**Spring 2018 – Systems Biology of Reproduction**
**Discussion Outline – Female Reproductive Tract Development & Function**
**Michael K. Skinner – Biol 475/575**
**CUE 418, 10:35-11:50 am, Tuesdays & Thursdays**
**February 8, 2018**
**Week 5**

## Female Reproductive Tract Development & Function

**Primary Papers:**
1. Mondejar, et al. (2012) Reproduction in Domestic Animals, 47(Suppl. 3) 22-29.
2. Hongling & Taylor (2015) CSH Persp Medicine, 6:a023002.
3. Shore & Rosen (2014) Int J Biochem & Cell Biol, 54:318-330.

## Discussion

Student 11: Contemporary Paper-Ref #1 above
- What are the functions of the oviduct?
- What methods were used?
- Are secretions important?

Student 12: Contemporary Paper-Ref #2 above
- What are HOX genes and role in development?
- What are endocrine disruptors and mechanism?
- How do they alter female reproductive tract?

Student 14: Contemporary Paper-Ref #3 above
- What are lncRNA and how identified?
- How do lncRNA influence mammary development?
- What is the role of lncRNA in breast cancer?

Reproduction in Domestic Animals

Reprod Dom Anim **47** (Suppl. 3), 22–29 (2012); doi: 10.1111/j.1439-0531.2012.02027.x
ISSN 0936-6768

# The Oviduct: Functional Genomic and Proteomic Approach

**I Mondéjar[1]*, OS Acuña[2]*, MJ Izquierdo-Rico[2], P Coy[1] and M Avilés[2]**

[1]*Department of Physiology, Veterinary Faculty and* [2]*Department of Cell Biology and Histology, Faculty of Medicine, University of Murcia, Murcia, Spain*

**Contents**

The mammalian oviduct is an anatomical part of the female reproductive tract, which plays several important roles in the events related to fertilization and embryo development. This review examines and compares several studies related to the proteomic and transcriptomic profile of the oviduct in different domestic animals. This information could be important for clarifying the role of oviductal factors in different events regulating fertilization and early embryo development, as well as for improving synthetic media for *in vitro* maturation/*in vitro* fertilization/embryo culture techniques (IVM/IVF/EC).

## Introduction

The concept of the oviduct as a passive structure involved in the transport of gametes has been substituted by that of a dynamic structure actively involved in several functions. The oviduct, also called the Fallopian tube in primates, is the organ in which fertilization takes place. Moreover, numerous studies have indicated that the oviduct and, especially, oviductal secretions play a key role in aspects related to gamete maturation, sperm capacitation and the development of the preimplantation embryo (Hunter 1998; Avilés et al. 2010).

Anatomically, the oviduct consists of four regions designated infundibulum, ampulla, isthmus and uterine-tubal junction. The mucosa of the oviduct shows primary and secondary folds of different height and orientation with a typical tree branch–like structure of varying degrees of complexity. Interspersed among these mucosal projections is a complex system of crypts, pockets and grooves (Hunter et al. 1991; Yániz et al. 2000). This complex anatomical structure contributes to sperm selection and probably participates in the regulation of the number of sperm that reach the site of fertilization, thus controlling polyspermy and providing different oviduct microenvironments (Hunter 2012). The epithelium is mainly formed by two different cell types: ciliated cells and non-ciliated cells or secretory cells (Fig. 1). The distribution and morphology of both cell types changes during the oestrous cycle, the anatomical region of the oviduct and even the specific region of the mucosa fold (apical or basal regions). Thus, it has been reported that ciliated cells are more abundant along lateral walls and in the apical region of longitudinal folds than in the basal regions among the mucosa folds (Abe 1996; Yániz et al. 2000; Yániz et al. 2006). In pig, a morphometric analysis even showed differences in the epithelial cells between two breeds (Abe and Hoshi 2008). In addition to these anatomical and histological differences, there are species-specific differences in the physiology of the oviduct. For example, in some species, ovulation is restricted to one of the two oviducts. It was previously reported that the concentration of different hormones (e.g. progesterone, prostaglandins) and the gene expression pattern in the ipsilateral oviduct differs from that observed in the contralateral oviduct (Wijayagunawardane et al. 1998; Bauersachs et al. 2003).

## The Oviduct Provides the Most Efficient Environment for the Success of Fertilization and Early Embryo Development

For most domestic animals, the fertility rate is generally higher than 50%. If a uniparous female animal is served during the oestrous period by a male with good sperm quality, the pregnancy rate may reach 60–70%. Among animals that deliver more than one offspring, in many cases the pregnancy rate can even exceed 90% (De Kruif 2003). However, in some cases, it is necessary to use assisted reproductive techniques (ARTs) to solve infertility and subfertility problems. For example, ARTs are linked to the protection and saving of species threatened by extinction, research and genetic improvement (Cseh and Solti 2000).

Nowadays, the production and development of embryos until the blastocyst stage in most of mammalian species can be achieved under *in vitro* conditions, with limitations that depend on the species (Table 1 and Data S1). In pig, for example, the developmental competence of *in vitro*-produced embryos is low compared with their *in vivo* counterparts (Kikuchi et al. 1999). An insufficient cytoplasmic ability for the development and polyspermy of *in vitro* matured oocytes and improper culture conditions for IVP embryos are thought to be responsible for this low efficacy (reviewed in Nagai et al. 2006). In cattle, although polyspermy is not a real problem in *in vitro* embryo production, the process is considered inefficient; while maturation and fertilization may appear to proceed normally, the proportion of embryos reaching the transferable stage is rarely over 40% and those that do reach this stage are often compromised in quality and competence. In equine species, IVF and development rates remain low (Hinrichs et al. 2002; Goudet 2011). The technology of *in vitro* oocyte maturation followed by the application of ICSI has been established to achieve fertilization *in vitro*. In this way, the rates of embryo development ranged from 5% to 10% in early studies but can reach 40% in later ones.

As mentioned above, fertilization and early embryonic development occurs in the oviduct. The quality of

*Both authors contributed equally to this work.

© 2012 Blackwell Verlag GmbH

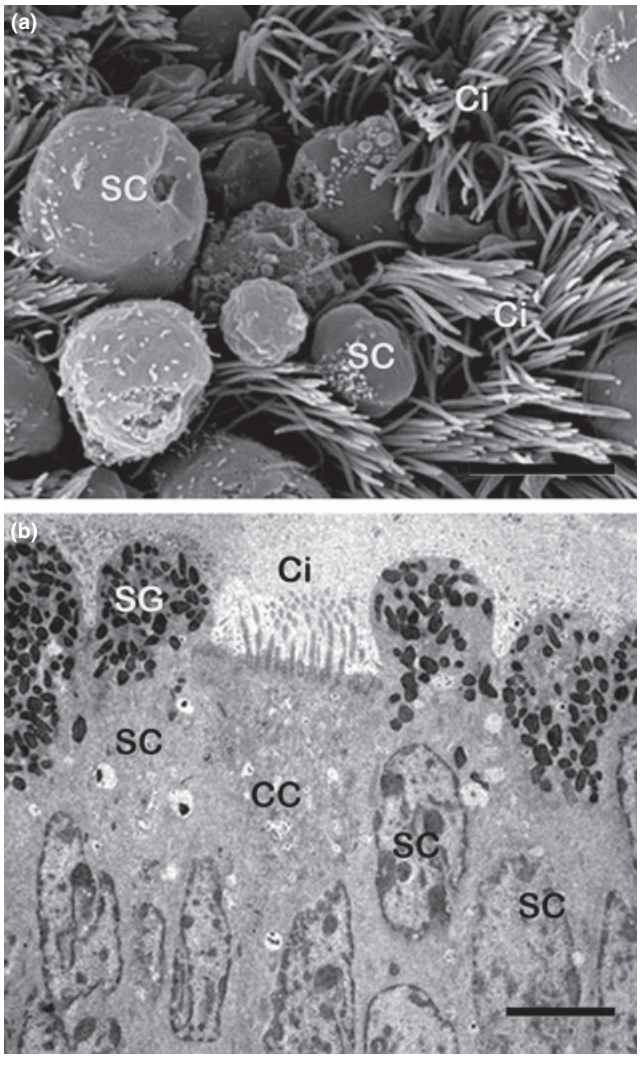


Fig. 1. Epithelial cells of the ampullary-isthmic junction (AIJ) of bovine oviduct. (a) Scanning electron micrographs of the epithelial surface of the ampulla of bovine oviduct in late follicular phase. (b) Electron micrograph of the epon-embedded AIJ. Ciliated cells (CC), secretory cells (SC), secretory granules (SG) and cilia (Ci). Bar: 5 $\mu$m

Table 1. Rate of *in vitro* fertilization (IVF), polyspermy, cleavage, morula and blastocyst formation in different domestic animals

| Species[a] | *In vitro* fertilization (IVF) (%) | Polyspermy (%) | Cleavage (%) | Morula (%) | Blastocyst (%) |
|---|---|---|---|---|---|
| Cat[1] | 46–80 | | 30–80 | 70–97 | 0–46 |
| Cow[2] | 60–92 | 34–45.6 | 64–95 | 60–80 | 32–65 |
| Dog[3] | 10–78 | 41.2–68 | 10 | 0–4 | 0 |
| Goat[4] | 32–37 | | 42–54 | | 10–20.8 |
| Horse[5] | 0–84 | 0–2.4 | 9–80[b] | | 4–40[b] |
| Pig[6] | 30–100 | 32–92.5 | 20–80 | | 2–40 |
| Rabbit[7] | 77–85 | | 64–92 | | 0–85 |
| Sheep[8] | | 3–21 | 30–80 | 50 | 3.9–57 |

[a]References used to prepare this table are included in the Supporting Information.
[b]Results obtained after ICSI.

the *in vitro*-produced embryos does not reflect the quality of their *in vivo* counterparts. Results of many studies suggest that culture conditions during *in vitro* embryo production may influence the developmental potential of the early embryo and its quality (Lonergan et al. 2007). Strategies developed to improve embryo development include the use of coculture media with epithelial cells, culture media supplemented with different proteins or growth factors and embryo culture in a foreign oviduct. The trans-species transfer of embryo to oviducts has been used to optimize early embryo development in different species, with the oviduct proving to be the best environment (Gandolfi and Moor 1987; Gutiérrez-Adán et al. 2004).

## The Oviduct is Involved in Regulation of Sperm, Oocyte and Embryo Physiology

The efficiency of fertilization is lower *in vitro* than *in vivo* for most species. However, it is unknown whether this is the result of (i) failures in final gamete maturation, (ii) deficient sperm–oocyte interaction or (iii) the lower ability of the recently formed zygote to develop. All these steps, which probably jointly affect the final outcome of *in vitro* procedures, take place in the oviduct under physiological conditions and, consequently, a study of the factors affecting them is of great importance for further advances in the reproductive biology field.

### Sperm

The role of the oviduct in male gamete capacitation mediated by binding of the spermatozoa to oviductal epithelial cells has been described in several species (Suarez 1998; Hunter 2012; Goudet 2011). In most cases, bound spermatozoa in the isthmus have been shown to decrease their movement and to prolong their survival, delaying the capacitation process (Suarez 2008; Fazeli et al. 2003). Partial identification of different proteins and carbohydrates involved in sperm binding and release from the oviduct (Suarez 2001; Talevi and Gualtieri 2010; Gualtieri et al. 2010; Talevi et al. 2010), as well as of the relationship between ovulation and the release of capacitated spermatozoa, has also been made (Gualtieri et al. 2005; Suarez 2007). However, a complete description of all the molecular pathways involved in these processes remains under research (Hunter 2012), and better understanding of these pathways will offer new tools for improving *in vitro* reproduction in domestic animals and also in humans.

Other possible roles of the oviduct as regards the male gamete have been related to the selection and guidance of spermatozoa towards the egg (Holt and Fazeli 2010). Moreover, the arrival of spermatozoa within the oviduct regulates gene expression in oviductal epithelial cells (Thomas et al. 1995; Fazeli et al. 2004; Georgiou et al. 2005, 2007). The oviduct may also be involved in a sperm selection process (Rodríguez-Martínez et al. 2005). After mating, where a large number of sperm are deposited in the vagina or uterus, very few are able to reach the site of fertilization. Severely deformed sperm cannot enter the oviduct (Styrna et al. 2002); however, sperm with a normal morphology or with few anomalies and a progressive linear movement can penetrate the uterotubal junction and enter the isthmus (Shalgi et al. 1992; Holt and Van Look 2004; Nakanishi

© 2012 Blackwell Verlag GmbH

et al. 2004). Then, sperm with appropriate receptors on their surface may bind to the epithelial cells for a period up to 30 h and form a preovulatory sperm reservoir (Rodríguez-Martínez et al. 2005; Hunter 2012). A seminal plasma protein called, BSP1 (or PDC-109), and annexin present in the apical membrane of the epithelial cells play a key role in this process (reviewed in Hung and Suarez 2010).

It has been reported that the female genital tract has a positive effect on the fertilization potential of spermatozoa that have been genetically altered (Kawano et al. 2010; Turunen et al. 2012). In mouse, Turunen et al. pointed to an 80% decrease in *in vitro* fertilization with sperm that lack CRISP4 compared with the wild type. These data indicated that, even if the physiology of the sperm is seriously compromised, the genetically modified mice were fertile, as wild-type animals, in normal mating. In our opinion, these results provide a new view of the uterine/oviductal contribution to sperm maturation in the genital tract.

The above studies could contribute to the development of sperm treatments with uterine and/or oviductal secretions (or uterine/oviductal tissue explants) to improve the sperm quality of animals with a low seminal quality, or in the case of damaged sperm after cryopreservation. We consider that this finding in mouse is worth investigating in other domestic animals and also in humans.

### Oocyte

With regard to the oocyte, the role of the oviduct on its final maturation, especially at the zona pellucida (ZP) level, has not been deeply studied, although it was suggested more than 20 years ago that oviductal glycoproteins may act to enhance the various functions of the ZP (Yang and Yanagimachi 1989). Recently, this role has been partially clarified when OVGP1 and heparin-like glycosaminoglycans from the oviductal fluid were seen to bind to the ZP and make it resilient to enzymatic digestion and to sperm binding and penetration (Coy et al. 2008). This mechanism represents a novel view of the so-called 'ZP hardening', which had been considered until now as a post-fertilization event associated with cortical granule exocytosis (cortical reaction). Now, it is known that the ZP undergoes those maturational changes in the oviduct before the arrival of spermatozoa and that these modifications may be crucial for any further oocyte response to sperm entry. As an example, polyspermy levels in the pig and cow are significantly affected by the contact of the ZP with oviductal secretions and, as a consequence, the final rate of fertilization is modified (Coy et al. 2008). A similar effect of the oviductal fluid on ZP maturation has recently been shown in the sheep and goat (Mondéjar 2011).

### Embryo

Finally, it cannot be forgotten that the recently formed zygote remains in the oviduct for a variable period of time, depending on the species but never $<48$ h. During this time, oviductal secretions are subjected to important changes derived from hormonal transition from an oestrogen-dependent to a progesterone-dependent environment. As we reported previously, the oviductal fluid protects the embryo against adverse impacts on mtDNA transcription/replication and apoptosis (Lloyd et al. 2009a). Moreover, a number of embryotrophic factors from the oviduct have been described (Lee and Yeung 2006; revised by Avilés et al. 2010), although functional experiments to clarify the specific role of each one and their potential use for improving *in vitro* culture systems remain to be performed. In the following paragraphs the identification and role of some of these factors will be discussed.

## Functional Genomic and Proteomic Analysis of the Oviductal Cells and Secretions

Oviductal fluid is a complex fluid formed by different metabolic and macromolecular components from blood plasma and epithelial cell secretions (reviewed in Buhi et al. 2000; Aguilar and Reyley 2005; Georgiou et al. 2005; Leese et al. 2008; Avilés et al. 2010). Most studies of the oviductal fluid have identified one, or a low number of, protein(s) in the oviduct by means of conventional analytical methods (Avilés et al. 2010). Other studies have tried to identify more components using complex technologies that include two dimensional electrophoresis (Gandolfi et al. 1989; Buhi et al. 2000). However, until now, it has been possible to identify only some of the proteins detected in the 2D gel by preparing specific antibodies. Fortunately, thanks to the development of the mass spectrometry instruments and the deciphering of the genome of different species, it is nowadays possible to identify a large number of proteins contained in complex body fluids and to study gene expression patterns in different tissues. Here, we describe the results previously reported in the literature and by our group obtained by using transcriptomic and proteomic analysis.

