*. This locus, located some 40 Mb away from the *XIC*, produces a large nuclear and mostly unspliced transcript that coats the active X chromosome in human ES cells (Vallot et al. 2013). Expression of *XACT* is restricted to pluripotent cells. Sequence conservation and expression analysis suggested this transcript to be a very recent addition, specific to the human lineage. Although the exact function of *XACT* remains to be identified, it is tempting to speculate that it might contribute to prevent *XIST*-mediated transcriptional repression during human pre-implantation development, thus providing a molecular explanation to the transient uncoupling of *XIST* coating and gene-silencing observed at these stages (Fig. 3.2).

3.9 Perspectives

We have described in this chapter how variable are the X-chromosome inactivation strategies within the therian lineage. It might appear at first glance surprising that so many routes have been taken in a limited evolutionary scale to lead to such an essential and similar outcome. While the origin of such diversity is poorly understood, it is likely that the very nature of the actors involved in the process has facilitated XCI plasticity. Abundance of ncRNAs linked to XCI probably reflects both the adaptable essence of these molecules and their highly localized effects. Indeed, long ncRNAs are under different evolutionary constraints compared to protein-coding genes and could thus represent versatile substrates during evolution. The "pervasive" transcription, which occurs throughout the genome, might constitute a reservoir of molecules that can easily acquire a function and be co-opted into biological processes. Long ncRNAs are on average lowly conserved at the sequence level, but much less is known regarding functional conservation. *Xist* and *Rsx* provide an example on how unrelated RNA molecules can achieve identical function. Conversely, it will be interesting to address the extent to which conserved ncRNAs, such as *Ftx* and *Jpx*, have similar activities in various species. Beyond evolutionary considerations, ncRNAs, which can act through their transcript itself or via the act of transcription, are also well suited to mediate localized effects, whether over small distance from their transcription site or on larger scales (at the chromosome level). As such, modification of *cis*-acting ncRNA will only impact a given process, allowing for increased flexibility and greatly attenuating the consequences of co-opting and losing these molecules.

With all these properties of ncRNAs in mind, it should not come as a surprise that these molecules were recruited during evolution to diversify developmental pathways. X-chromosome inactivation seems to be again at the forefront of this new discipline linking ncRNA and evolution, and might become a paradigm to decipher evolution's tinkering with such fundamental processes. Delineating the exact roles of these molecules in XCI holds the potential of revealing further exciting surprises.

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4 The piRNA Pathway Guards the Germline Genome Against Transposable Elements

Katalin Fejes Tóth, Dubravka Pezic, Evelyn Stuwe, and Alexandre Webster

Abstract

Transposable elements (TEs) have the capacity to replicate and insert into new genomic locations. This contributes significantly to evolution of genomes, but can also result in DNA breaks and illegitimate recombination, and therefore poses a significant threat to genomic integrity. Excess damage to the germ cell genome results in sterility. A specific RNA silencing pathway, termed the piRNA pathway operates in germ cells of animals to control TE activity. At the core of the piRNA pathway is a ribonucleoprotein complex consisting of a small RNA, called piRNA, and a protein from the PIWI subfamily of Argonaute nucleases. The piRNA pathway relies on the specificity provided by the piRNA sequence to recognize complementary TE targets, while effector functions are provided by the PIWI protein. PIWI-piRNA complexes silence TEs both at the transcriptional level – by attracting repressive chromatin modifications to genomic targets – and at the posttranscriptional level – by cleaving TE transcripts in the cytoplasm. Impairment of the piRNA pathway leads to overexpression of TEs, significantly compromised genome structure and, invariably, germ cell death and sterility.

The piRNA pathway is best understood in the fruit fly, *Drosophila melanogaster*, and in mouse. This Chapter gives an overview of current knowledge on piRNA biogenesis, and mechanistic details of both transcriptional and posttranscriptional TE silencing by the piRNA pathway. It further focuses on the importance of post-translational modifications and subcellular localization of the piRNA machinery. Finally, it provides a brief description of analogous pathways in other systems.

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4.1 Introduction

Of the 100 trillion cells in the body, germ cells are the only ones contributing to all future generations, and as such are essentially immortal. It is therefore of pivotal importance to preserve the integrity of the germ cell genome, as damage to it can result in an evolutionary dead end – sterility.

One of the greatest internal threats to genome stability is posed by transposable elements (TEs) – highly abundant and repetitive segments of DNA with the ability to replicate and insert into new locations, wreaking havoc in the process. New insertions cause breaks in the DNA, which lead to cell cycle arrest, disrupt genes and their regulatory regions, and increase the likelihood of non-homologous recombination (Slotkin and Martienssen 2007). Various systems have evolved in different organisms to combat and contain the expansion of TEs, but it is the elegant mechanism employed by the germ cells of metazoans that has attracted considerable interest in recent years: the piRNA pathway (Siomi et al. 2011).

The piRNA pathway is a germline-specific RNA silencing mechanism. The central effector complex of the pathway, called pi-RISC (piRNA-induced silencing complex) in analogy to canonical RNA interference pathways, consists of a protein from the PIWI subfamily of Argonaute nucleases, and a PIWI-interacting RNA, or piRNA. The piRNA pathway relies on the effector function of the Argonaute protein and the specificity provided by the piRNAs to restrict TE activity. PIWI proteins comprise a clade of the Argonaute protein family that is specific to metazoans and shows gonad-specific expression (Carmell et al. 2002; Cox et al. 1998; Kuramochi-Miyagawa et al. 2001, 2004; Lin and Spradling 1997; Houwing et al. 2007). PIWI, the protein that is the namesake of the PIWI clade of Argonautes, was discovered in a screen for factors affecting stem cell maintenance in the *Drosophila* germline. Mutants of this gene have very small gonads, and were therefore named <u>*P*</u>-element <u>*i*</u>nduced <u>*wi*</u>mpy testis, or *piwi* mutants (Cox et al. 1998; Lin and Spradling 1997).

The domain structure of PIWI proteins is similar to that of Argonautes (Fig. 4.1) (Song et al. 2004; Wang et al. 2008). Like Argonautes, the mid domain anchors the 5′ end of a piRNA, and the PAZ domain lodges the 3′ end of the piRNA. The PIWI domain contains catalytic residues within an RNase H-like nucleolytic fold, providing catalytic cleavage of target transcripts. Unlike Argonautes however, PIWIs have Arginine-rich motifs near their N-termini. The arginine residues are post-translationally symmetrically dimethylated. Arginine methylation allows PIWI proteins to interact with components of the pathway that contain Tudor domains (Kirino et al. 2009; Liu et al. 2010a; Nishida et al. 2009; Vagin et al. 2009). Tudor family proteins containing the eponymous Tudor domain can bind methylated arginines in PIWI proteins, and this relationship appears to be conserved in many species. Many Tudor proteins have several Tudor domains and act as a scaffold for the formation of higher-order complexes (Huang et al. 2011a; Mathioudakis et al. 2012). In germ cells of *Drosophila* and in mouse testis, this mode of protein interaction is the basis for the formation of distinct perinuclear granules similar to P-bodies, which contain many components of the piRNA pathway and have been known for a long

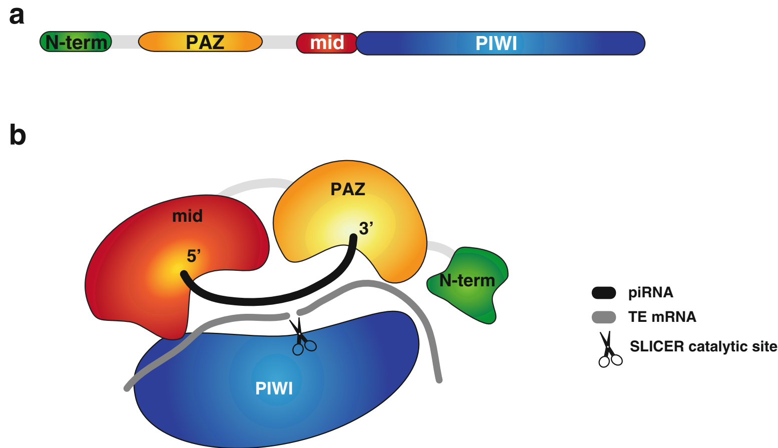

Fig. 4.1 The domain structure of PIWI-clade argonaute proteins. (**a**) The domains of PIWI proteins are organized in a similar fashion to other Argonaute proteins, comprised of the N-terminal region, PAZ (Piwi-Argonaute-Zwille), mid and PIWI domains. The N-terminal region consists of a notional domain that is characterized by arginine rich motifs that are targeted for methylation and in the case of PIWI in flies and MIWI2 in mouse contains the NLS signal. (**b**) The organization of protein domains in space shows how the mid-domain anchors the piRNA at its 5′ end and the PAZ domain holds the 3′ end of the piRNA. PIWI is the largest domain and its catalytic site responsible for 'slicer' activity is positioned to cleave the backbone of annealed target RNA exactly 10 nt relative to the 5′ end of the piRNA

time in cell biology as "nuage" (Aravin et al. 2008, 2009; Brennecke et al. 2007; Malone et al. 2009).

Based on shared domain structure with Argonaute proteins, it was inferred that PIWI proteins must also bind small RNAs. Indeed, at the emergence of high throughput sequencing techniques PIWI-associated small RNAs from several species were sequenced and characterized (Aravin et al. 2006; Girard et al. 2006; Grivna et al. 2006; Lau et al. 2006). piRNAs are typically 23–31 nucleotides (nt) in length and are longer relatives of ubiquitous short interfering RNAs (siRNAs) and microRNAs (miRNAs). Based on their sequences, piRNAs and the conserved role of PIWI proteins in taming transposable elements in the germline of animals were elucidated.

piRNAs are generated either from RNA transcripts of active TE copies or from transcripts originating from specialized loci in the genome called piRNA clusters. Clusters are loci containing dysfunctional remnants of TEs that form the basis of immunity against TE propagation (Aravin et al. 2007, 2008; Brennecke et al. 2007). The piRNAs that are generated from piRNA clusters are mostly antisense to TE mRNA sequences and serve as guides for PIWI proteins to find TE transcripts by complementary base pairing. In the cytoplasm, the identification of TE mRNAs by PIWIs leads to cleavage of targeted transcripts resulting in destruction of the TE message and the concomitant amplification of defensive sequences targeting active TEs, as the cleavage product itself is processed into piRNAs. The piRNA pathway has often been compared to an adaptive immune system, as it conveys memory of previous transposon invasions by storing TE sequence information in piRNA clusters. By amplifying piRNAs that are complementary to the active transposon sequence, the piRNA pathway can respond rapidly and specifically to acute TE activation. In addition to targeted cleavage of TE mRNAs within the cytoplasm, PIWI proteins also function at the chromatin level in the nucleus to silence TE transcription by directing deposition of repressive histone marks and DNA methylation to copies of TEs (Le Thomas et al. 2013; Sienski et al. 2012). Thus, the piRNA pathway acts on two levels to limit germline transposable element activity.

In this Chapter we review the most important findings about this indispensable pathway,

focusing mainly on discoveries in flies and mice. We first discuss the sources of piRNAs and known mechanistic details of their biogenesis, followed by the two main ways in which the piRNA pathway acts to repress TEs. Furthermore, we address the localization and composition of the protein complexes that operate within the piRNA pathway. Lastly, we examine several mechanisms similar to the piRNA pathway that repress TE activity in organisms other than *Drosophila* and mouse.

4.2 piRNA Biogenesis

piRNAs are classified into two groups based on their biogenesis: primary and secondary piRNAs. Primary piRNAs are generated by a yet poorly characterized biogenesis machinery, while secondary piRNAs arise in an amplification mechanism termed the Ping-Pong amplification loop to specifically enhance piRNA sequences targeting active elements. In this section we will concentrate on what is known about biogenesis of primary piRNAs focusing on their genomic origin, regulation of transcription and factors involved in their processing.

4.2.1 piRNA Cluster Architecture and Transcription

piRNA populations are highly complex: deep sequencing of piRNAs from mouse and fly germ cells revealed millions of individual, distinct piRNA molecules. Neither piRNAs nor their precursor sequences show any structural motif or sequence bias except for a preference for a Uracil as the first 5′ nucleotide (1U bias) of the piRNA. However, when mapped to the genome, this highly diverse population of piRNAs can be mapped to a few discrete genomic loci, called piRNA clusters (Brennecke et al. 2007; Aravin et al. 2006, 2007; Girard et al. 2006; Grivna et al. 2006; Lau et al. 2006; Ro et al. 2007). These clusters are transcriptional units of up to 200 kilobases that, at least in flies, are mostly located in pericentromeric and subtelomeric beta-heterochromatin and are highly enriched in transposable element sequences (Brennecke et al. 2007). Primary piRNAs are generated almost exclusively from these piRNA clusters. It is believed that upon new transposon invasion the TE eventually "jumps" into one of the clusters leaving a memory of the invasion. Once a transposable element leaves a trace of its sequence in a cluster, it can be targeted by the piRNA machinery, which will limit further propagation of the TE. Currently it is not known what causes active transposons to preferentially integrate into clusters but the chromatin features of clusters have been proposed to play a role.

In fly ovaries, clusters are expressed in two cell types: cells of germline origin that include the developing oocyte and associated nurse cells in addition to somatic support cells called follicular cells. Interestingly, the structure of clusters seems to differ depending on where they are expressed: Germline clusters are transcribed bidirectionally, generating both sense and antisense piRNAs in relation to the transposon mRNA. Conversely, somatic clusters appear to be transcribed unidirectionally, producing mostly piRNAs that are antisense to TE coding regions (Fig. 4.2a) (Brennecke et al. 2007). Overall, the source of a piRNA dictates how it is processed.

In mouse testes, spermatogenic cells contain two kinds of piRNA clusters: one class is transcribed during embryonic development and, just like in *Drosophila*, piRNAs derived from these clusters defend the germline against transposable elements. A second type of piRNA cluster is expressed in spermatogenic cells of adolescent mice during the first division of meiosis. These are called pachytene clusters, since piRNAs derived from these clusters are highly abundant in the pachytene stage of meiosis. Pachytene piRNAs are not enriched in transposon sequences and to date their function is unknown. An interesting feature of many pachytene clusters is that they are transcribed in both directions from a central promoter (Fig. 4.2b) (Aravin et al. 2006; Girard et al. 2006).

To date, the transcriptional regulation of piRNA clusters in flies and in embryonic stages of mouse spermatogenesis remains elusive.

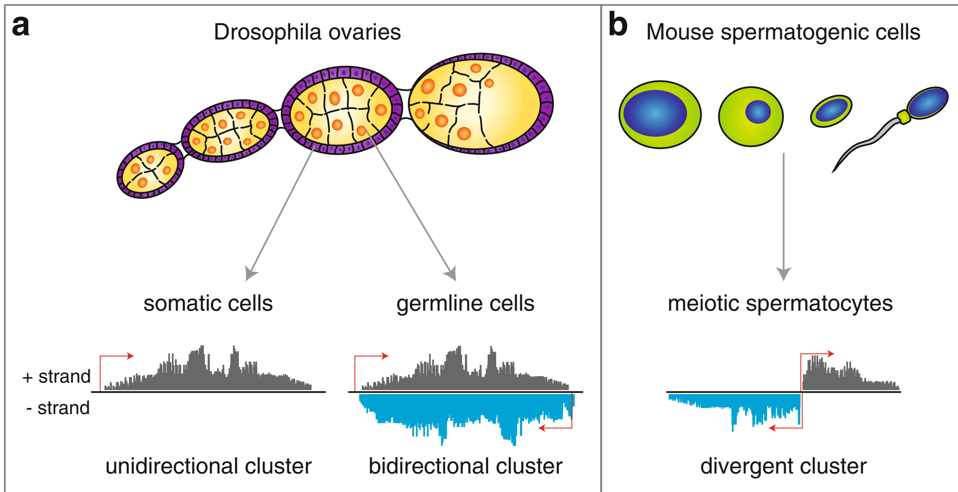

Fig. 4.2 The structure of piRNA clusters in *Drosophila* ovaries and mouse spermatogenic cells. (**a**) Clusters in *Drosophila* are divided into two groups based on their structure and they show differential expression in the two cell types of the ovary. Unidirectional clusters are expressed in follicular cells, which are the somatic support cells of the ovary. These clusters produce piRNAs from only one strand of their genomic locus, and are loaded into PIWI. Bidirectional clusters are expressed in germline cells of the ovary, and piRNAs from these clusters are preferentially loaded into AUB and PIWI. (**b**) Mouse spermatogenic cells contain unidirectional as well as divergent bidirectional clusters. Divergent clusters are transcribed from a central promoter into both directions. Putative transcription start sites are shown with *red arrows*. piRNA density profiles derived from the + and – strands are indicated in *grey* and *blue* as they would appear in small RNA-seq data

Chromatin immunoprecipitation (ChIP) analysis on histone modifications in the silkworm ovary-derived BmN4 cell line (this cell line contains a functional piRNA pathway and is used as an analogous model for the *Drosophila* piRNA pathway) revealed that piRNA clusters display features of euchromatin; they are enriched for histone modifications associated with transcriptional activity such as H3K4 di- and tri-methylation in addition to H3K9 acetylation and at the same time are devoid of repressive histone H3K9 di- and tri-methyl marks (Kawaoka et al. 2013). In ChIP performed on *Drosophila* ovaries, however, H3K9me3 marks are present on clusters and transposon loci (Rangan et al. 2011). The heterochromatin protein (HP1) homolog of *Drosophila*, Rhino, seems to bind those histone marks on piRNA clusters and is essential for piRNA cluster transcription (Klattenhoff et al. 2009). Still, it remains unresolved if these chromatin marks are a cause or consequence of cluster transcription, and transcription factors directly responsible for regulating cluster expression have not yet been identified. Taken together, data about piRNA clusters in *Drosophila* suggest the counterintuitive idea that piRNA clusters need to possess a repressive chromatin signature in order to generate piRNAs.

In mouse, a recent study has identified a transcription factor, A-MYB, which regulates expression of pachytene piRNA clusters. A-MYB also controls its own transcription and that of key genes in the piRNA pathway like *Miwi*, *Maelstrom* (*Mael*) and several Tudor domain-containing genes during the pachytene stage of spermatogenesis. The involvement of one transcription factor in the regulation of clusters and protein factors of the piRNA pathway suggests that a feed-forward loop orchestrates the transcription of piRNA clusters at the same time as the suite of proteins necessary to produce piRNAs from those cluster transcripts (Li et al. 2013). Whether a similar mechanism is at work in *Drosophila* and during embryonic stages of mouse spermatogenesis remains to be elucidated.

4.2.2 Biogenesis of Primary piRNAs

piRNAs in flies are 23–30 nt in length, whereas in mouse they are slightly longer, averaging 25–31 nt. Similar to other small RNA classes, piRNAs are processed from longer precursors, however, in contrast to miRNAs and siRNAs, the precursors are single stranded transcripts without obvious hairpin structures. It is thought that piRNA biogenesis begins with the endonucleolytic cleavage of the long precursor transcript, generating shorter piRNA precursors. This cleavage event specifies the 5′ end of the future piRNA. Genetic screens and structural studies suggest that the endonuclease, Zucchini (ZUC), might conduct this first cleavage of some piRNA precursors (Fig. 4.3a) (Czech et al. 2013; Handler et al. 2013; Muerdter et al. 2013; Olivieri et al. 2010; Ipsaro et al. 2012; Nishimasu et al. 2012; Voigt et al. 2012). After cleavage, the 5′ end of piRNA precursors gets loaded into a PIWI protein. In flies, primary piRNAs are loaded into two of the three PIWI proteins, PIWI and Aubergine (AUB). The factors that make up the piRNA loading machinery are currently unknown, however several studies have identified the proteins Shutdown (SHU), Vreteno (VRET), Brother-of-Yb (BoYb) and Sister-of-Yb (SoYb)

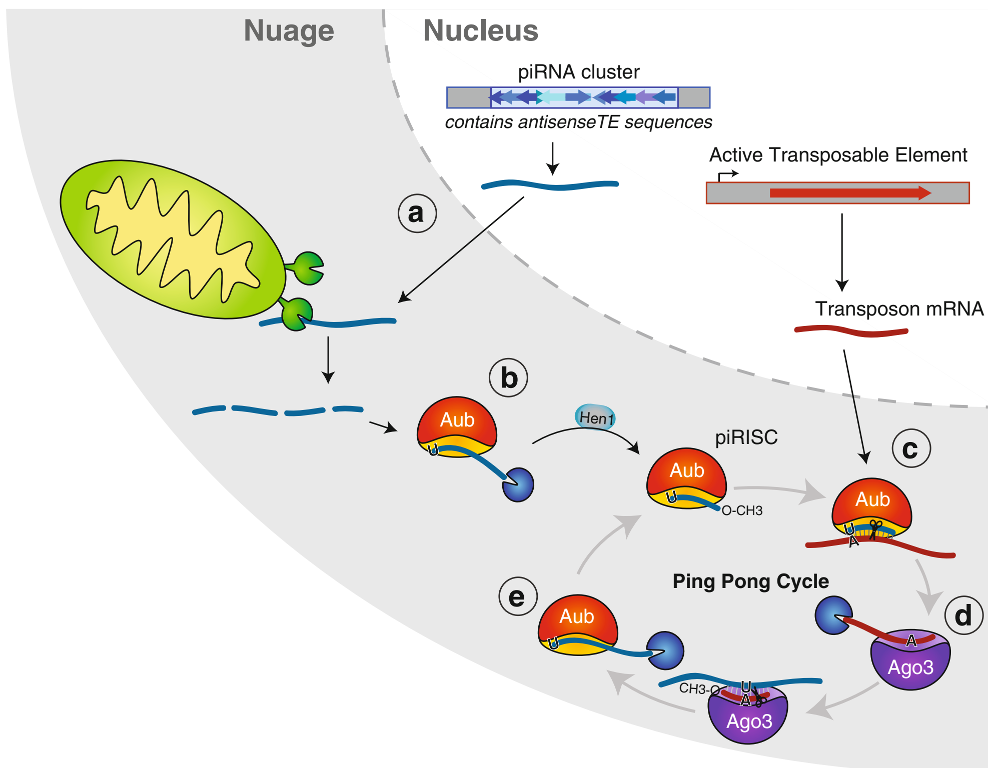

Fig. 4.3 Biogenesis of piRNAs in flies begins in the nucleus and initiates the ping-ping cycle in the cytoplasm. (**a**) Precursor transcripts originating from piRNA clusters that contain anti-sense TE sequences are exported from the nucleus and processed into smaller fragments, possibly by the mitochondrial-associated endonuclease, ZUC. (**b**) AUB binds precursor piRNA fragments with a preference for fragments that contain a 5′ terminal uracil (1U). Mature primary piRNAs are generated when an unknown 3′ > 5′ exonuclease trims the precursor piRNA fragment bound to AUB and the HEN1 methyltransferase catalyzes the 2'-O-methylation of the piRNA 3′ end. (**c**) When mRNA of an active transposon is exported from the nucleus, AUB piRNA anneals to complementary sequences within the mRNA and AUB cleaves the transcript. (**d**) The cleaved transcript is loaded into AGO3, followed by trimming and 2′-O-methylation of its 3′ end, to generate mature secondary piRNA. (**e**) AGO3 loaded with the secondary piRNA is believed to target and cleave newly generated precursor transcripts that are loaded into AUB to initiate a new round of Ping-Pong

as components that are necessary for loading of PIWI and AUB with primary piRNA (Handler et al. 2013; Olivieri et al. 2010) (Table 4.1). Loading of PIWI seems to require some additional factors including Armitage (ARMI) and Zucchini (ZUC) and, in somatic cells, the Tudor domain protein, Yb (Olivieri et al. 2010; Szakmary et al. 2009).

Primary piRNAs show a strong bias for a uridine as their 5′ terminal nucleotide. The step at which this bias is introduced is not clear, as there are two conceivable scenarios. First, cleavage of precursor transcripts could be random and the 5′ binding pocket of PIWI proteins could have a steric preference for binding piRNAs with a 5′ terminal 1U; or second, the nuclease conducting the first cleavage on precursor cluster transcripts could preferably cleave upstream of uridines, thereby creating the observed bias.

Once loaded into PIWI proteins, the piRNA precursor is trimmed at its 3′ end by an unknown 3′ > 5′ exonuclease. This nuclease is tentatively named "Trimmer" (Fig. 4.3b). The final length of the piRNA is determined by the piRNA-binding pocket of the PIWI protein: PIWI associated piRNAs have a median length of 25 nt, whereas piRNAs associated with AUB or AGO3 have median lengths of 24 nt or 23 nt, respectively. In mouse their length is slightly longer (25–31 nt) with median lengths of 26 nt, 28 nt and 30 nt for MILI, MIWI2 and MIWI-bound piRNAs, respectively (Aravin et al. 2006, 2008; Grivna et al. 2006). However, piRNAs can vary in length by one to two nucleotides, possibly as a consequence of imprecise trimming. Finally, after trimming, piRNAs acquire a characteristic 2′ O-methyl modification at their 3′ end that is introduced by the piRNA methyl-transferase HEN1 and leads to stabilization of the small RNA (Kawaoka et al. 2011).

Overall, while the exact biochemistry and the involved factors are still not fully known, piRNA biogenesis consists of multiple consecutive steps from transcription of precursors, through precursor cleavage and loading into Piwi proteins, to trimming and 2′ O-methylation. Defects in any of these steps leads to diminished piRNAs, upregulation of transposons and sterility.

4.3 Nuclear and Cytoplasmic Function of the piRNA Pathway

Transposons are selfish genetic elements that can be classified into DNA or retrotransposons based on the mechanism by which they transpose in the genome. DNA transposons mobilize by a 'cut and paste' mechanism that is accomplished with the help of an encoded transposase protein. Retrotransposons propagate by reverse transcription of an RNA intermediate. Here too, the required enzymes are encoded by the transposable elements. Another class of TE consists of semi-autonomous sequences, such as *Alu* repeats, that require the protein machinery of other transposons for their replication. In either case the transposon needs to be transcribed as an mRNA because it encodes the enzymes necessary for transposition, or is also required as a template for conversion to DNA by reverse transcription. Accordingly, transposition can be regulated on multiple levels, from the efficiency of transcription, to transcript stability and rate of translation of required factors, or by modulating the process of re-integration into the genome. The piRNA pathway seems to target two steps that are required for all transposons: In the nucleus, piRNAs are implicated in the regulation of chromatin structure that can affect transcription of targeted loci. In the cytoplasm, the piRNA pathway directly targets and destroys RNAs of transposons that escaped transcriptional silencing. In this section we will describe the details of these two levels of defence against transposable elements.

4.3.1 The Role of piRNAs in Regulating Chromatin Structure

Transcriptional regulation of a specific locus is influenced by its contextual chromatin architecture. In mammals, regulation is achieved at two levels: by methylation of DNA and by modifications of histone proteins or even incor-

Table 4.1 *Drosophila* proteins with known function in the piRNA pathway. Factors are grouped together by their localization or composition of their domains: The three Piwi proteins, Piwi, Aub and Ago3 that are central to the piRNA pathway; Nuclear Factors that localize and function in the nucleus; Cytoplamic Non-Tudor Proteins that localize to various compartments of the cytoplasm; and Tudor Proteins that contain at least one Tudor domain. Columns listed are: Protein name; synonymous names and abbreviations; tissue of expression; cellular localization; protein domains; known or putative functions; homology in mouse; and, references to literature

Gene	Synonyms	Tissue	Localization	Domains (M)	Function	Mouse	Ref.
Piwi proteins							
Piwi	*Piwi*	Germ; Soma	Nucleus	PAZ; PIWI	Argonaute; RISC effector	Mili, Miwi, or Miwi2	Cox et al. (1998) and Lin and Spradling (1997)
Aubergine	*Aub, Sting*	Germ	Cyto.: nuage, pole plasm	PAZ; PIWI	Argonaute; RISC effector	Mili, Miwi, or Miwi2	Schmidt et al. (1999) and Harris and Macdonald (2001)
Argonaute-3	*Ago3*	Germ	Cyto.: cytoplasmic granules:germ granules in flies:nuage	PAZ; PIWI	Argonaute; RISC effector	Mili, Miwi, or Miwi2	Li et al. (2009)
Nuclear factors							
Rhino	*Rhi; HP1D*	Germ	Nucleus	Chromo; chromo shadow	Chromatin factor	HP1a	Volpe et al. (2001) and Klattenhoff et al. (2009)
Eggless	*Egg; SetDB1*	Germ	Nucleus	MDB, SET	H3K9me3 HMT		Rangan et al. (2011)
Cutoff	*Cuff*	Germ	Nucleus	DOM3Z		DOM3Z	Pane et al. (2011)
Deadlock	*Del*	Germ	Nucleus	–			Czech et al. (2013)
UAP56	*Hel25E*	Germ; Soma	Nucleus	DEXDc; Hel-C	RNA helicase	UAP56	Zhang et al. (2012)
Asterix	*Arx; CG3893*	Germ; Soma	Nucleus	CHHC ZnF x 2	HMT cofactor		Muerdter et al. (2013)
Maelstrom	*Mael*	Germ	Nucleus and Cyto.	HMG		Mael	Findley et al. (2003), Sienski et al. (2012) and Sato et al. (2011)
Hsp90	*Hsp83*	Germ; Soma	Nucleus and Cyto.		Heat-shock response; chaperone	Hsp90	Olivieri et al. (2010) and Zhang et al. (2012)
Cytoplasmic non-tudor proteins							
Armitage	*Armi*	Germ; Soma	Cyto.	SDE3		Mov10L1	Cook et al. (2004)
Shutdown	*Shu*	Germ; Soma	Cyto.: nuage	PPIASE; TPR	Co-chaperone; piRNA loading	FKBP6	Olivieri et al. (2012) and Preall et al. (2012)

Hen-1	*Hen1; PIMET*	Germ; Soma	Cyto.	MeT	RNA 2′ O-methyl transferase		Saito et al. (2007) and Kirino and Mourelatos (2007)
Squash	*Squ*	Germ	Cyto.: Nuage	RNAase HII	RNA nuclease		Pane et al. (2007)
Zucchini	*Zuc*	Germ; Soma	Cyto.: Mito	HKD	RNA nuclease	PLD6	Pane et al. (2007) and Ipsaro et al. (2012)
Minotaur	*Mino; CG5508*	Germ; Soma	Cyto.: Mito., ER, ring canals		Glycerol-3-phosphate-O-acyltransferase (GPAT)		Vagin et al. (2013)
GASZ	*GASZ*	Germ; Soma	Cyto.: Mito.	Ankyrin x 3; SAM-2		GASZ	Ma et al. (2009), Handler et al. (2013) and Czech et al. (2013)
Vasa	*Vas*	Germ	Cyto.: nuage, pole plasm	DEXDc	RNA helicase	MVH	Liang et al. (1994)
Capsuléen	*Csul; dART5; PRMT5*	Germ	Cytoplasm	PRMT5	Argenine methyl transferase	PRMT5	Anne and Mechler (2005) and Anne et al. (2007)
Valois	*Vls*	Germ	Cytoplasm	MEP50; WD x 4	PRMT5 co-factor	Mep-50	Cavey et al. (2005)
Polo			Cyto.: nuage		Kinase		Pek et al. (2009)
Tudor proteins							
Tudor	*Tud*	Germ	Cyto.: nuage, pole plasm	Tud x 11 (Tud x 8)		Tdrd6	Arkov et al. (2006) and Anne (2010)
Qin	*Kumo*	Germ	Cyto.: nuage	ZnF RING E3 Ligase; Tud x 5	E3 ligase	Tdrd4	Annand et al. (2011) and Zhang et al. (2011)
Papi	*Papi*	Germ	Cyto.: nuage	KH x 2; Tud x 1		Tdrd2	Liu et al. (2011)
Tejas	*Tej*	Germ	Cyto.: nuage	LOTUS; Tud x 1		Tdrd5	Patil and Kai (2010)
CG8920		Germ		LOTUS; DSRM; LOTUS; Tud x 3		Tdrd7	Handler et al. (2011)
CG9925				ZnF-MYND; Tud x 3		Tdrd1	Handler et al. (2011)
Spndle-E	*SpnE; Homeless; Hls*	Germ	Cyto.: nuage	DEXDc; Hel-C; HA2; OB; RRM; ZnF; Tud x1	RNA helicase	Tdrd9	Gillespie and Berg (1995)
Krimper	*Krimp; Montecristo*	Germ	Cyto.: nuage	Tud x 1			Lim and Kai (2007)

(continued)

Table 4.1 (continued)

Gene	Synonyms	Tissue	Localization	Domains (M)	Function	Mouse	Ref.
Yb	*fs(1)Yb*	Soma	Cyto.	Hel-C like; DEAD; Hel-C; ZnF; Tud x 1			Szakmary et al. (2009)
Sister of Yb	*SoYb*	Germ; Soma	Cyto.: nuage	Tud x 2; DEAD; Hel-C; ZnF; CS		Tdrd12	Handler et al. (2011)
Brother of Yb	*BoYb*	Germ	Cyto.: nuage	Tud x 1; DEAD; Hel-C; ZnF; CS			Handler et al. (2011)
Vreteno	*Vret*	Germ; Soma	Cyto.: nuage	ZnF-MYND; RRM; Tud x 2			Zamparini et al. (2011) and Handler et al. (2011)

poration of special histone variants into nucleosomes. In *Drosophila melanogaster*, histone modifications and histone variants are mainly responsible for defining the properties of chromatin. In both flies and mice, one of the three PIWI proteins localizes to the nucleus, suggesting a nuclear role for the piRNA pathway possibly through transcriptional repression of targets (Cox et al. 1998; Aravin et al. 2008). Such a mechanism is further strengthened by the observation that in flies PIWI shows a banding pattern on polytene chromosomes of nurse cells, suggesting a direct interaction with chromatin (Le Thomas et al. 2013).

In mouse, DNA methylation plays an essential role in silencing of transposable elements (TEs). In mouse male germ cells, this silencing is established in a narrow developmental window during embryonic germ cell development, following global genome de-methylation. This is a critical point in the fate of spermatogenic cells, since the failure to methylate sequences of retrotransposons scattered throughout the genome leads to TE activation and subsequent meiotic failure and sterility (Bourc'his and Bestor 2004). Several lines of evidence indicate that piRNAs are responsible for directing DNA methylation to their genomic targets. First, several proteins involved in the piRNA pathway have been described to localize to the nucleus: MIWI2, TDRD9, and MAEL (Aravin et al. 2008, 2009; Shoji et al. 2009; Soper et al. 2008). While TDRD9 and MAEL are expressed throughout male germ cell development, MIWI2 is expressed in a narrow developmental time window exactly overlapping the time of *de novo* DNA methylation in the embryonic spermatocyte. Additionally, genetic data implicate piRNAs in establishing *de novo* DNA methylation patterns in the mouse male germline on regulatory regions of TEs (Aravin et al. 2008; Carmell et al. 2007; Kuramochi-Miyagawa et al. 2008). DNMT3L is an accessory factor for the *de novo* DNA methyltransferases DNMT3A and DNMT3B, and is essential for *de novo* DNA methylation. The defects observed in DNMT3L knockout animals are strikingly similar to those observed in animals deficient in many piRNA pathway components, including the two PIWI proteins, MILI and MIWI2: all result in meiotic arrest of spermatogenesis and apoptosis of germ cells. Furthermore, methylation patterns were not re-established on retrotransposon sequences in mice lacking MILI or MIWI2 (Kuramochi-Miyagawa et al. 2008). In contrast, piRNAs are still produced in DNMT3L mutants (Aravin et al. 2008). These observations indicate that the piRNA pathway directs DNA methylation of TEs and functions upstream of the methylation machinery.

A second PIWI protein, MILI is also expressed in the embryonic stage of spermatogenesis. While it localizes to the cytoplasm, its expression is necessary for piRNA loading and nuclear localization of MIWI2. The phenotype of *Mili* mutants is similar to that of *Miwi2* suggesting that piRNAs serve as sequence-specific guides that direct MIWI2 to sites where DNA methylation is established (Kuramochi-Miyagawa et al. 2001, 2004, 2008; Aravin et al. 2008). Biochemically, this process remains unexplored due to the restriction of *de novo* DNA methylation to a small number of germ cells in a narrow developmental window during embryogenesis. It is not clear whether MIWI2 associates directly with chromatin and it seems not to interact directly with DNA methyltransferases. In fruit flies, the piRNA pathway participates in deposition of repressive histone marks on TEs (see below). It remains to be determined whether this mechanism is conserved in mice. If so, it will be interesting to see if histone modifications lead to subsequent DNA methylation, or vice versa (Fig. 4.4).

Male germ cells also establish new methylation patterns at imprinted loci. Interestingly, the piRNA pathway is involved in methylation of at least one imprinted locus, *Rasgrf1* (Watanabe et al. 2011a). This process depends on a solo-LTR transposon located in the *Rasgrf1* locus and might be a consequence of piRNA-mediated retrotransposon methylation.

In contrast to mammals, DNA methylation in *Drosophila* does not play a major role in the regulation of chromatin structure and gene expression. In fruit flies it is believed that the piRNA pathway induces transcriptional repression by

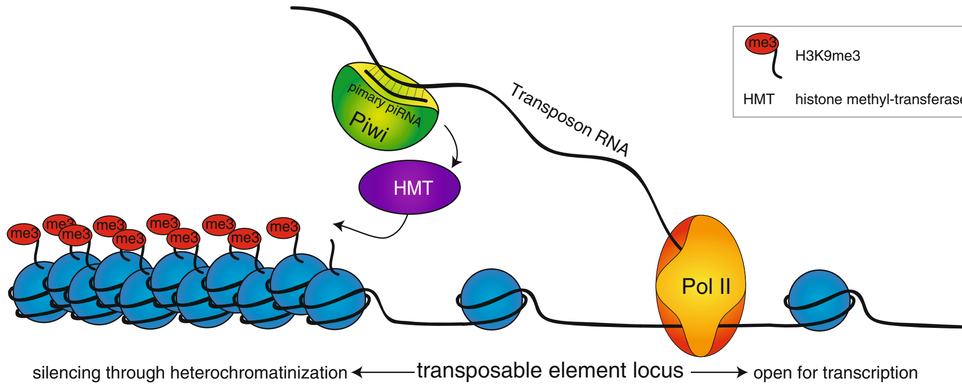

Fig. 4.4 The nuclear function of the piRNA pathway in flies. PIWI loaded with cluster-derived primary piRNAs enters the nucleus and scans nascent transcripts for complementarity to its piRNA. If a transposon RNA transcript is encountered, PIWI is believed to recruit chromatin factors such as histone methyl-transferases (HMT) to deposit repressive H3K9-methyl marks. The modifications induced by PIWI binding create sites for the assembly of additional factors that repress transcription of transposon loci

initiating deposition of repressive histone marks. Indeed, multiple studies have shown that the piRNA pathway transcriptionally represses at least some transposons. In cell culture experiments, deletion of PIWI's nuclear localization signal leads to failure of transposon silencing, even though PIWI proteins are loaded with piRNAs in the cytoplasm (Saito et al. 2009a). Similarly, in flies the deletion of the N-terminus of PIWI leads to its delocalization from the nucleus and a slight increase in active histone marks (di-methylation of H3K79 and H3K4) and a decrease of repressive marks (di-/tri-methylation of H3K9) over several transposons (Klenov et al. 2007). Additionally, an increase in transcription and accumulation of several telomeric retrotransposons was observed in *Drosophila* ovaries upon mutation of piRNA pathway components (Chambeyron et al. 2008; Shpiz et al. 2009, 2011). The telomeric transposons, which had increased transcription showed slight changes in their chromatin marks (Shpiz et al. 2011). These observations all indirectly pointed towards an involvement of PIWI in transcriptional regulation of transposable elements. Recently, three genome-wide studies confirmed this assumption: knockdown of *Piwi* leads to transcriptional derepression of a significant fraction of TEs both in cell culture and in ovaries as assessed by Pol II ChIP-seq and by GRO-seq (Le Thomas et al. 2013; Sienski et al. 2012; Rozhkov et al. 2013). Upon *Piwi* knockdown transcript levels of transposable elements increase significantly, indicating that PIWI primarily, if not solely, represses transposons at the transcriptional level.

While it seems likely that transcriptional silencing of transposable elements is achieved through establishment of a repressive chromatin state, this correlation between repressive chromatin marks and silencing has not been shown directly yet. Likewise, factors involved in mediating PIWI's repressive function are not known. Recently, two factors have been proposed to be involved in inducing transcriptional silencing. Maelstom (MAEL) is a conserved factor that has been implicated in TE silencing in both mouse and flies (Aravin et al. 2009; Sienski et al. 2012; Soper et al. 2008). Knockdown of *Mael* in flies leads to increased Pol II occupancy over TEs similar to changes observed upon *Piwi* knockdown (Sienski et al. 2012). Interestingly, while *Piwi* knockdown resulted in strong reduction of H3K9me3 signal at and downstream of target sequences, *Mael* knockdown resulted in increased spreading and only a modest reduction in H3K9me3 signal, indicating that MAEL does not act in establishing the repressive H3K9me3 mark over targets. Another recent study identified Asterix (CG3893) as a factor required for PIWI-mediated establishment of H3K9me3 mark over at least a subset of TEs (Muerdter et al. 2013).

A study using a cell culture model showed that PIWI silences a significant number of novel TE insertions through tri-methylation of H3K9 (Sienski et al. 2012). This silencing mark does not only cover the site of the novel TE insertion, but spreads up to 15 kb downstream in the direction of transcription. This observation indicates that transcription plays a role in establishing repressive chromatin. The spreading of repressive marks upon insertion of a transposable element can even lead to repression of proximal protein coding genes. In fly, however, similar new insertions in proximity to protein coding genes were not observed (Le Thomas et al. 2013). It is conceivable, that while in cultured cells the silencing of nearby genes is tolerated and can be detected, in a living organism there is a strong selective pressure against insertions that would compromise functional gene expression.

PIWI-bound piRNA sequences identify the sequences that are targeted for PIWI-mediated transcriptional repression. This is in agreement with the observation that host genes are generally unaffected by the pathway and that PIWI requires piRNAs to repress TEs (Le Thomas et al. 2013). Additionally, PIWI loaded with artificial sequences mapping to the lacZ transgene leads to silencing of lacZ-expressing loci in vivo. While the mechanism of target recognition remains unresolved, it is likely that piRNAs recognize nascent transcript targets through sequence complementarity. This is supported by the observation that *Piwi* knockdown results in decreased H3K9 tri-methylation of the genomic environment of active transposons, while non-transcribed fragments of transposable elements throughout the genome remained unaffected (Sienski et al. 2012).

Transcriptional repression by PIWI occurs in both germ cells and follicular cells of *Drosophila* ovaries. Follicular cells are the somatic support cells in fly ovaries, and while they are not germline cells, they do express a uniquely tailored version of the piRNA pathway. Out of the three PIWI proteins in *Drosophila*, only PIWI is expressed in follicular cells. In these cells it specifically loads with piRNAs generated from the unidirectional cluster, *Flamenco* (Malone et al. 2009). The *Flamenco* cluster appears to primarily target the LTR retrotransposon, *Gypsy*, whose expression can lead to the formation of viral particles that might infect the germ line (Song et al. 1997). Therefore, transcriptional repression of *Gypsy* loci by PIWI in follicular cells is crucial for germline survival.

In summary, while little is known about the mechanism of piRNA-mediated transcriptional silencing, it is apparent that in both mouse and *Drosophila*, PIWI proteins play an essential role in inhibiting TE transcription and establishing a repressive genomic environment.

4.3.2 Ping-Pong: The Cytoplasmic Function of the piRNA Pathway

The beauty and power of the piRNA pathway lies in its ability to store information of previous TE invasions within piRNA clusters and to specifically respond to TE activation by selectively amplifying sequences complementary to active elements. The latter function is achieved through the interplay of two cytoplasmic PIWI proteins – in *Drosophila*, Aubergine (AUB) and Argonaute 3 (AGO3) – in a process termed the Ping-Pong cycle. Since AUB and AGO3 are only expressed in the germline, the Ping-Pong cycle is also restricted to germ cells (Brennecke et al. 2007).

In *Drosophila*, AUB and AGO3 act as partners in the defence against active transposable elements, with AUB binding to piRNAs mainly coming from piRNA cluster transcripts and AGO3 enriched in piRNAs originating from transposon mRNAs. Both AUB and AGO3 can catalytically cleave RNA targets. At the beginning of the pathway, AUB loads with primary piRNA sequences that are derived from piRNA clusters and are antisense to TE transcripts (see Sect. 2.2). These primary piRNAs guide AUB to scavenge the cytoplasm for complementary transcripts. When a TE mRNA with sequence complementarity to an AUB-bound piRNA is identified, AUB cleaves the target RNA ten nucleotides downstream of the piRNA 5′ end. This cleavage of transposon mRNA serves two purposes: first, it destroys the transposon

transcript and second, it generates the 5′ end of a new piRNA precursor (Fig. 4.3c). This new precursor gets incorporated into AGO3 and is processed into a so-called secondary piRNA (Fig. 4.3d). Due to the fact that AGO3-bound piRNAs are sense to the transposable element, these piRNAs can promote the production of a new round of cluster-derived piRNAs by recognizing complementary cluster transcripts (which then get cleaved and incorporated into AUB) (Fig. 4.3e). As the cycle can only function if transposon mRNA is present, the Ping-Pong cycle shapes a specific piRNA repertoire targeting active TEs.