### Transcriptomic Analysis

A transcriptomic analysis of the oviduct has been performed in bovine, human and mouse species (Bauersachs et al. 2003; Fazeli et al. 2004; Bauersachs et al. 2004; Tone et al. 2008; George et al. 2011).

In cattle, the epithelial cells were obtained by scraping the mucosal epithelial layer of the complete oviduct using a glass slide from heifers in oestrous and dioestrous phases (Bauersachs et al. 2004) and in the postovulatory period (Bauersachs et al. 2003). During the postovulatory period, authors found differences for 35 genes when comparing gene expression in the ipsilateral and contralateral oviduct. Twenty-seven genes were up-regulated in the ipsilateral oviduct, and eight were down-regulated (Bauersachs et al. 2003). The comparative analysis of the gene expression between oestrous and dioestrous phase showed that 77 genes were differentially expressed; 37 and 40 genes were up-regulated in the oestrous and dioestrous phases, respectively (Bauersachs et al. 2004). These genes have been related to the immune response, protein secretion and

© 2012 Blackwell Verlag GmbH

modification, endocytosis, signalling and the regulation of transcription.

In women, a recent study compared the gene expression profile of epithelial cells of the Fallopian tube between the follicular and luteal phases (George et al. 2011). The authors identified five genes up-regulated and 15 down-regulated in the luteal phase (supplementary file in George et al. 2011). Some of these genes are of potential interest for different aspects related to fertilization and embryo development. For example, mRNA for heparanase was detected in the human oviductal mucosa. In a previous study, our group provide strong evidence to support a role for OVGP1 and heparin in blocking polyspermy (Coy et al. 2008). It was observed that heparin contributes to the stabilization of the OVGP1 effect. Our proteomic analysis also identified the existence of heparanase in the porcine oviductal fluid, agreeing with the results mentioned previously for the human oviduct. Heparin molecules have also been related to the release of bovine sperm bound to the oviductal epithelia (Gualtieri et al. 2010). Therefore, it seems possible that the heparanase present in the oviduct contributes to the regulation of these processes. Future experiments are necessary to confirm this hypothesis.

GPX3 mRNA, a glutathione peroxidase, is another gene differentially expressed in the human oviduct. This enzyme is involved in the redox balance and could make an important contribution to the control of DNA damage that affects gametes and the embryo (Aitken and De Iuliis 2010) and also to the sperm binding to the oviduct through the reduction of SS to SH (Gualtieri et al. 2009).

We have performed a detailed analysis of human specimens from the microarray experiment stored in the Gene Expression Omnibus (GEO) accessible through GEO Series accession number GSE10971 (Tone et al. 2008; Data S2). A total of 5703 genes of the original 54 675 probes present in the microarray were detected. This number of expressed genes is in accordance with a previous study performed in other human tissues using microarray analysis (Su et al. 2004). The list of genes was analysed and classified using the DAVID Bioinformatics Resource 6.7 (DAVID). Genes were classified according to the cellular localization, and genes encoding secreted proteins were also included (Fig. 2). It can be observed that 394 (8%) correspond to genes that codify for plasma membrane proteins and 245 (5%) of the expressed genes correspond to secreted proteins that can be classified into different groups (Fig. 3).

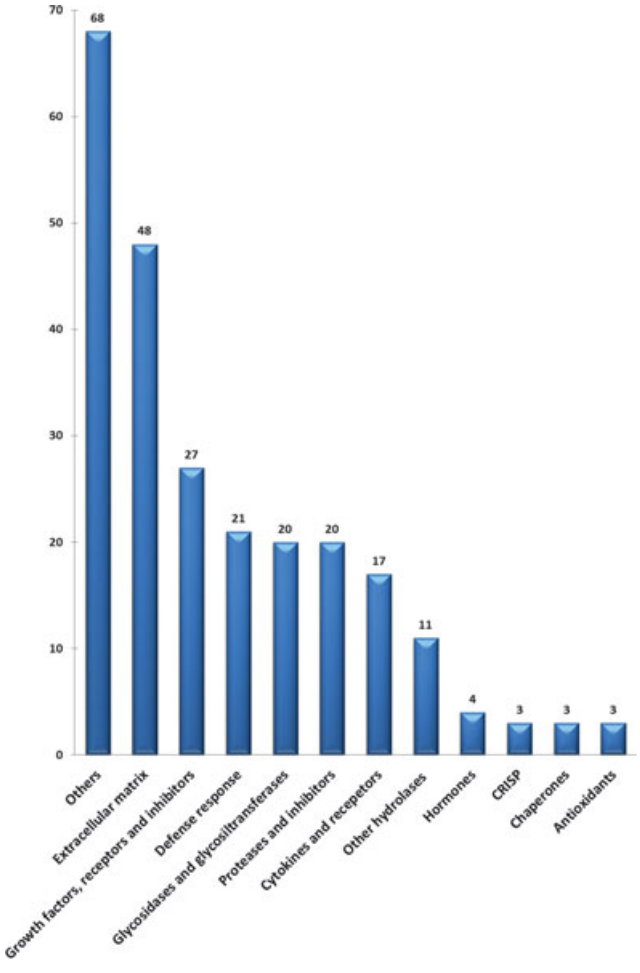


Fig. 3. Functional clustering of genes classified as 'secreted' using the DAVID bioinformatic tool using data from normal Fallopian tube reported in Tone et al. (2008)

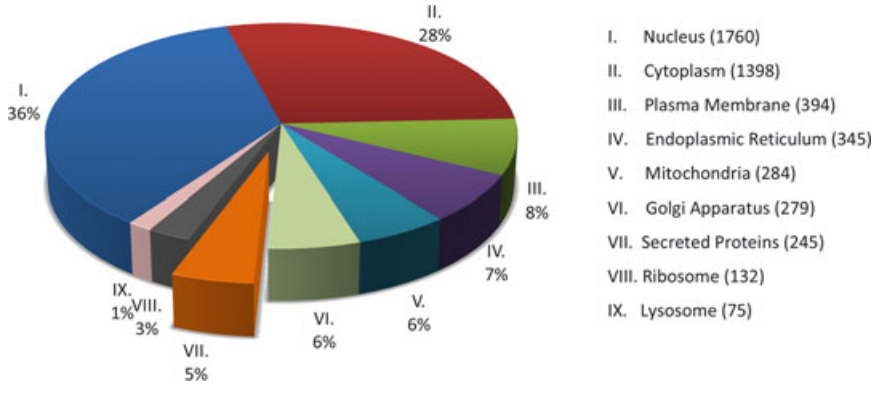


Fig. 2. Gene expression analysis of normal Fallopian tube (GEO: GSE10971). Gene clustering was performed according to the DAVID. The values represent the number and percentage of genes identified

© 2012 Blackwell Verlag GmbH

Some of the mRNA detected in this study has previously been identified in the oviductal epithelium in other species (Avilés et al. 2010); however, future studies are needed to confirm these data. The study by Tone et al. (2008) of gene expression analysis was performed using the oviductal mucosa and laser microdissection. Therefore, other cells (fibroblast, endothelial cells, lymphocyte, mast cells, etc.) present in the laminae propia are included in this analysis, and, consequently, some of the genes detected may not correspond to the epithelial cells covering the oviductal lumen. Other techniques like immunocytochemical and *in situ* hybridization analysis can be used to demonstrate the direct relationship between the expressed genes and the oviductal epithelium.

A gene expression study of the porcine oviduct in different phases of the oestrous cycle is currently in progress in our laboratories. In this review, we will provide unpublished information about the gene expression in the oviduct in the preovulatory phase of the cycle. For the analysis, the ampullary-isthmic junction region of the oviduct was selected because fertilization takes place in this region. The hybridization was performed using a microarray of 43 803 probes (Porcine (V2) 4x44K) from Agilent (Agilent Technologies, Madrid, Spain). Further details of the methodology employed is described in Data S2. Then, a total of 2968 genes were detected; this number is low compared with the human samples owing to incomplete annotation of the porcine genome. More than 480 genes are shared between the human and the porcine oviduct. Similarities between the porcine and bovine gene expression are even lower owing to the low number of gene available for comparative purposes (Bauersachs et al. 2007) (Fig. 4). The known genes expressed in the bovine oviduct epithelial cells are reduced owing to the fact that only differentially expressed genes were analysed using a subtracted library. In mice, a comparison of the only available data (125 genes) (Fazeli et al. 2004) showed that 82 of the genes are shared with the human oviduct, 17 are shared with the pig, and only two genes are expressed in the four species studied. In general, these results indicate that some genes are expressed in all four species, suggesting that basic components play a similar function and are evolutionarily conserved. These results are in agreement with functional events observed in a heterologous situation. Thus, embryo development from bovine species can be produced in the ewe oviduct (Rizos et al. 2007). Moreover, a pre-fertilization hardening of the ZP was observed when oocytes and oviductal fluids from different species are used (Mondéjar 2011); but this process is not always produced on the same scale or in all species. This finding is probably due to the existence of expressed genes that are not shared among the species.

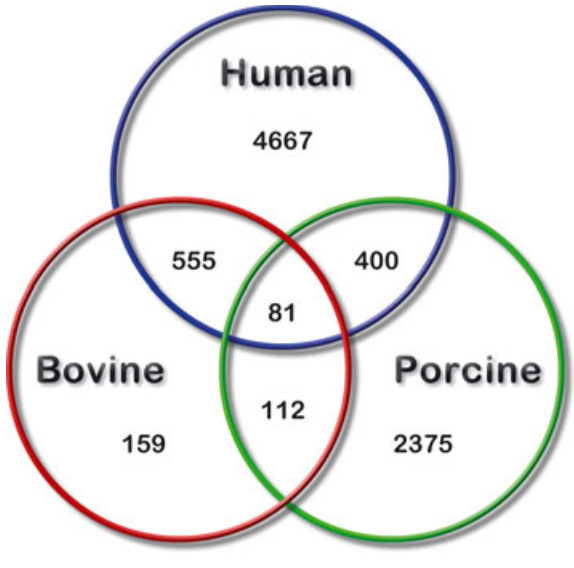


Fig. 4. Venn diagram showing overlapping and non-overlapping gene expression on human, bovine and porcine oviduct

## Proteomic Analysis

More than 160 proteins have been seen to be expressed or secreted by the oviduct of different species (Avilés et al. 2010). In pig, numerous proteins have been identified in the epithelial cells (Buhi et al. 2000; Georgiou et al. 2005; Sostaric et al. 2006; Seytanoglu et al. 2008) and also in the oviductal secretions (Georgiou et al. 2005, 2007; Mondéjar et al. 2009; Mondéjar 2011). At least 32 proteins from the oviductal fluid are affected by the presence of gametes, most of which are affected by the presence of the male gamete (Georgiou et al. 2007). In our laboratory, we performed an analysis of the porcine oviductal fluid from preovulatory phase. The oviductal fluid was obtained by luminal aspiration of previously dissected oviducts as previously reported (Carrasco et al. 2008). After centrifugation (5200 × ***g*** for 10 min) to remove the mucus and cellular debris, the samples were analysed by one dimensional SDS-PAGE electrophoresis under reducing conditions. The different bands were visualized by silver staining and were cut and digested with trypsin for subsequent proteomic analysis. The data were analysed by LC/MSD Trap Data Analysis Version 3.2 (Bruker Daltonik, GmbH, Germany), and the search for matches was conducted with the Spectrum Mill MS Proteomics Workbench (Agilent Technologies, Santa Clara, CA, USA) against the most recent version of the NCBInr database.

A total of 291 proteins were identified in the porcine oviductal secretions (Mondéjar 2011); however, only 35 of these proteins were detected in our microarray analysis (Mondéjar 2011), probably due to the incomplete array annotation. The different proteins can be classified into different groups as performed previously (Avilés et al. 2010) (Fig. 5). In this analysis, only 27 secreted proteins (9.3%) were identified; however, most of the proteins correspond to cellular components (90.7%). It is striking that a low number of the proteins identified correspond to typically secreted proteins compared with the total number of proteins. In the oviductal fluid, other proteins that are not typically secreted by the epithelial cells can be detected. These proteins may be divided into two main groups: proteins that come from the transudate of blood (for example, albumin and plasminogen) and other non-secreted cellular proteins. Some of them are proteins present in cellular organelles such as the nucleus, mitochondria,

© 2012 Blackwell Verlag GmbH

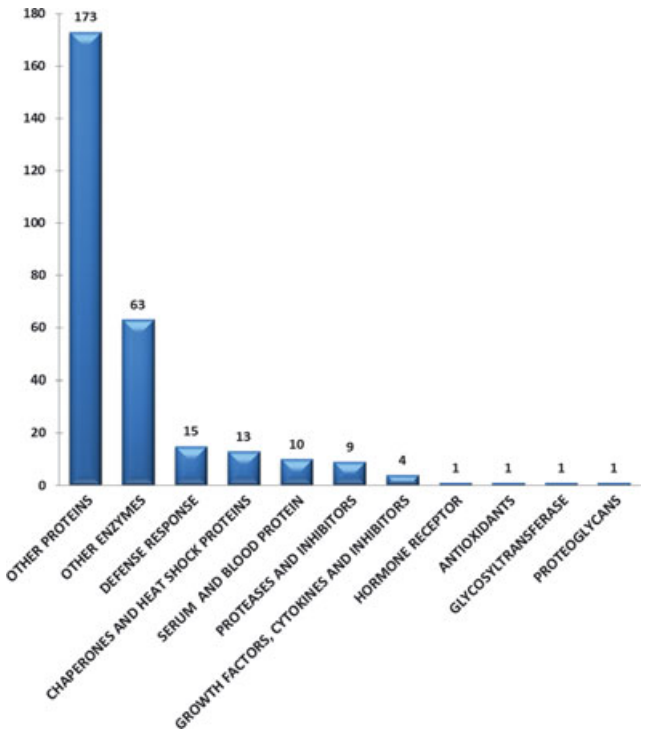


Fig. 5. Classification of the different proteins identified in the porcine oviductal fluid by proteomic analysis

endoplasmic reticulum and lysosomes. The origin of these proteins is probably related to different processes such as epithelial cell renewal or the secretory activity by apocrine and holocrine processes (Crow et al. 1994; Steffl et al. 2008); however, blood or oviductal cells contamination cannot be excluded.

The most abundant peptides identified correspond to albumin and the OVGP1 as expected owing to the fact that these proteins are abundant in oviductal fluid. Owing to space restriction, only three of the identified proteins are discussed in more detail.

Several heat shock proteins (HSP) were detected in our proteomic analysis, confirming their existence, as reported previously (Bauersachs et al. 2004; Georgiou et al. 2005). These proteins are usually considered to be intracellular proteins, although they have also been detected in the human serum and plasma (Molvarec et al. 2010). It was observed that HSP70 can be secreted by a non-classical pathway (Mambula and Calderwood 2006). A similar process, which remains to be confirmed, could exist in the oviduct. It was reported that HSPA8 and HSP60 are able to associate with spermatozoa, thus improving their survival in several species (Elliott et al. 2009; Lloyd et al. 2009b).

Another interesting protein identified in our study is annexin, which is considered a cytosolic protein; however, it was also reported that this protein can be secreted by a non-classical pathway (Christmas et al. 1991). It was previously reported that the bovine sperm interaction with the oviductal epithelium is mediated by BSP1 associated with the sperm membrane and with annexin molecules in the oviductal epithelium (Ignotz et al. 2007). Annexin in the oviductal fluid could regulate this type of interaction.

In our study, we detected, by both gene expression and proteomic analysis, the deleted in malignant brain tumours 1 (DMBT1) protein, confirming its presence at mRNA level in the bovine (Bauersachs et al. 2004) and human (Tone et al. 2008) oviduct. Very recently, it was reported that the DMBT1, previously called SPG, is expressed in the porcine oviduct (Teijeiro et al. 2012). In this study, the authors demonstrated the presence of this protein at the apical surface of the epithelial cells.

## Concluding Remarks

A large body of evidence strongly supports the complexity of and the important role played by the oviduct and its secretion in different aspects of gamete maturation, fertilization and embryo development. The oviduct undergoes important changes in several aspects, including its anatomy in different regions, changes in the histology and physiology of the mucosa during the ovarian cycle and a complex gene expression pattern that is modified according to the ovarian cycle status and also to the presence of gametes and embryos. More precise information is needed about the different genes expressed and proteins synthesized and secreted by the oviduct in its different regions, hormonal and other physiological conditions to clarify the role played by the oviduct. However, for this, knowledge of the complete genome annotation of different animals is necessary. The information obtained for different animals will also contribute to understanding the mechanisms conserved in the different species and also others that are responsible for species specificity. This information will contribute to improving different aspects of the methodology used in ARTs in domestic animals, endangered wildlife species and even human beings.

### Acknowledgements

This work was supported by a Spanish MEC grant (AGL2009-12512-C02-01-02) and Fundación Séneca (11996/PI/09 and 04542/GERM/06). The author OS Acuña received a training fellowship from the Mexican National Council for Science and Technology (CONACYT: Beca Bicentenario 2010 Id: 213560) and 'Doctores Jóvenes' fellowship from the Autonomous University of Sinaloa (UAS) México. We thank Dr. Horcajadas and Dr. Martinez-Conejero (Iviomics, Valencia, Spain) for the microarray analysis.

### Conflict of interest

None of the authors have any conflicts of interest to declare.

### Supporting information

Additional Supporting Information may be found in the online version of this article:

**Data S1.** References.

**Data S2.** Methods.

Please note: Wiley-Blackwell is not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

© 2012 Blackwell Verlag GmbH

## References


Abe H, 1996: The mammalian oviductal epithelium: regional variations in cytological and functional aspects of the oviductal secretory cells. Histol Histopathol **11,** 743–768.

Abe H, Hoshi H, 2008: Morphometric and ultrastructural changes in ciliated cells of the oviductal epithelium in prolific Chinese Meishan and Large White pigs during the oestrous cycle. Reprod Domest Anim **43,** 66–73.

Aguilar J, Reyley M, 2005: The uterine tubal fluid: secretion, composition and biological effects. Anim Reprod **2,** 91–105.

Aitken R, De Iuliis G, 2010: On the possible origins of DNA damage in human spermatozoa. Mol Hum Reprod **16,** 3–13.

Avilés M, Gutiérrez-Adán A, Coy P, 2010: Oviductal secretions: will they be key factors for the future ARTs? Mol Hum Reprod **16,** 896–906.

Bauersachs S, Blum H, Mallok S, Wenigerkind H, Rief S, Prelle K, Wolf E, 2003: Regulation of ipsilateral and contralateral bovine oviduct epithelial cell function in the postovulation period: a transcriptomics approach. Biol Reprod **68,** 1170–1177.

Bauersachs S, Rehfeld S, Ulbrich SE, Mallok S, Prelle K, Wenigerkind H, Einspanier R, Blum H, Wolf E, 2004: Monitoring gene expression changes in bovine oviduct epithelial cells during the oestrous cycle. J Mol Endocrinol **32,** 449–466.

Bauersachs S, Mitko K, Blum H, Wolf E, 2007: Technical note: bovine oviduct and endometrium array version 1: a tailored tool for studying bovine endometrium biology and pathophysiology. J Dairy Sci **90,** 4420–4423.

Buhi W, Alvarez I, Kouba A, 2000: Secreted proteins of the oviduct. Cells Tissues Organs **166,** 165–179.

Carrasco LC, Romar R, Avilés M, Gadea J, Coy P, 2008: Determination of glycosidase activity in porcine oviductal fluid at the different phases of the estrous cycle. Reproduction **136,** 833–842.

Christmas P, Callaway J, Fallon J, Jones J, Haigler HT, 1991: Selective secretion of annexin 1, a protein without a signal sequence, by the human prostate gland. J Biol Chem **266,** 2499–2507.

Coy P, Cánovas S, Mondéjar I, Saavedra M, Romar R, Grullón L, Matás C, Avilés M, 2008: Oviduct-specific glycoprotein and heparin modulate sperm-zona pellucida interaction during fertilization and contribute to the control of polyspermy. Proc Natl Acad Sci U S A **105,** 15809–15814.

Crow J, Amso NN, Lewin J, Shaw RW, 1994: Morphology and ultrastructure of fallopian tube epithelium at different stages of the menstrual cycle and menopause. Hum Reprod **9,** 2224–2233.

Cseh S, Solti L, 2000: Importance of assisted reproductive technologies in the conservation of wild, rare or indigenous ungulates: review article. Acta Vet Hung **48,** 313–323.

De Kruif A, 2003: Fertility and sterility in domestic animals. Verh K Acad Geneeskd Belg **65,** 189–202.

Elliott RM, Lloyd RE, Fazeli A, Sostaric E, Georgiou AS, Satake N, Watson PF, Holt WV, 2009: Effects of HSPA8, an evolutionarily conserved oviductal protein, on boar and bull spermatozoa. Reproduction **137,** 191–203.

Fazeli A, Elliott RM, Duncan AE, Moore A, Watson PF, Holt WV, 2003: *In vitro* maintenance of boar sperm viability by a soluble fraction obtained from oviductal apical plasma membrane preparations. Reproduction **125,** 509–517.

Fazeli A, Affara NA, Hubank M, Holt WV, 2004: Sperm-induced modification of the oviductal gene expression profile after natural insemination in mice. Biol Reprod **71,** 60–65.

Gandolfi F, Moor RM, 1987: Stimulation of early embryonic development in the sheep by co-culture with oviduct epithelial cells. J Reprod Fertil **81,** 23–28.

Gandolfi F, Brevini T, Richardson L, Brown C, Moor R, 1989: Characterization of proteins secreted by sheep oviduct epithelial cells and their function in embryonic development. Development **106,** 303–312.

George SH, Greenaway J, Milea A, Clary V, Shaw S, Sharma M, Virtanen C, Shaw PA, 2011: Identification of abrogated pathways in fallopian tube epithelium from BRCA1 mutation carriers. J Pathol **225,** 106–117.

Georgiou AS, Sostaric E, Wong CH, Snijders AP, Wright PC, Moore HD, Fazeli A, 2005: Gametes alter the oviductal secretory proteome. Mol Cell Proteomics **4,** 1785–1796.

Georgiou AS, Snijders AP, Sostaric E, Aflatoonian R, Vazquez JL, Vazquez JM, Roca J, Martinez EA, Wright PC, Fazeli A, 2007: Modulation of the oviductal environment by gametes. J Proteome Res **6,** 4656–4666.

Goudet G, 2011: Fertilisation in the horse and paracrine signalling in the oviduct. Reprod Fertil Dev **23,** 941–951.

Gualtieri R, Boni R, Tosti E, Zagami M, Talevi R, 2005: Intracellular calcium and protein tyrosine phosphorylation during the release of bovine sperm adhering to the fallopian tube epithelium *in vitro*. Reproduction **129,** 51–60.

Gualtieri R, Mollo V, Duma G, Talevi R, 2009: Redox control of surface protein sulphhydryls in bovine spermatozoa reversibly modulates sperm adhesion to the oviductal epithelium and capacitation. Reproduction **138,** 33–43.

Gualtieri R, Mollo V, Barbato V, Talevi R, 2010: Ability of sulfated glycoconjugates and disulfide-reductants to release bovine epididymal sperm bound to the oviductal epithelium *in vitro*. Theriogenology **73,** 1037–1043.

Gutiérrez-Adán A, Rizos D, Fair T, Moreira PN, Pintado B, de la Fuente J, Boland MP, Lonergan P, 2004: Effect of speed of development on mRNA expression pattern in early bovine embryos cultured *in vivo* or *in vitro*. Mol Reprod Dev **68,** 441–448.

Hinrichs K, Love CC, Brinsko SP, Choi YH, Varner DD, 2002: *In vitro* fertilization of *in vitro*-matured equine oocytes: effect of maturation medium, duration of maturation, and sperm calcium ionophore treatment, and comparison with rates of fertilization *in vivo* after oviductal transfer. Biol Reprod **67,** 256–262.

Holt WV, Fazeli A, 2010: The oviduct as a complex mediator of mammalian sperm function and selection. Mol Reprod Dev **77,** 934–943.

Holt WV, Van Look KJ, 2004: Concepts in sperm heterogeneity, sperm selection and sperm competition as biological foundations for laboratory tests of semen quality. Reproduction **127,** 527–535.

Hung PH, Suarez SS, 2010: Regulation of sperm storage and movement in the ruminant oviduct. Soc Reprod Fertil Suppl **67,** 257–266.

Hunter RH, 1998: Have the Fallopian tubes a vital role in promoting fertility? Acta Obstet Gynecol Scand **77,** 475–486.

Hunter RH, 2012: Components of oviduct physiology in eutherian mammals. Biol Rev Camb Philos Soc **87,** 244–255.

Hunter R, Fléchon B, Fléchon J, 1991: Distribution, morphology and epithelial interactions of bovine spermatozoa in the oviduct before and after ovulation: a scanning electron microscope study. Tissue Cell **23,** 641–656.

Ignotz G, Cho M, Suarez S, 2007: Annexins are candidate oviductal receptors for bovine sperm surface proteins and thus may serve to hold bovine sperm in the oviductal reservoir. Biol Reprod **77,** 906–913.

Kawano N, Kang W, Yamashita M, Koga Y, Yamazaki T, Hata T, Miyado K, Baba T, 2010: Mice lacking two sperm serine proteases, ACR and PRSS21, are subfertile, but the mutant sperm are infertile *in vitro*. Biol Reprod **83,** 359–369.

Kikuchi K, Kashiwazaki N, Noguchi J, Shimada A, Takahashi R, Hirabayashi M, Shino M, Ueda M, Kaneko H, 1999: Developmental competence, after transfer to recipients, of porcine oocytes matured, fertilized, and cultured *in vitro*. Biol Reprod **60,** 336–340.

Lee KF, Yeung WS, 2006: Gamete/embryo–oviduct interactions: implications on *in vitro* culture. Hum Fertil (Camb) **9,** 137–143.

Leese HJ, Hugentobler SA, Gray SM, Morris DG, Sturmey RG, Whitear SL, Sreenan JM, 2008: Female reproductive tract fluids: composition, mechanism of formation and potential role in the developmental origins of health and disease. Reprod Fertil Dev **20,** 1–8.

Lloyd RE, Romar R, Matas C, Gutiérrez-Adán A, Holt WV, Coy P, 2009a: Effects of oviductal fluid on the development, quality and gene expression of porcine blastocyst produced *in vitro*. Reproduction **137,** 679–687.

Lloyd RE, Elliott RM, Fazeli A, Watson PF, Holt WV, 2009b: Effects of oviductal proteins, including heat shock 70 kDa protein 8, on survival of ram spermatozoa over 48 h *in vitro*. Reprod Fertil Dev **21,** 408–418.

Lonergan P, Woods A, Fair T, Carter F, Rizos D, Ward F, Quinn K, Evans A, 2007: Effect of embryo source and recipient progesterone environment on embryo development in cattle. Reprod Fertil Dev **19,** 861–868.


© 2012 Blackwell Verlag GmbH

Mambula SS, Calderwood SK, 2006: Heat shock protein 70 is secreted from tumor cells by a nonclassical pathway involving lysosomal endosomes. J Immunol **177,** 7849–7857.

Molvarec A, Tamási L, Losonczy G, Madách K, Prohászka Z, Rigó J, 2010: Circulating heat shock protein 70 (HSPA1A) in normal and pathological pregnancies. Cell Stress Chaperones **15,** 237–247.

Mondéjar I, 2011: Estudio de la expresión génica y de la composición proteica del oviducto. Efectos del fluido oviductal sobre la resistencia de la zona pelúcida a la digestión enzimática en diferentes mamíferos. European Thesis, Murcia, Spain.

Mondéjar I, Saavedra MD, Avilés M, Coy P, 2009: Identification of different heat shock proteins in the porcine pre-ovulatory oviductal fluid. Maternal Communication with Gametes and Embryos. In: Fazelli A, Gandolfi F, Ledda S (eds), pp. 76.

Nagai T, Funahashi H, Yoshioka K, Kikuchi K, 2006: Up date of *in vitro* production of porcine embryos. Front Biosci **11,** 2565–2573.

Nakanishi T, Isotani A, Yamaguchi R, Ikawa M, Baba T, Suarez SS, Okabe M, 2004: Selective passage through the uterotubal junction of sperm from a mixed population produced by chimeras of calmegin-knockout and wild-type male mice. Biol Reprod **71,** 959–965.

Rizos D, Pintado B, de la Fuente J, Lonergan P, Gutiérrez-Adán A, 2007: Development and pattern of mRNA relative abundance of bovine embryos cultured in the isolated mouse oviduct in organ culture. Mol Reprod Dev **74,** 716–723.

Rodríguez-Martínez H, Saravia F, Wallgren M, Tienthai P, Johannisson A, Vázquez JM, Martínez E, Roca J, Sanz L, Calvete JJ, 2005: Boar spermatozoa in the oviduct. Theriogenology **63,** 514–535.

Seytanoglu A, Georgiou AS, Sostaric E, Watson PF, Holt WV, Fazeli A, 2008: Oviductal cell proteome alterations during the reproductive cycle in pigs. J Proteome Res **7,** 2825–2833.

Shalgi R, Smith TT, Yanagimachi R, 1992: A quantitative comparison of the passage of capacitated and uncapacitated hamster spermatozoa through the uterotubal junction. Biol Reprod **46,** 419–424.

Sostaric E, Georgiou AS, Wong CH, Watson PF, Holt WV, Fazeli A, 2006: Global profiling of surface plasma membrane proteome of oviductal epithelial cells. J Proteome Res **5,** 3029–3037.

Steffl M, Schweiger M, Sugiyama T, Amselgruber WM, 2008: Review of apoptotic and non-apoptotic events in non-ciliated cells of the mammalian oviduct. Ann Anat **190,** 46–52.

Styrna J, Bilińska B, Krzanowskaa H, 2002: The effect of a partial Y chromosome deletion in B10.BR-Ydel mice on testis morphology, sperm quality and efficiency of fertilization. Reprod Fertil Dev **14,** 101–108.

Su AI, Wiltshire T, Batalov S, Lapp H, Ching KA, Block D, Zhang J, Soden R, Hayakawa M, Kreiman G, Cooke MP, Walker JR, Hogenesch JB, 2004: A gene atlas of the mouse and human protein-encoding transcriptomes. Proc Natl Acad Sci U S A **101,** 6062–6067.

Suarez SS, 1998: The oviductal sperm reservoir in mammals: mechanisms of formation. Biol Reprod **58,** 1105–1107.

Suarez S, 2001: Carbohydrate-mediated formation of the oviductal sperm reservoir in mammals. Cells Tissues Organs **168,** 105–112.

Suarez S, 2007: Interactions of spermatozoa with the female reproductive tract: inspiration for assisted reproduction. Reprod Fertil Dev **19,** 103–110.

Suarez SS, 2008: Regulation of sperm storage and movement in the mammalian oviduct. Int J Dev Biol **52,** 455–462.

Talevi R, Gualtieri R, 2010: Molecules involved in sperm-oviduct adhesion and release. Theriogenology **73,** 796–801.

Talevi R, Barbato V, De Iorio S, Mollo V, Capriglione T, Ricchiari L, Samo A, Gualtieri R, 2010: Is there a role for endocannabinoids in sperm-oviduct interaction? Reproduction **140,** 247–257.

Teijeiro JM, Roldán ML, Marini PE, 2012: Molecular identification of the sperm selection involved porcine sperm binding glycoprotein (SBG) as deleted in malignant brain tumors 1 (DMBT1). Biochimie **94,** 263–267.