Since AUB-bound piRNAs have a strong bias for uracil at their 5′ ends, the piRNAs that are loaded into AGO3 have a bias for adenosine at position 10 (10A bias) and are complementary to the AUB-bound piRNAs over a 10-nucleotide stretch. This relationship between AUB piRNAs and AGO3 piRNAs is termed the Ping-Pong signature.

The mechanism by which AUB and AGO3 interact in the Ping-Pong cycle has only recently been partially elucidated in Drosophila. Multiple factors function in tandem to execute the various processes required for coordinating the Ping-Pong cycle. Krimper (KRIMP) is a Tudor domain protein that was shown to form a bridge between AUB that is loaded with piRNA and AGO3 that is not loaded, establishing the piRNA Ping-Pong processing (4P) complex (Webster et al. 2015). In this complex, targets recognized by AUB-associated piRNAs can be cleaved by AUB and the cleavage product can be incorporated into previously unloaded AGO3 enabling progression of the Ping-Pong cycle. The Tudor protein, Qin/Kumo (QIN) is also necessary for the interaction of AUB and AGO3 in ovarian germ cells and loss of QIN leads to impaired loading of AGO3 and TE activation (Zhang et al. 2011). Vasa (VAS) has also been proposed to function in the assembly of an ‘amplifier complex’ (Xiol et al. 2014) or to play a role in regenerating active piRISC complexes after target cleavage (Nishida et al. 2015).

In spermatogenic cells of mouse embryos, Ping-Pong occurs between the two PIWI proteins MILI and MIWI2. MILI binds primary piRNAs and MIWI2 is bound to secondary piRNAs. A difference to Ping-Pong in *Drosophila* is that the MILI-bound primary piRNAs are mainly in sense orientation relative to TE coding sequences, which suggests that in mouse, active transposable elements initiate the cycle (Aravin et al. 2008).

4.3.3 Suppressor of Stellate Is a Specialized piRNA Mechanism in *Drosophila* Testes

Beyond transposon control, the piRNA pathway seems to have been adopted for alternative functions: *Drosophila* testis employs a unique piRNA pathway mechanism whose primary role is to provide “quality control” during spermatogenesis. The *Stellate* locus on chromosome X is comprised of a tandemly repeated gene that encodes a protein, Stellate, whose hyperexpression is responsible for the formation of large crystalline needle-like fibers of Stellate protein that are toxic to the cell. Due to their high copy number, unchecked expression of Stellate kills primary spermatocytes and results in male sterility. The antidote to Stellate toxicity is provided by the *Crystal* locus on chromosome Y, which encodes another set of tandem repeats named *Suppressor-of-Stellate*, *Su*(*Ste*) (Aravin et al. 2001; Bozzetti et al. 1995). Interestingly, the *Su*(*Ste*) gene shares 90 % homology to *Stellate* and produces both sense and antisense transcripts. The antisense transcripts play an integral role in silencing Stellate mRNA and the mechanism of silencing requires many of the same components as the piRNA pathway in ovaries.

Repression of *Stellate* in testis requires AUB and AGO3 while PIWI appears to be dispensable (Li et al. 2009; Aravin et al. 2001; Schmidt et al. 1999; Vagin et al. 2006). Additionally, the testes of mutants for piRNA pathway components SPNE, QIN, ARMI, TEJ, and VRET also express *Stellate* and/or appear to have meiotic abnormalities (Zhang et al. 2011; Vagin et al. 2006; Aravin et al. 2004; Handler et al. 2011; Patil and Kai

2010; Stapleton et al. 2001). The piRNA pathway in testis also directs TE repression through both AUB and PIWI, with PIWI being required for fertility in males (Lin and Spradling 1997; Vagin et al. 2006). It is unlikely that Ping-Pong plays a significant role in testis biology, since AGO3 mutant males are sub-fertile, even though crystals of Stellate do accumulate within spermatocytes, and male germ stem cell maintenance is partially defective (Li et al. 2009; Kibanov et al. 2011). Many pathway components localize to perinuclear areas of spermatocytes forming piRNA nuage giant bodies, or piNG-bodies (Kibanov et al. 2011). These components likely have a similar structure to nuage in ovary, as discussed below.

The implied biological function of *Stellate* and *Su*(*Ste*) remains ambiguous. Certainly, this system does appear to ensure that spermatocytes develop with an intact piRNA pathway, in addition to preventing Y chromosome aneuploidy during spermatogenesis. Currently, a homologous "quality assurance" mechanism is not known to exist in the female germline of the fly, or in mouse. The existence of such alternative functions of the piRNA pathway raises the question whether it has unidentified functions in the germline or in other tissues. Although expression of the core components of the piRNA pathway – PIWI proteins – seems to be restricted to the germline, it can not be excluded that specified cells do express and employ the piRNA pathway for yet uncharacterized functions beyond transposon control.

4.4 Cytoplasmic Granules: Components, Structure and Function

4.4.1 Introduction to Germ Granules

Molecules involved in the same cellular process often assemble into granules – membraneless subcellular compartments – in order to increase the local concentration of factors and enhance the efficiency and specificity of processes. Germ granules are conserved germ cell components present in most if not all sexually reproducing metazoans, and are essential in ensuring the reproductive potential of the individual (Al-Mukhtar and Webb 1971; Eddy and Ito 1971; Mahowald 1968).

Germ granules were discovered more than a century ago, as darkly staining structures that could be traced from gametes of one generation into germ cells of the next in the fly (Hegner 1912). The importance of these granules for embryonic germ cell development and fertility was highlighted with experiments showing that their destruction by UV irradiation resulted in the so-called grandchild-less phenotype, i.e. sterility of the next generation (Hegner 1911; Hathaway and Selman 1961). Furthermore, the fertility of oocytes renderd sterile by UV irration could be rescued by transplanting germ plasm (which contains germ granules) from a healthy donor strain (Okada et al. 1974).

Detailed analysis of the constituents of germ granules reveals interesting relationships. In both mouse and fly, germ granules are frequently in close association with mitochondria and their constituent proteins have functions that are linked to the piRNA pathway (Aravin and Chan 2011). Interestingly, several mitochondrial proteins including Zucchini (ZUC) and GASZ are critical piRNA pathway components. Furthermore, many components that localize to germ granules contain Tudor domains (Ponting 1997). Tudor domains bind symmetrically di-methylated arginine (sDMA) residues, such as ones present in the N-terminal region of PIWI proteins. It is believed that Tudor domain proteins might serve as a scaffold to bring together PIWI proteins and other components of the piRNA pathway in germ granules.

4.4.2 Germ Granules in Flies

In flies, germ granules can be identified in almost all stages of germ cell development. Whether in testes or ovaries, granules have various names depending on their protein constituents, localization and stage of expression. Only recently has it

been uncovered that many components of the granules operate to ensure successful repression of transposable elements.

Nuage Are Composed of Many piRNA Pathway Components in Ovarian Nurse Cells

In nurse cells of the fruit fly ovary, nebulous perinuclear structures termed 'nuage' (after the French word for 'cloud') are visible in negative stained electron micrographs (Eddy and Ito 1971). The constituents of these granules include many cytoplasmic piRNA pathway components, including AUB and AGO3 (Fig. 4.5a; Table 4.1). The cytoplasmic functions of the piRNA pathway, such as piRNA loading and Ping-Pong processing are believed to occur within these granules. Delocalization of any of the piRNA pathway components from nuage leads to impairment of the pathway, indicating the significance of these granules in proper TE silencing.

The composition and requirements of nuage granule assembly is not yet fully understood. However, some general trends for nuage assembly have been worked out. First, the components of nuage granules seem to assemble in a fashion dependent on piRNA biogenesis. This is made evident by the fact that components including AUB and AGO3, which would normally localize to nuage, are cytoplasmically dispersed in ovaries mutant for piRNA biogenesis factors such as cluster-associated factors Rhino and Cutoff and the mRNA export protein UAP56 (Klattenhoff et al. 2009; Pane et al. 2011; Zhang et al. 2012). Second, the assembly of proteins that make up nuage granules appears to be hierarchical. For example, it is apparent from mapping pair-wise genetic interactions that some protein components only localize to nuage if other factors have already 'built' on the nuage complex. Some proteins may not localize to nuage in the absence of one or more upstream protein components, while the localization of these upstream components is unperturbed by mutations in downstream components (Malone et al. 2009; Anand and Kai 2012; Patil and Kai 2010; Lim and Kai 2007).

Lastly, arginine-rich motifs typically found near the N-termini of PIWI proteins are modified to symmetrical di-methyl arginines (sDMA) by the PRMT5 methylosome complex (Kirino et al. 2009, 2010a; Nishida et al. 2009; Anne and Mechler 2005). Methylated arginines can bind Tudor domains, which are a prevalent domain in piRNA pathway components (Table 4.1) (Kirino et al. 2010a, b; Liu et al. 2010b). It is believed that Tudor domain proteins might form a scaffold in nuage that helps bring together functional components of the pathway. While the role of arginine methylation in the function of the piRNA pathway is not entirely clear, AUB requires sDMA modifications to accumulate at the pole plasm during oogenesis indicating the requirement of arginine methylation for proper localization. Additionally, sDMA modifications on AGO3 appear to be required for interactions with another component, PAPI (Liu et al. 2011). Further information regarding the 'interactome' of nuage is currently lacking, but will likely be required to understand how nuage components operate together to execute their biological functions.

The Pole Plasm: Germ Granules in the Oocyte

The pole plasm is defined by an accumulation of maternally deposited RNA, mitochondria, and piRNA-associated granules at the posterior pole of the oocyte. Much of the material that adheres to the pole plasm will be inherited by pole cells, which are among the first cells to cellularize during embryogenesis (Hay et al. 1988). Pole cells migrate during gastrulation to the embryonic mid-gut and give rise to primordial germ cells of the developing larva. AUB is among the hand-full of piRNA pathway proteins that localize to the pole plasm (Fig. 4.5b; Table 4.1). The enrichment of AUB at the pole plasm depends on sDMA modifications that allow it to bind Tudor, which itself is anchored to the pole plasm by the PRMT5 methylosome complex and the body-patterning-associated factor Oskar (Anne and Mechler 2005; Anne 2010; Arkov et al. 2006; Anne et al. 2007; Thomson and Lasko 2004; Kugler and Lasko 2009; Liang et al. 1994).

The accumulation of AUB at the pole plasm by a devoted mechanism, i.e. post-translational sDMA modification, suggests that AUB is required for the biology of primordial germ cells.

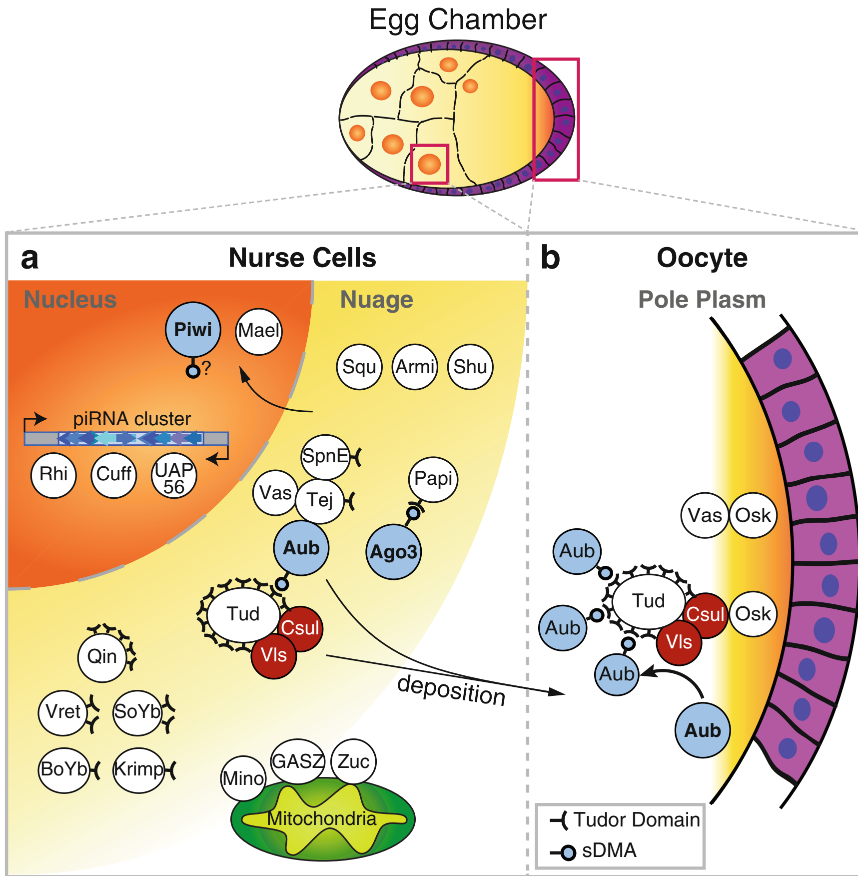

Fig. 4.5 Many protein factors make up nuage granules in *Drosophila* nurse cells and some accumulate at the pole plasm of the developing oocyte. (**a**) Nuage granules surround the nucleus of nurse cells and consist of many factors involved in the piRNA pathway. Nuage also shows close proximity to mitochondria, which contain piRNA pathway components (ZUC, GASZ) in their outer membrane. Nuage integrity depends on nuclear piRNA biogenesis factors (such as RHI, CUFF, UAP56) and a number of nuage components of unknown function. Many nuage components contain Tudor domains, which are receptors for proteins containing symmetrically di-methylated arginine (sDMA) residues. These Tudor proteins are believed to form a scaffold for the factors involved in the pathway. AUB and AGO3 contain sDMA motifs at their N-terminal regions and can interact with Tudor proteins. The enzyme complex responsible for methylation, PRMT5 methylosome, consisting of Capsuléen (CSUL) and Valois (VLS), is also present in nuage and associates with Tudor. Some nuclear proteins, such as PIWI and MAEL are believed to transiently visit the nuage and this is required for their function (Klenov et al. 2011; Saito et al. 2009b). Known interactions are indicated. (**b**) At later stages of oogenesis, nurse cells deposit their cytoplasmic contents, including piRNA pathway components, into the oocyte. Some components, including sDMA-modified AUB, accumulate at the posterior end of oocytes to form the pole plasm. The material that accumulates at the pole plasm becomes the cytoplasmic material of pole cells, which give rise to primordial germ cells of the embryo

Whether the role of AUB in primordial germ cells is solely palliative in destroying transposable element transcripts, as in the context of Ping-Pong, or whether its role in primordial germ cells serves a deeper purpose, one required for the establishment of the primordial gonad, remains to be understood. The idea that PIWI or AGO3 might also participate in germ cell development has been proposed, although direct evidence for their involvement in primordial gonads is still sparse (Nishida et al. 2009; Malone et al. 2009; Megosh et al. 2006).

4.4.3 Germ Granules in Mouse

Like in flies, mouse germ cells contain granular electron dense structures in their cytoplasm termed pi-bodies (Aravin et al. 2009). These structures were first cytologically described in rat germ cells and termed 'intramitochondrial cement' (IMC) (Eddy 1974). Studies using immunofluorescence microscopy showed that mouse piRNA pathway proteins, including MILI, MIWI2, MVH, and several Tudor proteins, accumulate in pi-bodies, which localize in the vicinity of mitochondria (Aravin et al. 2008, 2009; Shoji et al. 2009; Kuramochi-Miyagawa et al. 2010).

The functional importance of the integrity of pi-bodies and their spatial proximity to mitochondria was highlighted in studies that showed involvement of a mitochondrial protein mitoPLD in the piRNA pathway (Huang et al. 2011b; Watanabe et al. 2011b). MitoPLD is a mouse homolog of *Drosophila* protein ZUC, and was known to localize to the outer mitochondrial membrane and have a role in lipid signaling. In mitoPLD mutant, IMC formation is impaired, resulting in loss of localization of piRNA pathway components to the associated granules. As a consequence, TEs are upregulated, their regulatory regions lose DNA methylation, and the mice are sterile. A structural protein localized to IMC, GASZ, is also a conserved component of the piRNA pathway and its mutation leads to sterility. Loss of GASZ is detrimental for the integrity of the granules, resulting in loss of MILI and mislocalization of MVH and TDRD1 in embryonic testes (Ma et al. 2009).

The 'second arm' of the piRNA pathway, MIWI2 and its interacting partners TDRD9 and MAEL, occupy another discrete entity in the cytoplasm, partially co-localizing with the MILI-containing granules (Aravin et al. 2008, 2009; Shoji et al. 2009). These granules, which are larger and fewer than pi-bodies, also contain components of the ubiquitous P-bodies (such as GW182, XRN1 and DCP1A) and were termed piP-bodies. The integrity of these granules depends on the integrity of pi-bodies. Any disruption of the MILI-containing intramitochondrial cement results in loss of localization of MIWI2 and TDRD9 to piP-bodies and to the nucleus, downregulation of secondary piRNAs, and consequent TE overexpression.

Taken together, these data suggest that correct structure of these compartments and their spatial proximity heavily influences the efficiency of TE silencing, making them a pivotal subcellular compartment of embryonic male germ cells.

In postnatal germ cells, MILI, MIWI and their interacting proteins, such as TDRD6 and MAEL, are found in the chromatoid body, an entity thought to resemble the P-body of somatic cells. Chromatoid bodies are the proposed site of RNA storage and processing, but no specific function has been assigned to it yet. Given the localization of piRNA pathway components in this subcellular compartment, it has been proposed that pachytene piRNAs, in complex with MIWI and MILI, exert their function in the chromatoid body. This assumption still awaits experimental confirmation (Meikar et al. 2011).

4.5 Systems Similar to the piRNA Pathway in Other Organisms

A number of organisms contain small RNA pathways that share function with the germline-specific piRNA pathway of animals: protecting the host against harmful repetitive elements such as transposons by repressing their expression. In fact, small RNA pathways in yeast and in some single-cellular organisms are in many ways more similar to the germline-specific piRNA pathway than the ubiquitous miRNA or siRNA pathways of metazoans. In yeast and *Tetrahymena* the primary target seems to be transcriptional regulation of targets rather than the cytoplasmic posttranscriptional control, with the extreme being elimination of undesired genomic regions. Transcriptional control is also the main mechanism of action for the siRNA pathway in plants, which is responsible for DNA-methylation and repression of TE transcription. Interestingly, the germline of plants have an intriguing mechanism of small RNA-mediated TE silencing that seems to work non-cell autonomously. Finally, in *C. elegans* a system homologous to the piRNA pathway in flies and mouse closely interacts with the siRNA pathway to repress TEs in the germline.

Here we briefly discuss some interesting aspects of small RNA pathways in single-celled organisms and in the germline of plants and *C. elegans*, highlighting similarities and differences to the *Drosophila* and mouse piRNA pathway.

4.5.1 RNAi-Dependent Heterochromatin Formation in Fission Yeast

In *Schizosaccharomyces pombe*, RNAi functions to maintain transcriptional silencing at constitutively heterochromatic regions of the genome. The importance of the RNA pathway is underlined by the observation that defects in RNAi pathway genes and pericentromeric heterochromatin formation lead to aneuploidy and genomic instability that result from defective kinetochore assembly and chromosome segregation during mitosis (reviewed in Reyes-Turcu and Grewal 2012).

While heterochromatic DNA is mostly transcriptionally silent, during S-phase transcription of centromeric loci, including pericentromeric repeats, is enhanced, while the HP1 homolog, SWI6 is removed (Kato et al. 2005; Zofall and Grewal 2006). Interestingly, this transcription is required for the recruitment of the silencing machinery in two ways: first, transcripts are converted into a double-stranded RNA by the RNA-dependent-RNA polymerase complex (RDRC), which are subsequently processed by Dicer (DCR1) and loaded into Argonaute 1 (AGO1). Secondly, the siRNA loaded into AGO1 directs it to complementary nascent transcripts originating from pericentromeric loci. The AGO1 complex recruits the histone methyltransferase CLR4, the homologue of SU(VAR)3–9, that installs repressive H3K9 trimethylation marks over the locus. The complex containing CLR4 in turn recruits the RNA-dependent RNA polymerase thereby ensuring production of more siRNAs from the locus (Zhang et al. 2008). This leads to a positive feedback loop that results in strong repression of the locus throughout other stages of the cell cycle.

The yeast system is similar to the siRNA pathway in higher eukaryotes in terms of small RNA biogenesis. However, the nature of the targets, namely repetitive elements, and the mechanism of action, namely, transcriptional silencing through establishment of a repressive chromatin over target loci strongly resemble the mechanism employed by the piRNA pathway in the *Drosophila* germline.

4.5.2 Transcriptional and Posttranscriptional Control of Repetitive Elements in Neurospora

The filamentous fungus, *Neurospora crassa*, has virtually no known active transposons. The presumed reason for this is the existence of three different mechanisms that defend the *Neurospora* genome against repetitive sequences. One, known as ‘quelling’, acts in the vegetative phase, whereas repeat-induced point mutation (RIP) and meiotic silencing of unpaired DNA (MSUD) operate upon fertilization, in pre-meiotic and meiotic stages, respectively (reviewed in Caterina et al. 2006). Unlike RIP, which does not employ RNA silencing components, Quelling and MSUD are post-transcriptional silencing mechanisms that rely on homologs of known RNA silencing factors Dicer, Argonaute, and RNA-directed RNA polymerase (RdRP) (Romano and Macino 1992; Shiu et al. 2001). During vegetative growth, presence of arrays of repetitive sequences, such as transgenes, results in transcription of so-called aberrant RNA. Aberrant RNA is converted into double stranded form by the RdRP QDE1, and processed into small RNAs by a Dicer-like protein (Cogoni and Macino 1999; Catalanotto et al. 2004). Small RNAs join the Argonaute protein QDE2 to form RISC (RNA-induced silencing complex) and target all homologous RNAs for degradation (Catalanotto et al. 2002). MSUD is triggered by regions of unpaired DNA during chromosome pairing in meiosis. These regions may arise upon insertion of foreign DNA, like a transgene, a virus, or a transposon. Unpaired regions are transcribed, and a different RdRP,

SAD1, converts the transcripts into dsRNA. As in quelling, small RNAs are made by the Dicer-like protein and loaded into RISC, resulting in silencing of homologous RNA (Lee et al. 2003). The mechanism of quelling and MSUD strongly resemble siRNA biogenesis in yeast and plants in the requirement of RdRP for generation of dsRNA, yet unlike in those organisms both silence targets posttranscriptionally similarly to RNAi in animals and to the cytoplasmic piRNA pathway.

4.5.3 DNA Elimination in Ciliates

The most extreme form of gene silencing is seen in some unicellular organisms that literally eliminate substantial unnecessary genomic information from their "somatic" nuclei. *Tetrahymena*, Paramecium and other protozoans have an unconventional genetic configuration, possessing a germline genome, located in the micronucleus (mic), which is transcriptionally silent during vegetative growth, and a somatic genome, found in the macronucleus (mac) from which genes are expressed (Duharcourt et al. 1995, 1998; Jahn and Klobutcher 2002). The genomes of the two nuclei greatly differ both in content and in structure. The macronucleus contains multiple copies of fragmented chromosomes, which lack repetitive sequences that are common in the micronucleus. During the sexual process of conjugation, ciliated protozoans undergo programmed excision of excess DNA – the so-called internal eliminated sequences (IES), which are believed to be derived from transposons. Interestingly, *Tetrahymena* uses RNA guides to target the heterochromatin modifications to the loci to be excised (reviewed in Mochizuki and Gorovsky 2004a; Yao and Chao 2005). During conjugation, the entire micronuclear genome, which contains IES, is transcribed into double-stranded RNA. Small RNAs called scan RNA (scnRNA) are derived from these transcripts and loaded into a PIWI protein, TWI1 (Mochizuki and Gorovsky 2004a, b, 2005; Chalker and Yao 2001; Malone et al. 2005). TWI1-bound scnRNAs travel to the old macronucleus, from which the IES have been removed in the previous conjugation, and 'scan' the entire genome. scnRNA matching the macronuclear genome are degraded, and the remaining ones, matching IES, shuttle to the developing new macronucleus, where they induce heterochromatinization and subsequent excision of IES (Malone et al. 2005; Liu et al. 2004; Mochizuki et al. 2002). This process ensures that TEs are removed, while genomic sequences required for somatic functions are maintained in the mac of the next generation.

4.5.4 RNA-Dependent DNA Methylation in Plants

Similar to *S. pombe*, plants also utilize siRNAs to establish repressive chromatin at repetitive regions. Contrary to yeast, heterochromatin is marked by DNA methylation. Plant DNA methylation occurs throughout the genome, mostly on repetitive sequences, but not restricted to them. Constitutive expression of dsRNA mapping to promoter regions results in production of corresponding siRNAs and in de novo DNA methylation and gene silencing (Mette et al. 2000; Matzke et al. 2004). Additionally, in plants repeats can influence development by nucleating RNAi-dependent methylation and silencing of surrounding protein-coding genes. This indicates a more general function of small RNA pathways in plant transcriptional control.

Plants also seem to utilize a small RNA pathway to repress TEs in the germline, similar to piRNAs in animal germ cells (Slotkin et al. 2009). Unlike small RNA systems in animals, this process seems to function non-cell-autonomously and involves the supporter cells of the gonads. Pollen consists of two nuclei, one that will form the sperm and will be transmitted to the offspring and an accompanying vegetative nucleus, which does not contribute its DNA to the progeny. Due to demethylation of TE sequences in the vegetative nucleus of the pollen, TEs are reactivated, transpose and contribute to the formation of mature siRNAs, which can freely diffuse into the sperm nucleus. It is thought that this protects the sperm from activation of TEs and possibly directs establishment of the correct methylation pattern in the offspring

(Slotkin et al. 2009). A similar process – albeit using slightly different machinery – seems to be involved in silencing of transposons in the female gametophyte as well. This function of small RNAs transmitted to the germ cells would possibly correspond to the maternally deposited piRNAs observed in flies, which are thought to direct transposon repression in the offspring.

4.5.5 The Interplay of the piRNA and siRNA Pathways in *C. elegans*

C. elegans employs a remarkable system that uses trans-generational memory of gene expression to find and repress foreign sequences in germ cells (Ashe et al. 2012; Luteijn et al. 2012; Shirayama et al. 2012; Lee et al. 2012). The correct identification and silencing of non-self sequences in *C. elegans* requires the cooperation of three different small RNA pathways.

In the gonads of *C. elegans* a diverse population of so-called 22G-RNAs is generated from the transcriptome and loaded into the Argonaute CSR1 (Claycomb et al. 2009). CSR1 and the associated 22G-RNAs are transmitted into the progeny, where they recognize "self" transcripts that were present in the parental gonads. This recognition by CSR1 protects "self" transcripts against targeting by the second small RNA pathway, the *C. elegans* piRNA pathway, consisting of the *C. elegans* PIWI -clade protein PRG1 and the associated 21U-RNAs (the *C. elegans* piRNAs). Accordingly, recognition of non-self sequences seems to be achieved by targeting of all sequences that are not protected by the CSR1 system (Ashe et al. 2012; Luteijn et al. 2012; Shirayama et al. 2012; Lee et al. 2012). 21U-RNAs are very diverse and can likely bind transcripts through incomplete sequence complementarity allowing for recognition of any sequence (Ruby et al. 2006; Bagijn et al. 2012). Binding of the PRG1/21U-RNA complex to a target transcript leads to production of cognate 22G-RNAs (similar to the 22G-RNAs in CSR1, but mapping to the foreign sequence), which get loaded into WAGO9, part of the third small RNA pathway (Ashe et al. 2012; Luteijn et al. 2012; Lee et al. 2012). WAGO9 enters the nucleus and, together with other factors including chromatin proteins, induces transcriptional silencing of the sequence, along with deposition of the H3K9me3 mark. Once 22G-RNAs that incorporate into WAGO9 are generated against non-self, the WAGO pathway can maintain silencing in the absence of 21U-RNAs, thereby keeping memory of previously identified foreign sequences. Similarly to 22G-RNAs associated with CSR1 these are also trans-generationally inherited.

Together, these three systems, in concert with chromatin factors, allow for stable transgenerational transmission of information regarding self and non-self, and ensure an immediate silencing of new foreign sequences. The promiscuity of targeting guided by 21U-RNAs associated with PRG1 allows the piRNA pathway to recognize and induce silencing of new foreign elements of any sequence.

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5 Non-coding RNA in Ovarian Development and Disease

J. Browning Fitzgerald, Jitu George,
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Abstract

The ovary's primary function is to produce the mature female gamete, the oocyte that, following fertilization, can develop into an embryo, implant within the uterus and ultimately allow the mother's genetic material to be passed along to subsequent generations. In addition to supporting the generation of the oocyte, the ovary and specific ephemeral tissues within it, follicles and corpora lutea, produce steroids that regulate all aspects of the reproductive system, including the hypothalamic/pituitary axis, the reproductive tract (uterus, oviduct, cervix), secondary sex characteristics all of which are also essential for pregnancy and subsequent nurturing of the offspring. To accomplish these critical roles, ovarian development and function are tightly regulated by a number of exogenous (hypothalamic/pituitary) and endogenous (intraovarian) hormones. Within ovarian cells, intricate signalling cascades and transcriptional and post-transcriptional gene regulatory networks respond to these hormonal influences to provide the exquisite control over all of the temporal and spatial events that must be synchronized to allow this organ to successfully complete its function. This book chapter will focus specifically on the role of non-coding RNAs, their identification and described functional roles within the ovary with respect to normal function and their possible involvement in diseases, which involve the ovary.

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Folliculogenesis • MicroRNA • Premature ovarian failure • Ovarian cancer • Endo-siRNA • Long non-coding RNA

5.1 Introduction

Cell signalling networks and transcriptional factors within ovarian tissues have been extensively studied, however, studies of regulatory non-coding RNAs, which includes small RNA species such as piRNA, microRNA, endogenous small interfering RNAs (endo-siRNA), snoRNA and snRNA, and long RNA species including long intergenic noncoding RNA (lincRNA), long non-coding (lncRNA), have only recently (mid-2000s) been studied in ovarian tissues (Carletti and Christenson 2009; Lau et al. 2009). Much of the early work that continues today relates to the identification of the small RNA species within the organ, tissues and specific cell types of the ovary. Indeed as of yet, no systematic analysis of lncRNA has been completed in ovarian tissue. Current research is now beginning to elucidate the functional consequences these regulatory RNA have within ovarian tissues. This chapter will cover both of these aspects in normal physiology as well as discuss the relevance of regulatory non-coding RNAs with respect to several prominent ovarian diseases including ovarian cancer, polycystic ovarian syndrome, premature ovarian failure and ovarian hyperstimulation. Lastly, it should be noted that the majority of the published work on non-coding RNAs is centred on microRNA (miRNA), due primarily to their earlier identification, their high conservation across species, and their described regulatory mechanisms, which has allowed scientists to more easily study them. Therefore, this chapter will have a major focus on this class of non-coding RNAs, and mention briefly the few studies that look at other regulatory non-coding RNAs in the ovary.

5.2 Non-coding RNA in Ovarian Development

Ovarian development begins during gestation with primordial germ cells and somatic cells coming together to form a cellular mass known as the primordial follicles. These immature follicles contain the oocyte (the germ cell) and a surrounding layer of squamous granulosa cells (somatic cells). Subsequent to primordial follicle formation and prior to puberty, local factors within the ovary induce follicular waves that lead to recruitment, development and growth of some the primordial follicle pool causing progression from primordial to pre-antral and antral stage follicles. Currently, the presence and role for lncRNA in developing ovarian tissues has yet to be reported, although it is expected that these regulatory RNA will play mechanistic and functionally important roles similar to what has been observed in other tissues (Kung et al. 2013). Conversely, microRNA have been widely described in developing ovaries and we summarize those studies below.

Gene expression analysis studies have shown that miRNA are widely expressed in the mammalian ovary (Tripurani et al. 2010; Zhao and Rajkovic 2008; Ahn et al. 2010; Ro et al. 2007; Choi et al. 2007), with estimates ranging from 373 to 679 different miRNA being present in whole ovarian tissues using cloning or sequencing based technology. These early studies, while confirming presence of miRNA, were limited in their scope, as most did not address the cell types or stages of the ovarian cycle during which the ovaries were collected. Developmental studies in rodent, bovine and ovine models also examined miRNA expression in whole ovaries of newborn

and foetal animals (Tripurani et al. 2010; Zhao and Rajkovic 2008; Ahn et al. 2010; Choi et al. 2007; Torley et al. 2011; Veiga-Lopez et al. 2013; Luense et al. 2011). Using a microarray platform, Zhao et al. (Zhao and Rajkovic 2008) identified 173 microRNA that were significantly expressed in newborn mouse ovaries with members of the let-7 family and miR-199a being the most prevalent miRNA. Function and cell-specific localization of miR-199a and the let-7 family members within the developing ovary have yet to be determined. Furthermore, as few miRNA were known at the time of this study, only 373 different miRNA were evaluated. Cloning studies by Tripurani et al. (2010) and next generation sequencing studies by Ahn et al. (2010), which are not reliant on a priori identification of miRNA, indicated that newborn ovaries in cattle and mice have 679 and 398 expressed miRNA, respectively.

In the bovine foetal ovary, 8 miRNA of the 679 miRNA present (**bta-miR-99a**, **bta-miR-10b**, bta-miR-199a-3p, bta-miR-199a-5p, **bta-miR-424**, **bta-miR-100**, bta-miR-455 and bta-miR-214) had more than tenfold greater relative abundance in the foetal ovary when compared to other somatic tissues (brain, hypothalamus, spleen, heart, lung, kidney, small intestine and muscle), four of these miRNA (bolded) exhibited higher expression in human ovaries too (Tripurani et al. 2010; Liang et al. 2007). In their studies, characterizing newborn mouse ovaries, Ahn et al. (2010) found that miRNA were the most abundant (over 25 %) form of all small RNA species. Through quantifying the total number of reads, miR-320, let-7i and let-7d were identified as the most highly expressed miRNA in the newborn ovaries (Ahn et al. 2010). Comparison of ovarian tissue to brain, lung, heart, stomach, liver, muscle, kidney and uterus indicated that miR-202 and miR-298 were also enriched in mouse gonadal tissues (Ahn et al. 2010). Again as previously mentioned, isolation, expression and most importantly functional analysis of these miRNAs seen in whole tissue from specific ovarian cell populations are needed to determine whether these miRNA are critical players in ovarian development and function.

In two time course studies in foetal sheep, Torley et al. (2011) and Veiga-Lopez et al. (2013) examined miRNA expression during gonadal differentiation. These two studies compared the relative expression patterns of 128 and 742 different miRNA in foetal ovaries between gestational day (GD) 42 and 75 or GD 65 and 90, respectively. The time points span the period of meiotic resumption (GD 55) and primordial germ cell formation (GD 75) in the first study and are prior to primordial follicle assembly (GD 65) and towards the completion of the dramatic tissue remodelling associated with the ovigerous cord formation (GD 90) in the latter study. Interestingly, Torley et al. (2011) observed that 62 of 128 miRNA were differentially expressed with equal distribution over time (one-half being increased at GD 42 and the other half induced at GD 75), while Veiga-Lopez et al. (2013) observed that only 21 of the 742 miRNA examined in their study were differentially expressed with 13 being increased at GD 65 and 8 increased at GD 90. It is noteworthy that GD 65 and GD 90 are both after the onset of meiotic resumption, and thus, may present a more homogenous set of tissues. In addition to the developmental analysis, this latter study also examined the effect of maternal exposure of the environmental disruptor, bisphenol A (BPA), on foetal ovarian miRNA expression (Veiga-Lopez et al. 2013). Maternal exposure to BPA induced a dramatic decrease in 45 different miRNA in ovaries of GD 65 foetuses (Veiga-Lopez et al. 2013) and 11 miRNAs at GD 90, when compared to same age controls. Interestingly, BPA only caused miRNA to decline in foetal sheep ovaries, the authors evaluated several of the enzymatic genes (Drosha, Dicer and DiGeorge syndrome critical region gene 8) as a possible mechanism and found no differences (Veiga-Lopez et al. 2013). An attractive hypothesis includes the possibility that these miRNA have oestrogen response elements within their promoters that could interact with the weak estrogenic BPA molecule to thereby influence their expression. Together all of these studies provide evidence that miRNA change

during ovarian development, yet the role of specific miRNA and their functional impact on ovarian development remains to be determined.

5.3 Noncoding RNAs Involved in Ovarian Physiology

5.3.1 MicroRNA and Endo-siRNA on Oocyte Development

During the early stages of folliculogenesis, Dicer1 the RNase III enzyme that functions in the biogenic pathways of both endo-siRNA and miRNA exhibits robust and steady expression levels within the oocyte (Watanabe et al. 2008). Immediately following fertilization, however, Dicer1 levels are significantly reduced (Cui et al. 2007; Murchison et al. 2007). Coincident with decreased *Dicer1* expression, global miRNA levels were also reduced in the two-cell embryo subsequent to increased *Dicer1* expression and a return of increased miRNA expression (Tang et al. 2007). Consistent with these changes in *Dicer1* expression, examination of small RNA populations in oocytes by Watanabe et al. (2006) indicated that approximately 10 % and 11 % of the total non-coding RNAs are miRNA and endo-siRNA, respectively. Moreover, no observable differences between miRNA and siRNA expression patterns were seen between fully grown and metaphase II (MII) oocytes (Watanabe et al. 2006). Complete loss of Dicer1 activity by targeted deletion of the gene causes embryonic lethality (Bernstein et al. 2003), therefore to investigate miRNA/endo-siRNA activity multiple labs "floxed" Dicer1 (Harfe et al. 2005; Andl et al. 2006; Muljo et al. 2005) in order to selectively knock out *Dicer1* using cell-specific promoters to drive Cre recombinase.

Two studies using an oocyte-specific zona pellucida 3 (*Zp3*) Cre recombinase to knock out *Dicer1*, set out to determine if this biogenic enzyme functions within the oocyte (Murchison et al. 2007; Tang et al. 2007). In both studies there was near complete ablation of miRNA in the oocyte (Murchison et al. 2007; Tang et al. 2007). Murchison et al. (2007) observed disorganized spindles and chromosomal abnormalities, while Tang et al. (2007) reported that fertilized oocytes were unable to proceed through the first round of cell division and that half of the mutant oocytes showed cell fragmentation. Neither of these studies indicated that *Dicer1* and by inference miRNA (and endo-siRNA upon latter discovery) were critical in early oocyte development from the primary follicular stage (when *Zp3* expression begins) through the late maturation processes just prior to the ovulatory surge of luteinizing hormone (LH) (Murchison et al. 2007; Tang et al. 2007). Because these *Dicer1* knockout studies were inadequate to determine if *Dicer1* had any function at the earliest stages of folliculogenesis and oocyte growth, Mattiske et al. knocked out *Dicer1* using a Cre recombinase driven by the alkaline phosphatase liver/bone/kidney (*Alpl*) promoter, which is expressed in primordial germ cells (Mattiske et al. 2009). Similar to the results of the *Zp3* Cre models, this study identified chromosomal and meiotic abnormalities following the LH surge, while early folliculogenesis and oocyte growth were indistinguishable from wild type littermates (Mattiske et al. 2009). A particularly interesting observation from these oocyte-specific *Dicer1* deletions is that the well-established oocyte – somatic (cumulus) cell interactions, which are deemed vital for normal folliculogenesis (Gilchrist et al. 2004; Buccione et al. 1990; Eppig 2001), are not reliant of oocyte miRNA or endo-siRNA post-transcriptional mechanisms.

What remains unanswered from these studies is the extent to which miRNA or endo-siRNA are contributing to the observed phenotypes. In an effort to address this question, Suh et al. (2010) using the *Zp3*-Cre conditionally to knock out DiGeorge Syndrome critical region gene 8 (*Dgcr8*). This RNA-binding protein, in conjunction with Drosha, processes pri-miRNA to pre-miRNA within the nucleus. This protein is only present in the miRNA biosynthetic pathway (Suh et al. 2010). Similar to the previous *Dicer1* knockout experiments, miRNA expression was almost entirely ablated in the oocyte (Suh et al. 2010). However, in contrast to Dicer1 knockout models, oocyte maturation following the LH

surge and embryonic preimplantation development were normal (Suh et al. 2010). These results implicated endo-siRNA and not miRNA as the important players in late oocyte development. Additional confirmation of this hypothesis came from a study by Ma et al. (2010) who found that the 3′untranslated regions (3′UTRs) of mRNA upregulated after *Dicer1* deletion were not enriched in miRNA binding sites. The authors concluded from this finding that miRNA would therefore have a marginal impact on the maternal transcriptome in oocyte development (Ma et al. 2010). Further research investigated the ability of siRNA to regulate expression of retrotransposons (a class of genes known to be regulated in oocytes and embryos) during development (Watanabe et al. 2006). Co-transfection of EGFP linked to retrotransposons and EGFP linked to siRNA libraries into fully grown oocytes caused more degradation of the EGFP-retrotransposons when compared to the EGFP controls (Watanabe et al. 2006). Findings from this study suggest that siRNAs can post-transcriptionally regulated retrotransposons in the oocyte. However, whether siRNA regulates retrotransposons in vivo remains unknown, since the authors used an exogenous expression system (Watanabe et al. 2008).

5.3.2 MicroRNA and Endo-siRNA in Ovarian Somatic Cells

Folliculogenesis is characterized by rapid proliferation and dramatic morphological changes in granulosa cells as they transition from squamous to cuboidal cells during the transition from primordial follicles to primary follicles and beyond. These somatic changes are also coincident with alterations in oocyte growth, maturation, and differentiation, as well as the differentiation and identification of the thecal cell layer immediately adjacent to the basement membrane, separating the granulosa cells and oocyte from the vascular and stromal tissue of the ovary. In addition to the recruitment, growth, and selection processes commonly associated with folliculogenesis, the majority of these follicles that reach the preantral and antral stages will undergo atresia, a hormonally controlled apoptotic process that limits the number of follicles/oocytes that can be released to a number consistent with that species birth rate (monoovulatory or multiovulatory/litter bearing) (Tilly et al. 1991; Richards 1980).

Ovarian folliculogenesis is tightly regulated by the pituitary hormones, follicle stimulating hormone (FSH) and LH (Richards et al. 2002a, b). These hormones function as survival factors for follicles and increase the steroidogenic capacity of the follicles. At completion of folliculogenesis, the increasing oestradiol levels produced by the dominant follicle feeds back on the hypothalamic/pituitary to generate a surge of LH, which in turn initiates ovulation (release) of the cumulus oocyte complex from the dominant follicle, and subsequent uptake by the oviduct where fertilization can occur. Following the LH surge, the follicular granulosa and theca cells undergo a rapid and dramatic transformation into luteal cells, forming the parenchymal cells of the corpus luteum. The corpus luteum in turn secretes progesterone, a hormone critical for the establishment and maintenance of the pregnancy, if fertilization occurs, and for menstrual (human and primates) and oestrous (domestic animals, rodents) cyclicity in its absence. To accomplish these dramatic transformative events, beginning at the earliest stages of ovarian development all the way through to the process of luteolysis, the cellular mechanisms must be tightly regulated. MiRNA and endo-siRNA abilities to fine-tune gene regulation post-transcriptionally may be one of the mechanisms through which this regulation takes place.

Studies examining the role of miRNA and endo-siRNA in ovarian somatic cells have been conducted by three independent laboratories, who knocked out *Dicer1* in granulosa cells using the anti-Müllerian hormone receptor 2-Cre (*Amhr2*-Cre) (Hong et al. 2008; Gonzalez and Behringer 2009; Nagaraja et al. 2008; Pastorelli et al. 2009), which is expressed in early developing granulosa cells as well as throughout the female reproductive tract (Arango et al. 2008). Conditional deletion of *Dicer1* resulted in complete female sterility (Hong et al. 2008; Gonzalez and Behringer 2009; Nagaraja et al. 2008), pre-

dominantly caused by an inhospitable environment within the oviduct that killed early embryos. However, in addition to the marked morphologic and functional changes seen in the oviduct and uterus, Hong et al. (2008) observed markedly impaired ovulation rates both following natural cycles and following stimulated (PMSG+HCG) cycles. Confirmation of loss of ovarian function was also seen in the other studies (Gonzalez and Behringer 2009; Nagaraja et al. 2008). The partial loss of fertility is consistent with the lack of penetrance of the *Amhr2*-Cre within ovarian granulosa cells (Gifford et al. 2009).