Thomas PG, Ignotz G G, Ball BA, Brinsko S P, Currie WB, 1995: Effect of coculture with stallion spermatozoa on de novo protein synthesis and secretion by equine oviduct epithelial cells. Am J Vet Res **56,** 1657–1662.

Tone A, Begley H, Sharma M, Murphy J, Rosen B, Brown T, Shaw P, 2008: Gene expression profiles of luteal phase fallopian tube epithelium from BRCA mutation carriers resemble high-grade serous carcinoma. Clin Cancer Res **14,** 4067–4078.

Turunen HT, Sipilä P, Krutskikh A, Toivanen J, Mankonen H, Hämäläinen V, Björkgren I, Huhtaniemi I, Poutanen M, 2012: Loss of cysteine-rich secretory protein 4 (crisp4) leads to deficiency in sperm-zona pellucida interaction in mice. Biol Reprod **86,** 1–8.

Wijayagunawardane M, Miyamoto A, Cerbito W, Acosta T, Takagi M, Sato K, 1998: Local distributions of oviductal estradiol, progesterone, prostaglandins, oxytocin and endothelin-1 in the cyclic cow. Theriogenology **49,** 607–618.

Yang CH, Yanagimachi R, 1989: Differences between mature ovarian and oviductal oocytes: a study using the golden hamster. Hum Reprod **4,** 63–71.

Yániz JL, Lopez-Gatius F, Hunter RH, 2006: Scanning electron microscopic study of the functional anatomy of the porcine oviductal mucosa. Anat Histol Embryol **35,** 28–34.

Yániz JL, Lopez-Gatius F, Santolaria P, Mullins KJ, 2000: Study of the functional anatomy of bovine oviductal mucosa. Anat Rec **260,** 268–278.

**Submitted: 2 Feb 2012; Accepted: 28 Mar 2012**

**Author's address (for correspondence):** Manuel Avilés, Department of Cell Biology and Histology, Faculty of Medicine, Campus de Espinardo, E-30100, University of Murcia, Murcia, Spain. E-mail: maviles@um.es

© 2012 Blackwell Verlag GmbH

Downloaded from http://perspectivesinmedicine.cshlp.org/ at WASHINGTON STATE UNIV on January 19, 2016 - Published by Cold Spring Harbor Laboratory Press

# The Role of *Hox* Genes in Female Reproductive Tract Development, Adult Function, and Fertility

**Hongling Du and Hugh S. Taylor**

Department of Obstetrics, Gynecology and Reproductive Sciences, Yale University School of Medicine, New Haven, Connecticut 06520

*Correspondence:* hugh.taylor@yale.edu

Cold Spring Harbor Perspectives in Medicine
www.perspectivesinmedicine.org

*HOX* genes convey positional identity that leads to the proper partitioning and adult identity of the female reproductive track. Abnormalities in reproductive tract development can be caused by *HOX* gene mutations or altered *HOX* gene expression. Diethylstilbestrol (DES) and other endocrine disruptors cause Müllerian defects by changing *HOX* gene expression. *HOX* genes are also essential regulators of adult endometrial development. Regulated *HOXA10* and *HOXA11* expression is necessary for endometrial receptivity; decreased *HOXA10* or *HOXA11* expression leads to decreased implantation rates. Alternation of *HOXA10* and *HOXA11* expression has been identified as a mechanism of the decreased implantation associated with endometriosis, polycystic ovarian syndrome, leiomyoma, polyps, adenomyosis, and hydrosalpinx. Alteration of *HOX* gene expression causes both uterine developmental abnormalities and impaired adult endometrial development that prevent implantation and lead to female infertility.

*HOX* genes comprise a family of regulatory molecules that encode highly conserved transcription factors. In the past several decades, molecular and genetic evidence indicates that *HOX* genes are expressed along anterior–posterior axes and control morphogenesis and cell differentiation during normal embryonic axial development; this mechanism for assigning differential identity along previously uniform axes is used in species as diverse as *Drosophila* and humans (McGinnis and Krumlauf 1992). *HOX* genes have a similar role in the specification of the developmental fate in individual regions of the female reproductive tract, where they regulate developmental axis in the embryonic period. *HOX* genes also give specific identity to the developing endometrium during the menstrual cycle in adults. The cyclic growth of endometrium is dependent on the ordered production of estrogen and progesterone. *HOX* gene expression is regulated by sex steroids, and this regulated expression plays an important role in endometrial development and endometrial receptivity (Taylor et al. 1997, 1998, 1999b). Here, we review the role of *HOX* genes, specifically the *HOXA*/*Hoxa* genes, in reproductive tract development, endometrial cyclic growth and embryo implantation, and the alterations in *HOXA*/*Hoxa* gene expression that can lead to infertility.

Editors: Diana W. Bianchi and Errol R. Norwitz
Additional Perspectives on Molecular Approaches to Reproductive and Newborn Medicine available at www.perspectivesinmedicine.org

Copyright © 2016 Cold Spring Harbor Laboratory Press; all rights reserved; doi: 10.1101/cshperspect.a023002
Cite this article as *Cold Spring Harb Perspect Med* 2016;6:a023002

Downloaded from http://perspectivesinmedicine.cshlp.org/ at WASHINGTON STATE UNIV on January 19, 2016 - Published by Cold Spring Harbor Laboratory Press



Cold Spring Harbor Perspectives in Medicine
www.perspectivesinmedicine.org
CSH PERSPECTIVES

## *HOX* GENES AND THEIR ROLE IN THE BODY PLAN

### *HOX* Genes

Homeobox genes (as known as *HOX* genes) comprise a group of highly conserved genes that are essential regulators of anterior–posterior (A–P) axial pattern development. In 1978, the relationship between the location of a homeotic gene and positional development identity was first recognized in *Drosophila* (Lewis 1978). Six years later, the *HOX* genes were cloned and sequenced in the fruit fly *Drosophila melanogaster* (McGinnis et al. 1984a,b; Scott and Weiner 1984). Since then, multiple *HOX* genes have been identified in many species, including humans. *HOX* genes encode proteins that act as transcription factors. In each of the *HOX* genes, a 183-bp highly conserved sequence was identified, which encodes a 61-amino acid region, called the homeodomain (HD). Structural analyses have shown that the HD can self-fold, and form a structural motif called a "helix-turn-helix motif." Through this motif, the HD, a DNA binding domain, recognizes a typical core DNA sequence, typically TAAT or TTAT, and regulates the expression of target genes, many of which play a role in axial development (Gehring et al. 1994; Krumlauf 1994; Gruschus et al. 1999; Passner et al. 1999).

Like all other insects, *Drosophila* has eight *HOX* genes, which are clustered into two complexes in close proximity, the antennapedia (Ant-C) complex and bithorax (Bx-C) complex. In mice and humans, *Hox*/*HOX* genes are clustered into four unlinked genomic loci, *Hox a-d* (mouse) or *HOX A-D* (human); each locus contains nine to 13 genes and all four clusters contain a total of 39 *HOX* genes. Those four paralogues, classified by sequence similarity, are located on chromosomes 6, 11, 15, and 2 in mice and chromosomes 7, 17, 12, and 2 in humans. The clustered *HOX* genes are believed to have arisen from gross duplication of a single common ancestral cluster. Presently, none of the paralogues have 13 genes, so some duplicated genes must have been lost during the course of evolution (Krumlauf 1994).

### *Hox* Genes and Vertebrate Axial Development

In general, expression of the *HOX* genes follows a 3′ to 5′ order, which means, *HOX* genes at 3′ end are expressed earlier in development than their 5′ neighbors within the same cluster. The position in the cluster reflects both the timing and spatial position of developmental expression (Hunt and Krumlauf 1992; McGinnis and Krumlauf 1992). *HOX* genes have a well-characterized role in embryonic development, during which they determine identity along the A–P body axis. In vertebrates, gastrulation forms three germ layers: ectoderm, endoderm, and mesoderm. *HOX* genes are first expressed in the mesoderm during early gastrulation, and the 3′ genes are expressed first in anterior locations and then the 5′ genes are expressed later in the distal sacral regions. The role of mammalian *HOX* genes in regulating segmental patterns of hindbrain, skeleton axis and the limb axis is well established. In mice, gain- and loss-of-function experiments have revealed the spatiotemporal expression controlled by *Hox* genes in skeleton development (Ramirez-Solis et al. 1993; Horan et al. 1995; Fromental-Ramain et al. 1996; Favier and Dolle 1997). For instance, loss of *Hoxb4* expression leads to defects in the first and second cervical vertebrae. Targeted mutations of *Hoxa9* and *Hoxd9* result in anterior transformations of distinct lumbosacral vertebrae. There are transformations of sacral and first caudal vertebrae in *Hoxa11* knockout mice. In the vertebrate nervous system, the hindbrain or rhombencephalon develops under the regulating of such segmental patterning directed by *Hox* gene expression as well; regional expression of *Hox* genes in the hindbrain is thought to confer identity to rhombomeres (Carpenter et al. 1993; Mark et al. 1993; Goddard et al. 1996; Studer et al. 1996; Morrison et al. 1997; Manzanares et al. 1999; Ferretti et al. 2000; Yau et al. 2002). Mice harboring a *Hoxa1* mutation have alteration in hindbrain segmentation, deleting all or part of rhombomere5 (r5). The absence of *Hoxb1* function results in an apparent segmental transformation of r4 to an r2-like rhombomere identity. *Hox-* is essential for r4 development. *Hoxa3* and *Hoxb3* genes are segmentally ex-

Downloaded from http://perspectivesinmedicine.cshlp.org/ at WASHINGTON STATE UNIV on January 19, 2016 - Published by Cold Spring Harbor Laboratory Press



Cold Spring Harbor Perspectives in Medicine
www.perspectivesinmedicine.org

pressed in r4 and r6. *Hoxa4*, *Hoxb4*, and *Hoxd4* have anterior limits in the hindbrain, but map to the junction between rhombomeric segments r6 and r7. Vertebrate *HOX* genes not only specify positional identity along the A–P axis of the body plan, but also provide positional values on the axis of the developing limb (Davis and Capecchi 1996; Nelson et al. 1996; Goff and Tabin 1997; Scott 1997). The most 5′ members of the *Hoxa* and *Hoxd* clusters (*Hoxa9-13* and *Hoxd9-13*) are particularly important in vertebrate limb development. *Hoxa9* to *Hoxa10* and *Hoxd9* to *Hoxa10* are expressed in the developing upper arm/leg; *Hoxa11* and *Hoxd9* to *Hoxa13* are expressed in the development of the lower part of the arm/leg. *Hoxa13* and *Hoxd10* to *Hoxd13* are expressed during specification of the hand/foot. The first identified human limb malformation related to a defective *HOX* gene was synpolydactyly, which results from mutations in the *HOXD13* gene (Muragaki et al. 1996). The role of *HOX* genes in vertebrate axial patterning is similar to but more complex than that in *Drosophila*. In the mice and humans, *Hox*/*HOX* gene clusters provide a considerably overlapping expression pattern, which provides for the possibility of redundancy.

## THE ROLE OF *HOX* GENES IN FEMALE REPRODUCTION

### *HOX* Genes and Structure of Female Reproductive Tract

The female reproductive system is derived from the paramesonephric (Müllerian) duct, which ultimately develops into the fallopian tube (oviduct), uterus, cervix, and upper part of the vagina. The developing of female reproductive tract is patterned by the differential expression of *HOX* genes in the Müllerian duct.

In the developing Müllerian duct, a number of posterior *Abdominal B (AbdB) HOX* genes were found to be expressed in partially overlapping patterns along the A–P axis. In vertebrates, *HOX* genes in paralogous groups *Hoxa9-13* develop a characteristic spatial distribution throughout the Müllerian duct (Taylor et al. 1997; Taylor 2000; Goodman 2002). *AbdB* genes are expressed according to their 3′ to 5′ order in the *HOX* gene clusters. *Hoxa9* is expressed at high levels in areas that will become the oviduct, *Hoxa10* is expressed in the development of the uterus, *Hoxa11* is found in the primordial lower uterus and cervix, and *Hoxa13* is seen in the ectocervix and upper vagina. No gene exists in the *Hoxa* cluster that is a paralogue of *Hoxd12* or *Hoxc12*; hence, there is no *Hoxa12* gene. This expression pattern is conserved between mice and humans (Fig. 1). Targeted mutagenesis of these genes results in region-specific defects along the female reproductive tract. *Hoxa10* deficiency causes the homeotic transformation of the anterior part of the uterus into an oviduct-like structure. *Hoxa13* null embryos show a hypoplastic urogenital genital sinus and agenesis of the posterior portion of the Müllerian duct. When the *Hoxa11* gene is replaced by the *Hoxa13* gene, posterior homeotic transformation occurs in the female reproductive tract: the uterus, in which *Hoxa11*, but not *Hoxa13* is normally expressed, becomes similar to the more posterior cervix and vagina, in which *Hoxa13* is normally expressed (Satokata et al. 1995; Benson et al. 1996; Warot et al. 1997).

Although *HOX* genes were once considered to be expressed only during embryonic development, persistent *HOX* gene expression was first well characterized in the adult female reproductive tract (Benson et al. 1996; Taylor et al. 1997). The adult reproductive tract undergoes a continuing developmental process during each menstrual cycle; proliferation and differentiation of endometrium coupled with angiogenesis leads to a new endometrium in each estrus or menstrual cycle. In both mice and humans, the expression of *Hoxa9-13*/*HOXA9-13* in the adult reproductive tract has been described as the same regions as their expression in the embryo (Dolle et al. 1991; Favier and Dolle 1997; Taylor et al. 1997; Warot et al. 1997). Specifically, *Hoxa10*/*HOXA10* and *Hoxa11*/*HOXA11* are expressed in the endometrium of the adult mice and humans. The expression of these two genes varies in an estrus/menstrual cycle-dependent manner (Fig. 2). *Hoxa10*/*HOXA10* and *Hoxa11*/*HOXA11* are expressed in the proliferative phase of the endometrium and increase during the secretory phase (Taylor et al.

Downloaded from http://perspectivesinmedicine.cshlp.org/ at WASHINGTON STATE UNIV on January 19, 2016 - Published by Cold Spring Harbor Laboratory Press



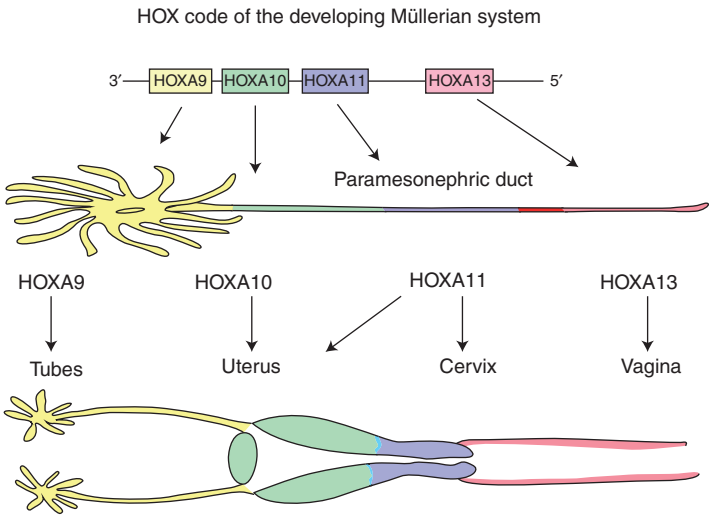


**Figure 1.** *HOX* code of the developing Müllerian system (adapted from Taylor 2000).

1997, 1998, 1999b). Persistent *HOX* gene expression in the adult may be a mechanism to retain developmental plasticity in the female reproductive tract.

*Emx2* is a divergent Homeobox gene, which is a mammalian homolog of the *Drosophila* empty spiracles (ems) gene. The vertebrate *Emx2* gene is located outside of the *Hox* cluster, and is expressed in the developing vertebrate brain as well as the urogenital system (Simeone et al. 1992a,b). In the embryo, *Emx2* is expressed in the epithelial components of the pronephros, mesonephros, ureteric buds, and the Wolffian and Müllerian ducts. In mouse embryos, *Emx2* expression is greatly diminished in male gonad, but strong expression remains detectable throughout the female gonad. Null mutants of *Emx2* mice fail to develop kidneys, gonads or a reproductive tract (Pellegrini et al. 1997; Svingen and Koopman 2007). In adults, *EMX2* has been detected in the human uterus. The expression of *EMX2* displayed a dynamic pattern that varied with the developmental phase of the human reproductive cycle (Fig. 2) (Troy et al. 2003).

## The Role of *HOX* Genes in Female Fertility

Female fertility is a broad term, which includes the ability to reproduce or become pregnant. Multiple factors influence female fertility, including normal aging and several disease processes. However, two processes are essential for normal female fertility: ovarian follicular maturation and embryo implantation. In vertebrates, *HOX* genes are involved in both of these processes.

Ovarian follicle development is a complex process in which many transcription factors participate. As described above, *HOX* genes containing the evolutionarily conserved HD sequence encode a family of DNA-binding transcription factors whose functions are crucial for embryonic development in vertebrates. In 1995, *HOXA4* and *HOXA7* expression was first described in the human unfertilized oocytes (Verlinsky et al. 1995). Sequence analysis of cDNA libraries generated from human unfertilized oocytes confirmed the expression of *HOXA7* (Adjaye and Monk 2000). Furthermore, in human ovarian folliculogenesis, *HOXA7* expression is nearly absent in primordial follicles but high in primary and mature follicles. During follicular maturation, the subcellular localization of *HOXA7* changes from nuclear to predominantly cytoplasmic. This differential localization indicates that *HOXA7* undergoes cell type- and stage-specific changes during the human ovarian folliculogenesis, and regulates proliferative

Cold Spring Harbor Perspectives in Medicine
www.perspectivesinmedicine.org
CSH PERSPECTIVES

Downloaded from http://perspectivesinmedicine.cshlp.org/ at WASHINGTON STATE UNIV on January 19, 2016 - Published by Cold Spring Harbor Laboratory Press



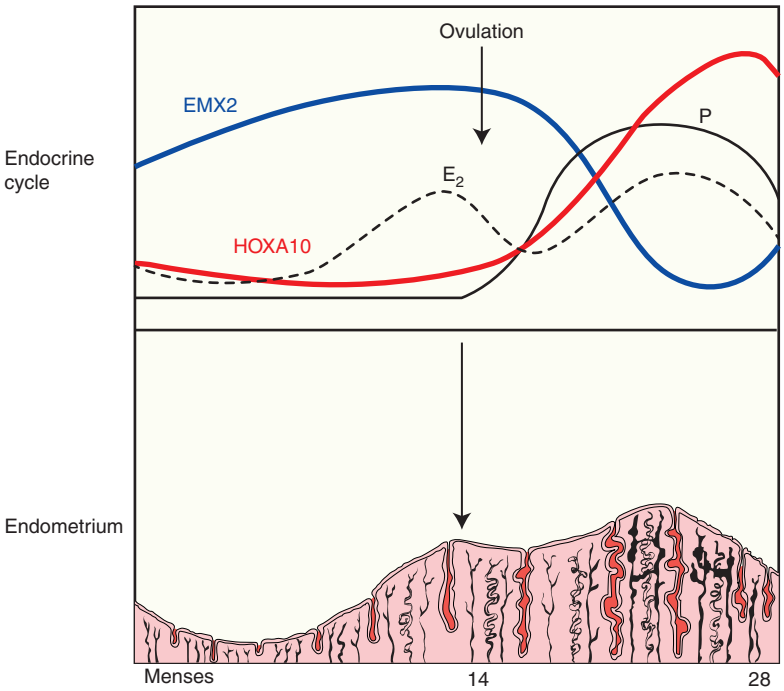


**Figure 2.** The pattern of *HOXA10* expression in the human endometrium through the menstrual cycle (adapted from Taylor 2000). *HOXA11* expression closely parallels that of *HOXA10*.

activities of ovarian follicles (Ota et al. 2006). Granulosa cells surround the developing oocyte, providing a critical microenvironment for follicular growth. During this process, the oocyte and the granulosa cells establish mutual interactions and their growth is regulated by coordinated paracrine mechanisms. *HOXA7* modulates granulosa cell growth and proliferation not only via the regulation of the epidermal growth factor receptor (EGFR), but also forms dimers with the *HOX* gene cofactor pre-B-cell leukemia transcription factor 2 (PBX2) to bind the specific promoter regions in the human granulosa cells. *HOXA7* plays an important role in ovarian follicular maturation (Ota et al. 2008; Zhang et al. 2010).

Embryo implantation is critical for female reproduction. This process is a complex event requiring synchronization between a developing embryo and receptive endometrium. Fundamental to this process is the dynamic and precisely ordered molecular and cellular events that drive and stabilize the interaction between the developing embryo and its host endometrium. As described above, *Hoxa10*/*HOXA10* and *Hoxa11*/*HOXA11* are expressed in endometrial glands and stroma throughout the estrus/menstrual cycle. These two *HOX* genes are essential for embryo implantation in both mice and humans (Hsieh-Li et al. 1995; Satokata et al. 1995; Benson et al. 1996; Gendron et al. 1997). Targeted mutation of either *Hoxa10* or *Hoxa11* in the mice leads to infertility related to defects in uterine receptivity. Embryos produced by *Hoxa10* deficient mice are viable and can successfully implant in wild-type surrogates. However, those embryos are not able to implant or survive in the uteri of *Hox* gene knockout mice. Although the uteri of these knockout mice appear anatomically normal, they do not support the development or implantation of their own embryos, nor of embryos from the wild-type mice. Histologic abnormalities were noted in the *Hoxa10* deficient mice, resulting in a homeotic transformation of the anterior part of the uterus into an oviduct-like structure. Similarly,

Cold Spring Harbor Perspectives in Medicine
www.perspectivesinmedicine.org
CSH PERSPECTIVES

Downloaded from http://perspectivesinmedicine.cshlp.org/ at WASHINGTON STATE UNIV on January 19, 2016 - Published by Cold Spring Harbor Laboratory Press



Cold Spring Harbor Perspectives in Medicine
www.perspectivesinmedicine.org

the mice with a homozygous mutation in the *Hoxa11* gene are infertile because of implantation defects. Those mice have reduced endometrial glands and decreased leukemia inhibitory factor (LIF) secretion. Targeted mutation of orthologous *Hox* genes such as both *Hoxd9* and *Hoxd10* in mice does not result in abnormalities on uterine structure or position (De La Cruz et al. 1999). Although no human females with mutations in *HOXA10* and *HOXA11* have been described, it has been reported that patients with lower implantation rates have lower *HOXA10* and *HOXA11* expression in the secretory phase, which indicates that maternal *HOX* gene expression is conserved and necessary for endometrial receptivity (Taylor et al. 1999b; Bagot et al. 2000; Taylor 2000).

## Estrogens and Progesterone Regulate *Hox* Gene Expression in the Reproductive Tract

So far, few regulators of *HOX* gene expression have been identified. Sex steroids have been investigated in the regulation of the *HOX* genes at the 5′ end of the cluster, which determine the posterior development, including the development of female reproductive tract (Taylor et al. 1997, 1998, 1999b; Ma et al. 1998; Cermik et al. 2001; Goodman 2002). During each reproductive cycle, endometrial epithelial and stromal cells display a well-defined cyclic pattern of functional differentiation under the influence of estrogen and progesterone. Menstrual cyclicity is regulated by timed expression of estrogen and progesterone, which act both independently and in concert to up-regulate *HOXA10* and *HOXA11* expression in the endometrium. In normal cycling women, *HOXA10* and *HOXA11* levels increase, reaching maximal expression during the mid-secretory phase, and remaining elevated throughout the secretory phase. In endometrial stromal cells, 17β-estradiol and progesterone significantly increase *HOXA10* and *HOXA11* expression. *HOXA9* is under the control of both estrogen and progesterone as well. The regulation of *HOX* gene expression in the adult uterus by ovarian steroids is related to its position within the cluster and mediated by the direct action of estrogen and progesterone receptors on these genes.

Humans are exposed to a wide variety of chemicals that have estrogenic properties. Those estrogenic compounds show profound and lasting effects on essential developmental genes in female reproductive tract. They have potential to alter the expression of estrogen responsive genes, such as *HOX* genes. These changes are likely to influence reproductive competence. Diethylstilbestrol (DES) is a nonsteroidal estrogen, a well-known teratogen. This chemical alters the localization of *Hox* gene expression along the axis of the developing murine reproductive tract, and induces developmental anomalies of female reproductive tract (Ma et al. 1998; Akbas et al. 2004). DES exposure in utero shifts *Hoxa9* expression from the oviducts to the uterus and leads to decreases in both *Hoxa10* and *Hoxa11* expression in the uterus. The decreased expression of the *Hoxa* genes may cause a "T-shaped" uterus, a structure that is characterized by branching and narrowing of the uterus into a tube-like phenotype. This phenotype is likely caused by expression of the *Hox* gene that controls tubal identity (*Hoxa9*) ectopically in the uterus. Because the multiple *HOX* gene clusters provide an overlapping expression pattern in the mice and humans, the complete transformation into an oviduct is probably prevented.

Studies on xenoestrogens, such as methoxychlor (MXC) and bisphenoyl A (BPA), have shown that exposure to these chemicals also alters the *Hoxa10* expression in female reproductive tract (Block et al. 2000; Suzuki et al. 2004; Fei et al. 2005; Markey et al. 2005; Sugiura-Ogasawara et al. 2005; Daftary and Taylor 2006; Smith and Taylor 2007). MXC is a pesticide and this chemical is associated with female reproductive defects after either prenatal or postnatal exposure. MXC specifically alters *Hoxa10* gene expression, specifically the *Hoxa10* gene expression. This *HOX* gene is responsible for normal uterine development and fertility, and its expression is permanently repressed in the uterus of mice exposed to MXC in utero. This effect is mediated through the *HOXA10* estrogen response element (ERE) in a dose-dependent pattern.

Downloaded from http://perspectivesinmedicine.cshlp.org/ at WASHINGTON STATE UNIV on January 19, 2016 - Published by Cold Spring Harbor Laboratory Press



Cold Spring Harbor Perspectives in Medicine
www.perspectivesinmedicine.org

BPA, another xenoestrogen, is a common component of polycarbonate plastics, epoxies used in food storage, canned goods, and dental sealants. BPA is also associated with adverse reproductive outcomes in both animal models and humans. After exposure to BPA in utero, *Hoxa10* expression is increased in female mice and this altered expression persisted in adults. The alternation of the gene expression persists long after exposure and alters the normally precise, temporal regulation of *Hoxa10* in reproductive tract development. This permanently modified expression of *Hoxa10* contributes to the decline in female reproductive potential. Despite its opposite effect on *HOX* gene expression in vivo, BPA behaves similarly to MXC in vitro by stimulating the *HOXA10* ERE. The difference seen after in utero exposure likely represents the unique molecular signals present in the embryo and underlies the increased risk of exposure to environmental chemicals during critical periods of development. Exposure to various xenoestrogens alters *Hoxa10* gene expression in the developing reproductive tract, and these exposures may lead to permanent alteration of gene expression in the adult (Fig. 3) (Taylor 2008).

## *HOX* GENES AND INFERTILITY

*HOX* genes are essential for endometrial development and embryo implantation in both mice and humans. As described above, the association between alteration of *Hoxa* gene expression and fertility is evident in animal models (Fig. 4) (Paria et al. 2002). The *Hoxa10*/*HOXA10* and *Hoxa11*/*HOXA11* genes act as important transcriptional moderators that either activate or repress the downstream target genes; these targets include β3-integrin and *Emx2*/*EMX2*, which are themselves important for embryo implantation. As discussed earlier, in normal cycling women, there is a surge of *HOXA10* and *HOXA11* expression during the mid-secretory phase; diminished *HOXA10* and *HOXA11* expression in the secretory phase leads to low embryo implantation rates. Impaired uterine receptivity has been studied in several gynecological diseases that lead to infertility. These include endometriosis, polycystic ovarian syndrome, leiomyoma, and hydrosalpinx. Compared with controls, there is diminished *HOXA10* and *HOXA11* expression in woman with each of those disorders (discussed in detail below). Although differential mechanisms may lead to decreased expression, it appears that altered *HOX* gene expression is so central to the process of implantation that decrease of their expression is required to diminish implantation. Alterations in the expression of *HOX* genes cause infertility in humans primarily by endometrial receptivity defects and impaired implantation.

### *HOX* Genes and Endometriosis

Endometriosis is an estrogen-dependent benign inflammatory disease defined by the presence of viable endometrial tissue outside the uterine cavity. The prevalence of endometriosis has been estimated as up to 10% to 15% of reproductive-age women and 30%–50% of women with endometriosis have infertility (Verkauf 1987; Olive and Pritts 2001). Multiple factors are considered to contribute to endometriosis related infertility, including altered folliculogenesis, impaired fertilization, poor oocyte quality, and defective implantation. Here, we will focus on the role of diminished implantation as it is related to diminished *HOX* gene expression. In patients with endometriosis, implantation rates are reduced during both natural and assisted reproductive technology cycles, even in patients with minimal disease (Barnhart et al. 2002). Two of the *HOXA* genes, *HOXA10* and *HOXA11*, involved in uterine embryogenesis and endometrial receptivity, have been implicated in the pathogenesis of endometriosis-associated infertility. In humans, the expression of both *HOXA10* and *HOXA11* rises dramatically during the implantation window and remains elevated throughout the secretory phase. However, patients with endometriosis do not show this rise in *HOXA10* and *HOXA11* (Taylor et al. 1999a; Kim et al. 2007; Lee et al. 2009).

*HOXA10* downstream target genes are also involved in this pathologic mechanism. As discussed above, *EMX2* is a divergent Homeobox gene, cyclically expressed in the adult

Downloaded from http://perspectivesinmedicine.cshlp.org/ at WASHINGTON STATE UNIV on January 19, 2016 - Published by Cold Spring Harbor Laboratory Press



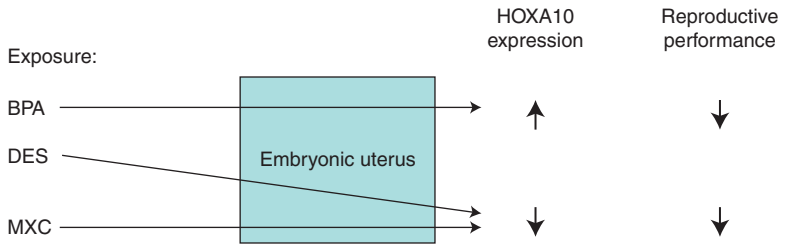


**Figure 3.** Exposure to various xenoestrogens alters *HOXA10* gene expression in the developing reproductive tract. BPA, bisphenol A; DES, diethylstilbestrol; and MXC, methoxychlor.

endometrium. Endometrial *EMX2* expression is directly regulated by endogenous endometrial *HOXA10*. Normally *EMX2* expression is down-regulated in the peri-implantation period; however, this regulated expression fails in women with endometriosis (Troy et al. 2003; Daftary and Taylor 2004). Further demonstrating the important role of this target gene, altering the endometrial *Emx2* levels is not only associated with defective implantation, but also reduces litter size in mice (Taylor and Fei 2005). Aberrant endometrial *EMX2* expression in women with endometriosis is mediated by altered *HOXA10* expression.