In a follow-up study of miRNA/endo-siRNAs' role in folliculogenesis, Lei et al. (2010) examined mouse ovarian tissues from 8-day, 8-week to 8-month old mice. Increased numbers of primordial follicles, accelerated follicular recruitment and more follicles that were degenerate were observed in the knockout animals versus control animals (Lei et al. 2010). In addition, several genes important in ovarian development, including 17alpha-hydroxylase (*Cyp17a1*), aromatase (*Cyp19a1*), growth/differentiation factor 9 (*Gdf9*), and bone morphogenetic protein 15 (*Bmp15*), amongst others, were differentially expressed in wild-type and conditional knockout ovaries (Lei et al. 2010). This study also evaluated expression of miR-503 an abundant ovarian miRNA (Takada et al. 2006), as expected loss of Dicer decreased miR-503 expression and temporal expression analysis indicated gonadotropin stimulation of follicle growth caused miR-503 levels to decrease (Lei et al. 2010). A specific role for miR-503 in ovarian function however awaits further study. In an alternative approach, Otsuka et al. (2008) used a hypomorphic *Dicer1* allele to globally deplete *Dicer1* to ~20 % of normal levels in mice. Female infertility was the major phenotype observed in this *Dicer1* depletion model and subsequent characterization identified that the corpus luteum had reduced vascularization and that progesterone production was reduced (Otsuka et al. 2008). In conclusion, these partial and complete knockout studies indicate that maintenance of the miRNA/endo-siRNA biogenic pathway within granulosa and luteal cells is important for folliculogenesis, ovulation and luteal function. Currently, no direct studies have implicated a role for miRNA/endo-siRNA in theca cells, the other major cell type within the follicle. However, it is expected that important regulatory roles for microRNA/siRNA occur in that cell type too. Additionally, it is unclear if the phenotypes derived from the Dicer knockouts described above are due to the loss of miRNAs and/or endo-siRNAs. However, both profiling and specific functional studies have shown that miRNA are important to ovarian function (described in detail below). Nonetheless, further research using selective depletion of *Dgcr8* in granulosa cells and theca-specific Cre lines should help address these open questions.

Additional evidence for miRNA-mediated post-transcriptional gene regulation as an important regulator of ovarian function can be obtained from a number of studies that have examined tissue (follicular and luteal) or cellular (granulosa) specific expression of miRNA over stages of development or from tissues at different stages during the oestrous/menstrual cycle or of pregnancy. Profiling studies have revealed that miRNA can vary their expression patterns depending on the stage of follicular development (Yao et al. 2009, 2010a; Ma et al. 2011; Fiedler 2008; Li et al. 2011; Yang et al. 2012a; McBride et al. 2012). Moreover, several of these gene expression studies have shown that certain miRNA can be under hormonal regulation in specific cell sub-types within the ovary.

Yao et al. (2009) investigated a small subset (6) of miRNA that were known to have low expression in primordial follicles, but are robustly expressed in primary follicles. These included let-7a, miR-125b and miR-143. To determine if miRNA expression is FSH regulated, an ovarian cell line, KK1, was subjected to FSH treatment for 6, 12, 24 and 48 h, after which miRNA expression was evaluated. MiR-143 was significantly reduced following FSH when compared to the untreated cells, while let-7a and miR-125b trended down (Yao et al. 2009). This study suggests that miRNA can be hormonally regulated. Subsequent work by the same laboratory using cultured rat granulosa cells identified 17 FSH-up-regulated and 14 FSH-down-regulated miRNA

(Yao et al. 2010b). However, because only a few select miRNA were chosen for examination or the method utilized was an in vitro approach, the overall relevance to ovarian function is limited. Using an unbiased in vivo approach to identify miRNA that are regulated by the LH surge, our laboratory treated mice with a standard follicular stimulation protocol (PMSG +HCG) and collected granulosa cells 4 h after the surge of LH (hCG) and compared them to PMSG treatment alone (Fiedler et al. 2008). We identified ten microRNA with decreased expression (miR-483, miR-491, miR-484, miR-329, miR-433-3p, miR-532, miR-431, miR-672, miR-99b and miR-351) and three dramatically up-regulated microRNA (miR-132, miR-212 and miR-21) in granulosa cells (Fiedler et al. 2008). These findings were the first to reveal that hormones can have in vivo effects on miRNA expression patterns in specific ovarian cells. Through extensive studies from our lab (discussed below) we have begun to elucidate miR-21's functional role and mechanism of action in granulosa cells and within the ovary.

In addition to hormonal regulation, studies have shown that intra-ovarian factors can regulate miRNA expression within ovarian cells. Yao et al. (2010a) investigated the effect of transforming growth factor β1 (TGFβ1), a factor known to play a role in follicular development and female fertility, on miRNA expression in pre-antral granulosa cells. After 6 h of TGFβ1 treatment of cultured pre-antral granulosa cells, 16 miRNAs were differentially expressed compared to control treated cells (Yao et al. 2010a). Of these 16, 13 were down-regulated and 3 were up-regulated including miR-712, miR-224 and miR-764-3p. Further investigation (described below in detail) implicated miR-224 in the regulation of granulosa cell proliferation (Yao et al. 2010a). In an alternative approach, granulosa cells were transfected with miRNA mimics and steroid synthesis was evaluated (Sirotkin et al. 2009). Of the 80 miRNAs tested, progesterone, testosterone and oestrogen synthesis decreased following transfection of 36, 57, and 51 of the individual miRNAs into cultured human granulosa cells respectively. Conversely, 10, 1 and no miRNAs were able to stimulate progesterone, testosterone and oestrogen synthesis, respectively. These experiments however are difficult to interpret, for several reasons: First, it is unclear if the changes in steroid output are a result of changes in production or if it is related to cellular viability, i.e. proliferation and apoptosis were not investigated, but were shown to be changed in a subsequent paper by the same group (Sirotkin et al. 2009) and secondly, the level of over-expression achieved was not reported. It is very likely that these cells were exposed to supra-physiologic levels of miRNA, which could lead to dramatic off-target effects.

Lastly, using microarray analysis, Ma et al. (2011) investigated miRNA expression patterns in regressed and non-regressed bovine corpora lutea (CL). They identified 13 differentially expressed miRNAs, 7 of which had preferential expression in non-regressed CL, while 6 miRNAs were elevated in regressed CL. MiR-378, a miRNA implicated in regulation of apoptosis, was one of the miRNAs that was up-regulated in the healthy non-regressing CL (Ma et al. 2011). Examination of miR-378 across the luteal phase indicated that its expression was greatest in mid and late stages and it dropped appreciably in regressing CL. Conversely a putative target gene, interferon gamma receptor 1, showed no change in mRNA levels, but its protein levels increased in regressing CL, prompting the conclusion that miR-378 might regulate this gene's expression (Ma et al. 2011). The failure to demonstrate an actual cause and effect relationship, through further experimentation, however should temper this conclusion. It remains to be shown if miR-378 is a regulator of apoptosis and interferon gamma receptor 1 expression or merely if miR-378 expression is correlated with regression. Additional studies from another lab (discussed below) implicated this miRNA in having a functional role in folliculogenesis.

Together the profiling studies implicate a number of miRNAs in functional roles throughout the ovarian cycle. The following paragraphs describe the miRNA that have been individual evaluated with respect to ovarian somatic cell function (Fig. 5.1).

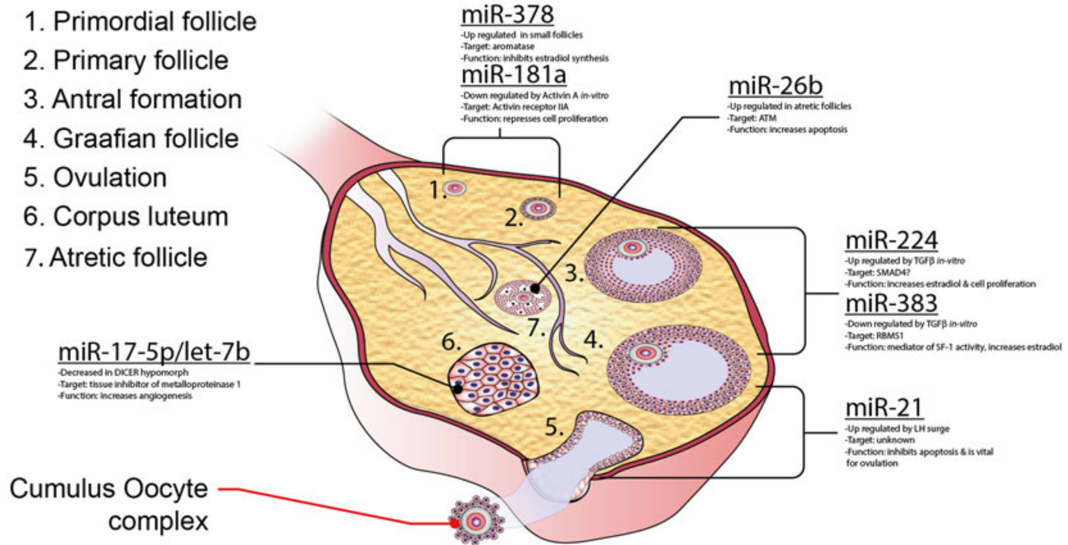

Fig. 5.1 Schematic showing the different stages of follicles, ovulation and the corpus luteum where individual miRNA are expressed or where loss of miRNA action (by targeted or pharmacologic depletion) has been implicated in ovarian somatic tissue function

MicroRNA-378 (miR-378) Bioinformatics analysis indicated the presence of several miRNA recognition sites within the 3′UTR of the porcine aromatase gene and expression analysis within small (1–3 mm) and large (3–6 mm) porcine follicles showed that miR-378 expression was approximately two-times greater in small follicles (Xu et al. 2011). To determine if miR-378 was able to directly regulate aromatase expression, miR-378 was overexpressed in granulosa cells isolated from both small and large follicles using a lentiviral expression system (Xu et al. 2011). Expression of miR-378 decreased aromatase levels with a concomitant decrease in oestradiol release (Xu et al. 2011). Similarly, inhibition of endogenous miR-378 levels increased aromatase levels and oestradiol production. Lastly, using the 3′UTR of aromatase linked to a luciferase reporter Xu et al. (2011) revealed that miR-378 regulates aromatase through direct binding to its 3′UTR. These observations therefore support a role for miR-378 in the regulation of aromatase, and point to the question of what regulates miR-378 expression in granulosa cells. Interestingly, recent studies of miR-378 and miR-378*, which are both are produced from the same pre-miRNA, but contain different seed sequences and thus target different genes, indicate that these miRNAs, which are located within the intron of the peroxisome proliferator-activated receptor γ coactivator 1β (*Pgc1β*) are equally co-expressed with this gene and counterbalance the metabolic actions of PGC1β, which includes metabolism and mitochondrial biogenesis (Carrer et al. 2012). Thus, this miRNA's ability to regulate ovarian aromatase linked with its direct connection to expression of PGC1β, a key metabolic regulatory protein, may provide a potential mechanism of integration of metabolism and ovarian function. It is well known that energy balance is essential for optimal ovarian function and that metabolic pathologies such as obesity, diabetes and other metabolic syndromes can detrimentally affect ovarian function (Brewer and Balen 2010).

MicroRNA-224 (miR-224) As mentioned above, the effect of the TGFβ superfamily and specifically TGFβ1 on cultured ovarian granulosa cell miRNA expression identified a number of differentially expressed miRNAs. The functional consequence of one of the three highly induced microRNAs, miR-224, was further evaluated in granulosa cells and the authors implicated miR-224 in the regulation of SMAD4 expression via a

series of miR-224 inhibitor and over-expression experiments (Yao et al. 2010a). The authors also found that loss or gain of miR-224 activity through inhibitors/mimics decreased and increased cell proliferation and oestradiol synthesis, respectively (Yao et al. 2010a). These cellular changes were associated with increased (miR-224 inhibitor) and decreased (miR-224 mimic) SMAD4 levels, respectively. Reconciling these results with the observations of Wang et al. (2011) is however difficult, as they show that direct loss of SMAD4 decreases cell proliferation and oestrogen synthesis, the opposite effect shown when miR-224 is used to modulate SMAD4 levels. It is possible that miR-224 may be mediating its effects on cell proliferation and oestradiol synthesis through the regulation of other genes. Interestingly, in a follow-up study examining what regulates miR-224 expression, Liang et al. (2013) clearly showed involvement of p53 and p65 in miR-224 expression. Surprisingly though, in this follow-up paper they only showed a minimal effect of miR-224 inhibition on SMAD4 levels. Ultimately, these in vitro experiments await confirmation in an in vivo context.

MicroRNA-383 (miR-383) Yin et al. initially confirmed that TGFβ signalling suppresses expression of miR-383 along with the sarcoglycan zeta (*Sgcz*) gene. *Mir-383* is embedded within one of the *Sgcz* introns, thus implicating the *Sgcz* gene promoter in miR-383 expression (Yin et al. 2012a). Transfection of miR-383 mimics into granulosa cells led to a dose-dependent increase in oestradiol (Yin et al. 2012a). In their comprehensive study, Yin et al. (2012a) further elucidated a possible mechanism of action for miR-383 within the follicle, identifying a direct target, RNA binding motif, single stranded interacting protein 1 (RBMS1), that is decreased at both the mRNA and protein levels following overexpression of miR-383 (Yin et al. 2012a). Additionally, *cMyc*, a downstream target of RBMS1, decreased following miR-383 overexpression and *cMyc* inhibition and over-expression in granulosa cells increased and decreased estradiol production, respectively (Yin et al. 2012a). Furthermore over-expression of *cMyc* was able to mitigate the effects of over-expression of miR-383 or silencing of RBMS1 on oestradiol synthesis. Lastly, the impact of steroidogenic factor 1 (SF1), a transcription factor known to regulate steroidogenesis in gonadal tissue, on *Sgcz*/miR-383 expression was examined (Yin et al. 2012a). Knockdown of *Sf1* decreased the primary and mature forms of miR-383 as well as *Sgcz* mRNA expression, follow-up luciferase and ChIP experiments confirmed that SF1 regulates *Sgcz* and *mir-383* gene expression via direct binding to its promoter (Yin et al. 2012a). This investigation reveals a unique role for miR-383 in mediating SF1 actions in granulosa cell oestradiol synthesis via a direct target, *Rbms1*, and a target of cMYC.

MicroRNA-21 (miR-21) miR-21 is up-regulated in virtually every known cancer and as a result has been the subject of many studies that have investigated both its function and its direct targets in tumours and cancer cell lines (Kumarswamy et al. 2011). Reports from our lab have revealed that, in addition to its pathological functions in cancer, miR-21 has a physiological role in the ovary (Fiedler et al. 2008; Carletti et al. 2010). Originally identified as an LH/hCG-regulated gene within murine ovarian granulosa cells (Fiedler et al. 2008), subsequent work has shown it to be up-regulated in ovine granulosa cells during the periovulatory period (McBride et al. 2012). Expression analysis throughout the periovulatory period (the time period between onset of LH/hCG stimulation and ovulation) in mice indicated that LH transcriptionally regulated *pri-mir-21* expression (Carletti et al. 2010). Furthermore, miR-21's potential regulatory role in cultured granulosa cells was examined and it was observed that complementary locked nucleic acid oligonucleotide inhibitors of miR-21 were able to induce apoptosis as evidenced by increased cleaved caspase 3 and annexin 5 staining (Carletti et al. 2010). These observations are consistent with miR-21's known anti-apoptotic role in cancer cells (Kumarswamy et al. 2011). Interestingly, analysis of a number of *bona fide* miR-21 targets identified in other cells and tissues were not found to change in granulosa

cells (Carletti et al. 2010). Lastly, we demonstrated that loss of miR-21 activity in vivo in mice undergoing ovarian stimulation (PMSG/hCG) caused a pronounced reduction in ovulation rates when compared to control treated animals, and the presence of trapped oocytes within corpora lutea (Carletti et al. 2010). Current work in the laboratory has identified additional pathways and target genes that are modulated by miR-21 expression (unpublished Carletti, Fitzgerald and Christenson). Linking these genes to ovarian function in vivo remains to be tested.

A study by Mase et al. (2012) also suggests that miR-21 is present in human granulosa cell lines, that it is highly expressed, and that over 80 % of the miRNA bound to EIF2C2 (a major component of the RNA induced silencing complex) was miR-21 (Mase et al. 2012). This latter finding suggests that a vast majority of miRNA-mediated post-transcriptional gene regulation may be occurring through miR-21 in these cell lines (Mase et al. 2012). Lastly, in a recent examination of GV and MII pig oocytes, miR-21 was shown to be substantially expressed in the MII oocytes (Yang et al. 2012a).

MicroRNA-181a (miR-181a) Activins, which are produced by the ovary, are important for follicular development and maintaining female fertility (Pangas et al. 2007). Zhang et al. (2013) recently showed that activin A treatment of cultured mural granulosa cells caused a reduction in miR-181a expression in a dose dependent manner. Over-expression of miR-181a in cultured granulosa cells resulted in decreased granulosa cell proliferation through direct interaction with the 3'UTR of the activin receptor IIA (*Acvr2a*) mRNA (Zhang et al. 2013). Additionally, target genes downstream of ACVR2A were shown to be regulated by miR-181a in the expected direction. These studies further showed that replacement of ACVR2A was able to compensate for miR-181a-mediated suppression. The authors also examined miR-181a expression in ovarian follicles, demonstrating that miR-181a expression decreased in murine follicles as they progressed from primary, preantral to antral stages. Lastly, these investigators observed in a very small sample of patients with premature ovarian failure (POF) that miR-181a was elevated 4.5-fold in the blood of the POF patients (Zhang et al. 2013). This study provides a wealth of information and points to miR-181a as an important regulator of ovarian function. It will be interesting to determine whether these effects are seen in other species and whether miR-181a mediates a similar effect in vivo.

MicroRNA-26b (miR-26b) Lin et al. (2012) compared miRNA expression in healthy, early and late stage atretic preovulatory porcine follicles. Using stringent conditions, 20 non-redundant miRNA were shown to be differentially expressed in healthy as well as both groups of atretric follicles with equal numbers being up- and down-regulated. Further characterization of miR-26b indicated that cultured porcine granulosa cells transfected with a miR-26b mimic were apoptotic as shown by anti-annexin V staining and evidence of dead cells in medium that did not occur in the controls treated cells. Lastly, the authors determined that ataxia telangiectasia mutated (*Atm*), a gene involved in repairing double-strand DNA breaks, was a direct target of miR-26b and that the number of DNA breaks increased in granulosa cells following treatment with miR-26b mimics (Lin et al. 2012). As noted by the authors, these experiments yielded a number of other interesting miRNA candidates that might be involved in atresia. Studies aimed at determining whether any of these identified miRNAs are involved in atresia and whether the atretic process is consistent for follicles of different stages remain to be performed.

Let-7b and miR-17-5p In their study using the *Dicer1* hypomorph, Otsuka et al. (2008) demonstrated that ovarian bursal injection of let-7b and miR-17-5p was able to partially restore the vascular defect caused by the overall loss of miRNAs. Additionally, the authors showed that the expression of a putative target, tissue inhibitor of metalloproteinase 1 (*Timp1*), changed in response to over-expression of either miRNA. Luciferase reporter assays showed that this is a direct effect on the 3′UTR of the *Timp1*

gene (Otsuka et al. 2008). The role of these particular miRNAs in ovarian function has not been further pursued, however, they would be interesting candidates for misregulation in luteal phase insufficiencies that result from neovascular defects (Boutzios et al. 2013).

Other microRNAs Implicated in Granulosa Cell Function The following two miRNAs, miR-23a and miR-145, have been demonstrated to have effects on cultured human granulosa cells, but as yet have not been shown to change in ovarian tissues, thus limiting the interpretation of whether these effects are confined to a cell culture system or whether these miRNA play important in vivo roles in ovarian function. Transfection of human granulosa cells with miR-23a caused a decrease in XIAP (X-linked inhibitor of apoptosis protein) and caspase-3 protein levels, but an increase in cleaved caspase-3. miR-23a expression in granulosa cells may be causing apoptosis through inhibition of XIAP (Yang et al. 2012b). Interestingly, the rationale to examine miR-23a, originated from a biomarker study examining serum miRNA in POF patients (Zhou et al. 2011). Yan et al. (2012) showed that miR-145 directly regulates the expression of the activin receptor 1b. Additionally, miR-145 was shown to regulate cultured granulosa cell proliferation mediated partially through the ACVR1B/SMAD2/Activin A pathway.

5.4 Long Non-coding RNA in Ovarian Somatic Tissues

Currently, no systematic analyses of lncRNA have been reported in ovarian tissues. However, with the advent of emerging technologies such as stranded sequencing and the identification of numerous lncRNA in other tissues and the subsequent development of PCR and hybridization arrays, this will be shortly rectified. Currently, the only specific reported evidence for an ovarian lncRNA is the steroidogenic acute regulatory protein natural antisense transcript *Star-NAT* (Castillo et al. 2011). This RNA was found to bear full sequence complementarity to the spliced *Star* sense 3.5-kb transcript and is expressed in steroidogenic tissues such as testis, adrenal gland, brain and ovary (Castillo et al. 2011). Over expression of *Star-NAT* followed by cyclic AMP stimulation led to a decrease in StAR protein expression and concurrent decrease in progesterone production, likely through a post-transcriptional mechanism (Castillo et al. 2011).

5.5 Non-coding RNAs Involved in Ovarian Diseases

5.5.1 Ovarian Cancer

A role for small RNAs in the aetiology of ovarian cancer has been hypothesized by numerous laboratories and the literature in this area is extensive (see review Di Leva and Croce 2013). In the following two paragraphs we discuss two comprehensive studies that provide strong evidence that non-coding RNAs play critical components to the aetiology of ovarian cancer. Zhang et al. (2006) analysed 283 miRNA loci in 93 primary ovarian cells and 16 cell lines derived from ovarian cancer. Of these loci, 105 (37.1 %) were shown to have significant copy number alterations. Significantly, the *mir-17–92* miRNA cluster located on chromosome 13q31, which has been described as oncogenic and is frequently amplified in multiple cancer types, was also increased in ovarian cancer (Knuutila et al. 1998). The *mir-15a* and *mir-16* locus located on human chromosome 13q14, was lost in 23.9 % of the ovarian cancer cell types tested (Zhang et al. 2006). Zhang et al. also reported that in 24.8 % of ovarian cancer cell types, *Dicer1* and *Argonaute 2* (*Ago2*), genes that are critical for miRNA biogenesis and function, had increased copy numbers (Zhang et al. 2006). In contrast to the study performed by Zhang et al. (2006), which showed increased copy numbers of *Dicer1* in ovarian cancer cell types, Merritt et al. (2008) show that there was variability in *Dicer1* and *Drosha* expression in ovarian cancer specimens and that low expression of these genes was highly correlative with advanced tumour stage and low survival rate. In support of these findings, previous

studies where *Dicer1* was knocked down increased tumour formation in vivo was observed (Kumar et al. 2007).

Numerous investigations (far beyond what can be covered in this chapter) have gone beyond profiling and identified specific miRNA that might participate in cancer or are implicated in chemotherapeutic roles in ovarian cancer (Li et al. 2010). As an example, Yang et al. (2008) identified that patients who were resistant to chemotherapy, exhibited significantly reduced expression of let-7i and expression of this miRNA was correlated with low survival rate in women undergoing chemotherapy. Overexpression of let-7i in cultured ovarian cancer cells increased resistance to the chemotherapy drug cisplatin when compared to control treated cells (Yang et al. 2008). Studies of miR-200a in ovarian cancer cells showed that restoration of this miRNA led to decreased binding of laminin and increased sensitivity to paclitaxel, a chemotherapeutic drug (Cochrane et al. 2010). In support of these findings, previous research has shown that miRNA can have pharmacologic/chemotherapeutic functions in tumours (Blower et al. 2008) and that chemoresistance in ovarian cancer and ovarian cancer cells is associated with a distinct miRNA profile (Sorrentino et al. 2008; Boren et al. 2009). Lastly, a number of lncRNA have identified by *in situ* hybridization and RT-PCR analysis to be mis-regulated in ovarian cancer cells, these include the lncRNA *XIST*, *H19* RNA, steroid receptor activator (*SRA*), *BC200* RNA, and, more recently, a *sno-lncRNA* (Ariel et al. 1995; Hussein-Fikret and Fuller 2005; Yin et al. 2012b).

5.5.2 MicroRNA in Ovarian Fertility Disorders

Polycystic ovary syndrome is the most prevalent female infertility disease with ~5–10 % of reproductive aged women being affected. This disease is characterized as an oligo- or anovulatory disease with elevated systemic androgen levels with or without presence of multiple cystic follicles on the ovary (Ehrmann 2005; Franks 1995). In a recent study examining miRNA within follicular fluid of patients undergoing assisted reproductive technology, Sang et al. (2013) identified ~120 miRNAs, including several highly upregulated (miR-132 and miR-21), which were previously identified as LH-regulated in murine granulosa cells (Fiedler et al. 2008). Characterization of seven of the more highly expressed miRNAs in control patients and in PCOS patients, indicated that miRNA-132 and miR-320 were significantly lower expressed in PCOS patients (Sang et al. 2013). The putative targets and any possible roles for these miRNAs remain to be determined, as does a more comprehensive analysis of the cellular source of these small RNAs.

In another study, miR-93, -133 and -223, which all target GLUT4, the insulin-sensitive glucose transporter, were evaluated in adipose tissue of PCOS patients (Chen et al. 2013). The authors go on to demonstrate a direct effect of miR-93 on GLUT4 and that this miRNAs is overexpressed in PCOS patients and women with insulin resistance and hypothesized that this miRNA may partially explain the overlap of these two conditions (Chen et al. 2013). In a rodent PCOS model, rats were given dihydrotestosterone (DHT) to induce a PCOS-like phenotype and ~25 highly expressed miRNAs were found in both control and DHT ovaries (Hossain et al. 2013). Interestingly, of the differentially expressed RNAs several were shown to be selectively expressed in theca cells (Hossain et al. 2013). This is an important observation as theca cells are the predominant source of elevated androgens in PCOS women and these studies provide evidence that dysregulation of miRNA expression should be further evaluated in human ovarian theca cells.

Currently, other diseases of ovarian origin such as POF and ovarian hyperstimulation, which occurs as a consequence of assisted reproductive technology, have not been carefully examined with respect to changes in small or long non-coding RNA as a causative agent or as reactive genes, i.e. potential biomarkers, with the exception of the serum miRNA biomarker study of POF previously described (Zhou et al. 2011).

In conclusion the current literature indicates that a number of regulatory small RNAs are pres-

ent within ovarian tissues. A select group of these small RNAs have been shown to be modulated by growth factors and steroids in cell culture systems, with only a few miRNAs having been shown to change in vivo and/or be hormonally (gonadotropin) regulated in vivo. Individual target genes for a select few miRNA have elucidated, yet it remains very likely that this represents a very small proportion of the target mRNAs these miRNA regulate. Lastly, the presence and study of long non-coding RNAs and the recently identified circular RNAs has not yet systematically studied in ovarian tissues and this area is sure to be an interesting topic in the coming years.

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6 Non-coding RNA in Spermatogenesis and Epididymal Maturation

J.E. Holt, S.J. Stanger, B. Nixon, and E.A. McLaughlin

Abstract

Testicular germ and somatic cells express many classes of small ncRNAs, including Dicer-independent PIWI-interacting RNAs, Dicer-dependent miRNAs, and endogenous small interfering RNA. Several studies have identified ncRNAs that are highly, exclusively, or preferentially expressed in the testis and epididymis in specific germ and somatic cell types. Temporal and spatial expression of proteins is a key requirement of successful spermatogenesis and large-scale gene transcription occurs in two key stages, just prior to transcriptional quiescence in meiosis and then during spermiogenesis just prior to nuclear silencing in elongating spermatids. More than 60 % of these transcripts are then stockpiled for subsequent translation. In this capacity ncRNAs may act to interpret and transduce cellular signals to either maintain the undifferentiated stem cell population and/or drive cell differentiation during spermatogenesis and epididymal maturation. The assignation of specific roles to the majority of ncRNA species implicated as having a role in spermatogenesis and epididymal function will underpin fundamental understanding of normal and disease states in humans such as infertility and the development of germ cell tumours.

Keywords

Noncoding RNA • Primordial germ cell • Gonocyte • Differentiation • Meiosis • Spermatogenesis • Spermiogenesis • Epididymis • Sertoli cell

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6.1 Overview of Spermatogenesis and Spermiogenesis, ncRNA Synthesis and Specific Targeting

6.1.1 Spermatogenesis and Spermiogenesis

The production of spermatozoa in mammals is a complex and highly regulated process of cell division and differentiation during which diploid spermatogonia develop into haploid gametes capable of fertilization. Spermatogenesis encompasses the period over which spermatogonia, first formed in the neonatal testis, undergo proliferation with a cohort entering meiosis to become spermatocytes. Following completion of the two meiotic divisions, the haploid germs, now termed spermatids, complete a differentiation program known as spermiogenesis, which culminates in the formation of a mature spermatozoon, structurally capable of transiting the female reproductive tract and fertilizing the egg (Fig. 6.1). The distinct developmental steps of male germ cell formation require strict spatial and temporal regulation of gene expression, which takes place at the epigenetic, transcriptional and post-translational levels.

Male germ cells are first specified in foetal life following sexual differentiation, when the primordial germ cells become gonocytes that undergo a burst of mitotic proliferation before entering G_0 arrest. At this time the gonocytes become closely associated with somatic Sertoli cells in seminiferous cords that later become the seminiferous tubules. Postnatally, the gonocytes migrate to the basement membrane of the seminiferous tubules and re-enter the cell cycle to become undifferentiated spermatogonia or spermatogonial stem cell cells (SSC). Junctions between the Sertoli cells form the 'blood-testis barrier' which compartmentalize the tubules into the basal compartment, where the spermatogonial cell population lies, and the adluminal compartment, which will contain the differentiating germ cells (Oatley and Brinster 2012; De Rooij and Russell 2000).

SSCs may retain the capacity to undergo self-renewal (designated A_{single}) or produce daughter cells (A_{paired}) that commit to differentiation by undergoing transit amplifying mitotic divisions as undifferentiated spermatogonia ($A_{aligned}$) to become differentiated $A_{1–4}$, intermediate and Type B spermatogonia before entering meiosis (Oatley and Brinster 2012; Hess and Renato de Franca 2008). The balance between the stem cell state and differentiation must be strictly regulated to maintain continuity of spermatogenesis and a number of secreted factors have been implicated in the regulation of SSC survival including GDNF, CSF1, FGF2, IGF1 and LIF (Oatley and

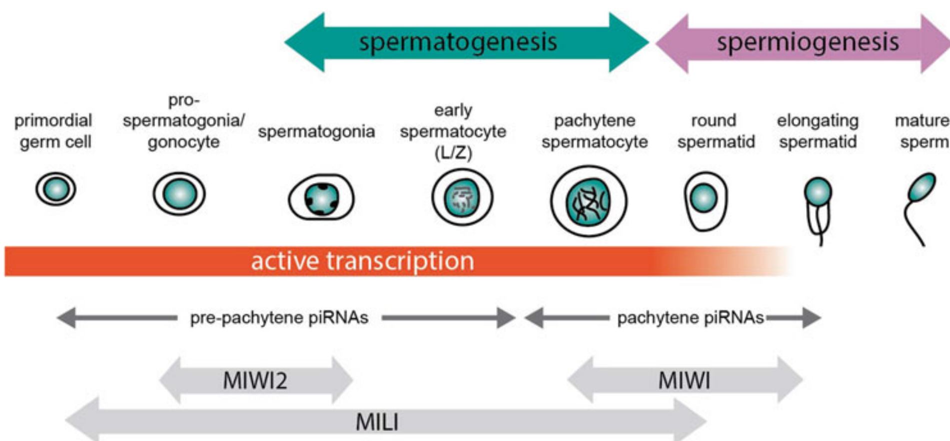

Fig. 6.1 Expression of piRNAs in germ cells during spermatogenesis and spermiogenesis

Brinster 2012). The action of GDNF upregulates a cohort of transcription factors thought to meditate roles in this process including Brachury and ETV5. Other GDNF-independent factors also support SSC renewal such as PLZF, FOXO, and POU5F1 (Oatley and Brinster 2012; Song and Wilkinson 2014). Activin A, BMP4, neuregulin 1, and members of the Notch signalling pathway act to promote SSC differentiation, potentially via activation of differentiation promoting transcription factors such as DMRT1, NGN3, SOHLH1/2 and SALL4 (Oatley and Brinster 2012; Song and Wilkinson 2014; Garcia et al. 2013; Kubota et al. 2004; Hobbs et al. 2012).

Type B spermatogonia enter meiotic Prophase I in response to retinoic acid (RA) produced by Sertoli cells, which increases as a result of downregulation of RA-degrading P450 enzyme CYP26B1 (Koubova et al. 2006, 2014; Bowles et al. 2006). Activation of RA-responsive genes, including *Stra8* and *Rec8*, initiates pre-meiotic S-phase replication of DNA in pre-leptotene spermatocytes (Koubova et al. 2014; Anderson et al. 2008). Homologous chromosome pairing and synapsis proceeds during prophase I, with SPO11 enzyme-induced double strand breaks allowing recombinatorial crossovers to take place (Mahadevaiah et al. 2001; Keeney et al. 1997). Double strand break (DSB) repair begins in the zygotene stage and by the pachytene stage, chromosomes are fully synapsed and completing the final stages of recombination (Baumann et al. 1996; Shinohara et al. 1992; Moens et al. 2002; Wang and Hoog 2006). A plethora of proteins regulate recombination through the phases of synapsis, DSB formation, strand invasion, DNA intermediate processing and crossover resolution (Baudat et al. 2013). Genetic disruption of many of these genes causes meiotic arrest in male germ cells, a response referred to as the 'pachytene checkpoint' (Handel and Schimenti 2010). Upon successful completion of prophase I, rising CDK1 levels promote the first meiotic division to yield diploid secondary spermatocytes, followed immediately by a second division that produces haploid spermatids ready to undergo spermiogenesis (Fig. 6.1).

Spermatids pass through four main phases during spermiogenesis, which are defined by the morphology of the developing nucleus, acrosome and sperm tail. The first phase, the Golgi stage, is initiated by the formation of a perinuclear Golgi apparatus that begins to accumulate proacrosomal vesicles that gradually coalesce to form a large acrosomal vesicle. During the capping phase, the acrosomal vesicle begins to flatten into a cap over the surface of the nucleus, which migrates over the ventral surface of the elongating nucleus in the third 'acrosomal' phase (Hess and Renato de Franca 2008). At this point the nucleus begins to condense as sperm-specific protamines replace the histones to tightly package the DNA, which induces transcriptional silencing in the elongating spermatid (Kimmins and Sassone-Corsi 2005; Kimmins et al. 2004). The nucleus continues to condense in the final maturation stage, the tail flagellum lengthens, and excess cytoplasm is removed forming residual bodies that are later discarded (Hess and Renato de Franca 2008). Upon completion of spermiogenesis, mature spermatozoa are released into the seminiferous tubule lumen and travel toward the epididymis.

After the first wave of spermatogenesis in the pre-pubertal testis, the seminiferous epithelium is organized into cyclic stages, such that a successive order of different stage germ cells are present along the length of the tubule. This arrangement ensures a constant supply of mature spermatozoa is available and allows the encompassing Sertoli cells to co-ordinate the development of germ cells at different stages (Hess and Renato de Franca 2008). In concert with testosterone-producing Leydig cells, Sertoli cells play a crucial role in spermatogenesis and spermiogenesis in response to FSH and LH cues (Ruwanpura et al. 2010). In addition to endocrine and paracrine control, the processes of spermatogenesis and spermiogenesis are regulated through intricate programs of transcriptional and post-transcriptional control within the germline. Over the last two decades, non-coding RNAs (ncRNAs) have emerged as important eukaryotic cell regulators that are implicated in this developmental

process. Here we will review the biosynthesis and targeting mechanisms for these regulatory classes before exploring their specific roles in governing the events of germ cell maturation in the testis and epididymis.

6.1.2 Small ncRNA Synthesis and Targeting in Male Germ Cells

Comprising a broad range of RNA species, ncRNAs can be divided in two major groups according to size: small RNAs (<200 nt) and long ncRNAs (>200 nt) (Pauli et al. 2011; Kung et al. 2013). Small non-coding RNAs of the miRNA and piRNA classes are now well established as key players in coordinating the cell cycle changes and differentiation events of spermatogenesis and spermiogenesis. MiRNAs function primarily as post-transcriptional regulators by altering target mRNA stability or translation, whilst piRNAs have been linked to the repression of transposable elements. piRNAs appears to be expressed predominantly in the male germ line, and whilst miRNAs are ubiquitous, a subset appear to be exclusively or predominantly expressed in the testes (McIver et al. 2012a; Yadav and Kotaja 2014). Long non-coding RNAs (lncRNAs) are less well characterized but have been linked to gene regulation through multiple mechanisms including chromatin modification, transcription and post-transcriptional processing and are abundantly expressed in the testes (Mercer et al. 2009; Luk et al. 2014).

6.1.3 MicroRNAs

Single stranded miRNAs are transcribed from regions throughout the genome from sites that lie within coding genes (intragenic) or are located at intergenic regions (Shomron and Levy 2009). Primary miRNA transcripts form hairpin loop structures that are cleaved by a complex of DGCR8 and Drosha enzyme to form a pre-miRNA of ~70-nucleotides. Following transport to the cytoplasm, the endonuclease Dicer processes the pre-miRNA into a 21–24 bp miRNA duplex. A single strand of this mature miRNA is loaded into the miRNA-induced silencing complex (miRISC) which allows the miRNA to base-pair with a specific target mRNA. Pairing of a miRNA usually occurs within the 3′UTR of the mRNA, although 5′UTRs and open reading frame pairing has also been reported (McIver et al. 2012b; Moretti et al. 2010). miRNA nucleotides 2–8 of the 5′ end of the RNA commonly form the ‘seed region’ of the sequence where perfect pairing takes place and which isessential for efficient target binding (Fig. 6.2). The position of the target site within an mRNA appears also be important – for example in 3′UTRs, AU-rich regions at least 15-nt away from the stop codon are preferred binding sites for miRNAs (Bartel 2009). In mammals, the overall complementarity between a miRNA and its target is usually imperfect, which allows each miRNA to potentially regulate multiple RNAs (Pasquinelli 2012).

The core components of the miRISC complex are the argonaute (AGO) family members 1–4 and the GW182 proteins (Fig. 6.2) which as act as platforms for the binding of numerous accessory proteins (Krol et al. 2010). The identity of the AGO protein and the degree of complementarity of the miRNA determines the fate of the mRNA target. In event of high miRNA/target complementarity in the presence of AGO2, the target will be cleaved by AGO2 and degraded by cellular exonucleases. Where complementarity is lower, as in mammalian systems, translational repression or destabilization without cleavage is the most common fate, which appears to be mediated by multiple potential mechanisms that may involve inhibition at any of the three key steps of translation (initiation, elongation and termination) (Gu and Kay 2010; Liu et al. 2008). However, the exact mechanisms and relative importance of each of these pathways in mammals remain controversial and the subject of much interest (Pasquinelli 2012). To date, one of the more well-defined pathways of non-cleavage RNA degradation is that of target de-adenylation involving GW182, which has been shown to recruit PABP, CCR4-NOT and PAN2-PAN3 com-

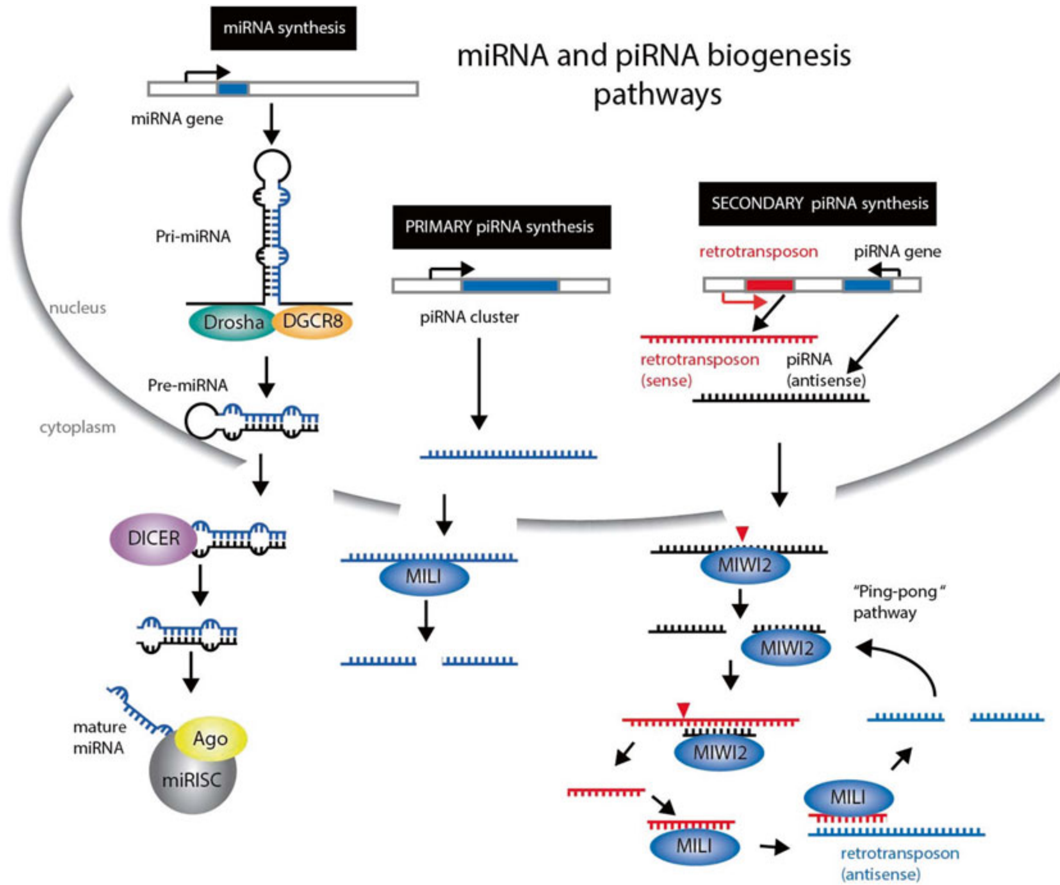

Fig. 6.2 MicroRNA and piRNA biogenesis pathways in spermatogenesis and spermiogenesis

plexes to induce shortening of the mRNA poly A tail (Huntzinger et al. 2013; Braun et al. 2011). Adding to the complexity of miRNA-mediated regulation, reports also exist for the role of miRNAs in stimulating translation (Orom et al. 2008; Vasudevan et al. 2007). For example, under conditions of nutrient deprivation, miR-10a has been shown to reduce translational repression of ribosomal protein mRNAs in human embryonic stem cells by interacting with the 5′ untranslated regions of the mRNAs (Orom et al. 2008).

6.1.4 PIWI-Interacting RNAs (piRNAs)

The piRNA classes of ncRNAs are slightly longer than miRNAs at 26–32 nucleotides in length, and rely on a distinctly different form of biosynthesis. Specific to the male germ line, piRNAs interact with the endonuclease PIWI (MIWI, MILI and MIWI2 in mouse), a subclass of the Argonaute family (Faehnle and Joshua-Tor 2007). Each PIWI protein is differentially expressed during male germ cell development and binds a specific cohort of piRNAs (Thomson and Lin 2009) (Table 6.1, Fig. 6.2). piRNA sequences themselves are poorly conserved between species, but the locations of piRNA clusters in the genome are conserved, which suggests chromatin regulation plays an important role in control of piRNA expression. Such clusters usually map to one genomic strand, which suggests that long, single-stranded precursors piRNAs are initially transcribed (Yadav and Kotaja 2014).

Table 6.1 Characteristics of the PIWI proteins expressed during male germ cell development in the mouse

PIWI family member	Cell type	Intracellular localization	piRNA class bound
MIWI2	Embryonic pro-spermatogonia	Nucleus	Pre-pachytene piRNAs
	Early post-natal spermatogonia	piP bodies	
MIWI	Pachytene spermatocyte	IMC bodies	Pachytene piRNAs
	Haploid/elongating spermatid	CB	
MILI	PGCs	Pi-bodies	Pre-pachytene and pachytene piRNAs
	Pro-spermatogonia, spermatogonia	IMC bodies	
	Spermatocytes		
	Round spermatids	CB	

IMC intermitochondrial cement bodies, *CB* chromatid body, *PGCs* primordial germ cells

Precursor piRNAs are polyadenylated and capped once transcribed, and transported to cytoplasmic granules known as 'nuage' bodies (Li et al. 2013). Here they are cleaved into shorter sequences by MILI in a 'primary synthesis' pathway that involves nucleolytic processing of both the 5′ and 3′ ends. The second more complex process of piRNA synthesis known as the 'ping-pong pathway' relies on a pool of piRNAs produced by the primary pathway that have homology to retrotransposons (Fig. 6.2). piRNAs that are complementary and in sense orientation to a retrotransposon can guide the PIWI protein to cleave the antisense strand of the retrotransposon. This generates new antisense piRNAs that can then cleave the sense-orientated retrotransposons in a MILI-MIWI2 dependent feed-forward loop (Yadav and Kotaja 2014; Hawkins et al. 2011).

piRNAs can be categorized into two main groupings according to when they are expressed during germ cell development: the pre-pachytene piRNAs and the pachytene piRNAs (Table 6.1). The major role of pre-pachytene piRNAs appears to be in guiding MILI and MIWI2 to cleave transposons to allow their degradation. Transposon silencing is also achieved through their ability to regulate DNA methylation (Aravin et al. 2008). In contrast, pachytene or late piRNAs derive from different genomic locations from pre-pachytene piRNAs and are expressed in great abundance – estimated at around one million piRNA per spermatocyte/spermatid (Aravin et al. 2006). Intriguingly, the majority of pachytene piRNAs are devoid of transposon sequences, suggesting they may play a different, as yet undefined role during germ cell development (Yadav and Kotaja 2014; Meikar et al. 2011). It has been proposed that they may in fact be degradation products from meiotic RNA products no longer required (Yadav and Kotaja 2014).