Furthermore, another biomarker of endometrial receptivity to embryonic implantation is also found to be decreased in endometriosis. Integrins are ubiquitous cell adhesion molecules that participate in cell–cell and cell–substratum interactions. These molecules undergo dynamic alterations during the normal menstrual cycle in the human endometrium. β3-integrin is expressed in endometrium at the time of implantation, and the disruption of in-

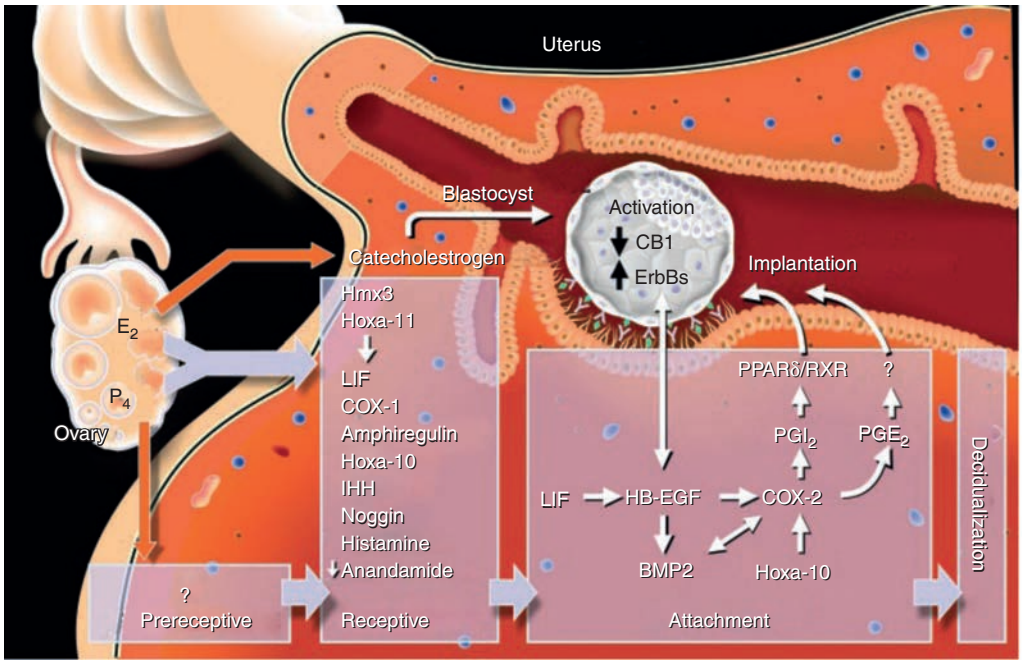


**Figure 4.** Molecular signaling during implantation in the mouse and human. (From Paria et al. 2002, reprinted, with permission, from The American Association for the Advancement of Science ©2002.)



Cold Spring Harbor Perspectives in Medicine
www.perspectivesinmedicine.org
CSH PERSPECTIVES

Downloaded from http://perspectivesinmedicine.cshlp.org/ at WASHINGTON STATE UNIV on January 19, 2016 - Published by Cold Spring Harbor Laboratory Press



Cold Spring Harbor Perspectives in Medicine
www.perspectivesinmedicine.org

tegrin expression is associated with decreased uterine receptivity and infertility (Lessey and Young 1997). Interestingly, β3-integrin subunit is a direct *Hoxa10* downstream target gene, and directly regulated by *HOXA10* in endometrial cells. Aberrant expression of both *HOXA10* and integrins have been described in the endometrium of women with endometriosis (Lessey et al. 1994; Lessey and Young 1997; Daftary et al. 2002; Klemmt et al. 2006; Cakmak and Taylor 2011).

Recent studies indicate that epigenetic modifications may play an important role in pathological process in endometriosis. Epigenetics refers to heritable alteration of DNA by long-lasting covalent methyl modification without DNA sequence changes. These epigenetic changes have been described in numerous studies including hypermethylation of *HOXA10*, progesterone receptor-β, and E-cadherin or hypomethylation of genes for estrogen receptor-β and steroidogenic factor 1 (Guo 2009; Senapati and Barnhart 2011). In both murine and baboon endometriosis models, hypermethylation of the promoter region of *Hoxa10*/*HOXA10* and decreased expression of *Hoxa10*/*HOXA10* genes were shown in eutopic endometrium (Kim et al. 2007; Lee et al. 2009). In humans, hypermethylation of *HOXA10* was identified in the endometrium of women with endometriosis (Wu et al. 2005). The DNA methyltransferase (DNMT) is a family of enzymes, which catalyze the transfer of a methyl group to DNA. DNMT 1, 3A, and 3B were found to be overexpressed in the epithelial component of endometriotic implants. However, only DNMT3A was found to be up-regulated in eutopic endometrium of women with endometriosis (Wu et al. 2007). A recently published study, using a genome-wide methylation array, shows that *HOXA10* expression was repressed and methylation of *HOXA10* gene was altered by 1.3-fold in human endometriosis (Naqvi et al. 2014). Other *HOX* genes, such as *HOXD10* and *HOXD11*, also showed significantly altered methylation in endometriosis (Naqvi et al. 2014). Epigenetic programming of *HOX* gene expression in endometriosis leads to lasting alterations in endometrial receptivity.

## *HOX* Genes and Polycystic Ovarian Syndrome

Polycystic ovarian syndrome (PCOS) is a common endocrine disease, afflicting 5% of women of reproductive age. It is characterized by anovulation and elevated androgen action. Infertility associated with PCOS derives from chronic anovulation. Despite the ability to correct ovulatory disorders, pregnancy rates remain paradoxically low, and spontaneous pregnancy loss rates are high. In women with PCOS, between 30% and 50% of all conceptions miscarry (Giudice 2006). Some data also suggest that poor oocyte quality, implantation failure, and higher rates of miscarriage further complicate achieving and maintaining a pregnancy in women with this disorder. Women with PCOS are also at significantly higher risk of endometrial hyperplasia (Niwa et al. 2000). PCOS may have complex effects on the endometrium, contributing to the infertility. Furthermore, increasing evidence and emerging data have shown that endometrial receptivity contributes to the infertility of PCOS even in the setting of ovulation induction (Giudice 2006). An increase in the expression of *HOXA10* in the endometrium is necessary for receptivity to embryo implantation. However, endometrial biopsies obtained from women with PCOS in ovulatory cycles have shown that *HOXA10* expression is decreased compared with normal fertile women during the secretory phase (Cermik et al. 2003). In vitro, *HOXA10* expression is repressed by testosterone (Cermik et al. 2003). Testosterone also prevents the increased expression of *HOXA10* induced by estradiol or progesterone. Dihydrotestosterone produced an effect similar to that of testosterone, whereas flutamide blocked the testosterone effect. Diminished uterine *HOXA10* expression may contribute to the diminished reproduction potential of women with PCOS, illustrating a significant effect of the disease on receptivity. Elevated androgen levels may induce infertility associated with PCOS by altering *HOX* gene expression.

As discussed above, β3-integrin, a biomarker of endometrial receptivity to embryonic implantation, is a *HOX* target gene that is

Downloaded from http://perspectivesinmedicine.cshlp.org/ at WASHINGTON STATE UNIV on January 19, 2016 - Published by Cold Spring Harbor Laboratory Press



Cold Spring Harbor Perspectives in Medicine
www.perspectivesinmedicine.org

directly regulated by *HOXA10* in endometrial cells. The expression of this biomarker is decreased in endometrium from women with PCOS compared with fertile controls (Apparao et al. 2002). Also, as described above, after ovulation induction treatment of infertility in PCOS, implantation rates remain low. In fertile women, when ovulation is induced with clomiphene citrate, the treatment provokes the expression of endometrial integrins at the implantation window. Interestingly, integrin is decreased in endometrial biopsy specimens from women with PCOS even after clomiphene citrate treatment (Gonzalez et al. 2001; Jakubowicz et al. 2001).

## *HOX* Genes and Leiomyoma

Leiomyomas (fibroids) are the most common benign uterine tumor of reproductive age women. The growth of leiomyoma is strictly related to sex steroids and their receptors. Their presence is associated with menorrhagia and poor reproductive outcomes. The prevalence of uterine fibroids approaches to 33% of women of reproductive age based on clinical assessment, and up to 50% on ultrasound scans. This disorder presents in 5%–10% of women with infertility (Payson et al. 2006; Revel 2012).

The presence of a distorted uterine cavity caused by leiomyomas significantly decreases in vitro fertilization (IVF) pregnancy rates. Fortunately, myomectomy can increase the pregnancy rates in patients with leiomyoma-related infertility (Bulletti et al. 1999; Surrey et al. 2001). However, the mechanisms by which leiomyoma cause infertility are not fully known. *HOXA10* is expressed in human myometrium and its expression also has a menstrual cycle-dependent pattern. In vitro, *HOXA10* expression is induced in endometrial stromal cells by progesterone, but in the primary myometrial cells, progesterone suppresses *HOXA10* expression (Cermik et al. 2001; Matsuzaki et al. 2009; Rackow and Taylor 2010; Sinclair et al. 2011). It is clear that there are different factors involved in the regulation of *HOXA10* by progesterone in myometrium than endometrium. Further, independent of any change in progesterone concentration, endometrial *HOXA10* and *HOXA11* expression are significantly decreased in uteri with submucosal myomas compared with controls. This effect is not localized to the endometrium overlying the myoma; rather the decreased *HOXA10* expression is seen throughout the endometrium. This global effect of the myoma on endometrium suggests the presence of a diffusible factor that would influence endometrial receptivity remote from the myoma itself. Indeed, we have recently reported that TGFβ secreted by myomas leads to decreased BMP receptor expression and subsequent *HOXA10* repression (Sinclair et al. 2011). Leiomyoma alter endometrial receptivity by secreting TGFβ and altering genes including *HOXA10* that are required for implantation.

## *HOX* Genes and Hydrosalpinx

Hydrosalpinx is an inflammatory disease involving the oviduct. The prevalence of hydrosalpinges in patients suffering from tubal disease is relatively common and ranges from 10% to 13% when diagnosed by ultrasound, and up to 30% when diagnosed by hysterosalpingography or at the time of surgery (Cakmak and Taylor 2011). Women with hydrosalpinges have decreased implantation rates in IVF, and their pregnancy rates can be improved with salpingectomy before IVF. The hydrosalpinx generates an inflammatory fluid that may interfere with endometrial receptivity and embryonic implantation mechanically or chemically (Zeyneloglu et al. 1998; Camus et al. 1999). Although a study has shown that culturing mice embryos in the medium containing hydrosalpinx fluid can suppress embryo maturation and promote degeneration, this toxic effect does not affect human embryos. (Mukherjee et al. 1996; Strandell et al. 1998) We performed an in vitro study demonstrating that hydrosalpinx fluid decreased endometrial *HOXA10* mRNA expression in a dose-dependent pattern. Subsequently, studies on women with hydrosalpinges show that the expression of *HOXA10* was significantly lower in women with hydrosalpinges compared with fertile controls. After salpingectomy, *HOXA10* expression in infertile women with hydrosal-

Downloaded from http://perspectivesinmedicine.cshlp.org/ at WASHINGTON STATE UNIV on January 19, 2016 - Published by Cold Spring Harbor Laboratory Press



Cold Spring Harbor Perspectives in Medicine
www.perspectivesinmedicine.org
CSH PERSPECTIVES

pinges was similar to that of age-matched fertile women, indicating that salpingectomy restores *HOXA10* expression to physiological levels (Daftary and Taylor 2002; Daftary et al. 2007).

As described above, β3-integrin subunit is a well-characterized endometrial receptivity marker, directly regulated by *HOXA10* in endometrial cells. In women with the presence of hydrosalpinges, the expression of β3-integrin is also reduced. Interestingly, two thirds of patients with hydrosalpinx who underwent salpingectomy also showed return of *HOXA10* and β3-integrin back to normal levels (Bildirici et al. 2001).

## SUMMARY

All metazoans use *HOX* genes to regulate embryonic patterning. *HOX* genes play a fundamental role in morphogenesis during embryonic development. Well-characterized examples include the role of *HOX* genes in the patterning of the vertebrate hindbrain, skeleton, and limbs. In reproduction, *HOX* genes determine positional identity during embryonic development of the female reproductive tract. Abnormalities in reproductive tract development are related to *HOX* gene mutations and to alterations in the normal *HOX* gene expression patterns. This has been clearly shown in mice with targeted *Hox* gene mutations as well as in mice exposed to chemicals with estrogenic properties such as DES. In the adult, the endometrium undergoes an ordered process of differentiation leading to receptivity to implantation. *HOX* genes are also essential to this process. As transcription factors, *HOX* genes control cyclical endometrial development and receptivity by activating or repressing the expression of target genes. *HOXA10* and *HOXA11* expression increases drastically in the mid-secretory phase, the time of implantation, and they remain elevated throughout the secretory phase. This increased expression is necessary for embryonic implantation; decreased *Hoxa10*/*HOXA10* and *Hoxa11*/*HOXA11* expression at this time leads to decrease implantation rates in both mice and humans. Impaired uterine receptivity has been studied in several infertility-related gynecological diseases, such as endometriosis, polycystic ovarian syndrome, leiomyoma, and hydrosalpinx. Alternation of *HOXA10* and *HOXA11* expression has been identified as a mechanism of the decreased implantation associated with these disorders. Alteration of *Hoxa* gene expression causes both uterine developmental abnormalities and impaired adult endometrial development that prevent implantation and lead to female infertility.

## REFERENCES

Adjaye J, Monk M. 2000. Transcription of homeobox-containing genes detected in cDNA libraries derived from human unfertilizedoocytes and preimplantation embryos. *Mol Hum Reprod* **6:** 707–711.

Akbas GE, Song J, Taylor HS. 2004. A *HOXA10* estrogen response element (ERE) is differentially regulated by 17 β-estradiol and diethylstilbestrol (DES). *J Mol Biol* **23:** 1013–1023.

Apparao KB, Lovely LP, Gui Y, Lininger RA, Lessey BA. 2002. Elevated endometrial androgen receptor expression in women with polycystic ovarian syndrome. *Biol Reprod* **66:** 297–304.

Bagot CN, Troy PJ, Taylor HS. 2000. Alteration of maternal *Hoxa10* expression by in vivo gene transfection affects implantation. *Gene Ther* **7:** 1378–1384.

Barnhart K, Dunsmoor-Su R, Coutifaris C. 2002. Effect of endometriosis on in vitro fertilization. *Fertil Steril* **77:** 1148–1155.

Benson GV, Lim H, Paria BC, Satokata I, Dey SK, Maas RL. 1996. Mechanisms of reduced fertility in *Hoxa-10* mutant mice: Uterine homeosis and loss of maternal *Hoxa-10* expression. *Development* **122:** 2687–2696.

Bildirici I, Bukulmez O, Ensari A, Yarali H, Gurgan T. 2001. A prospective evaluation of the effect of salpingectomy on endometrial receptivity in cases of women with communicating hydrosalpinges. *Hum Reprod* **16:** 2422–2426.

Block K, Kardana A, Igarashi P, Taylor HS. 2000. In utero diethylstilbestrol (DES), exposure alters *Hox* gene expression in the developing Müllerian system. *FASEB J* **14:** 1101–1108.

Bulletti C, De Ziegler D, Polli V, Flamigni C. 1999. The role of leiomyomas in infertility. *J Am Assoc Gynecol Laparosc* **6:** 441–445.

Cakmak H, Taylor HS. 2011. Implantation failure: Molecular mechanisms and clinical treatment. *Hum Reprod Update* **17:** 242–253.

Camus E, Poncelet C, Goffinet F, Wainer B, Merlet F, Nisand I, Philippe HJ. 1999. Pregnancy rates after in-vitro fertilization in cases of tubal infertility with and without hydrosalpinx: A meta-analysis of published comparative studies. *Hum Reprod* **14:** 1243–1249.

Carpenter EM, Goddard JM, Chisaka O, Manley NR, Gapecchi MR. 1993. Loss of *Hox-A1* (*Hox-1.6*) function

Downloaded from http://perspectivesinmedicine.cshlp.org/ at WASHINGTON STATE UNIV on January 19, 2016 - Published by Cold Spring Harbor Laboratory Press



Cold Spring Harbor Perspectives in Medicine
www.perspectivesinmedicine.org
CSH PERSPECTIVES

results in the reorganization of the murine hindbrain. *Development* **118:** 1063–1075.

Cermik D, Karaca M, Taylor HS. 2001. *HOXA10* expression is repressed by progesterone in the myometrium: Differential tissue-specific regulation of *HOX* gene expression in the reproductive tract. *J Clin Endocrinol Metab* **86:** 3387–3392.

Cermik D, Selam B, Taylor HS. 2003. Regulation of *HOXA10* expression by testosterone in vitro and in the endometrium of patients with polycystic ovary syndrome. *J Clin Endocrinol Metab* **88:** 238–243.