6.1.5 Long Non-coding RNA (lncRNAs)

Long ncRNAs can be classified according to their genomic origins relative to protein-coding genes, and fall into five main groups: long intergenic (lincRNAs), intronic, pseudogene, antisense and promoter and enhancer-associated lncRNAs (Kung et al. 2013). lncRNAs are normally transcribed by RNA polymerase II as mRNAs lacking protein-coding ability. Although they exhibit low sequence conservation, there is evidence for conserved secondary structure, splicing patterns and subcellular localization amongst lncRNAs. Recent microarray and high-throughput sequencing has revealed that together with piRNAs and miRNAs, this class of ncRNAs are abundant and highly expressed in the testis and are likely to play crucial regulatory role in the developing male germ cell at the chromatin, transcriptional and post-transcriptional levels (Mercer et al. 2009; Luk et al. 2014).

Amongst the lncRNAs characterized to date in other mammalian systems, the ability to recruit chromatin-modifying proteins represents a common mechanism of action. One of the most studied examples involves the lincRNA *Xist*, a 17 kB tran-

script implicated in X-chromosome inactivation in the female. *Xist* RNA acts a platform for the recruitment of chromatin silencing factors such as the polycomb repressive complex 2 (PRC2) to the X-chromosome that is destined to be silenced. In an example of lncRNAs acting as co-regulators, the lncRNA *Tsix* downregulates *Xist* on the active X-chromosome via a combination of mechanisms, one of which may be through targeting *Xist* for Dicer-dependent processing (Zhao et al. 2008; Ogawa et al. 2008). lncRNA- directed recruitment of histone methyltransferases (e.g. G9) and DNA methyltransferases (e.g. DNMT3A/B) appears to play a key role in other epigenetic changes at specific cell loci (Mercer et al. 2009).

Promoter and enhancer-associated lncRNAs are capable of interacting with various transcriptional regulators to either repress or enhance transcription. For example, lncRNAs associated with the cyclinD1 promoter can recruit RNA binding proteins that repress CREB activity and so silence cyclin D1 expression (Wang et al. 2008). Enhancer lncRNAs have been reported to recruit transcription factors to nearby genes to induce expression, whilst some lncRNA appear to interact with the RNA II polymerase complex to cause global transcriptional changes (Kung et al. 2013). More recently gene silencing by transcriptional interference has been demonstrated, whereby overlap of an antisense lncRNAs with its corresponding coding mRNA prevents target expression (Santoro et al. 2013).

At the level of post-transcriptional regulation, antisense RNAs are capable of masking splicing sites that may affect translational efficiency or mRNA isoform identity (Mercer et al. 2009). LncRNAs may also interfere with the miRNA pathway by acting as direct competitors for miRNA targets – a process which has been implicated in the regulation of human embryonic stem cell self-renewal and differentiation (Wang et al. 2013).

Non-coding RNA (ncRNA) molecules are short and long non-coding single stranded RNA molecules, which bind specifically, in conjunction with a protein complex, to several messenger RNA (mRNA) molecules to control their translation (Thomas et al. 2010). The first miRNA was identified in 1993 when scientists studying the development of *Caenorhabditis elegans* discovered a short non-coding RNA (lin-4), which contained sequences complementary to the 3′UTR (untranslated region) of the *lin-14* mRNA, which was known to encode a developmentally important temporal control protein. It was hypothesized that lin-4 controlled the expression of *lin-14* through RNA:RNA interactions. However it was not until the discovery of a second miRNA let-7 in 2000, which controls developmental timing and differentiation of *C. elegans* in a similar manner to that proposed for lin-4 that the scientific community acknowledged the importance of miRNAs in controlling gene expression. Since 2000, research on short and long non-coding RNA molecules has increased exponentially (Zhang et al. 2007). The expression of miRNAs is highly regulated and essential for normal development (Blakaj and Lin 2008). However dysregulation of miRNA expression has been linked to the development of several types of cancer including testicular cancer. For example the *mir-17–92* cluster which promotes cell survival and proliferation, is upregulated in most cancer types, and considered oncogenic (DeSano and Xu 2009).

During spermatogenesis the spatial and temporal regulation of gene expression is of vital importance. In particular, post-transcriptional regulation is essential as during spermatogenesis, germ cells are periodically transcriptionally silenced (Papaioannou and Nef 2010). In this review we now discuss short and long ncRNA regulation of mRNA, a key facilitator of development and differentiation in spermatogenesis, spermiogenesis, epididymal maturation and fertilization.

6.2 Role of ncRNA in Germ Cell Development

6.2.1 miRNA and Their Action in Male Germ Cell Development

Male germ cells express many classes of small ncRNAs, including Dicer-independent PIWI-interacting RNAs, Dicer-dependent miRNAs, and endogenous small interfering RNA. Several

studies have identified miRNAs that are highly, exclusively, or preferentially expressed in the testis and in specific germ and somatic cell types (McIver et al. 2012b).

Germ cell-specific *Dicer*-null mice demonstrate that miRNAs are essential for germ cell survival (Fig. 6.2). *Dicer* depletion in embryonic primordial germ cells resulted in a normal initial germ cell colonisation of the gonad followed by a rapid decline and a 50 % reduction in the spermatogonial pool in postnatal testes in null mice compared to wild type littermates (Hayashi et al. 2008). Importantly, miRNAs were also required for postnatal germ cell development as *Dicer*-null males retained active spermatogenesis in only a few tubules, with the fertility of young adult mice severely compromised. Histological analysis revealed that many seminiferous tubules were either harbouring abnormal germ cells or were completely devoid of germ cells (Hayashi et al. 2008; Maatouk et al. 2008a). Aged mouse testes had either predominantly Sertoli cell-only tubules or a subpopulation of tubules containing round or elongating spermatids (Maatouk et al. 2008a). Interestingly, the *Dicer*-null epididymal spermatozoa demonstrated significant tail defects resulting in pronounced impaired motility, contributing to the observed reduced fertility (Maatouk et al. 2008a). More recently using an early germ cell-specific *Dicer*-null model in which spermatogonial differentiation appeared unaffected, Korhonen and colleagues also demonstrated that haploid cell number was decreased and late haploid differentiation dysregulated in knockout testes with a concomitant increased number of apoptotic spermatocytes, and concluded that Dicer was critical for the normal chromatin organization and nuclear shaping of elongating spermatids (Korhonen et al. 2011). In addition, deletion of a second enzyme, Drosha, a nuclear RNase III enzyme, responsible for cleaving primary miRNAs into precursor miRNAs (Fig. 6.2), which is essential for the biogenesis of canonical miRNAs also results in male infertility. Drosha conditional null males displayed more severe spermatogenic disruptions than the *Dicer* germ cell null testes (Wu et al. 2012). In conclusion, collectively these results demonstrate that Dicer and Drosha, and hence miRNAs, are essential for early male germ cell proliferation and late spermiogenesis and contribute greatly to the production of functional spermatozoa (Hayashi et al. 2008; Maatouk et al. 2008a; Korhonen et al. 2011). Below we focus on specific miRNAs identified in the germline and the initial characterisation of the molecular function of miRNAs in germ cells.

6.2.2 miRNA Expression in the Testis and Germ Cells

Expression levels of miRNAs are significantly raised, compared to somatic cells, in primordial germ cells, developing germ cells and germ line stem cells (Buchold et al. 2010). As described above, in the germ line, mRNA transcript expression and the corresponding protein production is often significantly temporally uncoupled, and this has been linked to miRNA control of post-transcriptional regulation, a known role of small ncRNA molecules in development (Novotny et al. 2007). In a bid to identify key signalling pathways modulated by miRNAs many researchers have employed a variety of in silico target prediction analyses to refine lists of potential target genes underpinning successful spermatogenesis. This information must be treated judiciously as the predictive software program(s) incur both false positives and overlook miRNA targets with poorly conserved binding sites. Therefore, despite our enhanced understanding of miRNA binding sites, all computationally predicted target genes must be experimentally confirmed (Thomas et al. 2010; Huang et al. 2010; Witkos et al. 2011).

In contrast to female germ cells, during early male germ cell development, expression of the let-7 miRNA family together with miR-125a and miR-9 families increased (Hayashi et al. 2008). Both miR-125a and the let-7 miRNA family are known tumour suppressors, as miR-125a binds to HuR (Guo et al. 2009) and the let-7 family controls both FAS-regulated apoptosis and the RAS oncogenic pathway (Hayashi et al. 2008). Interestingly, let-7, miR-125a and miR-9 regulate a key con-

troller of stem cell pluripotency, LIN28, implicated in the establishment of testicular teratomas (Zhong et al. 2010). Comparison of miRNA expression profiles of ES cells and adult germline stem cells identified the *mir-290* and *mir-302* clusters as upregulated and central to the maintenance of pluripotency in both cell types (Zovoilis et al. 2008). During the transition from prospermatogonia to differentiating spermatogonia around birth and early postnatal life seven differentially expressed miRNAs were identified between gonocytes and spermatogonia (miR-293, miR-291a-5p, miR-290-5p and miR-294*, miR-136, miR-743a and miR-463*). Of the many potential targets of these miRNAs were members of the PTEN and WNT signalling pathways. These targets converge on the key downstream cell cycle regulator cyclin D1, indicating that a unique combination of male germ cell miRNAs may coordinate the differentiation and maintenance of pluripotency in germ cells (McIver et al. 2012a). In a similar fashion the *mir-17–92* (*Mirc1*) and *mir-106b–25* (*Mirc3*) clusters are thought to promote survival and proliferation of the early male germ cells, given that their expression is down-regulated in female germ cells following meiotic arrest, and in the postnatal testes they are involved in regulation of spermatogonial differentiation (Hayashi et al. 2008; Tong et al. 2012). The repression of miRNA clusters *mir-17–92* (*Mirc1*) and *mir-106b–25* (*Mirc3*) by retinoic acid could result in the increased expression of targets *Bim*, *Kit*, *Socs3*, and *Stat3*. While not essential for fertility, male germ cell-specific *mir-17–92* (*Mirc1*) knockout mice have small testes, reduced populations of epididymal spermatozoa, and mildly defective spermatogenesis. Interestingly, the depletion of *mir-17–92* (*Mirc1*) dramatically increases expression of its paralog *mir-106b–25* (*Mirc3*) cluster miRNAs in the germ cells, indicating that the *Mirc1* and *Mirc3* cluster miRNAs could function in a mutually cooperative manner to regulate spermatogonial development (Tong et al. 2012). Later developmental stages, i.e. meiotic and post-meiotic male germ cells, have been correlated with the down-regulation of expression of miR-141, miR-200a, miR-200c and miR-323, again this is associated with pluripotency and mitotic amplification (Hayashi et al. 2008).

Temporal and spatial expression of proteins is a key requirement of successful spermatogenesis. Large-scale gene transcription occurs in two key stages just prior to transcriptional quiescence in meiosis and then during spermiogenesis just prior to nuclear silencing in elongating spermatids. More than 60 % of these transcripts are then stockpiled for subsequent translation (Papaioannou and Nef 2010). Unsurprisingly, miRNA levels also increase to modulate both of these upturns in active gene transcription (Ro et al. 2007a; Yan et al. 2007). In initial studies, the dynamics of miRNA expression was investigated in whole testicular cell lysates over developmentally important periods. Yan and collaborators (Yan et al. 2007) compared the immature and mature testis in mice and identified nineteen differentially expressed miRNAs. This is a large underestimate of the changing population and was masked by the inclusion of both germ cells and somatic cells in the experimental samples. Using this approach, Yan and colleagues identified several important testicular miRNA target genes. For example *Sox5* and *Sox6* are postulated targets of miR-181c, and miR-355, miR-181c, miR-181b putatively targeted *Rsbn1*, a transcriptional regulation gene, expressed in haploid germ cells in adult testis (Yan et al. 2007).

Other animal model studies in non-human primates (Yan et al. 2009) and pigs (Luo et al. 2010) have also demonstrated differential miRNA expression between mature and immature testis with interestingly a high proportion of significant molecules down-regulated in adult tissue. In rhesus monkey testes, NOTCH1 a key regulator of germ cell differentiation and survival, is predicted to be targeted by miR-34b, miR-34c and miR-449, and BCL2 a key regulator of germ cell apoptosis is also a target of miR-449, which is highly expressed in mature testes (Yan et al. 2009). Using the porcine testis model, Lou et al. (2010) coupled miRNA expression to that of putative targets and identified candidate genes including *Dazl* (deleted in azoospermia like gene), essential for germ cell differentiation, as a target of miR-34b and miR-34c.

Similarly, cloning experiments have identified a suite of novel miRNAs that are exclusively expressed in testis (Ro et al. 2007a). Deep sequencing RNA analysis of neonatal mouse testis has identified novel miRNA species, unique splice variants and established validity of miRNA expression changes (Buchold et al. 2010). On identification of miRNA clusters on chromosomes 2 and X these miRNA clusters may play a role in germ cell development as they are upregulated in both day 14 testis and in the neonatal ovary are proposed to be active in meiotic cells (Buchold et al. 2010).

Studies conducted with whole testes clearly demonstrate that testicular expression of miRNAs is dependent on the stage of spermatogenesis. However, determining the relevance of these changes is complicated by the presence of germ cells at multiple stages of differentiation as well as significant populations of somatic cells. Initial investigations in a small scale pilot study revealed 28 testis expressed miRNAs in isolated testicular cell populations, i.e. spermatogonia, pachytene spermatocytes, round/elongating spermatids, as well as Sertoli cells and spermatozoa (Ro et al. 2007a). Microarray analysis on enriched spermatogonia, spermatocytes and spermatids populations has also been used to examine global miRNA expression (Marcon et al. 2008a). Although issues of cell purity complicated interpretation (Marcon et al. 2008a), the authors were able to conclude that the majority of miRNAs were preferentially expressed in germ cells during meiosis. When the investigators examined some potential roles for highly expressed miRNAs in germ cells (Marcon et al. 2008b) they concluded that miR-214 in meiotic cells was predicted to target heat shock proteins and miR-320, a germ cell universally expressed miRNA, was anticipated to modulate the cell adhesion molecules protocadherins.

Finally, as miRNAs have been identified in mature spermatozoa and seminal plasma and their expression is causally linked to men with subfertility or infertility, they are now considered potential biomarkers for the diagnosis and classification of male infertility (Yadav and Kotaja 2014). Of most interest is miR-34c, which in humans (Krawetz et al. 2011) is highly expressed and also found at high levels in mature sperm and zygotes in mice. This sperm contribution to the zygote is essential as miR-34c is required for the first zygotic cleavage (Liu et al. 2012).

6.3 The Elusive Roles of Long ncRNA in Testicular Function

Surveys of many deep sequencing and RNA sequencing projects have identified a number of lncRNAs expressed during male germ cell development (Luk et al. 2014), however only a limited subpopulation have been analysed. These include meiotic recombination hot spot locus (*Mrhl*) RNA, a 2.4 kb mono-exonic lncRNA located in the nucleus, which regulates spermatogenesis potentially by the cleavage of *Mrhl* by Drosha to form an 80-nucleotide RNA intermediate to directly interact with chromatin (Ganesan and Rao 2008), or by inhibiting the WNT signalling pathway through p68 (Arun et al. 2012). Testis-specific X-linked (*Tsx*) has recently been shown to be a lncRNA specifically expressed in pachytene spermatocytes and deletion resulted in apoptosis (Anguera et al. 2011). *Dmrt1*-related gene (*Dmr*) is a testis-specific functional lncRNA, which interrupts the expression of DMRT1 protein, which is essential for spermatogonial transit amplification and differentiation (Zhang et al. 2010a).

6.4 Role of Short and Long ncRNA in Epididymal Maturation

Following their morphological differentiation, spermatozoa are released from the germinal epithelium of the testes in a functionally immature state, incapable of movement or any of the complex array of cellular interactions that are required for fertilization (Hermo et al. 2010). In all mammalian species, the acquisition of functional competence occurs progressively during the cells descent through the epididymis, a long convoluted tubule that connects the testis to the vas deferens (Fig. 6.3). A remarkable feature of epi-

didymal maturation is that it is driven entirely by extrinsic factors in the complete absence of nuclear gene transcription or protein translation in the spermatozoa (Aitken et al. 2007; Cooper 1986).

The first region of the epididymis that immature sperm encounter is that of the caput, wherein they are concentrated by a mechanism of resorption that rapidly removes almost all the testicular fluid/proteins that enter the epididymis. As they leave this environment and enter the corpus epididymis, sperm begin to acquire their motility and fertilizing ability (Fig. 6.3). These attributes continue to develop as the sperm move through the corpus, and reach an optimal level as they enter the cauda region where they are stored in quiescent state prior to ejaculation (Fig. 6.3). Importantly, each of the epididymal regions possesses distinctive gene expression profiles (Jelinsky et al. 2007; Jervis and Robaire 2001; Johnston et al. 2005) that control the segment-specific secretion of proteins into the luminal fluid (Dacheux et al. 2006, 2009; Nixon et al. 2002) and thereby establish unique physiological compartments that directly affect sperm maturation (Cooper 1996).

The molecular mechanisms governing the postnatal development of the epididymis and the complex patterns of gene expression in the adult epididymis have yet to be fully elucidated. It is

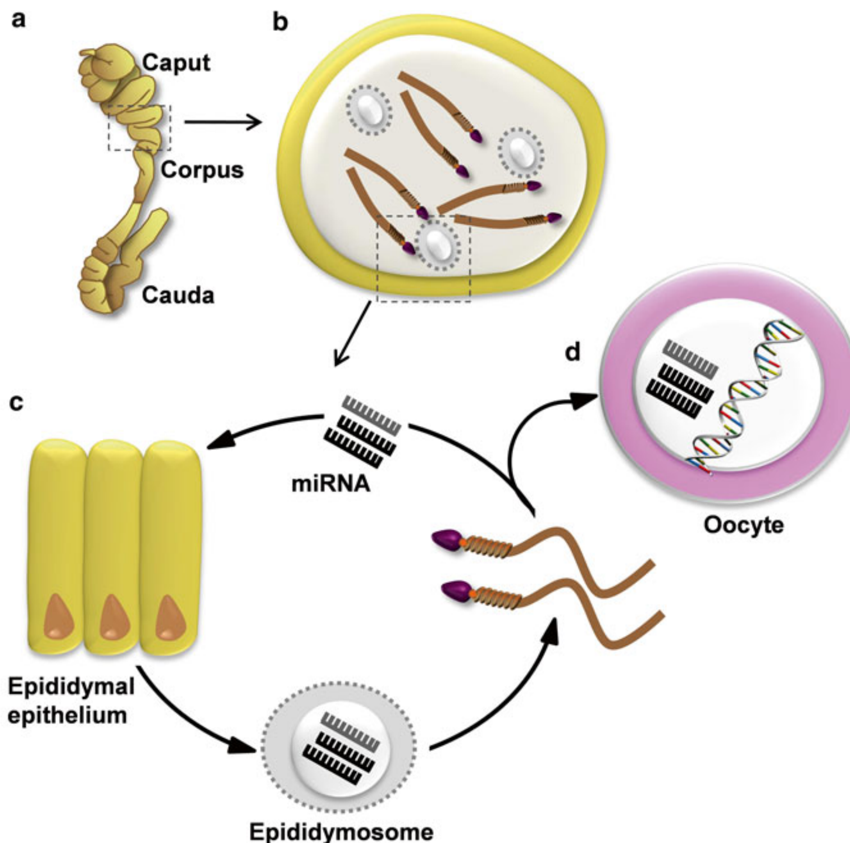

Fig. 6.3 Model of miRNA regulation of sperm maturation. (**a**) In addition to a putative role in the regulation of the dynamic epididymal microenvironment it has been shown that (**b**) the epididymis selectively secretes miRNA into the luminal environment via epididymosomes. (**c**) These exosome-like vesicles may mediate the transfer of miRNAs to either downstream epithelium and/or the maturing sperm cells. It is also conceivable that sperm may participate in the reciprocal transfer of miRNA to the epididymal epithelium and (**d**) that a portion of the sperm miRNA may be delivered to oocyte at the time of fertilization and contribute to embryo development

well established that androgens and testicular derived 'lumicrine' factors, including the spermatozoa themselves, can regulate epididymal gene expression in a region-specific manner (Hinton et al. 1998; Robaire and Hamzeh 2011). Indeed, most of the proximal caput regulation appears attributed to lumicrine factors (Lan et al. 1998), whereas genes in the more distal regions appear to be predominantly androgen-dependent (Sipila et al. 2006). However, the basis of this phenomenon is not known and it cannot be explained by unequal expression of 5α-reductase or androgen receptor since both are expressed uniformly throughout the epididymis (Viger and Robaire 1996; Zhou et al. 2002). Rather, recent evidence has highlighted a novel and potentially extremely important role for an additional tier of regulation involving non-coding RNAs (Ma et al. 2013; Ni et al. 2011; Zhang et al. 2010b, 2011; Belleannee et al. 2012a).

In this context, it has been shown that targeted ablation of the miRNA processing enzyme, DICER1, within the proximal segments of the mouse epididymis leads to epithelial de-differentiation (Bjorkgren et al. 2012). Interestingly, although the epithelium of mice bearing this conditional knockout was able to maintain its epididymal cell type identity, it lacked the characteristic profiles of segment-specific gene expression. Furthermore, the epithelium displayed a pronounced imbalance in the expression of sex steroid receptors, with excessive production of oestrogen receptor and significantly reduced androgen receptor (Bjorkgren et al. 2012). Taken together, these results offer support for the role of miRNAs as important mediators of steroid action in epididymal development and function. This notion is strengthened by studies of miR-29a, a critical regulator of homeostasis in a myriad of tissues. In this context, it has been documented that epididymal expression of miR-29a is susceptible to repression via androgen/androgen receptor signalling due to the targeting of an upstream conserved androgen response element (Ma et al. 2013). Interference of this repression by overexpressing miR-29a in transgenic mice resulted in disturbed epididymal development manifested in hypoplasia reminiscent of that observed in mice with an impaired androgen-androgen receptor signalling system. Furthermore, elevated miR-29a expression is able to reversibly suppress AR expression, thus arguing that it forms part of a regulatory circuitry between androgen/AR signalling pathway that underpins epididymal development and function (Ma et al. 2013).

However, a more direct role of miRNAs in the support of epididymal sperm maturation has been afforded by the demonstration that over-expression of a single, epididymis-specific miRNA is sufficient to elicit a marked reduction in sperm function (Ni et al. 2011). Indeed, through the use of an elegant in vivo model it was shown that microinjection of an antagomir, a chemically modified, single-stranded RNA analogue of the epididymal specific *mil-HongrES2* (a microRNA-like small RNA) directly into the epididymis of adult male rats, led to a significant down-regulation in the expression of its target gene *Ces7* (Ni et al. 2011). Furthermore, the spermatozoa recovered from these animals displayed a concomitant reduction in the amount of CES7 protein and were characterized by significant defects in their motility, ability to capacitate, and hence their fertility (Ni et al. 2011). Given the complexity of the miRNA profile identified within the epididymal epithelium it is likely that further work will uncover additional candidates as important regulators of sperm maturation.

6.5 ncRNA Expression in Somatic Cells of the Testis and Epididymis

Mammalian spermatogenesis is a characteristic model of stem cell function as it depends on self-renewal and subsequent differentiation of spermatogonial stem cells (SSCs). As with all stem cells, the fate of spermatogonia is shaped by the surrounding growth factor-rich microenvironment provided by the surrounding somatic support cells (Oatley and Brinster 2012). In the mammalian testis this microenvironment or niche is primarily comprised of Sertoli, Leydig and peritubular myoid cells. Non-coding RNAs are highly expressed in the testis and the use of

various transgenic mouse models has shown that these regulatory molecules offer an important level of translational control during all phases of the spermatogenic cycle (Korhonen et al. 2011; Deng and Lin 2002; Ma et al. 2009; Romero et al. 2011). It has also been hypothesized that this role extends to a 'restraint mechanism' that prevents germ cells prematurely entering meiosis (van den Driesche et al. 2014). As the somatic support cells of the niche play an essential role in directing germ cell differentiation, it is important to understand the role of ncRNAs in these cells.

6.6 The Role of ncRNAs in Sertoli Cells

Sertoli cells surround the proliferating and differentiating germ cells and are responsible for providing structural support, nutrients and seminiferous fluid, which collectively promote spermatogenesis (Papaioannou et al. 2009). Sertoli cells are columnar cells that originate in the basement membrane and project into the lumen of the seminiferous tubules (Fig. 6.4). These cells play an integral protective role owing to their ability to form the blood-testis barrier, a tight junction network that constitutes the fusion of adjacent Sertoli cells coinciding with the onset of puberty. Sertoli cells are themselves regulated by the pituitary follicle stimulating hormone (FSH) and, in turn, control the germ cell niche through two distinct mechanisms; firstly by direct contact with the spermatogonial stem cells, and secondly via an indirect pathway (Rossi and Dolci 2013). This latter form of control is achieved by the production of metabolites (lactate, androgen binding protein, transferrin), regulatory growth factors (stem cell factor, transforming growth factors, insulin-like growth factor 1, fibroblast growth factor) and developmental hormones (anti-Müllerian hormone and inhibin) (Skinner and Anway 2005).

To assess the global population and consequence of ncRNAs in the mouse Sertoli cell, Ortogero and colleagues used purified Sertoli cells to generate a small RNA transcriptome (Ortogero et al. 2013). This study identified a total of 2743 known ncRNAs, of which the primary population was comprised of miRNAs and piRNAs (62 %). tRNAs and snoRNAs were also abundantly represented in the analysis, which may reflect the Sertoli cells' requirement for diverse protein translation and ribosome processing machinery. Other ncRNA species such as endo-siRNAs, snRNAs and rRNAs, constituted less than 5 % of the global population. Interestingly, several of the identified miRNAs appeared to show preferential

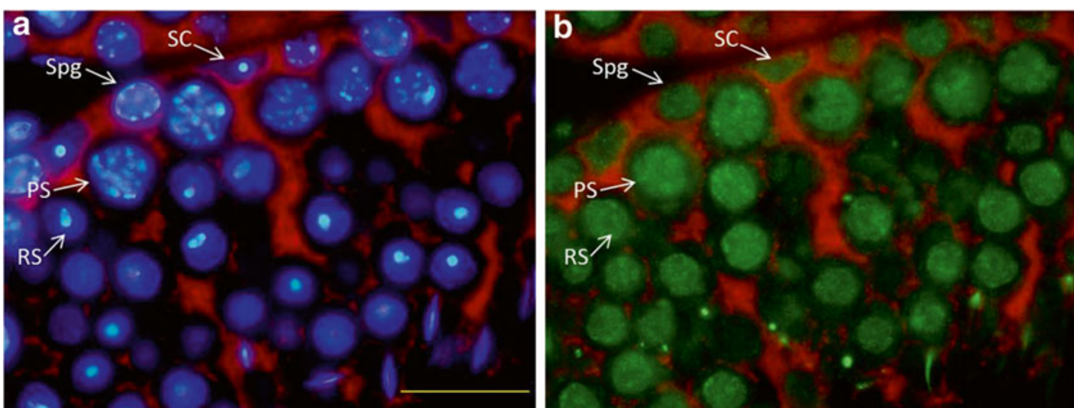

Fig. 6.4 Sertoli cell marker tyrosine tubulin and DICER1 expression in the mouse testis. (**a**) Adult mouse testis immunostained with cytoplasmic Sertoli cell marker tyrosine tubulin (*red*) and counterstained with DNA marker DAPI (*blue*) (*SC* Sertoli cell, *Spg* spermatogonial, *PS* pachytene spermatocyte, *RS* round spermatid). Scale bar = 50 μM. (**b**) DICER1 protein (*green*) localized to the nucleus of all germ cell and somatic cells of the testis including Sertoli cells identified by the cytoplasmic tyrosine tubulin localization (*red*)

binding capacity for targets involved in phosphorylation pathways and RNA-binding proteins. In contrast, the low abundance endo-siRNAs identified were found to target pathways involved in apoptosis (Ortogero et al. 2013), reinforcing the subsidiary role of Sertoli cells in phagocytizing degrading germ cells and excess cytoplasm during the later stages of spermatogenesis. The disparate range of targets infers a distinct division in function of sub-classes of ncRNAs in Sertoli cells and therefore diverse roles in controlling spermatogenesis.

Despite their substantial representation among the ncRNA population in Sertoli cells, very little is known regarding the role of pilRNAs in these somatic cells and consequently spermatogenesis. pilRNAs (or PIWI-interacting like RNAs) are a novel class of RNAs that are similar to piRNAs in their 5′ uridine sequence bias and their genome clustering (Yan et al. 2011). However, the pilRNAs differ in length (predominantly 29–30 nt), lack of germ cell specificity, and are characterized by both an absence of stem-loop formation in their precursor sequences and by a lack of interaction with PIWI (Ro et al. 2007b). The mouse testis has been shown to contain 496 novel pilRNAs, of which almost three quarters partially matched to known rodent piRNAs. Most pilRNAs mapped to intronic regions of the genome, were present on all chromosomes except chromosome 16, and tended to exist primarily in clusters (Ro et al. 2007b). Unlike piRNAs, approximately 70 % of the pilRNAs identified were expressed in multiple tissues and the pilRNA population as a whole increased in expression during development. The presence of pilRNAs in day 7 neonatal testis indicates a role for pilRNAs in spermatogonial and Sertoli cells in the mitotic phase of spermatogenesis (Ro et al. 2007b). pilRNAs have also been identified in the Drosophila and Macaque testis, epididymis and a range of somatic tissues. This varied expression profile across a wide range of species implies functional significance and the possibility that pilRNAs in somatic cells are performing a similar function to piRNAs in the germ line (Yan et al. 2011).

As one of the most highly abundant ncRNA species present in Sertoli cells, miRNAs have been shown to be essential for spermatogenesis and testicular function via knockout strategies involving key enzymes in the miRNA biogenesis pathway (Hayashi et al. 2008; Papaioannou et al. 2009). In this context, we have shown that DICER1 protein is expressed throughout the testis and epididymis in both germ and somatic cells (Fig. 6.4). Furthermore, Sertoli cell-specific *Dicer1*-knockout mice are viable, but exhibit infertility associated with reduced testis size and impaired spermatogenesis leading to a complete absence of late stage germ cells. The Sertoli cells in these mice were irregularly in shape, misplaced in the centre of the lumen and were unable to support meiosis and therefore spermiogenesis (Papaioannou et al. 2009). This phenotype was more pronounced following aging, such that at 6 months of age the testes had degenerated into a mass of interstitial cells with only a few tubules, each of which were completely devoid of germ cells. Moreover, proteomic analysis of the testicular proteins from these conditional *Dicer1*-knockout mice revealed a significant effect, including the dysregulated expression at least 50 proteins (Papaioannou et al. 2011). Interestingly however, targeted ablation of *Ago2* from the testis failed to elicit pronounced phenotypic effects, demonstrating that the AGO2 protein is dispensable for meiosis and spermiogenesis (Hayashi et al. 2008).

Once activated by FSH, Sertoli cells are responsible for control and secretion of hormones to facilitate spermatogenesis. It has been shown in rats, monkeys and humans that acute suppression of androgens and FSH results in failed spermiation whereby the round spermatids are retained by the Sertoli cells prior to being phagocytized (McLachlan et al. 2002). It has been proposed that testicular genes regulated by androgens are probably not the direct targets of the androgen receptor itself. Rather, these genes are likely to be indirectly regulated, via transcriptional activation or suppression, by androgen-sensitive factors (Panneerdoss et al. 2012). Such findings imply a role for miRNAs in hormonal regulation in Sertoli cells. In support of this notion, in vitro studies have shown that treatment of cultured Sertoli cells with testosterone and FSH has a pro-

nounced effect on their miRNA signature, with 122 upregulated and 41 down-regulated miRNAs identified. Several of these miRNAs have been previously identified as being hormonally responsive in the prostate (let-7d, let-7f, miR-15b and miR-125b), uterus (miR-20a, miR-23a/b) and ovary (miR-125b). Interestingly, miR-690 expression was shown to increase with removal of either testosterone or FSH, whereas expression of miR-23b was only influenced by the withdrawal of FSH indicting that testosterone and FSH can either act independently or synergistically to regulate miRNA expression in the testis (Nicholls et al. 2011). As miR-23b is hormonally regulated and has been shown to target PTEN and EPS15, both of which have proposed roles in cell junctions and intracellular signalling, it is thought that testicular hormones may act via specific miRNAs to regulate proteins central to junction restructuring and spermiation (Nicholls et al. 2011).

The effect of androgen suppression on Sertoli cell miRNA populations have also been studied in vivo by treating mice with the anti-androgen drug Flutamide and, with the GnRH antagonist Acyline, both of which arrested spermatogenesis at the level of spermatid formation (Panneerdoss et al. 2012). Following Flutamide/Acyline challenge, a total of 218 differentially expressed miRNAs were identified, the majority of which were upregulated in the Sertoli cells of treated animals, suggesting that targets of these miRNAs, such as FOXD1, would be down-regulated in the absence of androgens. Interestingly, many of the altered miRNAs (miR-471, miR-470, miR-463, miR-465, miR-743a/b, miR-883, miR-880, miR-201 and miR-547) were mapped to the X-chromosome implying that they have a synchronous role in androgen-dependent spermatogenic events. In addition, populations of testosterone-dependent and testosterone-responsive miRNAs were found to be developmentally expressed in a continuous fashion suggesting they may play an equally important role in post-meiotic germ cell development, Sertoli cell-spermatid adhesion, and sperm release from the testis (Panneerdoss et al. 2012).

6.7 ncRNAs in Leydig Cells and Peritubular Myoid Cells

Leydig cells are somatic cells present in the interstitial space between the seminiferous tubules and their primary role in spermatogenesis is to produce testosterone in response to LH stimulation. Testosterone acts as a paracrine factor that diffuses into the tubules and is essential for the maintenance of the blood-testis barrier, meiosis, Sertoli cell-spermatid adhesion and sperm release (as reviewed by Smith and Walker 2014). Peritubular myoid cells on the other hand form a single layer of flattened cells that surround the Sertoli cells, circumscribing the testis cords and, in conjunction with Sertoli cells, promote the movement of mature sperm through the seminiferous tubules. Androgens are essential regulatory factors for these cell types as demonstrated by a failure to complete spermatogenesis in androgen receptor conditional knockout mice models (Welsh et al. 2009, 2012). As demonstrated by studies of Sertoli cells, miRNAs can control and be controlled by both testosterone and other androgens. Despite the recent expansion of the literature in this area, little is known about the role of miRNAs or other ncRNAs in other somatic cells of the testis.

Investigations into the role of miRNAs in Leydig cells have been initiated in vitro using a mouse testicular Leydig tumor cell line, MLTC-1 (Hu et al. 2013). Treatment of these cells with a cAMP agonist, (Bt_2cAMP) to stimulate steroidogenesis via activation of the steroidogenic acute regulatory (*Star*) gene, resulted in an increased expression of miR-212, miR-183, miR-132, miR-182 and miR-96 and decreased expression of miR-138 and miR-19a (Hu et al. 2013). Predicted targets of these miRNAs include several genes known to play integral roles in lipid metabolism and steroidogenesis. This report of miRNAs in Leydig cells supports the notion that hormonally regulated miRNAs play a critical role in post-transcriptional regulation of steroidogenesis. Evidence that this role also extends to embryonic testis development has been provided by Rakoczy

and colleagues who sequenced the small RNA populations of XX and XY mouse foetal gonads and identified miRNAs that contribute to gonadal differentiation. This study identified miR-140-5p/3p as being highly upregulated in the developing testis. Furthermore, the generation of an miR-140-null embryo resulted in an increased Leydig cell phenotype; similar to that documented following disruption of Notch signalling, thus implying a functional role for miR-140-5p/3p in testis development due to its ability to regulate Leydig cell numbers (Rakoczy et al. 2013).

Human studies have focused on the sequence analysis of the *Dicer1* gene as hot spot mutations are detected in as many as 60 % of Leydig-Sertoli cell tumours (de Boer et al. 2012). Such mutations typically occur in the metal binding site of the RNase IIIb domain of DICER1 and result in reduced RNase IIIb activity. This mutational bias seems to be specific for Leydig-Sertoli cell tumours as is not represented in testicular germ cell tumours (de Boer et al. 2012). It is clear from the work in Sertoli cells, that ncRNAs play an essential role in somatic support of spermatogenesis. What remains to be determined is the role, scale and value of ncRNAs in Leydig and peritubular myoid cells and, if they are indeed necessary, for successful spermatogenesis.

6.7.1 ncRNA Expression in the Somatic Cells of the Epididymis

In addition to the complement of testicular expressed miRNAs, a number of studies have also documented the presence of miRNAs in the mammalian epididymis and shown that several are differentially expressed at juvenile and adult stages of development. For instance, more than 200 miRNAs have been detected in the human epididymis and several are significantly enriched in this tissue (Landgraf et al. 2007; Zhang et al. 2010c; Belleannee et al. 2012b). Furthermore, comparative profiling of epididymal miRNAs have revealed distinct temporal (Belleannee et al. 2012a) and spatial expression patterns (Zhang et al. 2011), thus supporting miRNA regulation of the unique epididymal environment and raising the possibility that they play a prominent role in promoting sperm maturation within this organ.

The somatic cells of the epididymis form a pseudostratified epithelial layer comprising four major cell types: principal, basal, clear and halo cells. The principal cells are the dominant cell type and play an integral role in the secretion and absorption of a myriad of small organic molecules, proteins and fluids. The clear cells are responsible for removing material from the lumen and the halo cells constitute part of the immune system (Robaire and Hermo 1988). Very little is known about the function of basal cells in the epididymis, but it has been hypothesized that they play a scavenging role by phagocytosing antigenic products in the epididymis that have been taken up the principle cells (Yeung et al. 1994). Recent work has also pointed to the existence of an additional population of dendritic cells that form a dense network located at the base of the epididymal epithelium and project long intraepithelial extensions between epithelial cells toward the lumen (Shum et al. 2014; Da Silva et al. 2011). The combined activity of these somatic cells creates an intraluminal environment that supports sperm maturation, transport and storage (Bischof et al. 2013).

Many recent studies have examined the role of ncRNAs, in particular miRNAs, in the epididymis but the majority of these have focused on the whole organ rather than the somatic constituents. The miRNA population in the human epididymis was identified as comprising a total of 281, 282 and 289 miRNA species in the caput, corpus and cauda regions, respectively. Of these miRNAs, 35 were identified as being differentially expressed in between the epididymal regions. Interestingly, miR-215 and members of the miR-888 cluster were found to be expressed at the highest levels in the distal region of the epididymis implying a role for these miRNAs in epididymal sperm maturation and storage (Belleannee et al. 2012a). Comparative studies have also been undertaken to establish the development expression profile of miRNAs in the newborn, adult and aged epididymis (Zhang et al. 2010b). This study detected a

limited number of miRNAs that were specifically enriched in the adult (hsa-miR-222, hsa-miR-221 and hsa-miR-29c) or aged epididymis (hsa-miR-193b and hsa-miR-374) (Zhang et al. 2010b). Such temporal differences in miRNA expression add support for the notion that these ncRNAs regulate the development of the epididymis and may play a role in age-dependent decline in male fertility.

In addition to studies focusing on miRNAs, deep sequencing strategies have also been employed to identify the entire population of ncRNAs in the human epididymis. This study revealed that miRNAs constituted the majority of the ncRNA population with a total of 527 known and 18 novel miRNAs identified. This screen also detected a small population of PIWI interacting RNAs (piRNAs) (Li et al. 2012). Although such findings are in contrast to previous work in the mouse that failed to detect any piRNAs in the cauda epididymis (Grivna et al. 2006), they nevertheless raise the possibility that human sperm retain a complement of piRNAs that are acquired during their testicular development. Alternatively, it is also possible that the somatic cells of the epithelium are housing the piRNA population. In support of this notion, pilRNAs have also been identified in the somatic cells of the macaque epididymis. Indeed, using an in situ hybridization strategy with 'anti-pilRNA' probes, transcripts were detected in the basal and principal epididymal cells but not in the peritubular tissue or spermatozoa (Yan et al. 2011).

As mentioned previously conditional deletion of *Dicer1* from the proximal regions of the mouse epididymis results in functionally compromised principal cells that display a significant reduction in segment specific gene expression and dedifferentiation. These changes appear to be attributed, in part, to alterations in the expression of sex steroid receptors within the epididymis suggesting that miRNAs play a critical role in modulating steroid signalling and maintenance of epithelial cell differentiation (Bjorkgren et al. 2012). This argument is strengthened by the demonstration that ablation of the androgen receptor from the caput epithelium, presents a similar phenotype to that of the *Dicer1* knockout (O'Hara et al. 2011). In this regard, the androgen receptor knockout mice lost their entire initial segment and displayed a progressive deterioration of the remainder of the caput epithelium culminating in complete infertility by the time the mice had reached 100 days of age (O'Hara et al. 2011). The critical role of miRNAs in meditating hormonal regulation of the epididymis also draws analogy with their function in a myriad of other cell types including that of the prostate (Sun et al. 2014; Todorova et al. 2013; Waltering et al. 2011).

Interestingly, in addition to their prominence in epididymal epithelial tissue, an impressive inventory of miRNAs has also recently been identified packaged within epididymosomes (Belleannee et al. 2013a), small exosome-like entities that are secreted into the epididymal lumen (Sullivan et al. 2005; Sullivan and Saez 2013). Such a finding shares analogy with several other tissue systems in which there is now compelling evidence that miRNAs are actively secreted in membrane-enclosed exosomes and delivered into recipient cells where they function as endogenous miRNAs (Lotvall and Valadi 2007; Mittelbrunn et al. 2011; Valadi et al. 2007). It is therefore conceivable that miRNAs participate in a paracrine-like form of intercellular communication to coordinate the activity of the different epididymal segments and/or are selectively conveyed to the maturing sperm cells for downstream roles in the regulation of embryo development (Belleannee et al. 2013a). In support of the former interpretation, distinct miRNA signatures have been documented in epididymosomes originating from different epididymal regions and these profiles also differ markedly from those of their parent epithelial cells (Belleannee et al. 2013a). Furthermore, in vitro assays have revealed that epididymosomes from the proximal epididymidies can associate with epithelial cells located downstream of their release site (Belleannee et al. 2013a). Although additional studies are needed to confirm the uptake of epididymosomes and release of their miRNA cargo within epididymal epithelial cells, it is noteworthy that several of the most abundant miRNAs found in epididymosomes have known

targets that display regionalized patterns of epididymal epithelial expression and possess key roles in epididymal function and male fertility (Belleannee et al. 2013a). For instance, miR-145, one of the most abundant miRNAs identified in caput epididymosomes is known to regulate the expression of key ion channels and tight junction proteins within the epididymal epithelial cells (Belleannee et al. 2012a; Chen et al. 2012).

Similarly, although direct evidence that epididymosomes can act as vehicles for the trafficking of specific miRNAs to maturing spermatozoa is currently lacking, these entities are nevertheless able to form intimate associations with the sperm membrane that facilitate the transfer of protein cargo to the maturing cells (Sullivan and Saez 2013; Sullivan et al. 2007). In addition, it has been known for some time that exogenous mRNA species, secreted by epididymal epithelial cells, can be acquired by sperm during epididymal transit (Boerke et al. 2007) and that sperm can efficiently incorporate exogenous DNA and RNA via artificial liposomes (Bachiller et al. 1991). Our own, unpublished data have also revealed that the complement of sperm-borne miRNAs is substantially modified as the cells descend through the epididymis. Thus, the miRNA species present in mature sperm cells are unlikely to be solely the remnants of the spermatogenic process, but instead represent exogenous miRNAs that have been actively delivered to the sperm during their passage through the epididymis. Such findings take on added significance in view of two key recent discoveries. Firstly, that miRNAs conveyed to the oocyte by spermatozoa are able to influence embryo development (Liu et al. 2012), and secondly that the profile of epididymosomes miRNA cargo is able be selectively modified by various environmental/physiological insults (Belleannee et al. 2013b). This raises the prospect that sperm borne miRNAs may also be susceptible to dynamic modifications and that such changes could influence fertility, contribute to the inheritance of acquired characteristics, and/or alter the developmental trajectory of the resulting embryo.

6.8 Can Testicular ncRNA Molecules Be Assigned a Function in Spermatogenesis, Fertility and Testicular Cancer?

6.8.1 Spermatogenesis

As documented previously, disruption of the miRNA synthesis pathway in the testis has proven that these entities are essential for Sertoli cell function (Papaioannou et al. 2009), and germ cell survival during both the colonization of the gonads and the later stages of spermatogenesis (Hayashi et al. 2008; Maatouk et al. 2008b). Differential screening has revealed a number of promising lead miRNAs postulated to have specific roles in spermatogenesis. In several cases the targets of differentially expressed miRNAs are now the subject of further experimental validation. For instance, miR-34c, a member of a family of miRNAs that are known to target cell cycle regulators are highly expressed in meiotic and post meiotic germ cells. Two spermatogenesis-related genes, *Tgif2* and *Notch2* are confirmed targets of miR-34c (Bouhallier et al. 2010), with Notch signalling known to promote germ cell differentiation from spermatogonia (Kostereva and Hofmann 2008) and *Tgif2* is a negative regulator of TGFβ signalling and thus inhibits the second meiotic division in spermatogenesis (Damestoy et al. 2005). Experimental overexpression of miR-34c in HeLa cells together with *DDX4* promoted the expression of germ cell markers and led to the hypothesis that miR-34c down-regulates somatic genes and enhances germ cell characteristics (Bouhallier et al. 2010). This correlates with work in humans, demonstrating that a reduction in several miRNAs in spermatozoa has been causally associated with subfertility. To this end, a recent study in mice found that deletion of both *mir-34b/c* and *mir-449* loci resulted in oligoasthenoteratozoospermia, a well-known correlate of human male infertility. This deletion of *mir-34b/c/449* concurrently impairs both meiosis and the final stages of spermiogenesis.

Analysis of the pachytene spermatocytes from *mir-34b/c*$^{-/-}$;*449*$^{-/-}$ mice, indicated a small cohort of miR-34 family target genes including the transcription factor FOXJ2 were deregulated indicating that the miR-34 family is functionally important in the origin of oligoasthenoteratozoospermia and infertility (Comazzetto et al. 2014).

Another miRNA, miR-18a, is thought to directly target the transcription factor HSF2 (heat shock factor 2) that influences many proteins required for successful germ cell development, and, unsurprisingly, the *Hsf2*-null male mice have small, morphologically abnormal testes with less elongating spermatids and severe spermatozoal abnormalities (Bjork et al. 2010). In much the same way, miR-122a has been shown to directly bind to and reduce the expression of *Tnp2*, encoding transition protein 2, one of the key transition proteins that replaces histones during the early phase of chromatin condensation that accompanies spermiogenesis (Yu et al. 2005). In summary, miRNAs are emerging as key players in germ cell function and the determination of cell fate. In this capacity miRNAs may act to interpret and transduce cellular signals to either maintain the undifferentiated stem cell population and/or drive cell differentiation during spermatogenesis. While further study is required to assign specific roles to the majority of miRNA species implicated as having a role in spermatogenesis, such work is warranted on the basis that it will likely have fundamental implications for our understanding of normal and disease states such as infertility and the development of germ cell tumours.

6.8.2 Testicular Germ Cell Tumours

Three types of testicular germ cell tumours (TGCT) have been documented that are largely distinguished on the basis of their age of onset (reviewed in McIver et al. 2013). Type I germ cell tumours consisting of benign mature teratomas or malignant yolk sac tumours occur in young children usually before the age of 4 and always before the onset of puberty. Type II TGCT, or seminoma and non-seminoma, are the most common cancer in men in their 20s–40s and are associated with a preinvasive lesion C or undifferentiated intratubular germ cell neoplasia. In contrast, type III TGCT, also known as spermatocytic seminomas, usually occur in older men (over 50 years of age). Such tumours are usually benign and slow growing, with genetic markers in common with type B spermatogonia. While the incidence of type I and III TGCT is very low and has remained steady within the developed world over the last 30 years, this same time period has seen an alarming increase in the incidence of type II germ cell tumours in this population (Looijenga and Oosterhuis 1999; Bahrami et al. 2007; Kristensen et al. 2008; Huyghe et al. 2007).

As dysregulated miRNA action is associated with the aetiology of many cancers (Li et al. 2009a, b; Gillis et al. 2007; Babashah and Soleimani 2011; Adams et al. 2014; Zhang et al. 2014), the presence and influence of miRNA in the development of TGCT has recently begun to be investigated (McIver et al. 2012b, 2013; de Boer et al. 2012; Yang et al. 2014; Syring et al. 2014; Tanaka et al. 2013; Liu et al. 2013; Kraggerud et al. 2013; Sabbaghian et al. 2013; van de Geijn et al. 2009; Duale et al. 2007; Zhu et al. 2007). Intriguingly, such studies have demonstrated that different type I and type III germ cell tumours and the histological subgroups of type II germ cell tumours (seminoma and non-seminoma) can be readily differentiated on the basis of their miRNA expression profiles (Gillis et al. 2007). In this context, the *mir-302* cluster, which is highly expressed in ES cells and thought to be involved in the maintenance of pluripotency, is elevated in seminoma tumours, but not in the other types of TGCTs that are more differentiated. Furthermore, the oncogenic miRNAs miR-21 (cell survival) and miR-155 (MYC regulation) are over-expressed in seminomas and type III germ cell tumours, while other miRNAs have been confirmed as tumour-specific. For example, over-expression of miR-19a has been documented in seminomas and type III tumours, over-expression of miR-29a occurs in type III tumours and under-expression of miR-133a and miR-145 is characteristic of both seminomas and type III tumours. Similarly, miR-146 expression is lower

in seminomas and type III tumours compared with normal testis and lower still in the more differentiated type II tumours such as embryonic carcinoma and teratomas (Gillis et al. 2007).

MicroRNAs have also emerged as playing important roles in testicular germ cell tumorigenesis and survival. For instance, Voorhoeve et al. (2006) identified miR-371, -372 and -373 as being overexpressed in TGCT expressing both oncogenic RAS and wild-type p53. Overexpression of these miRNA molecules was found to neutralize p53-mediated CDK inhibition, possibly through a mechanism involving the direct inhibition of the expression of the tumour-suppressor, large tumour suppressor homolog 2 (*Lats2*) (Voorhoeve et al. 2006). More recently, miR-373 has been implicated in promoting migration and metastasis, but not cell proliferation, in a breast cancer cell line (Huang et al. 2008). It is therefore conceivable that this miRNA could have a role in testicular cancer metastasis in addition to the roles identified by Voorhoeve et al. (2006). It is also of interest that the *mir-371–373* cluster was identified among a number of miRNAs that are up-regulated in several cisplatin-resistant germ cell tumour cell lines (Port et al. 2011). This suggests that the function of this cluster may extend to inhibition of cell death and promoting differentiation in response to cisplatin exposure (Port et al. 2011). Consequently the manipulation of miRNA levels within tumours has emerged as an attractive option in the development of new treatments for testicular cancer (Bhardwaj et al. 2010).

6.9 Fertility

Despite their transcriptionally inert state, spermatozoa have long been known to contain diverse RNA populations including mRNAs, antisense RNAs and miRNAs (Krawetz et al. 2011; Miller et al. 1999; Miller and Ostermeier 2006; Ostermeier et al. 2005). These RNA species were originally thought to be remnants of untranslated stores generated during spermatogenesis and therefore play a limited role in fertilization and early embryonic development. However, as documented above, it is now apparent that, together with the haploid genome, paternal mRNAs and miRNAs are delivered to the oocyte at the time of fertilization (Dadoune 2009) and contribute to modulation of the maternal transcripts prior to activation of the zygotic genome (Liu et al. 2012; Ostermeier et al. 2004). For instance, sperm-borne miR-34c has recently been shown to play a critical role in regulating the first cell division of mouse embryos (Liu et al. 2012). Such findings are of significance to the field of fertility regulation since each of these contributions from the fertilizing spermatozoon represents a source of potential sperm dysfunction. Indeed, aberrant embryo miRNA expression has been detected in human blastocysts derived from patients with male factor infertility, suggestive of a contribution from sub-fertile sperm that affected the phenotype of the resulting embryo (McCallie et al. 2010). Furthermore, different miRNA profiles have been identified in sperm samples exhibiting high levels of abnormal morphology and low motility compared to that of normal spermatozoa (Curry et al. 2011). In this context, Curry et al. (2011) have shown marked increases in the expression of four miRNAs, let-7a, -7d, -7e, and miR-22, along with a decrease in miR-15b, in abnormal semen samples. Two of these miRNAs, let-7d and let-7e, also displayed increased expression in the low motility group. Accordingly, bioinformatics analyses revealed that these miRNAs are predicted to target genes encoding proteins involved in spermatogenesis, sperm structure, motility, or metabolism. On the basis of this and a myriad of additional studies (Abu-Halima et al. 2013, 2014; Tscherner et al. 2014; Khazaie and Nasr Esfahani 2014; Salas-Huetos et al. 2014; Jodar et al. 2013; Hamatani 2012), it has been proposed that the complement of sperm-borne miRNAs could hold considerable diagnostic value as non-invasive molecular markers of male infertility.

As an extension of this work, it has also recently been shown that significant alterations to the epididymal miRNA expression profile can be evoked by exposure to stressors such as vasectomy (Belleannee et al. 2013b). The fact that such changes are only partially restored after vasovasostomy (reversal microsurgery) suggests that altered miRNA profiles may, in part, account for the

reduction in fertility potential of individuals that undergo surgically successful vasectomy reversal. At present, it remains to be established whether perturbations in epididymal miRNA expression also arise in response to other forms of insults and are capable of influencing the profile of miRNAs that are conveyed to spermatozoa, and subsequently onto the ovum to elicit epigenetic changes to inheritance. Nevertheless, it is noteworthy that conditions such as diet-induced paternal obesity have the potential to influence sperm miRNA content (Fullston et al. 2013) and thus contribute to the impaired metabolic and reproductive health observed in subsequent generations (Fullston et al. 2012, 2013). Similarly, exposure of either male mice to chronic stress (Gapp et al. 2014; Rodgers et al. 2013) or humans to cigarette smoke (Marczylo et al. 2012) is also capable of significantly altering their sperm miRNA content. Moreover, such changes appear to be linked to pronounced heritable epigenetic alterations in the offspring.

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7 Non-coding RNAs in Mammary Gland Development and Disease

Gurveen K. Sandhu, Michael J.G. Milevskiy, Wesley Wilson, Annette M. Shewan, and Melissa A. Brown

Abstract

Non-coding RNAs (ncRNAs) are untranslated RNA molecules that function to regulate the expression of numerous genes and associated biochemical pathways and cellular functions. NcRNAs include small interfering RNAs (siRNAs), microRNAs (miRNAs), PIWI-interacting RNAs (piRNAs), small nucleolar RNAs (snoRNAs) and long non-coding RNAs (lncRNAs). They participate in the regulation of all developmental processes and are frequently aberrantly expressed or functionally defective in disease. This Chapter will focus on the role of ncRNAs, in particular miRNAs and lncRNAs, in mammary gland development and disease.

Keywords

Breast cancer • Epithelial-stromal interactions • Hormonal regulation • Epithelial cell differentiation • EMT • Signalling pathways

7.1 Introduction

The function of the mammary gland is to provide the neonate with essential nourishment and to promote basic immunocompetence which is vital for early life (reviewed in Capuco and Akers 2009; Macias and Hinck 2012). The mammary gland consists of a bilayered configuration of two types of epithelial cells situated within the fatty stroma (as reviewed in Macias and Hinck 2012; Watson and Khaled 2008). The epithelial compartment includes an outer layer of basal myoepithelial cells that function as contractile units, and an inner layer of luminal epithelial cells, which further differentiate to form a hollow ductal tree terminating in individual secretory alveoli, both of which are a necessity for competent milk secretion and ejection during lactation (as reviewed in Macias and Hinck 2012; Mailleux et al. 2007, 2008; Ochoa-Espinosa and Affolter 2012; Van Keymeulen et al. 2011; Visvader 2009; Watson and Khaled 2008).

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In order to achieve its highly ordered structure, the mammary gland is subjected to an array of morphogenetic cues (reviewed in Macias and Hinck 2012; Piao and Ma 2012; Rijnkels et al. 2010; Watson and Khaled 2008). These cues control the major anatomical and physiological changes of the gland that occur through embryogenesis, puberty, pregnancy, lactation and involution, involving cyclical changes in cell proliferation, differentiation and apoptosis (Clarkson et al. 2004; Kendrick et al. 2008; Master et al. 2005; McBryan et al. 2007; Raouf et al. 2008; Rudolph et al. 2007; Stein et al. 2004; Topper and Freeman 1980). Molecules controlling this process include a range of steroid hormones and proteins that participate in signal transduction and the regulation of transcription. It is becoming increasingly clear that ncRNA molecules also play a major role in regulating mammary development.

Breast cancer arises as a consequence of malignant transformation of mammary epithelial cells resulting from an accumulation of pathogenic changes in multiple cancer genes, including those encoding ncRNAs. Many of the genes that are implicated in breast cancer also play key roles in mammary gland development and vice versa, with altered expression or function of these genes resulting in defective mammary cell proliferation, differentiation, apoptosis, migration and or invasion.

This chapter explores recent developments in the literature on the expression, regulation and function of ncRNAs, in particular miRNAs and lncRNAs, in mammary gland development and disease, and considers the future potential for these molecules to improve our understanding and ability to manipulate these biological processes.

7.2 NcRNAs in Mammary Gland Development

7.2.1 miRNA Expression during Mammary Gland Development

miRNAs are strongly implicated in mammary gland development (Table 7.1). Studies to date include expression profiling of whole mammary tissue throughout different developmental stages, expression profiling of different epithelial subcompartments of the mammary gland and expression analysis of the mammary stroma. Whilst most studies have focussed on the mouse, others have examined the expression of miRNAs in the mammary glands of other mammals.

The most comprehensive analysis of miRNA expression in the post-natal mouse mammary gland throughout mammary development revealed that they are expressed in seven distinct clusters throughout the various developmental stages (Avril-Sassen et al. 2009). The three largest clusters (cluster 1–3) included miRNAs that were repressed during lactation and early involution. Cluster 1, for example, includes multiple members of the let-7 family (let-7a-f), expression of which increases during puberty, mature virgin and early gestation developmental stages and decreases during lactation and involution (Avril-Sassen et al. 2009). In contrast, miRNAs in cluster 5, including miR-335, steadily decreased in levels during development, pregnancy, and lactation. Similar studies have been performed by others with outcomes including the demonstration that miR-126-3p is highly expressed in the virgin and involuting mammary gland, and repressed in the pregnant and lactating mammary gland and miR-200a which is increased in expression during mid-pregnancy and throughout lactation (Cui et al. 2011; Nagaoka et al. 2013).

Other studies have used in vitro models of mammary epithelial differentiation, such as the HC11 mouse mammary epithelial cell line, which differentiates and expresses milk protein genes in response to hormonal stimulation (Timmins et al. 2005). Expression array analysis of HC11 cells before and after treatment with dexamethasone, insulin and prolactin (DIP- which in combination induce milk protein gene expression) identified miR-23a, miR-27b, miR-101a, miR-141, miR-200a and miR-205 as significantly differentially expressed. Additionally, DIP induction of Eph4 mouse mammary epithelial cells, lead to increased expression of miR-200a (Nagaoka et al. 2013). MiRNA expression was also determined in a HC11 model of involution, which showed that

Table 7.1 NcRNAs implicated in mammary gland development

ncRNA	Expression/function in mammary gland development	Validated targets	References
Let-7 family members	Downregulated in mammary progenitor cells	*H-RAS*	Avril-Sassen et al. (2009), Ibarra et al. (2007), and Yu et al. (2007)
	Upregulated during, puberty, mature virgin, and early gestation	*HMGA2*	
	Downregulated during lactation and involution		
miR-17/92	Upregulated in late involution	*CDKN1A*	Avril-Sassen et al. (2009), Cloonan et al. (2008), and Feuermann et al. (2012)
	Dispensable for mouse mammary gland development	*BCL2L11*	
		PTEN	
miR-30b	Upregulated between puberty and mature virgin, mid and end pregnancy and late involution	*VIM*	Cheng et al. (2012), Le Guillou et al. (2012), Liu et al. (2012), and Zhong et al. (2010)
	Downregulated in early involution	*SNAIL1*	
	Remained stable in lactation	*LIN28*	
miR-93	Downregulated in mammary progenitor cells	TGFβR2	Ibarra et al. (2007) and Liu et al. (2012)
	Upregulated in MET	SMAD5	
miR-99 family members (miR-99a,miR-99b)	Upregulated in EMT – similar process occurs between lactation and involution	*MTOR*	Turcatel et al. (2012)
miR-101a	Upregulated in involution	*COX-2*	Tanaka et al. (2009)
miR-126-3p	Downregulated during pregnancy and lactation	*PR*	Cui et al. (2011)
miR-138	Regulates mammary epithelial proliferation and viability	*PRL-R*	Piao and Ma (2012) and Wang et al. (2008)
miR-146b	Upregulated in basal mammary epithelia	*IRAK1*	Avril-Sassen et al. (2009), Bhaumik et al. (2008), Bockmeyer et al. (2011), Elsarraj et al. (2012), and Kittrell et al. (2011)
	Upregulated in alveolar progenitor cells	*TRAF6*	
	Upregulated in lactation and early involution		
miR-200 family members	**miR-200a, miR-141,miR-429**	*ZEB1*	Avril-Sassen et al. (2009), Burk et al. (2008), Chang et al. (2011a), Gregory et al. (2008), Korpal et al. (2008), Nagaoka et al. (2013), Nam et al. (2008), Wright et al. (2010)
	Downregulated in EMT – similar process occurs between lactation and involution	*ZEB2*	
	Upregulated in mid pregnancy, lactation and early involution		
	miR-200c		
	Upregulated by p53 to mediate EMT-MET switch in mammary epithelial cells		
	Downregulated in late lactation and early involution		
miR-203	Upregulated in early development and gestation. Downregulated in lactation and involution	*SNAI2*	Avril-Sassen et al. (2009) and Ding et al. (2013)

(continued)

Table 7.1 (continued)

ncRNA	Expression/function in mammary gland development	Validated targets	References
miR-205	Upregulated in mammary progenitor cells	*PTEN ZEB1 ZEB2*	Avril-Sassen et al. (2009), Greene et al. (2010), and Ibarra et al. (2007)
	Upregulated up until mature virgin, with increasing expression in pregnancy and late involution		
miR-206	Upregulated in late involution	*ESR1*	Adams et al. (2007, 2009), Avril-Sassen et al. (2009), and Song et al. (2009)
		GATA3	
		SRC1	
		SRC3	
		NOTCH3	
miR-210	Upregulated during lactation and early involution	*FGFRL1*	Avril-Sassen et al. (2009) and Tsuchiya et al. (2011)
miR-212/132	Downregulated at pre-puberty and pregnancy	*MMP-9*	Ucar et al. (2010)
	Gradually increased throughout puberty and after parturition		
miR-221	Regulates mammary epithelial proliferation and viability	*GHR*	Lu et al. (2009) and Piao and Ma (2012)
miR-424/503	Activated by TGFβ and regulates mammary epithelial involution and TGFβ mediated cell cycle arrest	*BCL-2, IGF1R, CDC25*	Llobet-Navas et al. (2014a, b)
Neat1	Forms paraspeckles in the luminal epithelial cells of the mammary gland	–	Standaert et al. (2014)
	Regulates mammary epithelial branching, lobular alveolar development and lactation		
PINC	Upregulated in luminal and alveolar progenitor cells	–	Ginger et al. (2001. 2006) and Shore et al. (2012b)
	Upregulated in pregnancy, early involution and parous		
	Downregulated in virgin and early lactation		
Zfas	Upregulated in pregnancy and between lactation and involution	–	Askarian-Amiri et al. (2011) and Shore et al. (2012b)
	Downregulated between pregnancy and lactation		

miR-101a and miR-141 were also differentially expressed during involution. Consistent with the outcomes of these in vitro studies, miR-101a is also differentially expressed during mammary development in vivo (Tanaka et al. 2009).

The expression of miRNAs in different cellular sub-compartments of the mammary gland has also been determined. Such studies reveal complex patterns of miRNA expression in the tissue. For instance, miR-146b is upregulated in basal mammary epithelial cells and enriched in alveolar progenitor cells, compared with ductal and multipotent progenitor cells isolated from the mouse mammary epithelial cell line Comma-1D (Bockmeyer et al. 2011; Elsarraj et al. 2012; Kittrell et al. 2011). Furthermore, miR-205 is enriched in both luminal and basal cell populations during gestation and late involution (Avril-Sassen et al. 2009).

Genome-wide approaches have been used to elucidate the miRNA transcriptional profile

during lactation in other species, including *Bos taurus* (bovine, cow), *Capra hircus* (caprine, dairy goat), *Sus scrofa* (porcine, domestic pig) and *Rattus norvegicus* (rodent, brown rat). Li et al. identified 56 miRNAs that were significantly differentially regulated between lactation and non-lactation periods in bovine (Li et al. 2012). A total of 56 miRNAs were identified, 41 were expressed in both periods while only 6 were highly expressed in the non-lactation period and 9 during lactation (Li et al. 2012). MiR-138 was expressed most highly during lactation, *Stat5* and *Mapk* are known targets of miR-138, which ultimately inhibits the prolactin receptor (PRLR) and decreases proliferation of mouse mammary epithelial cells (Li et al. 2012). Recently miR-152 was found to target *DNMT1* thereby inhibiting global DNA methylation and enhancing lactation related genes/pathways such as the PI3K/AKT signalling pathway and *PPARγ* (peroxisome proliferator-activated receptor gamma) expression together with the secretion of β-casein, triglycerides and lactose in dairy cow mammary epithelial cells (Wang et al. 2014a). Moreover multiple miR-29 members have been found to regulate DNA methylation levels by inversely targeting *DNMT3A* and *3B* in dairy cow mammary epithelial cells where its inhibition causes global DNA hypermethylation at the promoters of lactation related genes such as *CSN1S1* (casein alpha s1), *ELF5* (E74-like factor 5), *PPARγ*, *SREBP1* (sterol regulatory element binding protein 1) and *GLUT1* (glucose transporter 1) (Bian et al. 2015). In addition, miR-486 was found to regulate phosphoinositide signalling in bovine mammary epithelial cells through down-regulating *PTEN* and altering the expression of downstream genes such as *AKT* and *MTOR*, which are vital for mammary gland function (Li et al. 2015). Proliferation of mammary epithelial cells as well as the capacity to secrete β-casein, lactose and lipids was also stimulated by miR-486 (Li et al. 2015). A similar approach was used in two separate studies done in dairy goat, however their respective most abundant and differentially expressed miRNAs were significantly different (Ji et al. 2012; Li et al. 2012).

In another study (Gu et al. 2012), the top ten most abundant miRNAs in the mammary gland of the dairy goat account for >87 % of miRNA expression including, miR-148a, miR-30 and miR-25. Of the ~150 miRNAs enriched in exosomes more than 30 % have immune-related function including miR-25, miR-30 family members, miR-200c and miR-574 (Gu et al. 2012; Ji et al. 2012; Li et al. 2012). The majority of miRNAs decreased in expression over the 28 days of lactation studied suggesting that the immune-related benefits from miRNAs may be a short lived burst when first breast feeding young (Gu et al. 2012). What remains unknown is the mechanism by which these miRNAs are up-regulated during lactation, what factors and hormones influence the expression patterns and ultimately how they function to bolster the immune system of the young.

MiRNAs have also been found to play a key role in pig lactation. Expression analysis was conducted in two different breeds of sows – Jinhua and Yorkshire, with miRNA profiling being highly correlated in both breeds (Peng et al. 2015). MiRNA target gene analysis shortlisted five genes that were involved in mammary epithelial proliferative capacity and epithelial cell viability (Peng et al. 2015). Furthermore, miRNAs have been identified to play a fundamental role in rat lactation whereby a total of 395 and 400 miRNAs were identified at day 1 and 7 postpartum respectively (Zhang et al. 2014a), out of which 27 miRNAs were differentially expressed between postpartum day 1 and 7 (Zhang et al. 2014a). Analysis of miRNA target genes demonstrated that many were involved in lipid biosynthesis, providing a potential avenue towards understanding poor lactation performance amongst women who breast feed (Zhang et al. 2014a).

7.2.2 Expression of lncRNAs in Mammary Gland Development

The expression of lncRNAs in mammary gland development is less well studied. The first

reported differentially expressed lncRNA was pregnancy-induced ncRNA (*PINC*; aka *G.B7*), an alternatively spliced, polyadenylated lncRNA that was identified by subtractive hybridisation analysis of the mammary glands of a rat model of simulated pregnancy (Ginger et al. 2001, 2006). A decrease in *Pinc* levels is observed when HC11 mouse mammary epithelial cells are induced to undergo lactogenic differentiation (Ginger et al. 2006; Shore et al. 2012b). In addition, *Pinc* together with the gene encoding retinoblastoma-associated protein 46 (*RbAp46*) have been shown to be upregulated in alveolar cells in pregnancy and persist in alveolar cells in regressed lobes in the involuted and parous gland (Ginger et al. 2001, 2006; Shore et al. 2012b).

Expression profiling of mouse mammary glands at pregnancy day 15, lactation day 7 and involution day 2 identified many additional lncRNAs that were differentially expressed in post pubertal mammary gland development including *Pinc* and a novel lncRNA *Zfas1* (Askarian-Amiri et al. 2011; Shore et al. 2012a). *Zfas1* is a lncRNA transcribed in the antisense direction from the promoter region of the *Znfx1* protein coding gene, and gives rise to three novel snoRNAs, which are differentially expressed in the pregnancy, lactation and involution stages of mouse mammary gland development (Askarian-Amiri et al. 2011; Shore et al. 2012a, b). *Zfas1* RNA is localised within the ducts and alveoli of the mammary gland where it is upregulated in pregnancy, downregulated between pregnancy and lactation and upregulated between lactation and involution (Askarian-Amiri et al. 2011; Shore et al. 2012a). *Zfas1* knockdown in HC11 cells induced lactogenic differentiation measured by dome formation and β-casein expression suggesting a role in normal lactation following pregnancy.

An integrative study of RNA-Seq profiles across numerous tissue types has identified thousands of large intergenic ncRNA transcripts. Cabili et al. collected over four billion RNA-Seq reads to iterate lincRNA (long intergenic ncRNA) transcripts across 24 human cell types and tissues (Cabili et al. 2011). A sample from a 29 year old female was used to identify transcripts within normal breast tissue showing previously identified lncRNAs such as *HOTAIR*, *GAS5-AS1* expression, which are described below.

7.2.3 Transcriptional Regulation of ncRNA Genes

Transcriptional regulation is a complex process involving the dynamic interaction of transcriptional control elements and transcription factors, together with epigenetic changes and chromatin looping (Fig. 7.1). MiRNA genes are transcribed predominantly by RNA polymerase II (Pol II) into long primary transcripts (pri-miRNA), which are processed by multiple enzymes including RNase III Dicer to their mature length (reviewed in Winter et al. 2009). Whole genome RNA Pol II ChIP sequence analysis has shown that most miRNA promoters are over 1 kb in length (range 400–4000 bp) and map to distances up to 10 kb from the miRNA sequence (Wang et al. 2010a). Recent advances in high throughput sequencing have shown that trimethylation of lysine 4 of histone 3 (H3K4me3) persist at the promoters of miRNAs and that such covalent modifications are restricted to sites of transcriptional initiation (Barski et al. 2009; Guenther et al. 2007). In addition, nucleosome depletion has been observed within 100–300 bp of the transcriptional start site (TSS) of transcriptionally active miRNA genes (Ozsolak et al. 2007). The development of computational models based on these observations has led to the annotation of hundreds of miRNA TSS and upstream regulatory regions in the human genome (Ozsolak et al. 2007).

LncRNAs are transcribed by either RNA pol II or pol III, and the resultant transcripts may or may not be spliced and may or may not be polyadenylated (Amaral et al. 2009). Histone modification marks are associated with the regulatory elements controlling lncRNA gene expression (Sati et al. 2012), and analysis has revealed a distinctive methylation pattern around the transcription start site (reviewed in Venkatesh and Workman 2012), strong evolutionary conservation and binding sites for transcription factors that play a key role in cell proliferation and differentiation

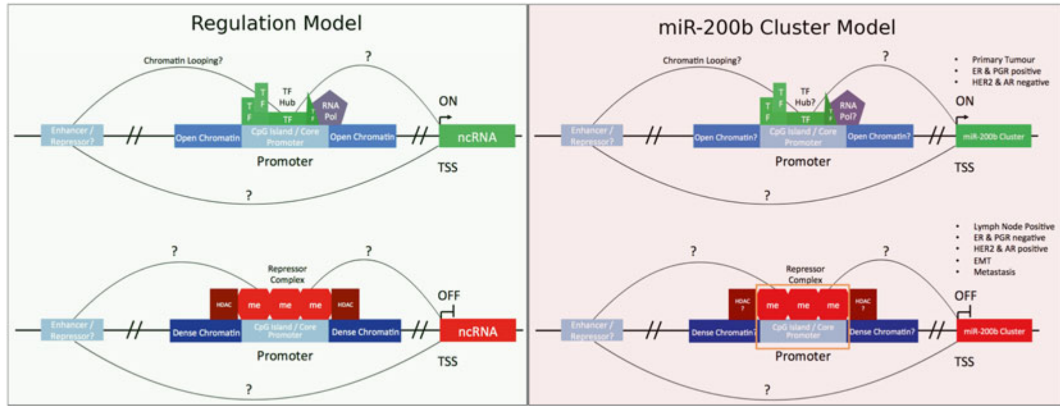

Fig. 7.1 Regulation of ncRNA transcription is a dynamic process mediated through epigenetic and transcriptional machinery. (**a**) The core promoter is responsible for driving the transcription of ncRNA genes and may be some distance from the start of transcription (*TSS*) (*double lines*). At the core promoter, a hub of transcription factors (*TF*) (*green*) binds facilitating the recruitment of RNA polymerase (II or III) (*purple*), to activate gene transcription. Transcription factors may also assemble on proximal or distal enhancer elements. Initiation of transcription may involve short or long-range interactions (*blue arrowed lines*). In the case of miR-200b, this state is more frequently observed in primary breast tumours, and in those tumours that are ER or PR positive. (**b**) Expression of ncRNAs can be suppressed by DNA methylation. Surrounding the core promoter, and distal elements, epigenetic machinery increases the density of chromatin through methylation and acetylation of Histone H3. Dense chromatin inhibits binding of transcription factors and RNA polymerase, and consequently chromatin looping that is dependent on transcription factor binding. In the case of the miR-200b promoter, the core promoter is more frequently methylated in lymph node metastasis and in ER or PR negative tumours

(Guttman et al. 2009). Algorithms have recently been developed to facilitate the analysis of ncRNA gene transcription, including ChIPBase (Yang et al. 2013).

7.2.4 Regulation of ncRNAs by Hormones That Control Mammary Gland Development

Mammary gland development involves a host of hormone receptors and co-activators that regulate global gene expression, including receptors for both oestrogen (ERα/ERβ) and progesterone (PR, (Macias and Hinck 2012), both of which have also been implicated in the regulation of ncRNAs.

The regulation of ncRNAs by hormones in mammary gland development is not well understood. *H19* is a lncRNA that is implicated in maternal imprinting and numerous cancers and diseases (Gabory et al. 2010). The levels of *H19* are elevated in breast cancer and highest in the mammary gland during the oestrus and metoestrus phases, and in response to oestradiol treatment in vivo (Adriaenssens et al. 1999). This induction was associated with increases to promoter activity in the presence of oestradiol in a luciferase reporter assay (Adriaenssens et al. 1999). There is also some evidence to suggest that the lncRNA *HOTAIR* is regulated by oestrogen (Bhan et al. 2013).

7.2.5 miRNAs Function in Mammary Epithelial Cell Differentiation

Many of the ncRNAs that are regulated in the mammary gland are functional in mammary epithelial cells. Experimental evidence ranges from phenotypes observed as a consequence of modulating the expression of ncRNAs in immortalised and tumour-derived mammary epithelial cell lines, through to in vivo studies in mice, involving xenograft and transgenic approaches.

A number of studies have employed the HC11 mouse mammary epithelial cell line, showing

that overexpression and repression of miRNAs can affect cell proliferation and hormone induced differentiation. For example, overexpression of miR-101a results in a decrease in cell proliferation, a decrease in hormone-induced β-casein mRNA levels, and an increase in *Cox2* mRNA (Tanaka et al. 2009). In a similar study, Cui et al. showed that miR-126-3p regulates the proliferation of mouse mammary epithelial cells, the expression of progesterone receptor and the expression of the β-casein (*Csn2*) gene in response to hormone treatment (Cui et al. 2011; Piao and Ma 2012). One study that employed the EpH4 cell line showed that the knockdown of miR-200a decreased β-casein, E-cadherin and β-catenin protein levels (Nagaoka et al. 2013). Furthermore, the tight junction protein ZO1 was mislocalised to the cytoplasm (Nagaoka et al. 2013).

Analyses of mouse models has provided further evidence that miRNAs play an important role in mammary epithelial cell differentiation. For example, miR-30b overexpressing transgenic mice display reduced mammary alveolar lumen size and a lipid droplet defect, which ultimately affects the growth of pups nursed by these mice (Le Guillou et al. 2012). These mice display a delay in mammary gland involution, due to the persistent presence of some differentiated mammary epithelial structures (Le Guillou et al. 2012). Other mouse model studies have shown that the *mir-17–92* cluster of miRNAs (miR-17, miR-18a, miR-19a, miR-19b, miR-20a and miR-92a) are dispensable for mouse mammary gland development, from pre-puberty to lactation (Feuermann et al. 2012). Recent studies have shown that the *mir-424–503* cluster is activated by TGFβ after weaning and regulates mammary epithelial involution by inducing secretory alveoli regression through targeting key genes (*Bcl2* and *Igf1r*) (Llobet-Navas et al. 2014a). Furthermore, the cluster was shown to regulate TGFβ-mediated cell cycle arrest through targeting *Cdc25* (Llobet-Navas et al. 2014b).

MiRNAs have also been implicated in the regulation of mammary stem cell function. For example, let-7 contributes to self-renewing properties of mouse mammary progenitor cells (Ibarra et al. 2007; Piao and Ma 2012). A recent review highlighted the role of several ncRNAs in the mammary epithelial hierarchy and in particular miRNAs that are associated with mammary stem cells (Fu et al. 2014). Another study has shown that the inactivation of the *mir-193b* locus, which also encodes miR-365-1 and miR-6365, a STAT5 target in the mammary epithelium, lead to an increase in mammary stem/progenitor cell activity in mice, where colonies derived from primary mammary epithelial cells (MEC) were larger and more proliferative (Yoo et al. 2014). This indicates a role for these miRNAs in the proper coordination of mammary alveolar differentiation (Yoo et al. 2014). The mammary epithelium was also more differentiated in mice during puberty and pregnancy, associated with a decrease in *Cav3* (Caveolin 3) and an increase in expression of the gene encoding the transcription factor ELF5 (Yoo et al. 2014). Interestingly, normal colony development was only achieved upon both the ectopic expression of *Cav3* and the reduction in *Elf5* using siRNAs (Yoo et al. 2014).

7.2.6 lncRNAs Function in Mammary Epithelial Differentiation

Knockdown of either isoform of the lncRNA *Pinc* in mouse HC11 cells leads to an increase in cell proliferation, differentiation and apoptosis (Ginger et al. 2006; Shore et al. 2012b), whilst ectopic expression of *Pinc* blocks HC11 lactogenic differentiation (Shore et al. 2012b). *Pinc* isoforms interact with the chromatin modifying protein RBAP46, suggesting a role for *Pinc* in the epigenetic regulation of mammary epithelial cell function (Shore et al. 2012b). Repression of the lncRNA *Zfas1* in the HC11 mouse mammary epithelial cell line results in a significant increase in cell proliferation and differentiation, the latter evidenced by dome formation and β-casein expression. Interestingly this phenotype was not associated with altered levels of its snoRNAs, suggesting a function independent of the snoRNAs it generates (Askarian-Amiri et al. 2011; Shore et al. 2012a, b). A separate study has highlighted that the lncRNA *Neat1* forms paraspeckles in the

luminal epithelial cells of the mammary gland and is involved in regulating mammary epithelial branching, lobular alveolar development and lactation, whereby loss of *Neat1* impairs all these processes (Standaert et al. 2014). Another paper found that the lncRNA *Ror* was overexpressed upon transcription factor NRF2 knockdown (Zhang et al. 2014b). Two NRF2 binding elements were predicted and validated using *Ror* reporter promoter deletion series, both of which were equally important towards the suppression of *Ror* transcription (Zhang et al. 2014b). H3K27me3 chromatin marks, which are usually found at condensed chromatin and repressed regulatory elements (Barski et al. 2007), as well as EZH2 binding, a subunit of the PRC2 complex which catalyses H3K27me3 addition to chromatin (Morey and Helin 2010), at the *Ror* promoter were also identified to be NRF2-dependent (Zhang et al. 2014b). NRF2 knockdown/*Ror* overexpression was found to lead to increased mammary stem cell self-renewal capacity and genotoxic stress induced by the oestrogen metabolite 4-OHE2 (Zhang et al. 2014b). Taken together, these studies suggest that lncRNAs as well as miRNAs play a crucial role in regulating mammary epithelial cells.

7.2.7 miRNAs Contribute to Mammary Epithelial-Stromal Interactions

Stromal interactions are essential in developing the mammary gland and maintaining epithelial cell states, where breakdown or changes to these interactions can often be the initiating step in tumourigenesis (Hanahan and Weinberg 2011). The mammary stroma has vital functions in mammary gland development as it provides the mammary epithelium with essential growth factors and extracellular matrix (ECM) proteins that are crucial for proper mammary epithelial morphogenesis (Parmar and Cunha 2004; Sakakura et al. 2013; Ucar et al. 2010; Wiseman and Werb 2002).

MiRNAs regulate epithelial-stromal interactions of the mammary gland. Multiple miRNAs have been found to be expressed in the mammary stroma, for example the miR-212-132 family, and using transplantation experiments Ucar and colleagues demonstrated that they are essential for stromal but not epithelial cell contribution to mammary gland development. Notably these miRNAs were shown to target matrix metalloproteinase 9 (*Mmp9*), and that their repression results in an accumulation of MMP9 (Piao and Ma 2012; Ucar et al. 2010). MiR-320 has also been implicated in regulating the mammary stroma, with ablation of miR-320 decreasing the effective establishment of tumour microenvironments through repression of key proteins needed for invasion and tumour establishment including ETS2 (Bronisz et al. 2012).

7.2.8 Concluding Remarks: ncRNAs in Mammary Gland Development

Global expression profiling has enabled significant progress to be made in mapping the relative expression of ncRNAs and how this changes during broad developmental processes, between the major epithelial subtypes and between normal mammary tissue and breast cancer. What has yet to be determined is how these levels change between more specific mammary developmental time points, more defined epithelial subpopulations and throughout the detailed and complex natural history of breast tumourigenesis. Whilst many elements and factors controlling ncRNA expression have been described, the significance of many of these has yet to be functionally validated and their individual and combined role in the context of complex biological processes has yet to be determined. It remains to be elucidated how transcription is fine-tuned in a temporal and spatial way through mammary development and how changes in this control, both at the genetic and epigenetic level contributes to disease and can be used to monitor and predict the outcome of the disease process.

7.3 ncRNAs in Mammary Gland EMT

7.3.1 miRNA Expression during Mammary EMT

Epithelial-to-mesenchymal transition (EMT) is important in mammary gland development (reviewed in Thiery et al. 2009; Wright et al. 2010). EMT occurs between the lactation and early involution stages of mammary gland development, where TGFβ pathways have been shown to increase with decreasing E-cadherin levels and the loss of epithelial cell-cell contacts (Andrechek et al. 2008; Avril-Sassen et al. 2009; Boussadia et al. 2002; Faure et al. 2000; Strange et al. 1992; Vallorosi et al. 2000; Wright et al. 2010). MiRNAs affect EMT through the regulation of the TGFβ pathway (Eades et al. 2011; Turcatel et al. 2012).

A study by Gregory et al. showed that members of the miR-200 family, which includes miR-200a, miR-141 and miR-429, are downregulated upon TGFβ induced EMT (Avril-Sassen et al. 2009; Gregory et al. 2008). In support of this observation are the results from another study, which used TGFβ to induce an EMT-like state in human mammary epithelial cells (HMEC), which showed that members of the miR-200 family were significantly repressed (Eades et al. 2011). A separate study by Zhang et al. revealed that miR-203 is epigenetically repressed during EMT (Zhang et al. 2011). Given the importance of EMT in normal developmental processes and in disease progression, it is imperative to unravel the role of ncRNAs in EMT and MET (mesenchymal-to-epithelial transition).

7.3.2 Regulation of ncRNAs during Mammary EMT

As noted above, the miR-200 family are known regulators of EMT, operating through the zinc finger E-Box binding homeodomain protein (ZEB) and other associated factors (Bracken et al. 2008). Moes et al. identified a double-negative regulatory loop where the expression of snail homologue 1 (SNAI1) decreased the expression of miR-203 through repression of its promoter activity, and miR-203 over-expression led to reduction in *Snai1* by targeting its 3′ UTR (Moes et al. 2012). A separate study found something similar using TGFβ, which inhibits epithelial cell proliferation and promotes EMT in tumourigenesis through repression of E-Cadherin and induction of SNAI2, ZEB1 and ZEB2 (Ding et al. 2013). Treatment of MDCK cells with TGFβ decreased expression of miR-203 by increasing levels of SNAI2, which bound to and repressed miR-203 promoter activity (Ding et al. 2013). MiR-203 also inhibits the translation of *SNAI2*, similar to *SNAI1*, creating another double-negative feedback loop (Ding et al. 2013).

A novel ncRNA called translational regulatory lncRNA (*treRNA*) is associated with the enrichment of *SNAIL* transcription (Gumireddy et al. 2013). It is upregulated in primary breast cancer and lymph node metastasis samples and is associated with the invasive capacity of breast cancer cell lines in vitro and metastatic capacity of breast tumours in vivo (Gumireddy et al. 2013). *treRNA* also represses E-Cadherin translation by targeting its 3′UTR (Gumireddy et al. 2013).

A study by Eades et al. has revealed a novel target of miR-200a, the class III histone deacetylase and epigenetic repressor SIRT1, through their studies on a TGFβ-driven model of normal human mammary epithelium (Eades et al. 2011). SIRT1 is enriched during EMT and epigenetic silencing of miR-200a contributes to its overexpression (Eades et al. 2011). The promoter of miR-200a becomes hypermethylated when HMECs are induced to undergo EMT by TGFβ1. Conversely, upon the restoration of miR-200a expression EMT is repressed (Eades et al. 2011). Interestingly, within breast tumours that overexpress *SIRT1*, miR-200a is often downregulated, possibly through the same epigenetic regulation (Eades et al. 2011).

7.3.3 The Role of miRNAs in Mammary EMT

A hallmark of EMT is cadherin isotype switching, often resulting from the loss of the epithelial cell

adhesion molecule, E-cadherin, a core component of the adherens junction complex (AJC), and concomitant up-regulation of mesenchymal markers, including vimentin and N-cadherin (Jeschke et al. 2005; Sreekumar et al. 2011; Tomita et al. 2000; Yoshida et al. 2001). Numerous miRNAs regulate E-cadherin (*Cdh1*) expression, either through direct repression of the *Cdh1* mRNA or indirect repression of the transcription factors that regulate *Cdh1* gene expression (Fig. 7.2, and reviewed in Sreekumar et al. 2011). *Cdh1* mRNA has been shown to be a direct target of miR-9 and a potential target of miR-10b (Liu et al. 2012; Ma et al. 2010; Sreekumar et al. 2011). MiR-200 and miR-205, both of which are repressed in metastatic breast cancers, have been shown to repress *ZEB1* and *ZEB2*, which are transcriptional repressors of *Cdh1* expression. As such, the miR-200 family prevents the loss of an epithelial phenotype in mammary epithelial cells (Burk et al. 2008; Gregory et al. 2008; Howe et al. 2012; Sreekumar et al. 2011). Expression of N-cadherin (*Cdh2*) is important in maintaining the switch from an epithelial to a mesenchymal state, and miR-199 and 218 have been suggested as potential modulators of N-cadherin expression (Sreekumar et al. 2011).