Daftary GS, Taylor HS. 2002. Hydrosalpinx fluid diminishes endometrial cell *HOXA10* expression. *Fertil Steril* **78:** 577–580.

Daftary GS, Taylor HS. 2004. *EMX2* gene expression in the female reproductive tract and aberrant expression in the endometrium of patients with endometriosis. *J Clin Endocrinol Metab* **89:** 2390–2396.

Daftary GS, Taylor HS. 2006. Endocrine regulation of *Hox* genes. *Endocr Rev* **27:** 331–335.

Daftary GS, Troy PJ, Bagot CN, Young SL, Taylor HS. 2002. Direct regulation of β3-integrin subunit gene expression by *HOXA10* in endometrial cells. *Mol Endocrinol* **16:** 571–579.

Daftary GS, Kayisli U, Seli E, Bukulmez O, Arici A, Taylor HS. 2007. Salpingectomy increases peri-implantation endometrial *HOXA10* expression in women with hydrosalpinx. *Fertil Steril* **87:** 367–372.

Davis AP, Capecchi MR. 1996. A mutational analysis of the 5′ *HoxD* genes: Dissection of genetic interactions during limb development in the mouse. *Development* **122:** 1175–1185.

De La Cruz CC, Der-Avakian A, Spyropoulos DD, Tieu DD, Carpenter EM. 1999. Targeted disruption of *Hoxd9* and *Hoxd10* alters locomotor behavior, vertebral identity, and peripheral nervous system development. *Dev Biol* **216:** 595–610.

Dolle P, Izpisua-Belmonte JC, Brown JM, Tickle C, Duboule D. 1991. *HOX-4* genes and the morphogenesis of mammalian genitalia. *Genes Dev* **5:** 1767–1776.

Favier B, Dolle P. 1997. Developmental functions of mammalian *Hox* genes. *Mol Hum Reprod* **3:** 115–131.

Fei X, Chung H, Taylor HS. 2005. Methoxychlor disrupts uterine *Hoxa10* gene expression. *Endocrinology* **146:** 3445–3451.

Ferretti E, Marshall H, Popperl H, Maconochie M, Krumlauf R, Blasi F. 2000. Segmental expression of *Hoxb2* in r4 requires two separate sites that integrate cooperative interactions between Prep1, Pbx and Hox proteins. *Development* **127:** 155–166.

Fromental-Ramain C, Warot X, Lakkaraju S, Favier B, Haack H, Birling C, Dierich A, Dolle P, Chambon P. 1996. Specific and redundant functions of the paralogous *Hoxa-9* and *Hoxd-9* genes in forelimb and axial skeleton patterning. *Development* **122:** 461–472.

Gehring WJ, Affolter M, Burglin T. 1994. Homeodomain proteins. *Annu Rev Biochem* **63:** 487–526.

Gendron RL, Paradis H, Hsieh-Li HM, Lee DW, Potter SS, Markoff E. 1997. Abnormal uterine stromal and glandular function associated with maternal reproductive defects in *Hoxa-11* null mice. *Biol Reprod* **56:** 1097–1105.

Giudice LC. 2006. Endometrium in PCOS: Implantation and predisposition to endocrine CA. *Best Pract Res Clin Endocrinol Metab* **20:** 235–244.

Goddard JM, Rossel M, Manley NR, Capecchi MR. 1996. Mice with targeted disruption of *Hoxb-1* fail to form the motor nucleus of the VIIth nerve. *Development* **122:** 3217–3228.

Goff DJ, Tabin CJ. 1997. Analysis of *Hoxd-13* and *Hoxd-11* misexpression in chick limb buds reveals that *Hox* genes affect both bone condensation and growth. *Development* **124:** 627–636.

Gonzalez RR, Palomino A, Vantman D, Gabler F, Devoto L. 2001. Abnormal pattern of integrin expression at the implantation window in endometrium from fertile women treated with clomiphene citrate and users of intrauterine device. *Early Pregnancy* **5:** 132–143.

Goodman FR. 2002. Limb malformations and the human *HOX* genes. *Am J Med Genet* **112:** 256–265.

Gruschus JM, Tsao DH, Wang LH, Nirenberg M, Ferretti JA. 1999. The three-dimensional structure of the vnd/NK-2 homeodomain-DNA complex by NMR spectroscopy. *J Mol Biol* **289:** 529–545.

Guo SW. 2009. Epigenetics of endometriosis. *Mol Hum Reprod* **15:** 87–607.

Horan GS, Ramirez-Solis R, Featherstone MS, Wolgemuth DJ, Bradley A, Behringer RR. 1995. Compound mutants for the paralogous *hoxa-4*, *hoxb-4*, and *hoxd-4* genes show more complete homeotic transformations and a dose-dependent increase in the number of vertebrae transformed. *Genes Dev* **9:** 1667–1677.

Hsieh-Li HM, Witte DP, Weinstein M, Brandford W, Li H, Small K, Potter SS. 1995. *Hoxa 11* structure, extensive antisense transcription, and function in male and female fertility. *Development* **121:** 1373–1385.

Hunt P, Krumlauf R. 1992. *Hox* codes and positional specification in vertebrate embryonic axes. *Annu Rev Cell Biol* **8:** 227–256.

Jakubowicz DJ, Seppälä M, Jakubowicz S, Rodriguez-Armas O, Rivas-Santiago A, Koistinen H, Koistinen R, Nestler JE. 2001. Insulin reduction with metformin increases luteal phase serum glycodelin and insulin-like growth factor-binding protein 1 concentrations and enhances uterine vascularity and blood flow in the polycystic ovary syndrome. *J Clin Endocrinol Metab* **86:** 1126–1133.

Kim JJ, Taylor HS, Lu Z, Ladhani O, Hastings JM, Jackson KS, Wu Y, Guo SW, Fazleabas AT. 2007. Altered expression of *HOXA10* in endometriosis: Potential role in decidualization. *Mol Hum Reprod* **13:** 323–332.

Klemmt PA, Carver JG, Kennedy SH, Koninckx PR, Mardon HJ. 2006. Stromal cells from endometriotic lesions and endometrium from women with endometriosis have reduced decidualization capacity. *Fertil Steril* **85:** 564–572.

Krumlauf R. 1994. *Hox* genes in vertebrate development. *Cell* **78:** 191–201.

Lee B, Du H, Taylor HS. 2009. Experimental murine endometriosis induces DNA methylation and altered gene expression in eutopic endometrium. *Biol Reprod* **80:** 79–85.

Lessey BA, Young SL. 1997. Integrins and other cell adhesion molecules in endometrium and endometriosis. *Semin Reprod Endocrinol* **15:** 291–299.

Downloaded from http://perspectivesinmedicine.cshlp.org/ at WASHINGTON STATE UNIV on January 19, 2016 - Published by Cold Spring Harbor Laboratory Press



Cold Spring Harbor Perspectives in Medicine
www.perspectivesinmedicine.org

Lessey BA, Castelbaum AJ, Sawin SW, Buck CA, Schinnar R, Bilker W, Strom BL. 1994. Aberrant integrin expression in the endometrium of women with endometriosis. *J Clin Endocrinol Metab* **79:** 643–649.

Lewis EB. 1978. A gene complex controlling segmentation in *Drosophila*. *Nature* **276:** 565–570.

Ma L, Benson GV, Lim H, Dey SK, Maas RL. 1998. *Abdominal B (AbdB) Hoxa* genes: Regulation in adult uterus by estrogen and progesterone and repression in Müllerian duct by the synthetic estrogen diethylstilbestrol (DES). *Dev Biol* **197:** 141–154.

Manzanares M, Cordes S, Ariza-McNaughton L, Sadl V, Maruthainar K, Barsh G, Krumlauf R. 1999. Conserved and distinct roles of kreisler in regulation of the paralogous *Hoxa3* and *Hoxb3* genes. *Development* **126:** 759–769.

Mark M, Lufkin T, Vonesch JL, Ruberte E, Olivo JC, Dolle P, Gorry P, Lumsden A, Chambon P. 1993. Two rhombomeres are altered in *Hoxa-1* mutant mice. *Development* **119:** 319–338.

Markey CM, Wadia PR, Rubin BS, Sonnenschein C, Soto AM. 2005. Longterm effects of fetal exposure to low doses of the xenoestrogenbisphenol-A in the female mouse genital tract. *Biol Reprod* **72:** 1344–1351.

Matsuzaki S, Canis M, Darcha C, Pouly JL, Mage G. 2009. *HOXA-10* expression in the mid-secretory endometrium of infertile patients with either endometriosis, uterine fibromas or unexplained infertility. *Hum Reprod* **24:** 3180–3187.

McGinnis W, Krumlauf R. 1992. Homeobox genes and axial patterning. *Cell* **68:** 283–302.

McGinnis W, Garber RL, Wirz J, Gehring WJ. 1984a. A homologous protein-coding sequence in *Drosophila* homeotic genes and its conservation in other metazoans. *Cell* **37:** 403–408.

McGinnis W, Levine MS, Hafen E, Kuroiwa A, Gehring WJ. 1984b. A conserved DNA sequence in homeotic genes of the *Drosophila* antennapedia and bithorax complexes. *Nature* **308:** 428–433.

Morrison A, Ariza-McNaughton L, Gould A, Featherstone M, Krumlauf R. 1997. *HOXD4* and regulation of the group 4 paralog genes. *Development* **124:** 3135–3146.

Mukherjee T, Copperman AB, McCaffrey C, Cook CA, Bustillo M, Obasaju MF. 1996. Hydrosalpinx fluid has embryotoxic effects on murine embryogenesis: A case for prophylactic salpingectomy. *Fertil Steril* **66:** 851–853.

Muragaki Y, Uragaki S, Upton J, Olsen BR. 1996. Altered growth and branching patterns in synpolydactyly caused by mutations in *HOXD13*. *Science* **272:** 548–551.

Naqvi HI, Ilagan Y, Krikun G, Taylor HS. 2014. Altered genome-wide methylation in endometriosis. *Reprod Sci* **21:** 1237–1243.

Nelson CE, Morgan BA, Burke AC, Laufer E, DiMambro E, Murtaugh LC, Gonzales E, Tessarollo L, Parada LF, Tabin C. 1996. Analysis of *Hox* gene expression in the chick limb bud. *Development* **122:** 1449–1466.

Niwa K, Imai A, Hashimoto M, Yokoyama Y, Mori H, Matsuda Y, Tamaya T. 2000. A case-control study of uterine endometrial cancer of pre- and postmenopausal women. *Oncol Rep* **7:** 89–93.

Olive DL, Pritts EA. 2001. Treatment of endometriosis. *N Engl J Med* **345:** 266–275.

Ota T, Choi KB, Gilks CB, Leung PC, Auersperg N. 2006. Cell type- and stage-specific changes in *HOXA7* protein expression in human ovarian folliculogenesis: Possible role of GDF-9. *Differentiation* **74:** 1–10.

Ota T, Asahina H, Park SH, Huang Q, Minegishi T, Auersperg N, Leung PC. 2008. *HOX* cofactors expression and regulation in the human ovary. *Reprod Biol Endocrinol* **6:** 49.

Paria BC, Reese J, Das SK, Dey SK. 2002. Deciphering the cross-talk of implantation: Advances and challenges. *Science* **296:** 2185–2188.

Passner JM, Ryoo HD, Shen L, Mann RS, Aggarwal AK. 1999. Structure of a DNA-bound ultrabithorax-extradenticle homeodomain complex. *Nature* **397:** 714–719.

Payson M, Leppert P, Segars J. 2006. Epidemiology of myomas. *Obstet Gynecol Clin North Am* **33:** 1–11.

Pellegrini M, Pantano S, Lucchini F, Fumi M, Forabosco A. 1997. *Emx2* developmental expression in the primordia of the reproductive and excretory systems. *Anat Embryol (Berl)* **196:** 427–433.

Rackow BW, Taylor HS. 2010. Submucosal uterine leiomyomas have a global effect on molecular determinants of endometrial receptivity. *Fertil Steril* **93:** 2027–2034.

Ramirez-Solis R, Zheng H, Whiting J, Krumlauf R, Bradley A. 1993. *Hoxb-4* (*Hox-2.6*) mutant mice show homeotic transformation of a cervical vertebra and defects in the closure of the sternal rudiments. *Cell* **73:** 279–294.

Revel A. 2012. Defective endometrial receptivity. *Fertil Steril* **97:** 1028–1032.

Satokata I, Benson G, Maas R. 1995. Sexually dimorphic sterility phenotypes in *Hoxa10*-deficient mice. *Nature* **374:** 460–463.

Scott MP. 1997. *Hox* genes, arms and the man. *Nat Genet* **15:** 117–118.

Scott MP, Weiner A. 1984. Structural relationships among genes that control development: Sequence homology between the Antennapedia, Ultrabithorax, and fushitarazu loci of *Drosophila*. *Proc Natl Acad Sci* **81:** 4115–4119.

Senapati S, Barnhart K. 2011. Managing endometriosis-associated infertility. *Clin Obstet Gynecol* **54:** 720–726.

Simeone A, Acampora D, Gulisano M, Stornaiuolo A, Boncinelli E. 1992a. Nested expression domains of four homeobox genes in developing rostral brain. *Nature* **358:** 687–690.

Simeone A, Gulisano M, Acampora D, Stornaiuolo A, Rambaldi M, Boncinelli E. 1992b. Two vertebrate homeobox genes related to the *Drosophila* empty spiracles gene are expressed in the embryonic cerebral cortex. *EMBO J* **11:** 2541–2550.

Sinclair DC, Mastroyannis A, Taylor HS. 2011. Leiomyoma simultaneously impair endometrial BMP-2-mediated decidualization and anticoagulant expression through secretion of TGF-β3. *J Clin Endocrinol Metab* **96:** 412–421.

Smith C, Taylor HS. 2007. Xenoestrogen exposure imprints expression of genes (*Hoxa10*) required for normal uterine development. *FASEB J* **21:** 239–246.

Strandell A, Sjögren A, Bentin-Ley U, Thorburn J, Hamberger L, Brännström M. 1998. Hydrosalpinx fluid does

Downloaded from http://perspectivesinmedicine.cshlp.org/ at WASHINGTON STATE UNIV on January 19, 2016 - Published by Cold Spring Harbor Laboratory Press



not adversely affect the normal development of human embryos and implantation in vitro. *Hum Reprod* **13:** 2921–2925.

Studer M, Lumsden A, Ariza-McNaughton L, Bradley A, Krumlauf R. 1996. Altered segmental identity and abnormal migration of motor neurons in mice lacking *Hoxb-1*. *Nature* **384:** 630–634.

Sugiura-Ogasawara M, Ozaki Y, Sonta S, Makino T, Suzumori K. 2005. Exposure to bisphenol A is associated with recurrent miscarriage. *Hum Reprod* **20:** 2325–2329.

Surrey ES, Lietz AK, Schoolcraft WB. 2001. Impact of intramural leiomyomata in patients with a normal endometrial cavity on in vitro fertilization-embryo transfer cycle outcome. *Fertil Steril* **75:** 405–410.

Suzuki M, Lee HC, Chiba S, Yonezawa T, Nishihara M. 2004. Effects of methoxychlor exposure during perinatal period on reproductive function after maturation in rats. *J Reprod Dev* **50:** 455–461.

Svingen T, Koopman P. 2007. Involvement of homeobox genes in mammalian sexual development. *Sex Dev* **1:** 12–23.

Taylor HS. 2000. The role of *HOX* genes in the development and function of the female reproductive tract. *Semin Reprod Med* **18:** 81–89.

Taylor HS. 2008. Endocrine disruptors affect developmental programming of *HOX* gene expression. *Fertil Steril* **89** e57–e58.

Taylor HS, Fei X. 2005. *Emx2* regulates mammalian reproduction by altering endometrial cell proliferation. *Mol Endocrinol* **19:** 2839–2846.

Taylor HS, Van den Heuvel GB, Igarashi P. 1997. A conserved *Hox* axis in the mouse and human female reproductive system: Late establishment and persistent adult expression of the *Hoxa* cluster genes. *Biol Reprod* **57:** 1338–1345.