Repression of miR-203 leads to the enrichment of Snail homologue 2 (SNAI2), an EMT promoting transcription factor (Zhang et al. 2011). Ding et al. highlighted that TGFβ signalling induced *SNAI2* expression through promoting miR-203 repression in a negative feedback loop formed between *SNAI2* and miR-203, to inhibit each other's expression (Ding et al. 2013). Work by Turcatel et al. revealed that both miR-99a and miR-99b are enriched during TGFβ-signalling-induced EMT in normal mouse mammary gland cells and that inhibition of these miRNAs decreases the activity of TGFβ through inhibiting SMAD3 phosphorylation, resulting in decreased

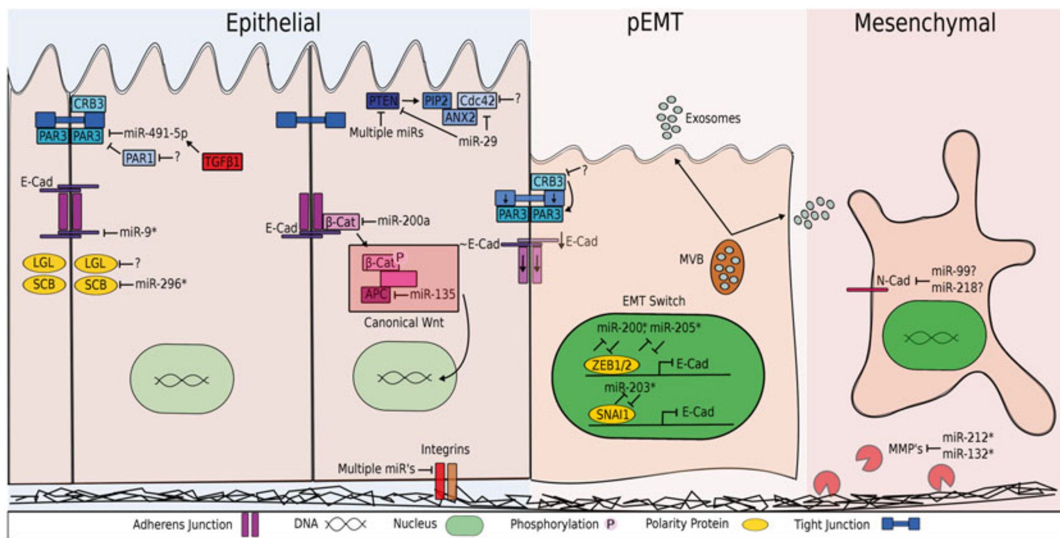

Fig. 7.2 miR regulation of epithelial polarity and EMT. miRNAs have been shown to regulate proteins involved in epithelial polarity and interaction with the basement membrane (*squiggly lines*) and thus contributing to EMT and tumourigenesis. Those miRNAs specifically implicated in breast development or disease have been highlighted with an asterisk. Given the high level of conservation of polarity machinery across species it is anticipated that these regulatory miR networks will contribute to the regulation of polarity signalling in breast. EMT-MET switch is a normal developmental process that is hijacked during disease progression. Cells that are undergoing transformation exhibit a partial EMT (*pEMT*) whereby they maintain histological features of polarised epithelium although they are undergoing biochemical, epigenetic and genetic changes that will ultimately result in EMT. This figure simplistically illustrates the miRNA regulation of conserved core polarity machinery and is not exhaustive. For full details of molecular pathways please refer to the relevant text

cellular migratory capacity (Turcatel et al. 2012). On the other hand, Liu et al. has reported that miR-93, previously shown to be repressed in mammary progenitor cells, functions to maintain normal breast stem cells in an epithelial state and induces a MET in normal breast epithelial cells (Ibarra et al. 2007; Liu et al. 2012).

p53 has been shown to regulate the EMT-MET switch in mammary epithelial cells. Chang et al. opened an entirely new aspect to p53 biology when they linked p53 to the control of EMT (Chang et al. 2011a). This study showed that in breast cancer p53 regulates EMT and stem cell characteristics through regulation of transcriptional transactivation of miR-200c (Chang et al. 2011a; Schubert and Brabletz 2011). Loss of p53 reduced expression of miR-200c, activating the EMT program via modulation of ZEB1, and stimulated an increase in the population of mammary stem cells via the modulation of the expression of polycomb ring finger oncogene *BMI1*. These studies raised the idea that therapeutic modulation of the p53-miR-200c axis could prove beneficial for targeting the tumour-initiating cell population. It has been noted that cancer cell mediated suppression of p53 expression is evident in fibroblasts adjacent to the tumour (Bar et al. 2009; Chatzistamou et al. 2010; Trimis et al. 2008). Given that the tumour microenvironment is considered to be pro-oncogenic due to its propensity to stimulate tumour cell survival and proliferation, uncovering a mechanism by which the p53-miR-200c axis operates in adjacent fibroblasts and neighbouring epithelial cells, will provide deeper understanding into tumour progression and survival of metastatic cells at distant sites (Farmaki et al. 2012).

7.3.4 The Role of miRNAs in Cytokine-Induced EMT

Oncostatin M (OSM), a member of the IL6 family of cytokines, is a driver of EMT in breast cancer (Guo et al. 2013). The LIN28/let-7/HMGA2 and miR-200/ZEB1 regulatory switches have been found to be controlled by OSM induced STAT3 activation. Reduction of let-7 and miR-200 levels maintains cytokine-induced EMT phenotypes; with high mobility group AT-hook 2 (HMGA2) acting as a master controller of cytokine-induced EMT. These studies highlight the importance of STAT3 as a driver of LIN28/let-7/HMGA2 and miR-200/ZEB1 circuits, highlighting the previously underappreciated role of inflammatory cytokines in cellular reprogramming in EMT and tumour progression (Biddle and Mackenzie 2012; Daheron et al. 2004; Guo et al. 2013; Ying et al. 2003).

7.3.5 miRNAs Regulate Mammary Epithelial Cell Polarity

Correct establishment and maintenance of epithelial polarity is essential for the development and homeostasis of all metazoans, is crucial for mammary development and is frequently defective in breast tumours. Three conserved polarity complexes contribute to the establishment of epithelial cellular polarity: PAR, SCRIB and CRB (Fig. 7.2) (Godde et al. 2010; Inman and Bissell 2010; Muthuswamy and Xue 2012). There have been a number of recent advances in our understanding of how miRNAs regulate the polarity machinery.

MiR-200a has also been implicated in the regulation of cell polarity. For example, knockdown of miR-200a decreases cell polarity in EpH4 cells that have been induced to polarise and form cavities in a 3D matrix (Nagaoka et al. 2013). This knockdown also results in a reduction in cavity formation and a decrease in expression of cell polarity associated genes – Claudin 3 (*Cldn3*), *Par6b* and E-cadherin (Nagaoka et al. 2013). A random distribution of ZO1, Claudin 3 and E-cadherin was also observed in miR-200a knockdown colonies that did not form colonies, suggesting a disturbance of intracellular polarity (Nagaoka et al. 2013).

MiR-296 transcriptionally represses the polarity and directional cell migration regulator *Scrib* and its expression suppresses tumour growth in vivo, while loss of miR-296 causes aberrantly

increased levels and mislocalisation of SCRIB in human tumours (Vaira et al. 2012). Deregulation of SCRIB levels and localisation caused exaggerated random cell migration and invasion (Dow et al. 2007; Osmani et al. 2006). Enhanced levels of *SCRIB* have been detected in primary lesions and metastases of lung, colon, breast, stomach cancer and hepatocellular carcinoma (Augello et al. 2009).

Other examples of miRNAs implicated in epithelial cell polarity include miR-375, which was identified in a miRNA expression screen of lobular breast tumours, and shown to disrupt the polarity of MCF10A cells when grown in matrigel 3D cultures (Giricz et al. 2012). In addition, miR-338-3p and miR-451 regulate the translocation of the epithelial-stromal interaction protein β1-integrin to the basolateral membrane in T84 lung epithelial cells (Tsuchiya et al. 2009) and thereby affecting cell polarity.

Given the importance of the polarity machinery in normal tissue development and in disease progression it will be invaluable to uncover the miRNA networks involved in the regulation of polarity and vice versa, since these kinds of studies will highlight novel modulators of epithelial plasticity.

7.3.6 Concluding Remarks: EMT Section

EMT is a vital process for mammary gland development and a hallmark for the invasion and metastatic progression of breast cancer. Although a significant number of ncRNAs have been found to be involved in mammary gland development, the roles that they play in EMT associated with the development of the mammary gland is still in its infancy and have yet to be fully explored. Nevertheless, the roles that ncRNAs, and particularly miRNAs, play in EMT associated with the invasion and metastasis of breast cancer has highlighted the potential for miRNAs to be therapeutic targets, if cell specificity can be achieved (reviewed in Wright et al. 2010).

7.4 NcRNAs in Breast Cancer

7.4.1 Expression of miRNAs and lncRNAs in Mammary Cancer

The expression of miRNAs has been determined in a large number of breast tumours. Early landmark papers include miRNA profiling of 76 breast tumours and 10 normal breast tissue, which identified 29 significantly altered miRNAs, 15 of which could predict the tumour state, including miR-125b, miR-145, miR-21, and miR-155 (Iorio et al. 2005). Blenkiron and colleagues subsequently identifed 133 miRNAs that could classify breast tumour subtype (Blenkiron et al. 2007). More recently, a study involving 1302 breast tumours with detailed clinical information assayed miRNA and mRNA expression (Dvinge et al. 2013). This study demonstrated that changes in miRNA expression are less likely to be due to copy number alterations (CNA) as compared to changes in mRNAs expression (Dvinge et al. 2013), suggesting that the majority of variation seen in miRNA expression is due to altered gene regulation (Dvinge et al. 2013). MiRNA/mRNA expression has also been determined across a variety of cellular processes including cell cycle, morphogenesis, hormone response and the immune response (Dvinge et al. 2013). Interestingly, when miRNAs that are enriched in such cellular process were combined, a much greater prognostic value and prediction of survival than individual miRNAs was observed (Dvinge et al. 2013). This study is a major advance in the identification of miRNAs as informative biomarkers for breast cancer, and highlights that combinations of dynamically expressed and functional miRNAs may be most useful in predicting disease prognosis.

Several lncRNAs are also dysregulated in breast cancer (recently reviewed by (Gutschner and Diederichs 2012). Gupta et al. profiled ncRNA transcripts within the four *HOX* loci from primary breast tumours and metastases (Gupta et al. 2010). The *HOX* transcript antisense RNA *HOTAIR* was found to be over-expressed, with one third of the tumours having at least 200-fold

higher levels of the *HOTAIR* transcript. The same study identified several other ncRNA transcripts from these loci that associated with breast cancer and metastasis and are yet to be fully explored.

The *H19* maternally imprinted lncRNA, which silences the expression of *IGF2*, is also overexpressed in breast cancer (Adriaenssens et al. 1998; Hark and Tilghman 1998; Shore et al. 2012a). *H19* overexpression is mostly detected in the stromal compartment as compared to the epithelial compartment of tumours and is associated with the expression of oestrogen receptor (ER) and progesterone receptor (PR) (Adriaenssens et al. 1998; Shore et al. 2012a). *H19* also displays oncogenic characteristics, for example when its expression is activated by E2F1, *H19* promotes cell cycle progression of breast cancer cells (Berteaux et al. 2005; Shore et al. 2012a). Under hypoxic conditions, *H19* is upregulated via HIF1α expression and simultaneous p53 suppression (Matouk et al. 2010; Shore et al. 2012a).

The steroid receptor RNA activator (*SRA1*) lncRNA, a transcriptional co-activator that is specific for the transactivation of steroid hormone receptors is significantly upregulated in breast tumours (Lanz et al. 1999, 2003; Shore et al. 2012a). Invasive breast cancer cell lines express *SRA1* at a higher level relative to non-invasive cell lines (Hube et al. 2006; Shore et al. 2012a). Consistent with this, MCF10A, a mammary epithelial cell line, expresses very low levels of *SRA1* (Hube et al. 2006; Shore et al. 2012a). *SRA1* expression was also shown to be associated with increasing progesterone levels in breast tumours (Cooper et al. 2009; Shore et al. 2012a).

The lncRNA growth arrest specific 5 (*GAS5*) acts as a decoy glucocorticoid response element (GRE) to repress glucocorticoid receptor (GR) transcriptional activity as well as other steroid receptors such as PR and androgen receptor (AR) (Kino et al. 2010). In breast cancers *GAS5* expression is significantly reduced when compared to normal breast epithelia (Mourtada-Maarabouni et al. 2009). *GAS5* and the snoRNA *RNU44*, which maps to an intron of the *GAS5* transcript, are both lowly expressed and associated with a more aggressive and prognostically poor phenotype (Gee et al. 2011). *GAS5* expression is also negatively regulated by miR-21, a miRNA whose expression is associated with good outcome in breast cancer (Yan et al. 2008; Zhang et al. 2013). Overexpression of *GAS5* induces growth arrest and apoptosis in breast cancer cell lines and also reduces tumour growth rate in mouse xenograft models, consistent with a tumour suppressive function for this lncRNA (Mourtada-Maarabouni et al. 2009; Zhang et al. 2013).

7.4.2 Regulation of miRNAs in Breast Cancer

Using a combination of bioinformatics prediction tools, which takes into account histone modification, DNA motifs and binding of RNA pol II, Wee et al. predicted promoter regions for 55 miRNAs (Wee et al. 2012). Of these 55, they experimentally validated 15 promoters using luciferase reporter assays. Focusing on two promoters in the *miR-200b* cluster, they demonstrated that increased CpG methylation significantly decreased their activity and that this region was hypermethylated in lymph nodes compared to primary tumours. Fine mapping of several individual CpG dinucleotides within the promoter demonstrated differential methylation across the CpG island. The same CpG island upstream of the *mir-200b* cluster was analysed for methylation changes during EMT (Davalos et al. 2012). The authors found that the CpG island became hypermethylated when MDCK cells, a model for epithelial morphogenesis, were treated with TGFβ to induce EMT. Hypermethylation of the same region was also seen in metastasis to the lungs of mice when injected with a metastatic breast cancer daughter cell line of MDA-MB-468 cells (Davalos et al. 2012). In a similar study, miR-31 which targets WAS Protein Family, Member 3 (*WAVE3*) to suppress metastasis in breast cancer, exhibits hypermethylation of its promoter decreasing expression and increasing the migration and invasion of breast cancer cell lines (Augoff et al. 2012). These studies highlight the importance of CpG methylation and the possibility of a biomarker to detect early stages of metastasis and the EMT phenotype in tumour cells.

7.4.3 Regulation of ncRNAs by Hormones in Breast Cancer

Several ncRNA genes are regulated by oestrogen (reviewed in Klinge 2012). For example miR-140 transcription is repressed via ERα binding to a response element in its promoter (Zhang et al. 2012), and treatment of MCF7 cells with oestradiol (E2) decreases expression of miR-16, miR-143 and miR-203 (Yu et al. 2012). Genome-wide expression analysis has also been used, identifying 52 oestrogen-responsive miRNAs in two different cell models (Ferraro et al. 2012).

An understanding of the regulation of ncRNAs has the potential to shed light on the mechanisms of therapeutic resistance in cancer. For example, a recent study identified a set of miRNAs that were differentially regulated in a cell model of oestrogen response relative to another cell model that displayed oestrogen-independent growth (Bailey et al. 2015). Among the 78 differentially expressed miRNAs was a cluster including let-7c, miR-99a and miR-125b. These miRNAs were shown to significantly decrease in expression upon deprivation of oestrogen. Interestingly, in this model *HER2* is upregulated and acts as a major signalling molecule for growth in these cells. This miRNA cluster was shown to directly target *HER2*, thus providing a mechanism by which silencing contributes to the acquisition of therapeutic resistance.

Enhancer-RNAs (eRNA) are a novel class of ncRNAs, which as the name suggests are transcribed from active enhancers. Using a global run-on sequencing technique Li et al. profiled eRNA transcripts in MCF7 cells treated with E2. This analyses revealed genome-wide enhancer transcription associated with E2 targeted genes (Li et al. 2013b). Knockdown of eRNA transcripts reduced levels of their associated genes, for example *FOXC1* and *TFF1*, which are two well established E2 response genes. ERNAs have been shown to be functionally significant across a range of cell models, although their role in mammary development remains uncharacterised (Kim et al. 2010; Wang et al. 2011a).

Studies on progesterone receptor-mediated expression of miRNAs have employed the progesterone responsive human breast cancer cell line, T47D. Analysis of T47D cells treated with medroxyprogesterone acetate (MPA) led to the identification of 59 differentially regulated miRNAs (Cochrane et al. 2012). This study also demonstrated that progesterone downregulated the miR-29 family members miR-29b and miR-29c, which target ATPase, Na+/K+ transporting, beta 1 polypeptide (*ATP1B1*), a gene upregulated in response to MPA (Cochrane et al. 2012). Progesterone receptor bound to the promoter of *ATP1B1* resulting in up-regulation and this in combination with repression of miRNA targets led to a dual mechanism of control (Cochrane et al. 2012).

7.4.4 Regulation of ncRNAs by Other Breast Cancer Associated Transcription Factors

GATA3, a transcription factor implicated in mammary development and breast cancer, has been shown to repress metastasis, regulate luminal epithelial cell differentiation and regulate oestrogen responsive genes (Cimino-Mathews et al. 2013). MiRNA profiling of 4T1 breast cells overexpressing *Gata3* identified miR-29b as a direct transcriptional target of GATA3 (Chou et al. 2013). GATA3 also induces the expression of miR-30c through binding upstream to its proximal promoter (Bockhorn et al. 2013).

Breast cancer 1 (*BRCA1*) is a breast cancer susceptibility gene that encodes a transcription factor that regulates DNA repair and progression through the cell cycle (Cressman et al. 1999). Ablation of *Brca1* in the mouse mammary gland leads to defective lobular-alveolar development and late onset mammary tumours. In humans, breast tumours arising from carriers of a pathogenic *BRCA1* mutation are often triple-negative basal-like with poor prognosis and survival (Bosch et al. 2010; Marquis et al. 1995; Valentin et al. 2012). Chang and colleagues demonstrated that wild-type BRCA1 is able to bind to the promoter of miR-155 and recruit HDAC2 to repress transcription (Chang et al. 2011b). Whereas a

R1699Q moderate risk variant of BRCA1 is unable to recruit HDAC2 and thus repress miR-155 expression, leading to increased levels of this miRNA (Chang et al. 2011b). This regulation by BRCA1 and its clinical implications in breast cancer have been reviewed recently by Stefansson and Esteller (Stefansson and Esteller 2012). A link between BRCA1 and the lncRNA *HOTAIR* (HOX antisense intergenic RNA) was recently identified through EZH2, a RNA binding component of the chromatin modifying complex PRC2, to which *HOTAIR* also binds to (Wang et al. 2013). In breast cancer cell lines, BRCA1 binds to the ncRNA-binding-domain of EZH2, and in doing so blocks the binding and thus the chromatin remodelling activity of *HOTAIR*. Although not highly conserved in sequence, mouse *Hotair* was recently shown to interact with PRC2 components during development and when deleted in vivo resulted in bone fusions within the embryo (Li et al. 2013a). In this study they focussed on embryonic development, which does not result in a fully functional mammary gland until after birth and pregnancy. Due to the role of *HOTAIR* in breast cancer and murine development future research should investigate whether *Hotair* can influence the developing mammary gland.

7.4.5 ncRNAs Contribute to Mammary Epithelial Cell Transformation and Breast Cancer

It is not surprising from the role ncRNAs have in epithelial differentiation, EMT and polarity that hundreds of these molecules have been implicated in the cellular processes underlying breast tumourigenesis. Indeed, this topic has been reviewed extensively (Hurst et al. 2009; Jiang et al. 2010). MiRNAs that promote breast tumourigenesis include miR-10b, miR-21, miR-103/107, and miR-373. Over-expression of miR-103/107 in vitro triggers an EMT in normal mammary epithelial cells (Martello et al. 2010). MiRNAs that repress breast tumourigenesis include miR-17/20, miR-31, miR-126, miR-145, miR-200, miR-205 and miR-335 (Gibbons et al. 2009; Gregory et al. 2008; Iliopoulos et al. 2010; Nam et al. 2008). A hallmark of malignant tumours is their ability to migrate by undergoing EMT to colonise distant tissue. Lymph node positivity is a commonly used clinical marker for metastasis (Vuong et al. 2014). Several studies have sought to understand ncRNA expression in lymph node metastasis. Avery-Kiejda and colleagues (Avery-Kiejda et al. 2014) compared miRNA expression profiles from primary breast cancer where patients were either lymph node negative or positive at time of collection, identifying 71 differentially expressed miRNAs. A more recent study calculated an optimal cluster of lncRNA expression profiles to determine the risk of metastasis occurring in lymph node negative patients (Sorensen et al. 2015). This set of 47 lncRNAs accurately classified 77 % of patients that went on to develop a metastasis, proposing a novel prognostic biomarker based on ncRNA expression. Both of these studies identify a core set of ncRNAs with prognostic and biological potential for early detection of metastasis in breast cancer.

Claudin-low breast tumours are a molecular subtype derived from basal cancers that have no targeted treatments and are highly resistant to chemotherapy (Sabatier et al. 2014). A recent study forced expression of miR-200c, a marker of luminal breast cancers, in a tumour model of claudin-low cancers (Knezevic et al. 2015). This resulted in suppression of tumour growth and a push from a stem-cell-like state to a more epithelial state resulting in a greater response to chemotherapeutic agents. This may present a novel therapeutic option, forcing expressing of miR-200c in patients with claudin-low tumours to force the cancers to remain epithelial, be more chemotherapy sensitive and reduce their propensity to metastasise.

Many lncRNAs have also been implicated in breast tumourigenesis. For example, overexpression of the lncRNA *HOTAIR* in breast cancer cells results in increased cell invasion in vitro and metastasis in vivo, whereas siRNA repression results in reduced tumour growth and invasion, increased apoptosis and increased sensitivity to cisplatin and doxorubicin (Gupta et al. 2010). LncRNAs can act as a scaffold of protein-protein

interactions or for chromatin modifying enzymes to bind to chromatin and exert their function (Khalil et al. 2009). *HOTAIR* acts as a scaffold for polycomb repressive complex (PRC1 and PRC2) proteins to actively repress chromatin through alteration in histone 3 methylation (H3K27me3 and H3K4me2). In the invasive breast cancer cell line MDA-MB-231, *HOTAIR* over-expression promoted expression of *ABL2, SNAIL, LAMB3* and *LAMC2* while repressing *JAM2, PCDH10 and PCDHB5* to promote metastasis both in vitro and in vivo in mice.

Initial characterisation of *SRA1* demonstrated coactivator function with SRC1/SRC2 in the ER pathway (Lanz et al. 1999). To broaden the spectrum of *SRA1* mediated regulation of target genes, *SRA1* was repressed in the HeLa and MCF7 (cervical and breast cancer) cell lines and expression analysed through a cDNA microarray (Foulds et al. 2010; Shore et al. 2012a). Results revealed that gene expression was majorly reduced following *SRA1* depletion, which highlights its role as a transcriptional co-activator (Foulds et al. 2010). As *SRA1* interacts with co-activators of ERα, it was deemed to potentially co-activate ERα, nevertheless the expression of only a small subset of ERα associated target genes were affected in oestradiol treated MCF7 cells depleted of *SRA1* (Foulds et al. 2010). Furthermore, *SRA1* depletion in MDA-MB-231 cells lead to reduced cell invasion, highlighting a potential role for *SRA1* in the regulation of breast cancer associated invasion and metastatic capacity (Foulds et al. 2010). Overexpression of *SRA1* exclusively in the mammary gland results in epithelial hyperplasia although it was not shown to progress into a tumour potentially due to the observed increased apoptosis (Lanz et al. 2003). Although there is evidence for the role of *SRA1* in promoting breast tumourigenesis the mechanisms it utilises require further elucidation (Shore et al. 2012a).

7.4.6 Regulation of Hormone Receptors by ncRNAs

The regulation of hormone receptors in breast cancer by ncRNAs is a relatively untouched area of research. Recently a study identified a region of high transcription within intron 2 of the *ESR1* (ERα) gene and upstream of the gene promoter (Tomita et al. 2015). This RNA, housing several ncRNAs, was more highly expressed in a model of long-term oestrogen deprivation, mimicking aromatase inhibition in breast cancer patients. These novel ncRNA transcripts were named *Eleanors* (*ESR1* locus enhancing and activation noncoding RNAs). Depletion of u-Eleanor (located upstream of the *ESR1* promoter) decreased *ESR1* mRNA and led to growth inhibition of these long-term oestrogen deprived cells. These data present a novel mechanism for the regulation of hormone receptor gene expression in breast cancer and an area of research for further focus in the future.

7.4.7 Concluding Remarks: Breast Cancer

Although the role of ncRNAs in breast cancer development and progression is less well understood than that of protein coding genes, it has shown great promise for elucidating the mechanism and improving the diagnosis, prognosis and treatment of breast cancer. For example, hormone receptors, the key regulators of the major growth pathways in the majority of breast cancer, act through a dynamic and complex transcriptome that includes a plethora of ncRNAs, including lncRNAs, miRNAs, eRNAs and others. Recent findings have shown that ncRNAs can in turn regulate the hormone receptors *ESR1* and *HER2*. NcRNAs are broadly categorised as regulatory molecules and many studies have shown that they play a key role in the development of tumours, response to both chemotherapy and endocrine therapy and present as novel targets for therapies. Recent advances have included genome-wide mapping of miRNA targets using small molecular interactions following by a traditional chromatin immunoprecipitation assay (Imig et al. 2015). The development of domain-specific chromatin isolation by RNA purification (dChIRP) (Quinn et al. 2014) has further enabled the simultaneous mapping of lncRNA-RNA, lncRNA-protein and

lncRNA-DNA interactions. These methodologies and many others will pave the way for advances in our understanding of breast cancer biology and the role of ncRNA in the development of the mammary gland.

7.5 Signalling Pathways Are Impacted by ncRNAs in Breast Development and Disease

This section briefly considers recent insights of the impact of ncRNAs upon three of the key signalling pathways involved in mammary gland development and disease, namely: NOTCH, PTEN and HIPPO. In-depth reviews of the literature on these signalling pathways is available elsewhere (Bernascone and Martin-Belmonte 2013; Guo et al. 2011).

7.5.1 miRNAs Regulate NOTCH Signalling

In the developing mammary gland, NOTCH activation specifies luminal cell fate, with NOTCH signalling activity detected at sites of pubertal ductal morphogenesis, where luminal cell fate is determined (Yalcin-Ozuysal et al. 2010). The NOTCH signalling pathway has been shown to induce EMT in vitro and is de-regulated in many human cancers, including breast cancer (Brabletz et al. 2011; Hurlbut et al. 2007; Koch and Radtke 2007; Leong et al. 2007; Rizzo et al. 2008; Sahlgren et al. 2008). A number of miRNAs contribute to NOTCH pathway regulation in breast cancer, including the *mir-1–206*, *mir-34*, *mir-29*, *mir-146*, *mir-199* and *mir-200* families (reviewed in de Antonellis et al. 2013; Li et al. 2011; Wang et al. 2010b, 2014b). For example, enforced expression of miR-206 (a *mir-1* family member) in MCF-7 breast cancer cells inhibits cell growth and suppresses cell proliferation and colony formation. MiR-206 also reduces cyclin D1 and D2 expression at both the mRNA and protein levels, supporting a tumour suppressive role of miR-206 in the progression of breast cancer (Elliman et al. 2014; Zhou et al. 2013).

Brabletz and colleagues demonstrate that the EMT inducer ZEB1 indirectly triggers NOTCH signalling in cancer cells by inhibiting miR-200 expression, and this leads to stabilisation of NOTCH pathway components and activation of the NOTCH signalling pathway (Brabletz et al. 2011). Similarly, Vallejo et al. identified that the miR-8–200 microRNA family targeted the NOTCH pathway using a Drosophila over-expression screen (Vallejo et al. 2011). In this system, miR-8 directly inhibits the NOTCH ligand *Serrate*, while in prostate cancer cells miR-200c and miR-141 directly inhibits *Jagged1*, which impedes proliferation.

Survivin is a member of the inhibitor of apoptosis family of proteins (IAP) and is implicated in suppression of cell death, regulation of mitosis, checkpoint surveillance and adaptation of cells to inhospitable environments (reviewed in Altieri 2010). *BIRC5* (Survivin) is a target gene of multiple oncogenic signalling nodes, including NOTCH (Lee et al. 2008a). Let-7a expression is elevated in response to NOTCH activation, revealing evolutionarily conserved cross talk between the let-7 developmental timing loop and the NOTCH signalling pathway (Lee et al. 2008a; Solomon et al. 2008). NOTCH is activated in ER negative breast cancer cells, resulting in up-regulation of survivin (Lee et al. 2008b). These studies reveal the reliance of triple negative breast cancer cells on the NOTCH-Survivin axis for their survival in vivo (Lee et al. 2008a, b). Regulation of the NOTCH pathway in development and disease is an area of intensive investigation, across all aspects of cell and developmental biology.

7.5.2 ncRNAs Regulate PTEN Signalling

The cytoplasmic cell polarity regulator PTEN is lost in many cancers including breast cancer, and miRNAs have been shown to regulate PTEN expression status (reviewed in Sreekumar et al. 2011). *PTEN* together with *Maspin*, *TPM1* (tropomyosin 1) and *PDCD4* (programmed cell death 4) have been shown to be direct targets of miR-21, a miRNA that is significantly overex-

pressed in invasive breast cancers (Meng et al. 2007; Tang et al. 2012; Zhu et al. 2007, 2008). Huang et al. revealed that PTEN was inversely correlated with miR-21 expression status in invasive breast cancer (Huang et al. 2008, 2009; Tang et al. 2012). Other studies have reported that the overexpression of miR-21 promoted tumour invasiveness, whereas its repression induced loss of invasion and metastasis of mammary epithelial cells (Qi et al. 2009; Tang et al. 2012; Zhu et al. 2007, 2008). *TPM1*, *SERPINB5* and *PDCD4* repression by miR-21 have shown to enhance invasion and metastasis in mammary epithelial cells (Tang et al. 2012; Zhu et al. 2007, 2008), however it remains to be determined whether *PTEN* is a direct target of miR-21 in invasive breast cancer.

MiR-21 and miR-155 activate the protein kinase AKT via targeting *PTEN*, a negative regulator of AKT activation, and phosphatase protein phosphatase 2A catalytic subunit *α* – *PPP2CA*, a suppressor of AKT phosphorylation in colon carcinoma (Bakirtzi et al. 2011). Interestingly, miR-155 is over-expressed in breast cancer (Tang et al. 2012; Volinia et al. 2006). A study conducted by Kong et al. demonstrated the upregulation of miR-155 in murine mammary epithelial cells, through the activation of the TGFβ pathway, which induced EMT and mammary epithelial invasion (Kong et al. 2008; Tang et al. 2012). EMT and mammary epithelial invasion was induced by dissolution of tight junctions, leading to a more migratory phenotype in mammary epithelial cells (Kong et al. 2008; Tang et al. 2012). MiR-155 was ultimately found to target *Rhoa*, encoding a GTPase protein known to regulate cell adhesion, motility and polarity through actin cytoskeletal rearrangement (Bryant and Mostov 2008; Kong et al. 2008; Shewan et al. 2005; Tang et al. 2012). The targeting of *Rhoa* by miR-155 recapitulated the phenotype of tight junction dissolution, cell migration, EMT and invasion observed in miR-155 overexpressing mammary epithelial cells (Kong et al. 2008; Tang et al. 2012). Therefore, both miR-21 and miR-155 appear to be potent metastasis activators in breast cancer. Additionally there are other miRNAs such as miR-301a and miR-374a that are upregulated in metastatic breast cancer cell lines and primary tumours of patients with distant metastasis (Cai et al. 2013; Ma et al. 2014). Both miRNAs were shown to target *PTEN* and thereby activate the WNT/β-catenin signalling pathway, thus producing invasiveness and metastatic capacity (Cai et al. 2013; Ma et al. 2014). MiR-374a also targets the negative regulators of the WNT/β-catenin signalling pathway *WIF1* and *WNT5A* to stimulate WNT signalling pathway activity (Cai et al. 2013). Further work to define additional cellular targets of the above-mentioned miRNAs will be invaluable for informing the development of targeted therapeutics for the treatment of breast cancers. Also on the therapeutics front, several miRNAs have been implicated in breast cancer related drug sensitivity (miR-21 and miR-200c) and resistance (miR-222, miR-29a, miR-19, miR-221) through modulating *PTEN* expression (Chen et al. 2013; Liang et al. 2011; Wang et al. 2011b; Ye et al. 2014; Zhong et al. 2013)

As stated above, miRNAs are critical regulators of PTEN. Additionally, there is evidence that a lncRNA *PTENP1*, a pseudogene of *PTEN*, acts as a decoy to compete for miRNA binding with *PTEN* (Poliseno et al. 2010; Shore et al. 2012a). *PTENP1* was also found to have tumour suppressive properties and its expression was lost in cancer (Poliseno et al. 2010). Similar to *PTENP1*, also protein-coding RNAs can act as ceRNAs (competing endogenous RNAs) and compete for miRNA binding with *PTEN* (Karreth et al. 2011; Shore et al. 2012a). *ZEB2* is one such ceRNA, which modulates PTEN protein levels in a miRNA-dependent manner (Karreth et al. 2011).

More recent molecular profiling experiments have shown that *PTENP1* produces an anti-sense transcript called *PTENPG1*-antisense RNA (asRNA) (Johnsson et al. 2013). This asRNA is encoded within the sense *PTENP1* transcript and extends beyond the 5′end producing two isoforms α and β. The α isoform epigenetically regulates *PTEN* transcription through recruitment of chromatin modifying enzymes that suppress gene expression. The β isoform binds to *PTENP1*, which stops it acting as a miRNA sponge for miRNAs targeting *PTEN* mRNA resulting in miRNA-mediated suppression of *PTEN*. The contribution of the asRNA in cancer however remains unexplored.

7.5.3 miRNAs Regulate HIPPO Pathway

The HIPPO and mTOR signalling pathways are critical regulators of organ size, playing a key role in regulation of cell proliferation and growth, epithelial development and homeostasis, and are frequently defective in cancer (reviewed in Bernascone and Martin-Belmonte 2013; Csibi and Blenis 2012; Nishio et al. 2013). YAP, the nuclear effector of the HIPPO pathway, was recently shown to induce the expression of miR-29. Furthermore, transient expression of miR-29 in YAP-depleted cells reduces PTEN levels and activates mTOR signalling (S6K1) (Tumaneng et al. 2012). MiR-9 targets *LIFR* in E-cadherin negative breast tumour cells, thereby promoting metastasis through the HIPPO-YAP pathway (Chen et al. 2012). These studies provide clear evidence of molecular cross talk between the HIPPO and mTOR pathways, and the importance of miRNAs in this process.

The expression of Vestigial-like 1 (VGLL1, a Drosophila transcriptional co-activator bearing structural homology to TAZ and YAP) and its intronic miRNA miR-934 was recently investigated in a cohort of grade 3 invasive ductal breast carcinomas; 37 ER-positive and 23 triple negative breast carcinomas (Castilla et al. 2014). Expression analysis of fresh frozen tumour samples revealed 28 miRNAs that were differentially expressed between these two groups, and *VGLL1* expression directly correlated with miR-934 levels (consistent with simultaneous transcription). The main conclusion of this study was that *VGLL1* and miR-934 were predominantly expressed in ER-negative breast carcinoma, including triple-negative breast cancers (TNBC), sporadic, and BRCA1-associated TNBC. In addition, *VGLL1* expression associated with reduced overall patient survival. *VGLL1* and miR-934 expression may also be associated with maintenance of luminal progenitors. *ESR1*, *FOXA1* and *GATA3* (luminal lineage determinants) were among the top 10 genes most negatively correlated with miR-934 and *VGLL1* expression, while strong positive associations were reported for *VGLL1* expression and the luminal progenitor genes *FOXC1*, *PROM1*, *SLC6A14* and *BBOX1*. Moreover, their expression may be mediated via direct modulation of *ESR1*, with miR-934 acting similarly to miR-18a and miR18b in regulation of *ESR1*. These data clearly highlight the importance of the HIPPO pathway in mammary gland development and its contribution to basal-like and triple negative breast cancers.

7.5.4 Concluding Remarks: Signalling Pathways

An extensive body of research has implicated many ncRNAs in the regulation of mammary development and disease. A range of approaches and systems has been employed, although many studies have relied on experimental modulation of individual gene products to dissect their role in individual pathways and cellular functions. Whilst informative, there are limitations associated with the necessarily linear outputs. An increasing engagement with network analysis will overcome this issue and provide a more global view of the role, regulation and interaction of the pathways controlling mammary epithelial cell function and dysfunction, and the contribution of ncRNAs to these processes.

7.6 Future Perspectives

In this chapter, we have reviewed progress on elucidating the role of ncRNAs in mammary gland development and disease. With the availability of new genomic and cell separation and analysis tools, this field has accelerated in the last few years and the current findings have demonstrated the pivotal roles of these RNA molecules in the regulation of the anatomy as well as the physiology and pathology of the mammary gland.

We anticipate that future studies will refine the expression profiles of ncRNAs. This will include broadening the data as more ncRNAs, especially lncRNAs are discovered, and deepening the data as the tools for investigating, differentiating and separating the plethora of mammary epithelial cell types, in both normal mammary biology and

pathology, improve. These studies will ultimately reveal patterns of ncRNA expression that robustly identify different cell types representing key stages in development and disease. The aim is to be able to track miRNA levels from an embryonic mammary epithelial cell, through all the stages of puberty and lactational development, and then use this information to dissect and control mammary morphogenesis ex vivo. For mammary cancer, the knowledge of the significant molecular drivers will improve differential diagnosis and prognosis. The potential to target ncRNAs, e.g. with antagomirs and bioactive small molecules (Velagapudi et al. 2014), provides the potential for these molecules to form the basis of new targeted therapies. Mapping the regulatory pathways controlling the expression of ncRNAs will in the first instance expand our understanding on ncRNA biology and mammary gland biology, and will also enable the identification of another layer of genetic and epigenetic biomarkers for improved detection and prediction of prognosis.

Understanding transcriptional and epigenetic regulation is crucial for mapping pathways and understanding of gene function. Within the mammary gland the hormones receptors ER, PR and PRLR are master regulators of transcription, defining transcriptomes at major developmental time points and within breast cancer. Mammary gland biology is primarily studied to further our understanding of breast cancer, from initiation to progression and treatment, however fundamental understanding of the gland and how transcription is altered is mostly uncharted territory. A recent advance in this area was profiling of the epigenetic marks H3K27me3 and H3K4me2, repression and activation respectively, during development of mammary stem cells and luminal cells through puberty and pregnancy (Pal et al. 2013). This study shows how epigenetic marks mirror the transcriptional profile of genes involved in development, and also demonstrates that when the epigenetic regulation is lost, development goes awry. Our ability to understand and treat cancer lies in the understanding of the mammary gland itself and future work should aim to comprehensively characterise regulation of ncRNAs and protein-coding genes during the developmental stages of the breast.

MiRNAs are also regulated by miRNA 'sponge' transcripts, which are competing endogenous RNAs for miRNA interaction (Hansen et al. 2013). MiRNA sponges have been used to specifically ameliorate miRNA expression in vivo and in vitro (Ma et al. 2010; Mandal et al. 2012; Pellegrino et al. 2013). Of note, Hansen and colleagues identified a highly expressed circular RNA (circRNA) from human and mouse brain that contains greater than 70 conserved miRNA target sites. The circRNA was resistant to miRNA-mediated destabilisation and strongly suppressed miR-7 activity, resulting in enhanced levels of miR-7 targets. In addition, expression of the *CD44* 3′UTR, a target of miR-491, enhances breast cancer metastasis in vivo and in vitro, raising the possibility that the *CD44* 3′UTR modulates the ability of miR-491 to regulate its other targets, including the extracellular matrix proteins, fibronectin and collagen type 1 α1 (Jeyapalan and Yang 2012; Rutnam and Yang 2012). The *HOTAIR* lncRNA has also been shown to act as a sponge for miR-34a (Chiyomaru et al. 2013). The significance of miRNA sponges in mammary development and breast cancer however remains to be determined and is an area that deserves further investigation.

Several lncRNAs have been associated with breast cancer, including *HOTAIR*, metastasis associated lung adenocarcinoma transcript 1 (*MALAT1*) and growth arrest specific 5 (*GAS5*), which are involved in metastasis, tumour formation and apoptosis respectively (Piao and Ma 2012). Research in this area has focussed on the functional characterisation of these transcripts and with few studies identifying upstream mediators of transcription. A future priority will be the identification of functional control elements and trans-acting factors that regulate the expression of lncRNA genes in health and disease.

Further studies into the function of ncRNAs in mammary gland development will enhance our understanding of this field. The development of improved systems to control and monitor the global expression of ncRNAs in mammary epithelial cells will enable this goal to be reached

with outcomes including genome-wide portraits of the signalling pathways in which ncRNAs can and do participate. In many cases, we have made linear connections between individual transcriptional factors, a ncRNA that it regulates and individual downstream targets of that ncRNA, and find inconsistencies in the functional studies, likely indicating the complex reality that each transcription factor and ncRNA actually target multiple molecules and pathways. Resolving the detailed networks in which these molecules participate will be crucial for fully understanding the role and potential of these molecules.

'Field cancerisation' describes the occurrence of genetic, epigenetic and biochemical anomalies in structurally intact cells that appear as histologically normal cells lying adjacent to cancerous lesions (Slaughter 1953). The field effect is proposed to be the driver behind the occurrence of multifocal tumours and also for the formation of locally recurrent cancer following surgical resection. Recent studies point to field cancerisation effects in breast cancer (Foschini et al. 2013; Maggrah et al. 2013; Trujillo et al. 2011a, b), (Rivenbark and Coleman 2012). Although the incidence of field cancerisation is reportedly low in breast cancer, local recurrence in other tumours presents as incurability, so developing a comprehensive understanding of the mechanistic basis of field cancerisation is imperative (Trujillo et al. 2011a). Whilst several studies have identified coding genes that contribute to this process in breast cancer (Castro et al. 2008), the role of ncRNAs is yet to be investigated and will likely to be another important area of future investigation. We speculate that ncRNAs will contribute to this process by influencing cell polarity and thus maintaining apparent morphological normality, acting as a tumour suppressor mechanism whilst the cell is accumulating defects in its genome and encoded signalling pathways (reviewed in Lee and Vasioukhin 2008).

Studies into the potential for miRNAs to be either therapeutics or therapeutic targets are showing enormous promise and are likely to develop significantly in the future. MiR-34 is frequently deleted in a range of cancers, resulting in activation of *Myc*, *Bcl2* and *Met* pathways. Using miRNA mimics embedded in lipid nanoparticles, Xue et al. has shown significant suppression of hyperplasia in a K-Ras mouse model of lung cancer and a dramatic increase in the survival of the more aggressive K-rRas G12D x p53$^{flox/flox}$ mouse model (Xue et al. 2014; Misso et al. 2014). These mimics are now en route to the clinic (reviewed in Bouchie 2013). MiR-155, on the other hand, is an 'oncomiR' that is overexpressed in breast and other cancers and functions to regulate cell adhesion and polarity via RhoA (Kong et al. 2008). Recent studies have shown that antisense-miR-155 using pHLIP technology can suppress tumour growth in two models of lymphoma (Cheng et al. 2015). Future priorities in this area include improving the modes of miRNA and antisense miRNA delivery.

Exosomes are small membrane-derived vesicles (40–100 nm) that are able to export proteins, lipids, mRNAs, and microRNAs that can be delivered to recipient cells via fusion to target cell membranes (Mathivanan et al. 2010; Rana et al. 2013). These small vesicular carriers are promising candidates for early detection of cancer, including prostate cancer (Khan et al. 2012). Exosomes are also important in mediating cell-cell communication. MiR-214, a miRNA that controls endothelial cell function and angiogenesis, has been shown to be critical in exosome mediated signalling between endothelial cells (van Balkom et al. 2013). Endothelial exosomes containing miR-214 are able to stimulate migration and angiogenesis in recipient cells, where miR-214 depleted exosomes fail to activate these processes. It is possible that mammary gland development related microRNAs such as miR-212 and miR-312, play a similar role in exosomal signalling by modulation of stromal interactions of breast cancer, a hypothesis that will be worth exploring in the future (Ucar et al. 2010). In cervical and prostate cancer cells the NOTCH pathway target, Survivin, is secreted via exosomes (Khan et al. 2011, 2012). The extracellular pool of Survivin was reported to enhance cell proliferation, growth and tumour cell invasive potential (Khan et al. 2012). These observations raise the interesting idea that exosomes participate in field cancerisation events, delivering exosome

cargos (including: proteins, miRNA, and mRNAs) to cells in the region, and potentially to those further afield – transforming the landscape to a pre-cancerous field. Whether this is the case in breast cancer remains to be established but is certainly an exciting possibility.

This is a very exciting time in ncRNA research. With the availability of comprehensive datasets of ncRNA expression and genome status in multiple cellular environments, including tumours, together with extensive data on the role of ncRNAs in multiple pathways and cellular processes, there is no doubt that ncRNAs are key players in normal biology and disease. For the mammary gland biologist and breast cancer researcher, ncRNAs contribute to mammary cell differentiation, polarity, epithelial to mesenchymal transition and their expression and function is defective, predictive, prognostic and potentially targetable in breast cancer. Future research outcomes for breast cancer will also include novel and more accurate biomarker panels and be targets for new and more effective cancer treatments.

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8 Non-coding RNAs in Prostate Cancer: From Discovery to Clinical Applications

Yvonne Ceder

Abstract

Prostate cancer is a heterogeneous disease for which the molecular mechanisms are still not fully elucidated. Prostate cancer research has traditionally focused on genomic and epigenetic alterations affecting the proteome, but over the last decade non-coding RNAs, especially microRNAs, have been recognized to play a key role in prostate cancer progression. A considerable number of individual microRNAs have been found to be deregulated in prostate cancer and their biological significance elucidated in functional studies. This review will delineate the current advances regarding the involvement of microRNAs and their targets in prostate cancer biology as well as their potential usage in the clinical management of the disease. The main focus will be on microRNAs contributing to initiation and progression of prostate cancer, including androgen signalling, cellular plasticity, stem cells biology and metastatic processes. To conclude, implications on potential future microRNA-based therapeutics based on the recent advances regarding the interplay between microRNAs and their targets are discussed.

Keywords

MicroRNAs • Prostatic neoplasms • Androgen receptor • Epithelial-mesenchymal transition • Neoplasm metastasis

8.1 Introduction

Annually, close to 900,000 new prostate cancer cases are diagnosed worldwide making it the second most frequently diagnosed cancer in men, mainly affecting elderly men. Even though the incident is much higher in developed regions, the mortality is similar in developed and developing

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regions, and constitutes the sixth leading cause of cancer related deaths in men (Ferlay et al. 2010). Prostate cancer is a multifocal and heterogeneous disease, making characterisation challenging (Arora et al. 2004). An in depth examination of the small non-coding RNA (ncRNA) content in prostatic tissues using next generation sequencing identified microRNA (miRNA), small nucleolar RNA (snoRNA), small nuclear RNA (snRNA), ribosomal RNA (rRNA), transfer RNA (tRNA) and fragments of large ncRNA (Martens-Uzunova et al. 2012). The most abundant class of ncRNA detected in the prostate was miRNAs, constituting 95 % of the RNA pool. The first systematic profiling of miRNAs in prostate cancer was presented by Porkka et al. in 2007, and since then several screening studies, and a multitude of individual expression analyses, have been published using different methodology (Volinia et al. 2006; Ambs et al. 2008; Ozen et al. 2008; Lu et al. 2005; Prueitt et al. 2008; Mattie et al. 2006; Tong et al. 2009; Martens-Uzunova et al. 2012; Szczyrba et al. 2010; Wach et al. 2012; Schubert et al. 2013; Porkka et al. 2007). The collective effort towards elucidating the function and mode of action of miRNAs in prostate cancer is increasing, and the impact of individual miRNAs on prostate cancer initiation and progression has been studied from different biological aspects (summarised in Table 8.1). The consensus is that ncRNAs may represent novel therapeutic targets and biomarkers for prostate cancer, but more basic research and clinical studies are needed. In this review, the main biological processes reported to be regulated by miRNAs will be outlined. This includes regulation of androgen signalling and the androgen receptor (AR), the transition to castration resistant prostate cancer (CRPC), cellular plasticity, stem cells and metastases. The best characterised miRNAs regulating these processes in the prostate, or miRNAs suggested to have therapeutic potential, will be presented together with ncRNAs used for diagnostic purposes.

Table 8.1 Regulatory miRNAs confirmed in studies of prostate cancer cells

miRNA	Chromosomal location	Function	Targets	References
Tumour suppressors				
miR-205	1q32	Proliferation, motility, invasion, EMT, adhesion	AR, PKC-ε	Hagman et al. (2013b) and Gandellini et al. (2009, 2012)
miR-133a	18q11, 20q13	Proliferation, invasion, migration	EGFR, PNP	Kojima et al. (2012) and Tao et al. (2012)
miR-143	5q32	Proliferation, migration	KRAS, ERK5, MMP13, FNDC3B	Xu et al. (2011), Clape et al. (2009), Fan et al. (2013), and Wu et al. (2013)
miR-145	5q32	EMT, motility, cell cycle, apoptosis	ERG, CCNA2, Oct4, c-Myc and Klf4	Hart et al. (2013), Ren et al. (2013), and Wang et al. (2009)
miR-16	3q25, 13q14	Apoptosis, proliferation	BCL2, CCDN1, WNT3a	Bonci et al. (2008)
miR-34a	1p36	Proliferation, EMT, stemness	AR, CD44, NOTCH,	Liu et al. (2011a), Ostling et al. (2011), and Kashat et al. (2012)
miR-34c	11q23	Proliferation, apoptosis, EMT, migration,	AR, E2F3, BCL2 and MET	Hagman et al. (2010, 2013a) and Ostling et al. (2011)
miR-200a	1p36	EMT	ZEB1/2	Kong et al. (2009)
miR-31	9q21	Apoptosis, cell cycle	AR, E2F1	Lin et al. (2013)
miR-124	8p23, 8q12, 20q13	Proliferation	AR	Shi et al. (2013)
miR-221	Xp11	Proliferation, androgen independence, migration	p27, HECTD2 and RAB1A, DVL2	Sun et al. (2014), Zheng et al. (2012), and Galardi et al. (2007)

(continued)

Table 8.1 (continued)

miRNA	Chromosomal location	Function	Targets	References
Onco-miRNAs				
miR-32	9q31	apoptosis	BTG2	Jalava et al. (2012)
miR-21	17q23	Invasion, EMT	RECK, BTG2	Coppola et al. (2013) and Reis et al. (2012)
miR-125b	11q24, 21q21	Apoptosis, proliferation	P53, puma, bak1, p14 (ARF), NCOR2	Amir et al. (2013), Yang et al. (2012), and Shi et al. (2011)
miR-106b	7q22	Apoptosis, cell cycle	Caspase-7, E2F1, p21	Hudson et al. (2013) and Ambs et al. (2008)
miR-141	12p13	AR activity, proliferation	Shp, JAG1	Xiao et al. (2012) and Vallejo et al. (2011)

8.2 Androgen Receptor

Androgen signalling through the AR is vital for the development and maintenance of the prostate, as well as governing the initiation and progression of prostate cancer. It has been shown that miRNAs are mediators of androgen action in the prostate and the existence of feedback loops between miRNAs, AR, and AR co-repressors, has been suggested (Narayanan et al. 2010). The AR seem to be able to act through different mechanisms in addition to the AR being recruited to the promoter of target genes, there also seem to exist a three-step pathway including miRNA activation, co-repressor suppression and DNA interaction. In LNCaP cells treated with siRNA against Dicer the induction of androgen-regulated prostate specific antigen (PSA) upon androgen stimulation was abolished, and tissue-specific knockout of Dicer in mouse models significantly reduced the activity of AR resulting in androgen insensitivity syndrome (Narayanan et al. 2010). The AR interaction with Dicer seems to be dependent on the conformational change brought upon by ligand interaction, indicating that the interaction between miRNAs and AR is signal specific. So far only a relatively modest number of miRNAs has been found to be regulated by androgens, compared to the large number of androgen-regulated mRNAs (Jalava et al. 2012; Hagman et al. 2013b; Ostling et al. 2011). In contrast, the regulation of AR seems to be complex. In a functional miRNA library screen using reverse phase protein array 71 unique miRNAs was found to affect AR levels in human prostate cancer cells (Ostling et al. 2011). Fifteen miRNAs down regulating AR, including miR-34a and miR-34c, was further confirmed to decrease androgen-induced proliferation. There are also several examples of indirect regulation of the AR activity e.g. the androgen-regulated miR-27a regulates the AR co-repressor prohibitin, and miR-21 and AR are involved in a positive feedback loop possibly through the phosphatase and tensin homolog (PTEN) (Fletcher et al. 2012; Mishra et al. 2014). Together this corroborates that miRNAs are important regulators of the AR and androgen signalling in normal prostate development, as well as in prostate cancer progression (summarized in Fig. 8.1).

8.2.1 miR-34 Family

The miR-34 family are known as master regulators of tumour suppression. The family is comprised of *mir-34a* located at chromosome 1p36, and *mir-34b* and *-c* clustered at chromosome 11q23. In prostate cancer, all miR-34 family members have been shown to be down regulated, and the expression of miR-34c correlates with the tumour grade, occurrence of metastases, and overall survival (Hagman et al. 2010; Kong et al. 2012). This down regulation has been linked to methylation of the CpG islands in the promoter of *mir-34a* and *mir-34b/c*, loss of het-

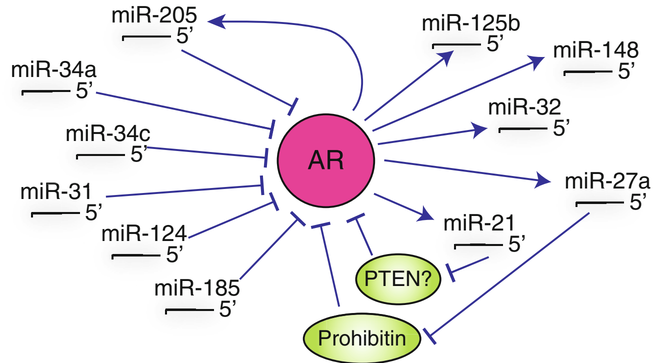

Fig. 8.1 Summary of miRNAs regulating AR and androgen regulated miRNAs (Qu et al. 2013a; Shi et al. 2007, 2013; Lin et al. 2013; Ostling et al. 2011; Hagman et al. 2013b; Jalava et al. 2012; Fletcher et al. 2012; Mishra et al. 2014)

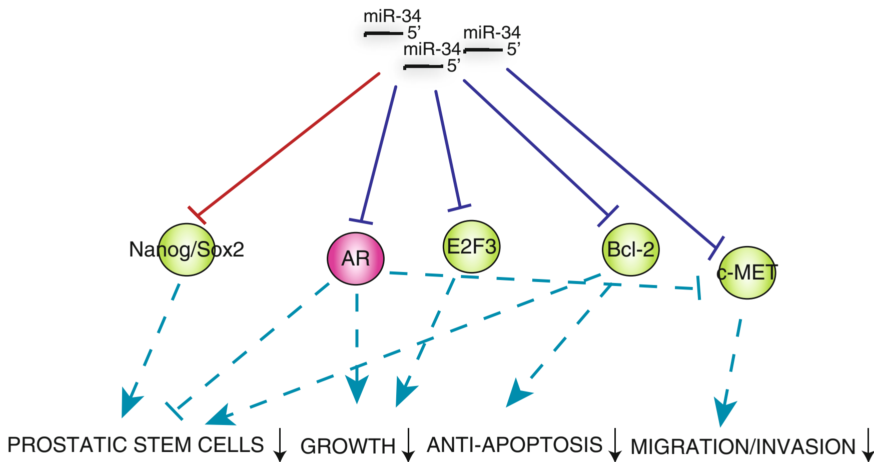

Fig. 8.2 The regulation of processes important for prostate cancer progression by the miR-34 family through key intermediates

erozygosity, and also direct regulation by the cellular tumour antigen p53 in response to DNA damage, hypoxia, and oncogenic stress, or by an alternative ATM-dependent pathway involving the p38-MAPK/MK2 pathway (Cannell et al. 2010; Toyota et al. 2008; Dahiya et al. 1997; Corney et al. 2007). Reconstituted levels of miR-34 can induce changes in proliferation, apoptosis, EMT and migration, and invasiveness of prostate cancer cells in vitro (Fig. 8.2). The family members have overlapping, but not identical targets. In prostate cancer cells, miR-34a has been shown to regulate AR, CD44, and NOTCH (Liu et al. 2011a; Ostling et al. 2011; Kashat et al. 2012), miR-34b regulates RAC-alpha serine/threonine-protein kinase (AKT) and the proto-oncogene protein MYC (Majid et al. 2013; Benassi et al. 2012), and miR-34c regulates AR, the apoptosis regulator BCL2, transcription factor E2F3, hepatocyte growth factor receptor cMET, and MYC (Hagman et al. 2010, 2013a; Ostling et al. 2011; Benassi et al. 2012). The miR-34 family has also been shown to be involved in epithelial-to-mesenchymal transition (EMT), caught up in a double negative feedback loop with Zinc finger protein SNAI1, and a similar feedback loop with the target ZEB1 (Siemens et al. 2011).

It has also been suggested that miR-34 acts as a barrier for somatic cell transition to stem/progenitor cells. Knockout mice of *mir-34a-c* show increased number of induced pluripotent stem cells and reprogramming efficiency without com-

promising self-renewal (Choi et al. 2011). In contrast, miR-34a also has been reported to decreased self-renewal capacity of prostate cancer cells (Kashat et al. 2012). It is possible the miR-34 family members individually have different functions that are modulated when the whole family is altered together. One hypothesis is that the expression of miR-34 family members is one of the mechanisms that keep the normal prostatic stem cells in control, but when the levels decrease upon prostate cancer initiation, the stem cell population is activated. Reintroduction of miR-34a or -b has been shown to significantly decrease androgen-independent prostate xenograft tumour growth in nude mice (Majid et al. 2013; Yamamura et al. 2012). In addition, miR-34a has been shown to reduce prostate cancer metastasis and increase the lifespan of xenografted mice (Liu et al. 2011a). Surprisingly, *mir-34a-c* knockout mice do not show increased effect on induced or spontaneous tumourigenesis (Concepcion et al. 2012). It is possible that this is due to the entire family being altered or that their function is compensated by other systems activated by the feedback loops the family is involved in.

8.3 CRPC

While confined to the prostate gland, the cancer is curable by either prostatectomy or radiation therapy. As the tumour progresses, it develops the abilities to invade surrounding tissue, induce angiogenesis, and metastasize. Androgen deprivation therapy, either chemical or surgical castration, is the gold standard treatment for advanced prostate cancer. Androgen depletion induces apoptosis of prostate cancer cells resulting in tumour regression, but this is followed by subsequent progression to castration resistance. To survive and resume growth in an androgen depleted surrounding, the cells must either adapt the AR pathway or induce alternative survival and growth pathways. Mechanisms underlying adaptation of the AR can be increased expression of AR, increased local production of androgens, hypersensitivity or constitutively active, truncated forms of the AR, promiscuity and/or ligand independent activation through kinase cross-talk. To bypass the AR pathway the epithelial cells might also be able to switch to autocrine production of growth factors such as epidermal growth factor (EGF), insulin-like growth factor (IGF1), hepatocyte growth factor (HGF), keratinocyte growth factor (KGF) or interleukin 6 (IL-6) (Jenster 1999). It has also been reported that individual miRNAs can promote androgen independent growth e.g. miR-21 (Ribas et al. 2009).

8.3.1 miR-21

The miRNA *mir-21* is an oncomiR located at 17q23.1, which is amplified in prostate cancer (Kasahara et al. 2002). The expression of miR-21 is induced by androgens through binding of the AR complex to its promoter region and is highly upregulated in CRPC (Jalava et al. 2012; Ribas et al. 2009). It has been reported that miR-21 promotes androgen resistance, but it is active in both androgen-dependent and -independent prostate cancer, and has been shown to stimulate prostate cancer xenograft growth in mouse models in both a ligand-dependent and -independent manner (Ribas et al. 2009). However, others report that ectopic expression of miR-21 only has a limited effect on cellular proliferation and invasiveness of prostate cancer cells, and contrary to in other cancer settings, miR-21 does not regulate the tumour suppressors PTEN and programmed cell death protein 4 (PDCD4) in prostate cancer cells (Folini et al. 2010). Nevertheless, it has been suggested that miR-21 has an effect on differentiation of prostate cancer cells as expression of miR-21 decreases B-cell translocation gene 2 (*Btg2*) levels, instigating the expression of luminal markers and a switch from epithelial to a mesenchymal phenotype in prostate cancer cells (Coppola et al. 2013). Hypoxia increases the expression of miR-21 and overexpression of miR-21 increases the levels of hypoxia-inducible factor 1-alpha (HIF1α) and vascular endothelial growth factor (VEGF), as well as AKT and extracellular signal-regulated kinase (ERK) through targeting of *Pten* leading to increased tumour angiogenesis (Liu et al. 2011b; Bao et al. 2012).

Another target of miR-21 is *Reck*, a known regulator of tumour cell invasion (Reis et al. 2012). In conclusion, miR-21 might contribute to the tumourigenesis by affecting several pathways leading to increased proliferation, EMT, angiogenesis and invasion. In addition, serum and plasma levels of miR-21 have been shown to be associated with aggressive prostate cancer, including castration resistant growth and metastases (Shen et al. 2012; Zhang et al. 2011; Yaman Agaoglu et al. 2011).

8.4 EMT and Cellular Plasticity

The transition from an epithelial to a mesenchymal phenotype plays a key role in prostate cancer progression. Tumour cells undergo EMT to be able to disseminate, yet the resulting metastases exhibit epithelial phenotypes. Hence, after seeding at the secondary sight, the cell has to undergo the reversal of EMT, i.e. mesenchymal-to-epithelial transition (MET), in order to proliferate and establish macrometastases. A finding supporting this plasticity scenario was reported in a murine prostate model in which 24 transcripts were compared in GFP-tagged PC3 primary tumour cells, circulating tumour cells (CTCs) and metastases. The primary and metastatic signatures were close to identical, whereas the CTCs signature stood out, for example *Bcl2* expression was increased in CTCs compared to the primary and metastatic tumour cells (Helzer et al. 2009). Disseminated metastatic cells can remain dormant for a variable period of time before forming secondary tumour sites. These secondary tumours may then shed novel metastatic cells to the blood stream resulting in multiple metastatic sites even if the primary tumour has been removed. It seems that the cells must exhibit phenotypic plasticity, a transient EMT-MET process, in response to the changing microenvironment as they invade through the basement membrane, enter and exit the circulation, and survive and grow at distant locations. Transcription factors, including SNAI1, SNAI2, TWIST and ZEB1/2, have been shown to regulate epithelial-mesenchymal plasticity. But recently, miRNAs have also been shown to be crucial regulators of EMT and cancer cell invasion. In fact, several studies suggest that cellular plasticity is governed by reciprocal feedback loops between miRNAs and their EMT-inducing targets (Fig. 8.3); e.g. the miR-200 family and ZEB1/2 (Burk et al. 2008; Bracken et al. 2008), miR-203 and SNAI1/2 (Qu et al. 2013b) and the miR-34 family and SNAI1 (Siemens et al. 2011). It is possible that these and similar feedback loops regulate the reversible phenotypic switch that allows tumour cells to exhibit EMT/MET plasticity in response to the microenvironment.

8.4.1 miR-200

The miR-200 family members have been described to act as tumour suppressors. As mentioned, miR-200 can repress expression of ZEB1 and ZEB2 transcription factors through direct tar-

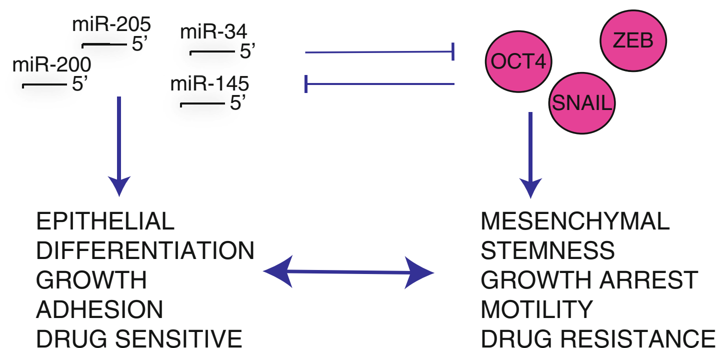

Fig. 8.3 Simplified illustration of the reciprocal feedback loops between certain miRNAs and their targets resulting in phenotypic plasticity

geting, leading to enhanced E-cadherin expression and inhibition of EMT. Conversely, ZEB1 and ZEB2 repress miR-200 expression by binding to the promoter of the *mir-200* encoding gene cluster, forming a double negative feedback loop controlling expressions of both during EMT. *Snai2* is another target of miR-200, and conversely SNAI2 is a direct repressor of miR-200 expression (Liu et al. 2013). In benign prostate cells, SNAI2 was found to be important for EMT initiation, while ZEB in cooperation with the miR-200 family opposed the reversal of the EMT (Slabakova et al. 2011). By preventing EMT, miR-200 prevents invasion and distant metastasis. Depletion of SNAI2 inhibits EMT during tumourigenesis, whereas reintroduction of miR-200 inhibited both EMT and tumourigenesis in human and mouse model systems (Liu et al. 2013). In concordance with these findings, decreased miR-200 expression has been associated with the acquisition of cancer stem cell traits and tumour-initiating capacity in other cancer settings. For example, the miR-200 family members also target Notch pathway components, such as *Jag1*, *Notch1*, mastermind-like gene 2 (*Mam2*) and *Mam3* (Brabletz et al. 2011; Kong et al. 2009). Further, it has been suggested that miR-200 plays a key role in linking the characteristics of cancer stem-like cells with EMT-like cells in prostate cancer. Cells with the EMT phenotype display stem-like cell features and decreased expression of miR-200, but re-introduction of miR-200 lead to reversal of EMT, reduced prostasphere formation, and expression of *Notch1* and *Lin28b* (Kong et al. 2009). It is possible that miR-200 is essential for cellular plasticity of cancer stem cells and hence of driving cancer progression towards metastasis. The levels of this potent molecule is tightly regulated by multiple reciprocal feedback loops with their targets and this might explain why miR-200 has not been found to be deregulated when identifying general expression patterns in larger prostate cancer cohorts (Porkka et al. 2007; Martens-Uzunova et al. 2012). However, in a study identifying the miRNA profile of primary prostate cancers using deep sequencing, miR-200c was found to be the most common transcript representing approximately 10 % of all miRNAs in pooled prostate cancer tissue (Szczyrba et al. 2010).

8.5 Stem Cells

The prostate contains a subpopulation of cells that do not depend directly on androgens for their survival; the prostate epithelial stem cells. Prostate stem cells are proposed to be present in the basal cell layer and, when dividing, give rise to another stem cell and a daughter progenitor cell/transit amplifying cell, which after a few divisions differentiate into end stage secretory luminal cells. They have the capacity to make the tumour recur from a single cell (Leong et al. 2008). Androgen deprivation therapy leads to expansion of the existing population of stem/progenitor cells (Lee et al. 2013). miRNAs, e.g. miR-145, have been suggested to play a central role in stem cell biology and regulate vital features such as self-renewal, pluripotency and differentiation. Further, exposure to increasing concentrations of chemotherapy has been shown to correlate to cancer stem cell-like traits and induction of EMT through downregulation of miRNAs such as miR-205 (Puhr et al. 2012).

8.5.1 miR-145

The intergenic *MIR145* is located on chromosome 5q32, and is co-transcribed with *MIR143*. The level of miR-145 is consistently reported to be decreased in prostate cancer (Larne et al. 2013; Wach et al. 2012; Ozen et al. 2008). A possible mechanism for the down regulation of miR-145 is methylation of the promoter as has been reported for the prostate cancer cell lines PC3, DU145 and LNCaP (Suh et al. 2011). miR-145 has also been suggested to be transcriptionally activated by p53, which frequently is mutated in advanced prostate cancer cells, and repressed by IL6, which is commonly up regulated in prostate cancer (Sachdeva et al. 2009; Suh et al. 2011; Zaman et al. 2010). Several reports also indicate that miR-145 is further decreased in metastatic prostate cancer, especially bone metastases

compared to localised prostate cancer (Leite et al. 2013; Peng et al. 2011). This agrees well with the described tumour suppressive functions of miR-145 in prostate cancer. It has been shown that miR-145 target oncogenic pathways such as C-myc (Sachdeva et al. 2009) and Ras (Kent et al. 2010), and is involved in the regulation of EMT and invasion (Peng et al. 2011; Guo et al. 2013). miR-145 also inhibits tumour growth and bone metastases of PC3 cells by repressing cancer stem cell properties in vivo, and in cooperation with miR-143, mir-145 supress prostatic tumour sphere formation and stemness markers in PC3 cells (Huang et al. 2012). Further, miR-145 inhibit stem cell renewal and pluripotency by targeting OCT4, SOX2 and Krueppel-like factor 4, and the reciprocal inhibition of miR-145 and OCT4 is believed to establish an irreversible switch priming cells to enter the differentiation program (Jain et al. 2012; Xu et al. 2009; Hu et al. 2012).

8.6 Metastases

Metastatic disease is the major cause of cancer-related deaths in men with prostate cancer, the 5 year survival rate is only 32 % compared to almost 100 % in localised early stages (Jemal et al. 2008). The development of metastases is a complex and dynamic process, involving detachment of the tumour cells from the primary site, entering and surviving in the bloodstream, migrating to distant locations where they extravasate and establish secondary tumours. Only a small fraction of the tumour cells in circulation give rise to distant metastasis, it has been suggested that cellular plasticity and transient acquisition of stem cell characteristics is necessary. The prostate cancer metastases are predominantly detectable in bone; autopsies reveal the presence of bone metastases in ~90 % of men with spread prostate cancer (Bubendorf et al. 2000). In bone, the prostate cancer induces the formation of lesions that are primarily osteoblastic in nature, causing the patient to experience severe bone pain and skeletal fragility. The prostate cancer cells at the metastatic site might still respond to androgen ablation but will also at the distant sites transform to CRPC. It has been shown that androgen deprivation therapy induces EMT and expansion of the existing population of stem cells, as a consequence the transition to CRPC is associated with increased incidence of metastases (Sun et al. 2012; Lee et al. 2013). While it is clear that the tumour microenvironment play a crucial role in determining the lethal phenotype of cancer cells, the molecular events associated with metastasis, homing to bone and colonization, invasion and survival at the secondary site are not well understood. It has been shown that miRNA expression in the primary tumour correlates to metastatic disease and also that a certain miRNA signature, the miRNA index quote (miQ), is an independent predictor of metastases events occurring 0.5–10 years after the removal of the primary tumour (Larne et al. 2013).

8.6.1 miR-205

The miR-205 encoding gene is located within the gene *LOC642587* of unknown function at chromosome 1q32, and has been shown to have decreased expression in prostate cancer (Majid et al. 2010; Gandellini et al. 2009). The miR-205 expression is regulated by p53, p63 and epigenetic silencing (Piovan et al. 2012; Wiklund et al. 2011; Hulf et al. 2013). In addition, the miR-205 expression is mainly localized to the basal epithelial cells, but as these cells disappear or differentiate during prostate cancer progression, this conceivably result in a loss of miR-205 expression (Gandellini et al. 2012; Zhang et al. 2010; Hagman et al. 2013b). It is reasonably a combination of these regulatory events that are responsible for the decrease of miR-205 levels corresponding to prostate cancer progression that is described in several independent studies (Majid et al. 2010; Gandellini et al. 2009; Hagman et al. 2013b). The expression of miR-205 is also inversely correlated to occurrence of metastases, castration resistant and shortened overall survival (Hagman et al. 2013b). In concordance with this, there are several reports indicating that miR-205 act as a tumour suppressor in prostate cancer

cells. miR-205 has been shown to directly regulate PKC-epsilon resulting in an effect on migration and invasiveness of prostate cancer cells (Wu et al. 2009). The expression of miR-205 decreases 100-fold when cells undergo EMT, but also contribute to EMT by targeting ZEB1/2, the transcriptional repressors of E-cadherin, this also contribute to enhanced migration (Gregory et al. 2008; Tucci et al. 2012). In addition, miR-205 has been shown to directly target the AR, a finding that was corroborated in a patient cohort were miR-205 expression inversely correlated to AR immunostaining in malignant prostate cells and to serum levels of the androgen regulated PSA (Hagman et al. 2013b). During prostate cancer progression, miR-205 levels decrease and this seem to result in activated AR signalling and increased migratory potential.

8.6.2 miR-15a/16

The first miRNA encoding genes identified to be frequently deleted in cancer was MIR15A and −16–1 located at chromosome 13q14 (Calin et al. 2002). They have also been found to be homozygously deleted in a subset of prostate cancers and to correlate with tumour progression (Porkka et al. 2011; Dong et al. 2001; Hyytinen et al. 1999). Loss of miR-15a/16 induce cellular proliferation in prostate cancer cells and restoration of miR-15a/16 result in growth arrest, apoptosis, and regression of prostate tumours in xenograft models (Cimmino et al. 2005; Bonci et al. 2008). These miRNAs promote apoptosis by targeting among others *Bcl2* (Bonci et al. 2008). Prostate luminal stem cells express *Bcl2* (Ceder et al. 2008) and it is plausible that miR-16 has an impact on the sensitivity to apoptosis in these cells. miR-15/16 have also been found to be down regulated in fibroblasts surrounding prostate tumours, and to repress fibroblast growth factor 2 (FGF2) and its receptor, which act on both stromal and tumour cells to enhance cancer cell survival, proliferation and migration (Musumeci et al. 2011). In a xenograph bone metastasis model, injection of miR-16 via the tail vein significantly inhibited the growth of prostate tumours in bone (Takeshita et al. 2010).

8.7 ncRNAs with Diagnostic Potential

A special characteristic of prostate cancer is that the latent form of the disease is very common; microscopic lesions are found in more than 50 % of 70–80 year old men (Gronberg 2003). However, most cases will never experience cancer symptoms during their lifetime. The current clinical practice for diagnosis and decision making of prostate cancer involve digital rectal examination, serum PSA and subsequent biopsies for histopathological staging and Gleason scoring. Each of these methods has its shortcomings and today a momentous problem is over diagnosis and treatment of patients with indolent prostate cancer. The management of prostate cancer would benefit from better tools for detection, prognosis and treatment response. The miRNAs are technically suitable as biomarkers as they are deregulated in prostate cancer, easy to detect and found to be stable in plasma, serum, fresh frozen, and formalin fixed paraffin embedded tissues. Many studies highlight the diagnostic and prognostic potential of individual miRNAs, however, no single miRNA has been consistently validated or implemented as a biomarker in clinical management of prostate cancer.

8.7.1 miRNAs Signatures

Lately several studies focusing on different miRNA signatures have been published. By extensive microarray analyses Martens-Uzunova et al. derived a miRNA diagnostic classifier that distinguishes prostate cancer from benign specimens; this classifier contains 54 miRNAs and gives an area under the curve of 0.95. The same team also constructed a prognostic signature consisting of 25 miRNAs that was able to independently predict postoperative outcome (Martens-Uzunova et al. 2012). Even smaller

number of prostate derived miRNAs has successfully been combined into quotes e.g. the miQ; ((miR-96 × miR-183)/(miR-145 × miR-221)), was found to successfully predict diagnosis with high accuracy in several independent cohorts, and also has prognostic power to predict aggressiveness of tumours, metastatic status, and overall survival (Larne et al. 2013). The advantage using a quote is increased discrimination, no need for housekeepings, and most important it may be an advantage considering the heterogeneity of the disease. There have also been preliminary data of non-invasive miRNA signatures that have prognostic properties e.g. miR-141 + miR151-3p + miR-16, that could discriminating between metastatic CRPC and localized prostate cancer in a cohort of 50 men (Watahiki et al. 2013). However, recent reports highlight the need for caution in the interpretation of the cancer-specificity of circulation miRNAs. It has been reported that 58 % of the published circulating miRNA biomarkers are highly expressed in hematopoietic cells, e.g. miR-16 is expressed in red blood cells, and that the levels of miR-21 and miR-141 in circulating are unaltered by radical prostatectomy raising questions of the origin of these miRNAs (Pritchard et al. 2012; Egidi et al. 2013).

8.7.2 *PCA3*

The only ncRNA that has made it all the way into clinical practice is the long ncRNA, prostate cancer antigen 3 (*PCA3*). *PCA3* is only expressed in the prostate, and it is highly overexpressed in 95 % of prostate cancer cells. Although the mechanisms of action are still unknown, *PCA3* has shown to be a useful prostate cancer biomarker. It can be detected by PCR in urine obtained after digital rectal examination (Bussemakers et al. 1999; Hessels et al. 2003). The urinary based test *PCA3*-to-PSA transcript has been approved for use in men suspected to have prostate cancer due to digital rectal examination and PSA but the first prostate biopsies is negative, as it has been shown to be useful to predict the presence of malignancy in this setting, and can thus reduce the number of unnecessary prostate biopsy (Haese et al. 2008; Marks et al. 2007). The *PCA3* score has also been shown to predict tumour volume, which might help in selecting prostate cancer patients for active surveillance (Ploussard et al. 2011).

8.8 Conclusions and Future Perspective

A decade ago small ncRNAs was first discovered to be involved in carcinogenesis (Chan et al. 2005; Calin et al. 2002). Now they are at the centre of attention and suggested to be suitable in the clinical management for most cancer forms. The miRNAs are promising biomarkers as they are deregulated, stable in both tissue and body fluids and easy to detect. As the technology will become even cheaper and more accessible the knowledge of the role of the ncRNAs and their potential applications will increase. It is conceivable that the miRNA signatures in circulating tumour cells or cancer-cell secreted microvesicles can enable personalized and patient tailored treatments for prostate cancer patients.

Individual miRNAs have evolved to coordinate the regulation of groups of molecules involved in essential cellular functions in the prostate. Prostate cancer progression is driven by not one but an array of genetic mutations and epigenetic alterations, individual miRNAs might provide a strategy to target systems rather than one molecule at a time in a fine-tuned manner. During the multi-step cascade of transient processes the tumour cells have to go through to enable e.g. metastatic spread, it is reasonable to speculate that reversible epigenetic modification is more likely to be regulating the balance between these events rather than fixed genetic alterations. In this context, it is interesting to note that the majority of miRNAs discussed in this review are epigenetically regulated. This also points to the very interesting interference option to reverse these processes by targeting the miRNAs.

However, it has lately become apparent that the miRNAs interact with their targets in more intricate manners than initially recognized. It has been discovered that many miRNAs are involved in both positive and negative feedback loops with their targets, hypothetically enhancing the robustness of gene regulation by creating a homeostasis between miRNAs and their targets. The tumour suppressor miR-34a is involved in a positive feedback loop with p53; miR-34a inhibits the expression of NAD-dependent protein deacetylase sirtuin-1 (SIRT1) that activates p53 (Yamakuchi and Lowenstein 2009). Loss or mutation of p53 is rare in primary prostate cancer but frequent in advanced cases. It is possible that reintroduction of miR-34a in the prostate cells with disturbed p53 function would lead to the expected tumour inhibition, but in adjacent cells with WT p53 it could potentially give negative long term effects of increased mutation rates leading to accelerated tumourigenesis. Then there is the heterogeneity of the 3′UTRs of the targets to take into considerations. It has lately been shown that over 50 % of all genes have alternative polyadenylation signals, and shorter 3′UTRs would lead to altered miRNA susceptibility in a specific setting (Tian et al. 2005). However, with increase knowledge about these changes, it is possible that this could be an advantage when designing specific gene targeting. Another factor complicating future miRNA based therapeutics might be target competition (Poliseno et al. 2010). Each miRNA targets several genes, as the levels of one of the targeted transcripts are increased in malignant tissues the available level of the inhibiting miRNA are decreased, and the repressive effect this is having on other transcripts might be relieved through target competition. This highlights the importance of targeted expression in the right setting and also the importance of selection of eligible patients. More in depth pre-clinical studies are needed to investigate the interplay between miRNAs and their targets in prostate cancer cells and the long term effects of manipulation of these delicate networks, as well as improved strategies for deregulation of the miRNAs in a prostate specific manner.

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9 Non-coding RNAs in Uterine Development, Function and Disease

Warren B. Nothnick

Abstract

The major function of the uterus is to accept and provide a suitable environment for an embryo, ultimately leading the birth of offspring and successful propagation of the species. For this occur, there must be precise coordination of hormonal signalling within both the endometrial and myometrial components of this organ. Non-coding RNAs, specifically, microRNAs (miRNAs) have been shown to be essential for normal uterine development and function. Within this organ, miRNAs are proposed to fine-tune the actions of the female steroid hormones estradiol and progesterone. Not surprising, mis-expression of miRNAs has been documented in diseases of the endometrium and myometrium such as endometriosis and leiomyomas, respectively. In this chapter, I will review the current understanding on the role, regulation and function of non-coding RNAs focusing on miRNAs in both the normal physiology of the endometrium and myometrium as well as in pathologies of these tissues, namely endometriosis and leiomyomas.

Keywords

Uterus • Endometrium • Myometrium • Endometriosis • Leiomyoma • miRNA

9.1 Introduction

The uterus, often referred to as the womb, is an essential female organ necessary for successful reproduction. The proper development and function of the uterus is dependent on the proper interactions of a complex system involving gene transcription, post-transcriptional regulation and protein translation. This review will focus on the

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role of non-coding RNAs (ncRNAs) in the development and function of the uterus, with particular emphasis on the role of short RNAs such as microRNAs (miRNAs).

Non-coding RNAs (ncRNAs) are functional RNA molecules that are not translated into proteins and can be classified into long ncRNAs and short ncRNAs. While ncRNAs do not code for proteins, many of these molecules none the less have an important role in modulation of gene and protein expression. By far the examination of small RNAs, such as miRNAs, has gained the most attention of all of the ncRNAs (Taft et al. 2010). Not surprisingly, the majority of research conducted to understand the expression and function of small RNAs within the female reproductive tract has focused primarily on miRNAs. The objective of this chapter is to highlight the expression, regulation and functional role of miRNAs in uterine development and diseases.

9.2 Role of miRNAs/Small RNAs in Uterine Development and Function

The uterus is composed of three tissue layers, the endometrium, the myometrium and the perimetrium. The endometrium is the inner most layer or the lining of the uterine cavity. The endometrium is hormone-responsive and, in response to successive action of oestrogen and progesterone, provides the necessary environment for embryo attachment and establishment of pregnancy. The myometrium is the muscular layer, which separates the endometrium from the outer most layer, the perimetrium. While the myometrium remains relatively quiescent during the course of the reproductive cycle, the major function of the myometrium is to provide the contractile force necessary for expulsion of the foetus at the time of parturition.

Using animal models, which are genetically depleted of specific components of the small RNA/miRNA biogenesis pathway, it has been clearly established that this class of ncRNAs is essential for normal uterine development and function. DICER (DICER1 in mice) is an RNAse III enzyme that cleaves pre-miRNAs into transient RNA duplexes, which results in the generation of two strands of RNA. One of the strands is degraded while the remaining strand is the mature miRNA (Bartel 2004). Nagaraja and colleagues (2008) demonstrated that female mice in which *Dicer1* was inactivated in Müllerian duct mesenchyme-derived tissues of the reproductive tract were sterile and displayed decreased ovulation rates, altered oocyte/embryo integrity, oviductual cysts and shorter uterine horns. Similarly, Hong and coworkers (2008) observed similar oviduct, ovulatory and uterine defects, but no significant impact of *Dicer1* deletion on embryo development in vitro. However, embryos collected from day 3 of pregnancy in vivo were developmentally delayed in *Dicer1*-deficient mice compared to wild-type counterparts. Gonzalez and Behringer (2009) also observed similar defects within the oviduct, degenerated/unfertilized oocytes within the oviductal cysts and an inability to establish pregnancy. Further histological analysis demonstrated that *Dicer1*-deficient uteri contained less glandular tissue and exhibited what appeared to be early stage adenomyosis or growth of endometrial glands within the myometrial tissue layer. Taken together, the infertility and reproductive tract abnormalities characteristic of *Dicer1*-deficient female mice strongly suggest that DICER1 function and miRNA mediated post-transcriptional gene regulation are essential for normal female reproductive tract development and function.

With respect to the role of miRNAs in uterine tissue, although the first reports were in the areas of endometriosis (Pan et al. 2007) and uterine fibroids/leiomyomas (Wang et al. 2007), the majority of research has focused on the role of miRNAs in endometrial cancer. As it is beyond the scope of this review to cover in detail the role of these small RNAs in endometrial cancer, the reader is referred to reviews on this topic (Banno et al. 2013; Gilabert-Estelles et al. 2012; Lee et al. 2011). In this chapter, we will focus on the expression of the small non-coding RNAs, miRNAs, and their function within the uterus, with emphasis in endometrium and myometrium under normal and pathological states.

9.3 miRNAs in Endometrial Physiology and Pathology

9.3.1 Expression and Function of miRNAs in the Endometrium

The endometrium is the innermost layer of the uterus and functions to provide a suitable environment for establishment of pregnancy (embryo implantation) in response to the changing sex steroid levels that occur during the course of the menstrual/reproductive cycle. The first assessment of endometrial miRNA expression was conducted by Pan and colleagues (2007) using endometrial tissue from "normal" women compared to endometrium from women with endometriosis obtained during the early to mid-secretory stage of the menstrual cycle. Sixty-five miRNAs were detected above a predetermined threshold level. In endometrium from "normal" women (women without endometriosis in this study), miR-125b, miR-21, miR-145, miR-26a, miR-23b, miR-29a, and miR-99a were among the most abundantly expressed miRNAs. In ectopic (implant) and eutopic endometrium from women with endometriosis, there was a significant reduction in the expression of all of these miRNAs as well as several other miRNAs, with miR-451 being one of the most reduced miRNAs in the tissues from women with endometriosis. MicroRNA expression in normal endometrium was further characterized into miRNA expressed by endometrial stromal cells and glandular epithelial cells. Thirty-two miRNAs were shown to be differentially expressed between cell types. Of these, miR-20a, miR-21, and miR-26a were further assessed for steroidal regulation (Table 9.1).

This same group (Toloubeydokhti et al. 2008) conducted a follow-up study in which miR-17-5p, miR-23a, miR-23b, and miR-542-3p expression was assessed (Table 9.1). Findings

Table 9.1 Endometrial miRNAs whose expression is regulated by estradiol and/or progesterone

miRNA	Cell/tissue type	Steroidal regulation	References
miR-20a	Human stromal cells[a]	*E2[b] ↓ / MPA[c] ↓*	Pan et al. (2007)
miR-21		*E2 ↓ / MPA ↓*	
miR-26a		*E2 ↓ / MPA ↓*	
miR-20a	Human glandular epithelial cells	*E2 ↓ / MPA ↑*	Pan et al. (2007)
miR-21		*E2 ↓ / MPA ↓*	
miR-26a		*E2 ↑ / MPA ↑*	
miR-17-5p	Human stromal cells	*E2 ↑ / MPA ↑*	Toloubeydokhti et al. (2008)
miR-542-3p		*E2 ↑ / MPA ↑*	
miR-23a		*E2 ↓ / MPA ↓*	
miR-23b		*E2 ↓ / MPA no affect*	
miR-17-5p	Human glandular epithelial cells	*E2 ↑ / MPA ↑*	Toloubeydokhti et al. (2008)
miR-542-3p		*E2 ↓ / MPA ↓*	
miR-23a		*E2 ↓ / MPA ↑*	
miR-23b		*E2 ↑ / MPA ↑*	
miR-155	Mouse uterus[d]	*E2 ↑*	Nothnick and Healy (2010)
miR-429			
miR-451			
miR-181b	Mouse uterus	*E2 ↓*	Nothnick and Healy (2010)
miR-204			

[a]All studies using human endometrial stromal or epithelial cells were conducted in vitro
[b]E2 = estradiol 17beta
[c]MPA = medroxyprogesterone acetate
[d]All studies using mouse uterine tissue were conducted in vivo

from this study revealed that miR-23b and miR-542-3p were expressed in lower levels and that miR-17-5p was expressed at higher levels in paired eutopic and ectopic endometrial tissue compared to "normal" eutopic endometrium and that oestradiol and MPA could regulate expression of all three of these miRNAs (Table 9.1). In this and the previously cited study (Pan et al. 2007), steroidal regulation could be blocked for some of the miRNAs using the oestrogen receptor antagonist ICI-182780, as well as the progesterone receptor antagonist RU-486. These observations suggest that steroidal regulation of miRNAs in endometrial stromal and glandular epithelial cells appears to occur via complex mechanisms.

A similar experimental design was implemented to compare miRNA expression between early secretory endometrium from women with and without endometriosis (Burney et al. 2009). Significantly lower levels of miR-34c-3p, miR-34c-5p, miR-9, miR-9*, miR-34b*, and the unannotated miRPlus_42 780 were expressed in the early secretory endometrium from women with endometriosis. Although additional miRNA profile information was not provided in this report, one may conclude that these miRNAs are expressed in early secretory endometrium from women without endometriosis and may play a role within the endometrium during the menstrual cycle.

The first report to examine hormonal regulation of miRNAs in the human endometrium to gain insight into the mechanisms of the opposing action of progesterone on that of oestradiol during the period of embryo implantation was conducted by Kuokkanen and colleagues (2010). Using isolated endometrial epithelial cells from women in the late proliferative versus the mid-secretory stages of the menstrual cycle, it was demonstrated that 12 miRNAs were expressed at significantly higher levels in the late proliferative stage (miR-29b, miR-29c, miR-30b, miR-30d, miR-31, miR-193a-3p, miR-203, miR-204, miR-200c, miR-210, miR-582-5p, and miR-345) compared to the mid-secretory stage. Based upon the up-regulation of these miRNAs coupled with the fact that these miRNAs are proposed to target cell cycle genes, it is tempting to speculate that these miRNAs may suppress cell proliferation during the secretory phase of the menstrual cycle.

In subsequent studies, miR-30b and miR-30d were further proposed to regulate human endometrial receptivity. First, Sha and colleagues (2011) performed a genome-wide analysis of small RNAs/miRNAs associated with endometrial receptivity in a population of women undergoing in vitro fertilization treatment. Comparing 7 days post LH surge to 2 days post LH surge (with 7 days post LH surge being representative of the "receptive" state of the endometrium), 20 miRNAs were determined to be differentially expressed. Of these, miR-30d, miR30b*, miR-31, and miR-30b showed the most significant up-regulation (6.92, 3.97, 3.32, and 2.99-fold, respectively).

Secondly, Altmäe and coworkers (2013) also compared 7 days post LH surge to 2 days post LH surge and again found that miR-30b and miR-30d were significantly up-regulated in the former group compared to the latter. Further, miR-494 and miR-923 were both down-regulated and predicted to target leukemia inhibitory factor (LIF), which has been proposed as a major factor necessary for embryo implantation in mammals (reviewed in Stewart 1994).

It should be noted that in both of these studies, the up-regulation of miR-30b and miR-30d were in agreement with the work of Kuokkanen et al. (2010). Collectively, both studies suggest that miR-30b and miR-30d up-regulation during the window of implantation may function to dampen expression of potential protein targets associated with cell cycle regulation such as CCNB1, RASSF2, and MMP7. A limitation to these studies as a whole was that data were restricted to miRNA profiling and prediction of their target genes by *in silico* algorithms. Functional studies demonstrating that these miRNAs target these proposed genes within the endometrium remain to be conducted.

Additional information with respect to miRNA mis-expression and altered embryo implantation was reported in 2011 by Petracco and colleagues. Homeobox A10 (HOXA10) is essential for normal uterine function/embryo implantation

(Taylor et al. 1998) and is mis-expressed in the endometrium from women with endometriosis (Taylor et al. 1999). As *HOXA10* is a proposed target of miR-135a/b, Petracco and colleagues (2011) examined miR-135a/b expression in endometrium from women with and without endometriosis and found that miR-135a/b was significantly increased in eutopic endometrial tissue from women with endometriosis and this elevated expression was associated with reduced levels of *HOXA10* transcript expression (Fig. 9.1). Additional studies demonstrated that miR-135a/b bound to the 3′ UTR of *HOXA10* as well as modulated *HOXA10* transcript and protein expression in cultured endometrial stromal cells. These studies suggest that the over-expression of miR-135a/b in endometrium of women with endometriosis, a disease associated with impaired embryo implantation, may lead to reduced levels of HOXA10 expression which is necessary for embryo implantation and is one of the few studies demonstrating expression and function for a given miRNA in human endometrial tissue (Fig. 9.1).

In addition to information gleaned from studies that incorporated human endometrial tissue, there is also limited information on miRNA profiles in mouse uterine tissue, with the bulk focusing on identifying miRNAs that are associated with and may play a role in embryo implantation. Initial characterization was performed by Chakrabarty and colleagues (2007) in which expression profiling was performed with oligonucleotide microarrays and compared the expression patterns of 380 miRNAs common to humans, mice, and rats, in day 1 (pre-receptive; oestrogen dominance) and day 4 (receptive; progesterone dominance) pregnant mouse uteri, with day 1

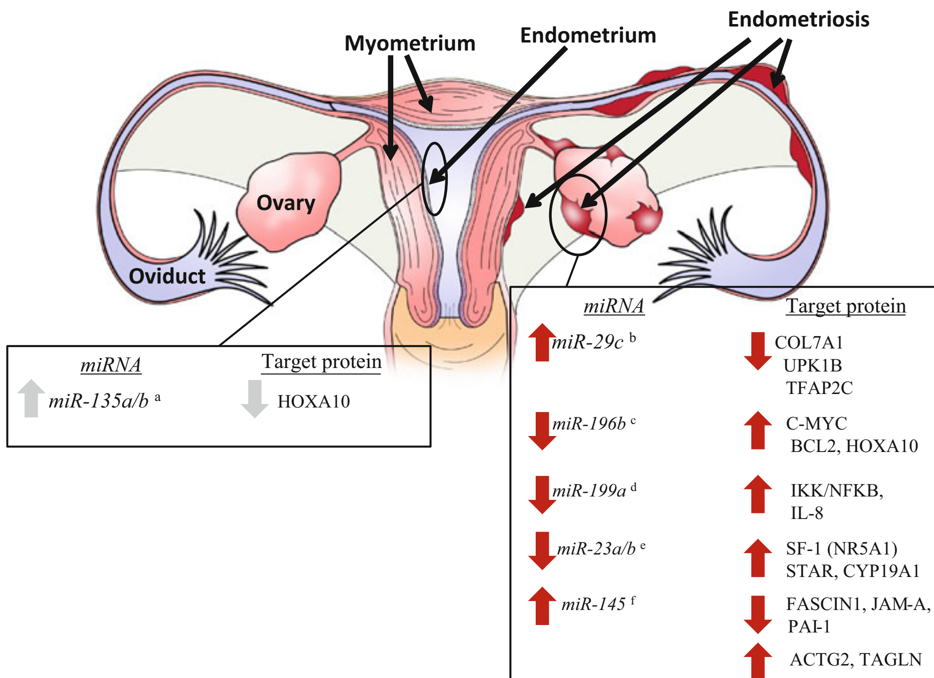

Fig. 9.1 miRNAs and their validated targets in endometrial and endometriotic tissue. *Left table* summarizes miRNAs and their predicted transcript/protein in endometrial tissue (indicated as the *gray lining* of the uterus in the figure) derived from studies incorporating human endometrial cells which have been validated. *Right table* summarizes miRNAs and their predicted transcript/protein in endometriotic tissue (indicated as *red* ectopic tissue in the figure) derived from studies incorporating human cells which have been validated. Superscripts refer to references with *a* = Petracco et al. 2011; *b* = Hawkins et al. 2011; *c* = Abe et al. 2013; *d* = Dai et al. 2012; *e* = Shen et al. 2013; *f* = Adammek et al. 2013. Validation refers to either assessing cellular events and/or mRNA and protein levels by modulation of endogenous miRNA levels

representing the presence of a vaginal plug. Thirty-two miRNAs were up-regulated during the receptive phase (progesterone dominance) and five were down-regulated compared to the pre-receptive (oestrogen dominance) phase. Of those up-regulated, miR-101a and miR-199a* were further examined and verified to regulate cyclo-oxygenase-2 (*Cox2*) expression, encoding an enzyme which is essential for embryo implantation. Additional studies have suggested that miR-21 (Hu et al. 2008), let-7a (Xia et al. 2010a) and miR-320 (Xia et al. 2010b) may be important for embryo implantation in rodents.

My laboratory (Nothnick and Healy 2010) recently utilized miRNA arrays followed by qRT-PCR validation to demonstrate that oestradiol up-regulates miR-155, miR-429, and miR-451 expression in mouse uterine tissue, but decreases the expression of miR-181b and miR-204 (Table 9.1). Use of ICI 182,780 validated that this regulation was mediated via the classical oestrogen receptor pathway. Additional unpublished observations from our research group demonstrate that within the mouse uterus from ovariectomized mice, miR-709 is the most abundant miRNA followed by the let-7 family members and miR-26a. Mis-expression of miRNAs such as miR-709 has been proposed to contribute to the discordant pattern of expression between MMP9 protein and transcript expression detected in oestradiol-primed mouse uterine tissue (Nothnick 2008).

Oestradiol is a known proliferative agent within the endometrium and its actions thought to be fine-tuned through miRNAs (Lessey 2010). Oestradiol increased oestrogen receptor positive endometrial adenocarcinoma cell proliferation which was associated with an up-regulation of BCL2 and concurrent down-regulation of *Bax* (Zhang et al. 2012). As *Bax* repression was determined to occur post-transcriptionally, miRNA assessment revealed that members of the let-7 family (let7a–g) and miR-27a targeted *Bax* transcript. Surprisingly, as discussed above, none of these miRNAs were up-regulated in human endometrial cells or mouse uterus in response to oestrogen administration.

From our current knowledge, it appears that endometrial miRNAs are regulated by both oestradiol and progesterone. Some of these miRNAs appear to be modulated through the classical nuclear steroid receptor pathway while the regulation of others appears to be more complex. Future assessment will need to focus on the specific mechanisms by which oestradiol and progesterone modulate endometrial miRNA expression and how steroid modulation of these miRNAs in turn "fine-tune" steroid action downstream within this tissue.

To deepen our understanding on the role of miRNAs in uterine physiology and/or pathology, the function of any given miRNA needs to be understood. Functional data on the role of miRNAs within the uterus are beginning to accumulate with the majority of these studies using endometrial carcinoma cell lines. Pan and colleagues (2007) were perhaps the first investigators to postulate the possible function of miRNAs in endometrial/uterine tissue based upon algorithms for the identification of miRNA targets. Based upon this assessment, endometrial miRNAs were proposed to modulate diverse pathways including cellular proliferation, apoptosis, cell differentiation, and inflammation all of which are essential to normal endometrial biology. Further, it was proposed that dysregulation of these miRNA-modulated pathways could lead to the pathogenesis of endometrial pathologies such as endometriosis and endometrial cancer. Our current understanding on the functional roles of specific miRNAs in endometrial cells is derived primarily from studies incorporating human endometrial adenocarcinoma cell lines and as such, should be interpreted with caution as it is well established that mis-expression of miRNAs are associated with the malignant phenotype in a variety of cancers (Fabbri 2013). Nonetheless, our current understanding on the function of endometrial miRNAs from studies utilizing these models is outlined below and summarized in Table 9.2.

Cellular proliferation is one of the most-well-studied miRNA-mediated cellular events within the endometrium. Of the miRNAs to date, the miR-200 family is the most studied. miR-200a/b/c, miR-141, and miR-429 are up-regulated in both endometrioid endometrial adenocarcinoma and

Table 9.2 Endometrial carcinoma miRNAs, their expression level, proposed function and transcriptional targets

miRNA	Cell/tissue type	↑/↓	Function	Proposed target	Validation[a]	References
miR-200a/b/c	HEC-1A, Ishikawa cell lines	↑	Proliferation	NA[b]	Yes	Lee et al. (2011)
miR-141, *miR-429*						
miR-200c	HEC-1A, Ishikawa cell lines	↑	Proliferation	BRD7	Yes	Park et al. (2012)
miR-9, *miR-27*,	Endometrial carcinoma tissue, Ishikawa cells	↑	ND[c]	FOXO1 (decreased)	ND	Myatt et al. (2010)
miR-96, *miR-153*,						
miR-182, *miR-183*,						
miR-186	HEC-1B cells		Proliferation	FOXO1	Yes	Myatt et al. (2010)
miR-7, *miR-149*,	Endometrial carcinoma tissue	↑	ND	NA	ND	Myatt and Lam (2007)
miR-449b, *miR-204*						
miR-204	HEC-1A cell line		Migration, invasion	FOXC1	Yes	Myatt and Lam (2007)
miR-125b	Type II endometrial carcinoma cells	↑	Proliferation, invasion (in vitro and in vivo)	TP53INP1	Yes	Jiang et al. (2011)

[a]Target validation was performed by 3″ UTR reporter construct assays and/or, miRNA transfection and Western analysis
[b]NA = function or proposed target was not assessed in the cited study
[c]ND = not determined

complex atypical hyperplasia compared to normal control endometrium (Snowdon et al. 2011). Since this report, subsequent studies (Lee et al. 2011; Park et al. 2012) further documented a functional role for these miRNAs in cancerous tissues as well as endometrial adenocarcinoma cell lines. Incorporation of anti-miR technology revealed that each of these miRNAs suppressed HEC-1A cell proliferation, but only anti-miR-141, -200c, and -429 inhibitors reduced growth of Ishikawa cells (Lee et al. 2011). Park and co-workers (2012) further demonstrated that miR-200c modulated HEC-1A and Ishikawa cell proliferation, survival and apoptosis and that this was associated, at least in part, via down-regulation of transcript for the tumour suppressor protein BRD7.

Additional investigations examined the potential role of miRNAs in modulation of tumour suppressor genes and their role in proliferation. FOXO1 expression is reduced in endometrial carcinoma (Goto et al. 2008; Myatt et al. 2010). Using miRNA target prediction programs, Myatt and colleagues (2010) identified a panel of highly conserved miRNAs, which could potentially target the 3′ UTR of *FOXO1* transcript. Of these, miR-9, miR-27, miR-96, miR-128, miR-153, miR-182, miR-183, and miR-186 up-regulation correlated with loss of *FOXO1* expression in both endometrial carcinoma tissue and Ishikawa cells. Individual over-expression of all of these miRNAs in HEC-1B cells, except *miR-128*, reduced FOXO1 protein expression. When inhibitors of miR-9, miR-27, miR-96, miR-153, miR-183, and miR-186 were transfected as a pool into Ishikawa cells, cell cycle arrest and apoptosis were induced.

FOXC1 belongs to the same family of forkhead box transcription factors as FOXO1 and its mis-expression has also been associated with carcinogenesis (Myatt and Lam 2007). Through analysis of differential expression, endometrial cancer expressed a set of dysregulated miRNAs, which included miR-7, miR-149, miR-449b, and miR-204. Of these, miR-204 was shown to target *FOXC1* and functionally regulate endometrial adenocarcinoma (HEC-1A) cell migration and invasion (Chung et al. 2012). Similarly, the tumour suppressor, TP53INP1 is also modulated by miRNAs and its miRNA-induced suppression is associated with enhanced cell proliferation (Jiang et al. 2011). More specifically, miR-125b was found to up-regulated in type II endometrial carcinoma cells. Over-expression and inhibition studies using adenocarcinoma cell lines revealed that miR-125b modulated in vitro and in vivo cell proliferation and invasion and that these events were mediated via TP53INP1.

Within the field of endometrial biology, miRNA profiles have been generated for both human and rodent uterine tissue. Steroidal regulation of uterine/endometrial miRNAs has been investigated using both in vivo (rodent) and in vitro (human) models. Unfortunately, very few miRNAs and their targets have been validated in endometrial biology and/or disease with miR-135a/b being one of the few (Fig. 9.1). Currently, the majority of our functional understanding on the role of miRNAs within the uterus/endometrium has been formulated from studies that employed human endometrial adenocarcinoma cell lines. Collectively, this body of knowledge supports the concept that endometrial miRNAs regulate cellular proliferation, migration and/or invasion, and that members of the miR-200 family appear to be major players in these regulatory pathways. However, care must be taken when extrapolating miRNA regulation and/or functional data from cancerous cell types to that of "normal" endometrial cells that reside within the endometrium as there is strong existing data which demonstrates alterations between miRNA profiles in "normal" endometrium and endometrial adenocarcinoma tissue (reviewed in Fabbri 2013).

9.3.2 miRNAs Are Mis-expressed in Endometriosis

As discussed previously, it appears that within the endometrium, miRNAs function to modulate downstream gene expression of steroid-regulated genes important for normal reproductive cycles and fertility/embryo implantation. Endometriosis is a significant disease in which endometrial tissue grows ectopically within the pelvic cavity. This disease is associated with pelvic pain and infertility with the latter thought to be associated with embryo implantation insufficiencies among other pathological mechanisms. Over the last several years, miRNA expression profiles have been generated comparing miRNA expression within endometriotic implant tissue (ectopic endometrium) compared to that of the eutopic endometrium. The first study to profile endometriotic implant miRNA expression was conducted by Pan and colleagues (2007). Endometrial biopsy and endometriotic tissue was obtained from women of reproductive age during the early to mid-secretory stage (rising progesterone levels) of the menstrual cycle. Using miRNA array analysis, 48 miRNAs were identified as differentially expressed between ectopic and eutopic endometrium with the majority exhibiting approximately 40–60 % lower levels of expression in the ectopic endometrial (endometriosis) tissue. The reduced expression of these miRNAs in the endometriotic tissue was proposed to play a role in allowing target over-expression and subsequent enhancement of cellular events conducive to endometriotic implant survival and growth.

Filigheddu and co-workers (2010) conducted a similar miRNA profiling experiment comparing miRNA expression between ectopic and eutopic endometrium using tissue obtained during the proliferative stage of the menstrual cycle (cycle days 6–12, associated with rising estradiol levels). Fifty miRNAs were considered differentially expressed between tissue types. Again, the mis-expression of these miRNAs was postulated to allow for mis-expression of various cytokines, enzymes, growth factors, receptors and transcription regulators, all of which are proposed to play

a role in the pathogenesis of endometriosis. In comparing the data presented in the study by Pan and colleagues to those of Filigheddu and co-workers, an interesting observation may be noted. Of the miRNAs profiled in the two studies, eight miRNAs were common to both reports; miR-100, miR-126, miR-143, miR-145, miR-17-5p, miR-29c, miR-30e-5p, and miR-99a. Interestingly, in the first study, which collected samples during rising endogenous progesterone levels, all of these miRNAs showed lower expression in the endometriotic tissue compared to the corresponding (same patient) eutopic endometrium, while in the latter study in which samples were collected during rising endogenous oestradiol levels, the expression of all eight miRNAs was markedly higher in the endometriotic implant tissue compared to corresponding eutopic endometrium. This may suggest altered steroidal regulation of these miRNAs in the ectopic tissue between the proliferative and secretory stages of the menstrual cycle.

Using endometriotic and corresponding eutopic endometrial tissue from patients in both the proliferative and secretory stages of the menstrual cycle, Ohlsson-Teague and colleagues (2009) identified 14 differentially expressed miRNAs that were up-regulated in the endometriotic implant tissue and 8 that were down-regulated in the endometriotic tissue. ANOVA analysis of the microarray data according to stage of menstrual cycle revealed no significant differences in miRNA profiles based upon stage of menstrual cycle. Of the 14 up-regulated miRNAs in this study, 6 miRNAs (miR-145, miR-143, miR-99a, miR-126, miR-100, and miR-29c) were common to the studies of Pan et al. (2007) and Filigheddu et al. (2010). These six miRNAs were all up-regulated in the study by Filigheddu and colleagues, but down-regulated in the Pan and co-workers study. The finding that the menstrual cycle stage did not appear to influence the level of these six miRNAs (unlike the earlier suggestion) may be due to the small sample sizes in the study by Ohlsson-Teague et al. (2009) in which only four and three subjects from the proliferative and secretory stages of the menstrual cycle were enrolled.

The first transcriptome-miRNA analysis of endometriotic endometriomas (endometriotic cysts of the ovary) was conducted in 2011 (Hawkins et al. 2011). The top 30 miRNAs expressed in endometriomas and the corresponding abundance of transcript in non-endometriosis control endometrium was determined. Many of the miRNAs identified in the previous three reports (Pan et al. 2007; Ohlsson Teague et al. 2009; Filigheddu et al. 2010) were again identified in the report by Hawkins and colleagues (2011). Further, in the study by Hawkins et al. the potential function of miR-29c, which exhibited the highest expression differential between tissue types, was evaluated. An in vitro cell culture system using primary human endometrial stromal cells was employed in which levels of miR-29c were either inhibited or up-regulated. Putative extracellular matrix (ECM) protein gene targets of miR-29c, *COL7A1*, *UPK1B* and *TFAP2C*, were down-regulated in cells over-expressing miR-29c and the direct effect on the 3′ UTR of the genes was confirmed (Fig. 9.1). Thus, mis-expression of miR-29c in endometriomas appears to functionally contribute to the mis-expression of ECM proteins associated with the disease.

Most recently, Abe and colleagues (2013) examined the miRNA profiles in endometriotic versus eutopic endometrial stromal cells. Stromal cells were isolated from ovarian endometriomas, while endometrial stromal cells were obtained from eutopic endometrium of women with leiomyomas but no visible signs of endometriosis. Twelve miRNAs were demonstrated to be differentially expressed between endometrioma and endometrial stromal cells, eight down-regulated in endometrioma stromal cells (miR-199b-5p, miR-503, miR-424, miR-196b, miR-199a-3p, miR-214, miR-29b, and miR-455-3p) and four up-regulated (miR-210, miR-100, miR-132*, and miR-181a). Of these, miR-196b that showed approximate 70 % reduction in expression was selected for further evaluation. Transfection of endometrioma stromal cells with *mir-196b* precursor, which leads to subsequent expression of mature miR-196b, resulted in rounded/polygonal cells that poorly adhered to culture vesicles as opposed to the normal dendritic/stellate shape

that adhere to the cell culture plate. Increased expression of miR-196b was also associated with reduced endometrioma stromal cell viability and cell proliferation as well as increased apoptosis, which was associated with elevated caspase-3 and -7 activity. To evaluate the miR-196b-mediated pathways, *cMYC*, *BCL2*, and *HOXA10* expression were examined. First, *cMYC* expression was higher in endometrioma stromal cells compared to cells from eutopic endometrium. Transfection of endometrioma stromal cells with *mir-196b* precursor revealed that the anti-proliferative and pro-apoptotic activities are likely mediated through *cMYC* and *BCL2*, respectively, as miR-196b suppressed transcript levels of these factors. *HOXA10* expression was lower in endometrioma stromal cells compared to cells from eutopic endometrium, which was correlated with lower levels of miR-196b in these cells. Lastly, it was determined that the suppressed expression of miR-196b in endometrioma stromal cells is a result of DNA hypermethylation. Collectively, these studies suggest that aberrant miR-196b expression plays a role in the events conducive to the cellular processes associated with the pathogenesis of endometriosis, and that this mis-expression may be epigenetic in nature and results in increased expression of cMYC, BCL2 and HOXA10 protein (Fig. 9.1).

Ramón and colleagues (2011) assessed the expression of miR-15b, miR-16, miR-17-5p, miR-20a, miR-21, miR-125a, miR-221, and miR-222 and correlated the levels of expression for these miRNAs with the angiogenic factors vascular endothelial growth factor A (VEGFA) and thrombospondin I (THBS1). When analysing paired specimens, ovarian endometriomas exhibited significantly lower levels of pro-angiogenic *VEGFA* mRNA and protein and higher levels of miR-125a and miR-222 compared to corresponding eutopic endometrium. In contrast, levels of the angiogenesis inhibitor THBS1 were significantly higher in endometriomas and this was associated with reduced levels of miR-17-5p. Significant inverse correlations were noted between miR-222 and VEGFA protein expression and miR-17-5p and THBS1 protein levels suggesting that the mis-expression of these miRNAs may at least in part contribute to the observed altered expression of these angiogenic factors and in doing so contribute to the pathogenesis of the disease.

Additional miRNAs, which have been shown to be mis-expressed in endometriotic tissue include miR-199a (Dai et al. 2012), miR-126 (Liu et al. 2012), miR-23a/23b (Shen et al. 2013) and miR-145 (Adammek et al. 2013). It is well established that endometriosis is an invasive disease associated with increased angiogenesis, which often draws comparison to the malignant process. Of these microRNAs miR-199a is down-regulated in several types of cancer (Shen et al. 2010; Cheung et al. 2011). Based upon these observations, Dai and coworkers (2012) evaluated miR-199a expression in matched ovarian endometriomas and eutopic endometrium as well as endometrium from women free of endometriosis. Compared to eutopic endometrium from women without endometriosis, miR-199a expression was lower in both the eutopic endometrium from women with endometriosis and ovarian endometriomas. Forced expression of miR-199a in endometrial stromal cells resulted in dampened cell adhesion, invasion and migration, which was associated with suppression of the IKK/NFκB pathway and reduced interleukin 8 expression suggesting a possible functional role for the mis-expressed levels of miR-199a in the pathogenesis of endometriosis (Fig. 9.1).

miR-126 is a proposed regulator of cell growth, adhesion, invasion and angiogenesis (Guo et al. 2008; Feng et al. 2010), all processes essential for endometriosis development and progression. miR-126 expression is significantly reduced in ovarian endometriomas and eutopic endometrium from women with endometriosis compared to eutopic endometrium from control subjects. Associated with this reduction in miR-126 was a significant increase in *CRK* mRNA and protein. Furthermore, a highly significant association was found between miR-126 expression in endometriomas and eutopic endometrium from endometriosis subjects and stage/score of endometriosis suggesting that the worse the disease, the lower the expression of miR-126. Unfortunately, the ability of miR-126 to modulate

the events conducive to endometriosis pathogenesis such as proliferation, adhesion, invasion, and angiogenesis, and if these events are associated with altered *CRK* expression in endometriosis were not evaluated.

There is considerable evidence that steroidogenic factor 1 (SF1 or NR5A1) expression is increased in stromal cells from endometriotic tissue, but the mechanisms for this mis-expression are largely unknown. miR-23a/b expression is reduced in endometriotic and eutopic endometrium from women with endometriosis. As miR-23a/b is proposed to target *NR5A1*, the potential mechanistic link between miR-23a/b and *NR5A1* expression in the pathogenesis of endometriosis was examined (Shen et al. 2013). Reduced levels of miR-23a/b expression was confirmed in ectopic endometriotic tissue and eutopic endometrium from women with endometriosis compared to eutopic endometrium from women free of endometriosis and this reduction was associated with elevated transcript levels of *NR5A1* (*SF1*), *STAR* and *CYP19A1* (Fig. 9.1). To confirm that miR-23a/b directly regulated *NR5A1* (*SF1*) expression, luciferase reporter assays were conducted. Despite the fact that transfection with *mir-23a/b* precursor suppressed *NR5A1* (*SF1*) transcript, miR-23a/b did not bind to the 3′ UTR of *NR5A1* (*SF1*) suggesting that miR-23a/b mediation of *NR5A1* expression is via an indirect mechanism.

miR-145 has been shown to be mis-expressed in endometriotic tissue compared to eutopic endometrium (Pan et al. 2007; Ohlsson-Teague et al. 2009; Filigheddu et al. 2010). Coupled with the fact that miR-145 putatively targets factors involved in cellular events (Götte et al. 2010) conducive to endometriosis survival and progression, Adammek and colleagues (2013) examined the expression and potential function of miR-145 in the pathogenesis of endometriosis. Transfection of the human immortalized epithelial endometriotic cell line 12Z with miR-145 resulted in reduced cell proliferation and invasion while in primary ectopic and eutopic endometrial stromal cells from endometriosis patients, miR-145 over-expression resulted in inhibition of cell proliferation. Further analysis revealed that miR-145 induced post-transcriptional down-regulation of proposed targets *FASCIN1*, *PAI1*, and *JAMA* at the transcript and protein level in 12Z cells. Direct binding of miR-145 to the 3′ UTR of *JAMA* was confirmed by luciferase reporter construct assays. In contrast, only *FASCIN1* was shown to be modulated by miR-145 in primary ectopic and eutopic endometrial stromal cells from endometriosis patients. Cytoskeleton proteins ACTG2, TAGLN, and MYL9 were shown to be differentially regulated by miR-145 with over-expression of miR-145 leading to decreased *ACTG2* transcript expression but increased *TAGLN* transcript expression while *MYL9* expression was unaffected. Similarly, miR-145 over-expression in 12Z and stromal cells from both eutopic and ectopic endometrial cells from women with endometriosis resulted in decreased transcript expression of pluripotency and stemness-related markers. Modulation of protein expression or verification of direct binding via 3′ UTR reporter assays to further validate these targets were not performed. Although initial results on miR-145 expression in different endometriosis samples are conflicting (Pan et al. 2007; Ohlsson-Teague et al. 2009; Filigheddu et al. 2010), these data suggest that miR-145 appears to inhibit endometriotic cell proliferation and invasion as well as regulation of stem cell properties.

As discussed earlier, miRNAs are proposed to regulate steroid action within the endometrium to control the cellular events necessary for "normal" endometrial function. Mis-expression of miRNAs is in turn thought to contribute to the pathogenesis of diseases of endometrial tissue origin such as endometriosis. Within the field of endometriosis research, several miRNAs have emerged as potential players in modulating the events conducive to the establishment, survival and progression of the ectopic implant. Of these, functional data exists for only a few (Fig. 9.1). To truly understand which miRNAs are important in the pathogenesis of endometriosis and how they contribute to the disease, greater effort must be put forth to standardize what constitutes true "control" groups and what constitutes "endometriotic tissue". Reported and unpublished observations

strongly suggest that not only does miRNA expression vary based upon the "type" of implant, i.e. ovarian endometrioma versus peritoneal implant, among study subjects but that this expression also varies by type of implant within study subject.

9.4 miRNAs in Myometrial Physiology and Pathology

9.4.1 Expression and Function of miRNAs in the Myometrium

The role of miRNAs in normal myometrial physiology and function is just beginning to be explored. The primary function of the myometrium is to aid in the expulsion of the foetus during the birthing process via contraction. During the period of pregnancy, the myometrium is quiescent due to the high progesterone levels associated with pregnancy.

Renthal and colleagues (2010) first examined the potential role and regulation of miRNAs in myometrial function. In this study, microarray analysis was utilized to examine myometrial miRNA expression in term labour and non-labour human and mouse uterine tissue (pre-term and term). miR-200a, miR-200b, miR-200c, miR-141 and miR-429 (collectively referred to as the miR-200 family) were up-regulated in mouse myometrium from the day 18.5 group compared to the day 15.5 group, while in human tissue a similar pattern of expression was detected in labouring human myometrium compared to myometrium from term, non-labouring subjects. Expression of zinc finger E-box-binding homeobox 1 (ZEB1) and ZEB2, which are targets of the miR-200 family, was decreased demonstrating an inverse pattern of expression between miRNA and target protein in both mouse and human tissues. Further, an increase in expression of connexion 43 (CXN43) and oxytocin receptor (OXTR) was also detected in both mouse and human myometrium. Additional mouse models of preterm labour were used and revealed that there was an up-regulation of the miR-200 family and down-regulation of ZEB1/ZEB2. It was further revealed that ZEB1 is directly up-regulated by progesterone and that ZEB1 and ZEB2 inhibit the expression of contractile proteins CXN43 and OXTR. Collectively, these findings suggest that progesterone regulates the expression of the miR-200 family of microRNAs. In turn, the miR-200 family regulates ZEB1 and ZEB2 to modulate uterine contractility during pregnancy and labour (Fig. 9.2).

Subsequent studies by this research group (Williams et al. 2012a) further demonstrated that miR-200a plays a key role in initiating uterine contractility by enhancing the metabolism of progesterone in the myometrium in turn leading to reduced progesterone receptor function. Specifically, miR-200a represses STAT5b, which in turn leads to an increase in the expression of 20α-hydroxysteroid dehydrogenase (20α-HSD), a key progesterone-metabolizing enzyme. From these findings it was concluded that miR-200a plays a key role in the decline in progesterone action, which is necessary for initiation of labour through down-regulation of STAT5b (Fig. 9.2).

More recently, the same research group (Williams et al. 2012b) determined that while an increase in miR-200a plays a necessary role in labour induction, a decline in the expression of the miR-199a/214 cluster might also be important in the labour process. Both miR-199a-3p and miR-214 decline during labour in both mouse and human models employed in this study and this decrease was associated with an up-regulation of COX2 protein in both species during labour. It was further determined that oestrogens levels increase near term and are capable of stimulatory pro-inflammatory cascades, decrease miR-199a/214 expression and increase COX2 levels. These events could be blocked by progesterone, which often antagonizes many of the effects of oestrogens. Over-expression of miR-199a-3p and miR-214 inhibited COX2 protein and blocked tumour necrosis factor α-induced myometrial cell contractility. Together, these data could be interpreted to suggest that miR-199a-3p and miR-214 regulate myometrial contractility through the modulation of COX2 expression (Fig. 9.2).

Collectively, the studies from this research group provide strong insight into the role of the

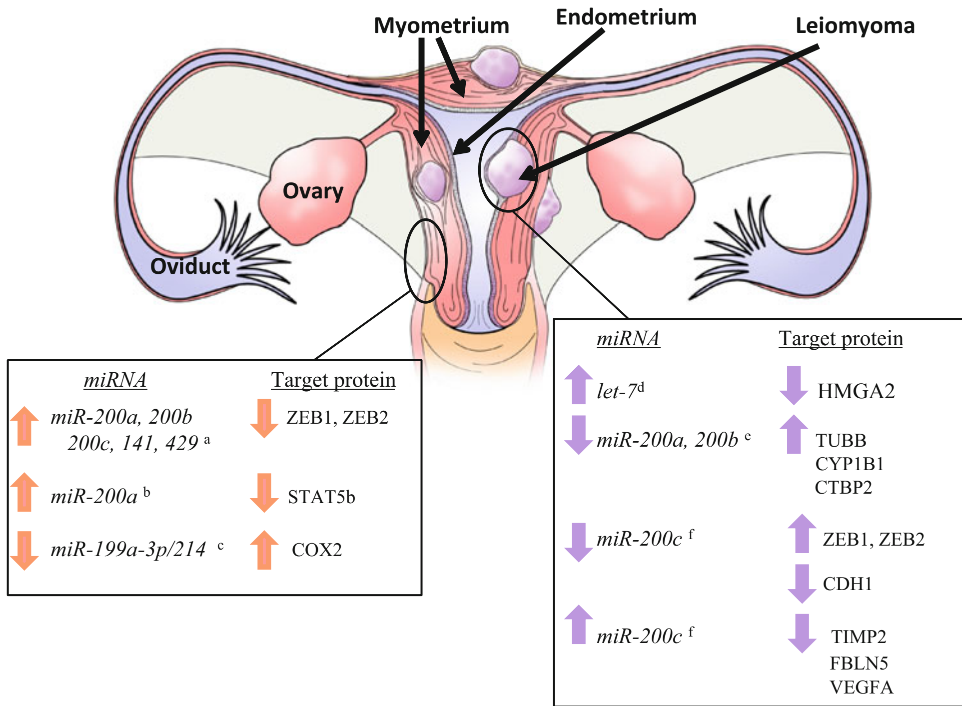

Fig. 9.2 miRNAs and their validated targets in myometrial and leiomyoma tissue. *Left table* summarizes miRNAs and their predicted transcript/protein in myometrial tissue (indicated as the *pink* layer of tissue in the figure) derived from studies incorporating human myometrial cells which have been validated. *Right table* summarizes miRNAs and their predicted transcript/protein in leiomyoma tissue (indicated as the *lavender* tissue within the myometrium) derived from studies incorporating human cells which have been validated. Superscripts refer to references with *a* = Renthel et al. 2010; *b* = Williams et al. 2012a; *c* = Williams et al. 2012b; *d* = Wang et al. 2007; *e* = Zavadil et al. 2010; *f* = Chuang et al. 2012. Validation refers to either assessing cellular events and/or mRNA and protein levels by modulation of endogenous miRNA levels

miR-200 and miR-199a/214 families in the process of uterine contractility. From these studies it appears that these miRNA families regulate the expression of ZEB1, ZEB2, STAT5B, and PTGS2 and of these, ZEB1 appears to be a central mediator of these opposing actions of progesterone and oestradiol on myometrial contractility.

9.4.2 miRNAs Are Mis-expressed in Uterine Leiomyomas and Contribute to Disease Pathogenesis

As discussed in the preceding paragraphs, we are just beginning to dissect the regulation of miRNAs in the myometrium and their role in the process of parturition. To date, there is no information on the potential role of miRNAs in abnormal labour/labour dystocia. With respect to miRNA function in abnormal myometrium, our current understanding is derived primarily from studies focusing on the role of miRNAs in the pathogenesis of uterine fibroids or leiomyomas. Uterine leiomyomas are benign smooth muscle (myometrium) neoplasms, which develop in women of reproductive age via uncertain mechanisms. As altered gene expression and/or function are predicted to play a role in the pathogenesis of leiomyomas, it is not surprising that miRNA expression has been examined in this tissue. The first study to assess myometrial miRNA expression was conducted by Wang and colleagues in 2007, in which they generated a miRNA signature associated with race, tumour size and target gene activity. Two hundred and six (206) miRNAs

were examined and 45 were found to be dysregulated in uterine leiomyomas. Of these, the let-7 family, miR-21, miR-23b, miR-27a, and miR-30a were the most significantly up-regulated leiomyoma miRNAs. Let-7 expression was significantly higher in small (<3 cm) compared to large (>10 cm) leiomyomas and exhibited an inverse correlation with one of its proposed targets, *HMGA2*. The putative regulation of *HMGA2* by let-7 was confirmed by transfection of leiomyoma cells with let-7 miRNA. In this same study, miR-29b, miR-32, miR-144, miR-197 and miR-212 were shown to be significantly down-regulated in leiomyomas. In contrast to the functional studies performed to validate let-7 regulation of *HMGA2*, only the predicted targets for these miRNAs were reported by Wang and coworkers (2007) without further validation.

Marsh and colleagues (2008) identified 46 miRNAs, which were differentially expressed in leiomyomas compared to myometrial tissue. Of the 46 detected, 19 were up-regulated and 27 were down-regulated. miR-542-3p was the most significant up-regulated microRNA with approximately 12-fold increase, while miR-498 was the most significantly down-regulated (approximately 2.5-fold). The authors further confirmed expression of miR-21, miR-34a, miR-125b, miR-139 and miR-323 by qRT-PCR, but did not examine either the regulation or function of these miRNAs in their study. The observation that miR-542-3p expression was the most significantly up-regulated miRNA in leiomyoma tissue was interesting in that this miRNAs has been proposed and validated to target survivin which suppresses cell growth (Yoon et al. 2010). If true, one may speculate that elevated miR-542-3p and subsequent reduction of survivin would lead to increased cell proliferation in fibroid tissue. miR-498 has been proposed to target *ZEB2* (based on upon TargetScan analysis), which has been linked to cell proliferation and tumour growth (Qi et al. 2012). Thus, one may speculate that reduced levels of miR-498 in leiomyomas may be associated with increased levels of *ZEB2* and enhancement of cellular proliferation.

Pan and colleagues (2008) used a multifaceted approach incorporating paired myometrial and leiomyoma tissue, myometrial (MSMC) and leiomyoma (LSMC) isolated cells, as well as leiomyoma cell lines T-LSMC and SK-LMS-1 to examine myometrial/leiomyoma miRNA expression. Ninety-one (91) miRNAs were identified which were expressed above myometrium thresholds with miR-20a, miR-21, miR26a, mir-18a, miR-206, miR-181a, and miR-142-5p expression confirmed by qRT-PCR. Further, steroidal regulation of these specific miRNAs was assessed in MSMC, LSMC, T-LSMC, and SK-LMS-1 cells. Compared to myometrium, leiomyomas expressed higher levels of miR-20a, miR-21, miR-26a, and miR-206, but expressed lower levels of miR-142-5p from Caucasians, but not in African Americans. African Americans also expressed lower levels of miR-181a in leiomyomas compared to matched myometrium while Caucasians expressed higher levels of miR-181a in leiomyoma tissue versus myometrium.

Comparison of primary cells LSMC and MSMC to T-LSMC and SKLM cell lines demonstrated that both cell lines expressed significantly higher levels of expression of miR-20a and miR-26a compared to both MSMC and LSMC. However, expression of these miRNAs was lower in the LSMC cells compared to that in MSMC. In contrast, miR-21 expression was lower in LSMC, tLSMC, and SKLM compared to MSMC but the cell lines exhibited significantly greater levels of miR-21 expression compared to MSMC. The potential impact of oestradiol and progesterone on the regulation of miR-20a, miR-26a, and miR-21 was evaluated. Oestradiol decreased miR-21 expression in MSMC but not in LSMC. The progesterone analogue, MPA, increased miR-21 expression in LSMC but not in MSMC. When combined, oestradiol and MPA increased miR-26a expression in MSMC but reduced its expression in LSMC. miR-20a expression was influenced by neither oestrogen nor MPA in either MSMC or LSMC cells.

Using uterine leiomyomas, Zavadil and coworkers (2010) profiled and analysed miRNA expression and evaluated the predicted target products of miR-21, miR-23b, miR-27a, miR-30a, and let-7s, which were the most significantly

up-regulated, and miR-29b, miR-32, miR-144, miR-197, and miR-212, which were the most significantly down-regulated. Two hundred and forty-nine (249) down-regulated putative mRNA targets were identified which corresponded to the 5 up-regulated miRNAs and 97 up-regulated putative mRNA targets were identified which corresponded with the 5 down-regulated miRNAs. Protein localization and relative level of expression in leiomyoma versus myometrium were assessed for EGFR, ERα, GRIP1, Hamartin, HMGA1, HMGA2, IGF1, IGF2, Ki67, PDECGF, PI3K, PRA, RARα, RXRα, TGFα, and TSC2. Of these proteins, RXR, TFGα, TSC2, and PRA showed higher expression in myometrial tissue with RXR exhibiting the highest expression, while the remaining proteins exhibited greater expression in leiomyoma tissue with HMGA2 exhibiting the highest level of expression. Further, there was an inverse correlation between Ki67 expression and that of let-7. Moderate negative correlations were found between EGFR and miR-194-1 as well as between TGFα and miR-199a-2. Overall, most gene products and their corresponding miRNAs exhibited negative correlations.

In addition to the miRNAs listed above, miR-200a and miR-200b, the *mir-15/mir-16* cluster and the let-7 family were also found to be significantly down-regulated in leiomyomas (Zavadil et al. 2010). Evaluation of putative targets of these miRNAs, which are over-expressed in leiomyoma tissue, revealed a number of over-expressed targets that may potentially contribute to the pathogenesis of the disease. Of these, the authors assessed *TUBB*, *CYP1B1*, *CTBP2*, *TNPO1*, and *ATXN1*, which are all putative targets of miR-200a, for validation and functionality. miR-200a suppressed transcript expression of *TUBB*, *CYP1B1*, and *CTBP2* but not that of *TNPO1* or *ATXN1*, which was also associated with reduced cell proliferation of leiomyoma cells and reversed their phenotype from fibroblast-like to that of a more pronounced epithelial phenotype. Further, let-7c was found to repress transcript expression of *PPP1R12B*, *STARD13*, *TRIB1*, *BTG2*, *HMGA2*, and *ITGB3*, but the impact on cell function or phenotype was not assessed.

Of the miRNAs studied in leiomyomas, miR-21 is one of the more studied yet its role in the pathogenesis of the disease is still largely unknown. Fitzgerald and colleagues (2012) recently examined the association between miR-21 and one of its targets, programmed cell death 4 (*PDCD4*) in leiomyomas and myometrial tissue. Consistent with the previously reported observations, leiomyoma tissue exhibited significantly higher levels of miR-21 expression compared to normal myometrium. However, the increased expression of miR-21 was associated with elevated (not reduced) levels of PDCD4 protein expression, which is in contrast to previous observations for miR-21 and PDCD4 in HeLa cells (Yao et al. 2009). Transfection of either immortalized myometrial or leiomyoma cells with blocking oligonucleotides to miR-21 resulted in increased PDCD4 protein expression. The authors suggested that these contrasting results indicate that PDCD4 may be regulated by a complex mechanism in which miR-21 may play a minimal role.

In addition to miR-21, the miR-200 family has also gained considerable interest in the pathogenesis of leiomyomas. miR-200c was recently demonstrated to be down-regulated in leiomyomas compared to myometrial tissue (Chuang et al. 2012). Using isolated cells from leiomyoma (LYO) and myometrial tissue (MYO) as well as the leiomyomasacrcoma cell line, SKLM-S1, it was demonstrated that miR-200c transfection (gain-of-function) repressed protein expression of *ZEB1* and *ZEB2* mRNA and protein, but increased E-cadherin (*CDH1*) transcript and protein expression (Fig. 9.2). Also associated with miR-200c gain-of-function was a change in cell morphology from elongated to round shape in both MYO and LYO (less so in SKLM-S1) and a reduction in cell viability and proliferation. Using luciferase reporter constructs, Chuang and colleagues (2012) also determined that miR-200c is capable of binding to the 3′ UTR seed sequence of tissue inhibitor of metalloproteinase 2 (*TIMP2*), fibulin 5 (*FBLN5*), and vascular endo-

thelial growth factor A (*VEGFA*). Further, gain of miR-200c function studies indicated that miR-200c repressed *TIMP2*, *FBLN5*, and *VEGFA* mRNA and protein levels (with only SKLM-S1 cells exhibiting repressed *VEGFA* mRNA; Fig. 9.2). The authors concluded from this series of studies that miR-200c, via regulation of ZEBs, VEGFA, FBLN5, and TIMP2 expression, may contribute to leiomyoma growth and maintenance of their cellular characteristics (Fig. 9.2).

Myometrial miRNAs (miR-200 family) appear to be necessary for normal pregnancy and labour where they regulate uterine contractility (Fig. 9.2). Outside of this report, the bulk of our information on myometrial miRNAs is derived from studies that have focused on differential profiles between myometrium and leiomyomas (fibroids). Along these lines, current information is primarily limited to assessment of cell growth and proliferation as well as steroidal regulation of myometrial/leiomyoma miRNAs. As myometrial function is essential to parturition, additional studies are required to enhance our understanding on the regulation and function of miRNAs within this muscle layer. This analysis should also expand into myometrial dysfunction not only associated with the pathogenesis of uterine leiomyomas but also abnormal myometrial function/contraction during abnormal labour/birth.

9.5 Summary and Conclusions

In summary, the majority of research that has evaluated the role of non-coding RNAs in uterine development and function has been concentrated in the area of short non-coding RNAs, predominately miRNAs. Within the context of the uterus, miRNA profiles have been generated for human and rodent myometrial and uterine/endometrial tissue. Steroidal regulation of some of these miRNAs has been examined in isolated stromal and glandular epithelial systems. Emerging data from studies which have incorporated human endometrial adenocarcinoma cell lines has shed some insight into the potential function of these miRNAs within the cells of the endometrium and the pathways by which they may do so. The majority of the current information suggests that myometrial miRNAs appear to mediate myometrial contractility during pregnancy and labour, while uterine/endometrial miRNAs appear to regulate cellular proliferation, migration and/or invasion especially in the context of the case of endometriosis. The members of the miR-200 family appear to be major players in these processes in both myometrium and uterus/endometrium. Research on miRNA regulation and function within the endometrium and myometrium is occurring at a rapid rate. One can fully anticipate that future study will increase focus on dissecting the mechanisms which contribute to the expression/mis-expression of these miRNAs in endometrial and myometrial tissue/cells as well as further defining their specific functions and the mediators by which they modulate these cellular events. Forthcoming information and application of novel approaches will allow for an expansion of our knowledge on which specific miRNAs play a role in the normal and abnormal events within these tissues.

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