Taylor HS, Arici A, Olive D, Igarashi P. 1998. *HOXA10* is expressed in response to sex steroids at the time of implantation in the human endometrium. *J Clin Invest* **101:** 1379–1384.

Taylor HS, Bagot C, Kardana A, Olive D, Arici A. 1999a. *HOX* gene expression is altered in the endometrium of women with endometriosis. *Hum Reprod* **14:** 1328–1331.

Taylor HS, Igarashi P, Olive D, Arici A. 1999b. Sex steroids mediate *HOXA11* expression in the human peri-implantation endometrium. *J Clin Endocrinol Metab* **84:** 1129–1135.

Troy PJ, Daftary GS, Bagot CN, Taylor HS. 2003. Transcriptional repression of peri-implantation *EMX2* expression in mammalian reproduction by *HOXA10*. *Mol Cell Biol* **23:** 1–13.

Verkauf BS. 1987. Incidence, symptoms, and signs of endometriosis in fertile and infertile women. *J Fla Med Assoc* **74:** 671–675.

Verlinsky Y, Morozov G, Gindilis V, Strom CM, Freidin M, Rechitsky S, Verlinsky O, Ivakhnenko V, Zdanovsky V, Kuliev A. 1995. Homeobox gene expression in human oocytes and preembryos. *Mol Reprod Dev* **41:** 127–132.

Warot X, Fromental-Ramain C, Fraulob V, Chambon P, Dolle P. 1997. Gene dosage-dependent effects of the *Hoxa-13* and *Hoxd-13* mutations on morphogenesis of the terminal parts of the digestive and urogenital tracts. *Development* **124:** 4781–4791.

Wu Y, Halverson G, Basir Z, Strawn E, Yan P, Guo SW. 2005. Aberrant methylation at *HOXA10* may be responsible for its aberrant expression in the endometrium of patients with endometriosis. *Am J Obstet Gynecol* **193:** 371–380.

Wu Y, Strawn E, Basir Z, Halverson G, Guo SW. 2007. Aberrant expression of deoxyribonucleic acid methyltransferases DNMT1, DNMT3A, and DNMT3B in women with endometriosis. *Fertil Steril* **87:** 24–32.

Yau TO, Kwan CT, Jakt LM, Stallwood N, Cordes S, Sham MH. 2002. Auto/cross-regulation of *Hoxb3* expression in posterior hindbrain and spinal cord. *Dev Biol* **252:** 287–300.

Zeyneloglu HB, Arici A, Olive DL. 1998. Adverse effects of hydrosalpinxon pregnancy rates after in vitro fertilization-embryo transfer. *Fertil Steril* **70:** 492–499.

Zhang Y, Huang Q, Cheng JC, Nishi Y, Yanase T, Huang HF, Leung PC. 2010. Homeobox A7 increases cell proliferation by up-regulation of epidermal growth factor receptor expression in human granulosa cells. *Reprod Biol Endocrinol* **8:** 61.

Cold Spring Harbor Perspectives in Medicine
www.perspectivesinmedicine.org

Downloaded from http://perspectivesinmedicine.cshlp.org/ at WASHINGTON STATE UNIV on January 19, 2016 - Published by Cold Spring Harbor Laboratory Press

Cold Spring Harbor Perspectives in Medicine

# The Role of *Hox* Genes in Female Reproductive Tract Development, Adult Function, and Fertility

Hongling Du and Hugh S. Taylor

*Cold Spring Harb Perspect Med* 2016; doi: 10.1101/cshperspect.a023002 originally published online November 9, 2015

**Subject Collection** Molecular Approaches to Reproductive and Newborn Medicine

**A Molecular Perspective on Procedures and Outcomes with Assisted Reproductive Technologies**
*Monica A. Mainigi, Carmen Sapienza, Samantha Butts, et al.*

**The Role of *Hox* Genes in Female Reproductive Tract Development, Adult Function, and Fertility**
*Hongling Du and Hugh S. Taylor*

**Whole-Exome Sequencing and Whole-Genome Sequencing in Critically Ill Neonates Suspected to Have Single-Gene Disorders**
*Laurie D. Smith, Laurel K. Willig and Stephen F. Kingsmore*

**Molecular Cross-Talk at the Feto–Maternal Interface**
*Gendie E. Lash*

**Molecular Regulation of Parturition: A Myometrial Perspective**
*Nora E. Renthal, Koriand'r C. Williams, Alina P. Montalbano, et al.*

**Genome-Wide Sequencing for Prenatal Detection of Fetal Single-Gene Disorders**
*Ignatia B. Van den Veyver and Christine M. Eng*

**Noninvasive Prenatal Screening for Genetic Diseases Using Massively Parallel Sequencing of Maternal Plasma DNA**
*Lyn S. Chitty and Y. M. Dennis Lo*

**Confrontation, Consolidation, and Recognition: The Oocyte's Perspective on the Incoming Sperm**
*David Miller*

**Noninvasive Antenatal Determination of Fetal Blood Group Using Next-Generation Sequencing**
*Klaus Rieneck, Frederik Banch Clausen and Morten Hanefeld Dziegiel*

**The Neonatal Salivary Transcriptome**
*Jill L. Maron*

**Potential Uses and Inherent Challenges of Using Genome-Scale Sequencing to Augment Current Newborn Screening**
*Jonathan S. Berg and Cynthia M. Powell*

**Molecular Regulation of Parturition: The Role of the Decidual Clock**
*Errol R. Norwitz, Elizabeth A. Bonney, Victoria V. Snegovskikh, et al.*

**Molecular Mechanisms of Preeclampsia**
*Tammy Hod, Ana Sofia Cerdeira and S. Ananth Karumanchi*

**Effects of Maternal Obesity on Fetal Programming: Molecular Approaches**
*Caterina Neri and Andrea G. Edlow*

**MicroRNA in Ovarian Biology and Disease**
*Lynda K. McGinnis, Lacey J. Luense and Lane K. Christenson*

**The Function of TrophomiRs and Other MicroRNAs in the Human Placenta**
*Yoel Sadovsky, Jean-Francois Mouillet, Yingshi Ouyang, et al.*

For additional articles in this collection, see http://perspectivesinmedicine.cshlp.org/cgi/collection/

Copyright © 2016 Cold Spring Harbor Laboratory Press; all rights reserved

The International Journal of Biochemistry & Cell Biology 54 (2014) 318–330

Contents lists available at ScienceDirect

The International Journal of Biochemistry & Cell Biology

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

Review

# Regulation of mammary epithelial cell homeostasis by lncRNAs☆

Amy N. Shore*, Jeffrey M. Rosen

*Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX, United States*

ARTICLE INFO

*Article history:*
Received 20 September 2013
Received in revised form 14 March 2014
Accepted 18 March 2014
Available online 26 March 2014

*Keywords:*
lncRNAs
Mammary gland
Development
Differentiation
Breast cancer

ABSTRACT

The epithelial cells of the mammary gland develop primarily after birth and undergo surges of hormonally regulated proliferation, differentiation, and apoptosis during both puberty and pregnancy. Thus, the mammary gland is a useful model to study fundamental processes of development and adult tissue homeostasis, such as stem and progenitor cell regulation, cell fate commitment, and differentiation. Long noncoding RNAs (lncRNAs) are emerging as prominent regulators of these essential processes, as their extraordinary versatility allows them to modulate gene expression via diverse mechanisms at both transcriptional and post-transcriptional levels. Not surprisingly, lncRNAs are also aberrantly expressed in cancer and promote tumorigenesis by disrupting vital cellular functions, such as cell cycle, survival, and migration. In this review, we first broadly summarize the functions of lncRNAs in mammalian development and cancer. Then we focus on what is currently known about the role of lncRNAs in mammary gland development and breast cancer.

This article is part of a Directed Issue entitled: The Non-coding RNA Revolution.

© 2014 Elsevier Ltd. All rights reserved.

**Contents**

1. Introduction ..... 319
   1.1. LncRNAs and development ..... 319
   1.2. LncRNAs and cancer ..... 319
2. LncRNAs: form and mechanism ..... 320
3. Development of the mammary gland ..... 321

*Abbreviations:* lncRNA, long/large noncoding RNA; *Xist*, X-inactive specific transcript; mESC, mouse embryonic stem cell; *Evf2*, embryonic ventral forebrain 2; *Six3os*, sine oculis-related homeobox 3 homolog, opposite strand; *Dlx1as*, distal-less homeobox 1, antisense; *EGO*, eosinophil granule ontogeny; *HOTAIRM1*, HOX antisense intergenic RNA myeloid 1; *LincRNA-EPS*, lincRNA erythroid prosurvival 1; *Lnc-MD1*, long noncoding RNA, muscle differentiation 1; *ANCR*, anti-differentiation noncoding RNA; *TINCR*, terminal differentiation-induced ncRNA; *Bvht*, braveheart; *Fendrr*, Fetal-lethal noncoding developmental regulatory RNA; SCNA, somatic copy number alteration; *PRNCR1*, prostate cancer noncoding RNA 1; *PCGEM1*, prostate cancer gene expression marker 1; *PANDA*, p21 associated ncRNA DNA damage activated; NF-YA, nuclear transcription factor Y, alpha; *MALAT1*, metastasis associated lung adenocarcinoma transcript 1; *BC200*, brain cytoplasmic RNA 200; miRNA, microRNA; piRNA, PIWI-interacting RNA; snoRNA, small nucleolar RNA; eRNA, enhancer RNA; PRC2, polycomb repressive complex 2; LSD1, lysine-specific demethylase 1; REST, repressor element-1 (RE1) silencing transcription factor; CoREST, co-repressor for REST; PRC1, polycomb repressive complex 1; MLL1, mixed lineage leukemia 1; TEB, terminal end bud; *SRA*, steroid receptor RNA activator; *mPINC*, mouse pregnancy induced noncoding RNA; *Zfas1*, Znfx1 antisense RNA 1; *Znfx1*, zinc finger, NFX1-type containing 1; *RbAp46*, retinoblastoma associated protein 46; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; TNBC, triple negative breast cancer; ADH, atypical ductal hyperplasia; DCIS, ductal carcinoma *in situ*; IDC, invasive ductal carcinoma; GRO-seq, global run-on sequencing; *MEG3*, maternally expressed gene 3; *PTENP1*, phosphatase and tensin homolog pseudogene 1; PTEN, phosphatase and tensin homolog; MRE, microRNA recognition element; asRNA, antisense RNA; *GAS5*, growth arrest-specific transcript 5; GR, glucocorticoid receptor; AR, androgen receptor; *UCA1*, urothelial carcinoma-associated 1; hnRNP I, heterogeneous nuclear ribonucleoprotein I; ATM, ataxia-telangiectasia mutated; *LSINCT5*, long stress-induced noncoding transcript 5; *NEAT1 and 2*, nuclear enriched abundant transcript 1 and 2; PSPC1, paraspeckle component 1; BRCA1, breast cancer 1, early onset; DDR, DNA damage response; H4, histone 4; HG, high grade; NHG, non-high grade; *treRNA*, translational regulatory RNA; EMT, epithelial-to-mesenchymal transition; SRSF1, 2 and 3, serine/arginine-rich splicing factor 1, 2 and 3; Pc2, polycomb 2 homolog; *HOTAIR*, HOX antisense intergenic RNA; EZH2, enhancer of zeste homolog 2; *PCDH*, protocadherin; *EPHA1*, ephrin type-A receptor 1; *IGF2*, insulin-like growth factor 2; IGN, imprinted gene network; *Igfr1*, insulin-like growth factor type 1 receptor; *RB1*, retinoblastoma 1; NR, nuclear receptors; MMTV LTR, mouse mammary tumor virus long terminal repeat; *PCA3*, prostate cancer gene 3.

☆ This article is part of a Directed Issue entitled: The Non-coding RNA Revolution.

* Corresponding author. Tel.: +1 713 798 6211.
*E-mail address:* as148002@bcm.edu (A.N. Shore).

http://dx.doi.org/10.1016/j.biocel.2014.03.012
1357-2725/© 2014 Elsevier Ltd. All rights reserved.

4. LncRNAs and mammary gland development ..... 321
4.1. Mammary gland lncRNAs of unknown function ..... 321
4.2. LncRNAs that regulate mammary epithelial differentiation ..... 321
5. The current state of breast cancer research ..... 323
6. LncRNAs associated with breast cancer ..... 323
6.1. Global lncRNA expression in breast cancer ..... 323
6.2. Tumor suppressor lncRNAs ..... 324
6.3. Oncogenic lncRNAs ..... 325
6.4. Controversial lncRNAs in mammary tumorigenesis ..... 326
6.5. Potential role of lncRNAs in breast cancer progression ..... 327
7. Perspectives and future directions ..... 327
7.1. In vivo function of lncRNAs ..... 327
7.2. Identification of novel lncRNAs in mammary development and tumorigenesis ..... 327
7.3. LncRNAs as therapeutic targets in breast cancer ..... 327
Acknowledgements ..... 327
References ..... 328

## 1. Introduction

### 1.1. LncRNAs and development

A fundamental goal in developmental biology is to understand how stem and progenitor cells differentiate and perform specialized functions in each tissue type of a particular organism. Cells proceed toward differentiation through highly coordinated, stepwise changes in gene expression that are largely regulated by cell-type dependent transcription factor networks, resulting in a mixed population of stem, progenitor, and differentiated cell types. The precise regulation of this cellular hierarchy is essential for creating and maintaining the structure and function of an organ. Additionally, the preservation of each cell type in a given tissue requires maintenance of their unique patterns of gene expression, which is thought to be regulated predominantly by epigenetic mechanisms. In the past decade, long noncoding RNAs (lncRNAs) have emerged as significant regulators of tissue- and cell-specific gene expression by diverse mechanisms, many of which result in targeted epigenetic modifications. Therefore, it is not surprising that there is growing evidence of an instructional role for lncRNAs in mediating key processes in cellular differentiation and development (Hu et al., 2011).

Relatively few lncRNAs have identified functions. However, a large portion of these regulate developmental processes in embryonic and adult mammalian tissue. Some of the earliest characterized lncRNAs regulate distinct epigenetic processes that are critical for embryonic development, such as the regulation of genomic imprinting by *H19* (Bartolomei and Ferguson-Smith, 2011) and X-inactivation by *Xist* (Jeon et al., 2012). Imprinting and X-inactivation are both mediated by multiple lncRNA-chromatin modifying complexes that target and silence genes in *cis* (Lee and Bartolomei, 2013). In addition, large-scale analyses using mouse embryonic stem cells (mESCs) have identified hundreds of lncRNAs, some of which are differentially expressed in various stages of mESC differentiation (Dinger et al., 2008; Guttman et al., 2009). Loss-of-function studies of dozens of mESC lncRNAs show that they act to repress lineage commitment programs to maintain the mESC pluripotent state (Guttman et al., 2011). Other lncRNAs, such as *Mira*, may be induced by, and necessary for, mESC differentiation (Bertani et al., 2011). Taken together, these data support a central role for lncRNAs in regulating key processes during embryogenesis, including genomic imprinting and dosage compensation, as well as mESC pluripotency and differentiation.

In the adult, lncRNAs often show precise spatiotemporal expression patterns (Cabili et al., 2011; Derrien et al., 2012; Djebali et al., 2012; Ravasi et al., 2006), reflecting their potential role in regulating lineage commitment and differentiation. Consistent with this proposal, several lncRNAs have been shown to regulate cell fate decisions across a broad range of tissues. For example, global analyses have identified a large number of lncRNAs that show discrete patterns of expression in the central nervous system (Mercer et al., 2008; Ng et al., 2012; Ponjavic et al., 2009; Qureshi et al., 2010). Further studies have shown that several lncRNAs are required for proper neural differentiation and development, such as *Evf2* (Bond et al., 2009), *Six3os*, and *Dlx1as* (Ramos et al., 2013). In addition, the expression of several lncRNAs is induced during, and necessary for, the differentiation of distinct hematopoietic lineages, including *EGO* (Wagner et al., 2007), *HOTAIRM1* (Zhang et al., 2009), and *LincRNA-EPS* (Hu, Yuan, 2011). Another lncRNA called *Linc-MD1* promotes muscle differentiation by binding and sequestering miRNAs that repress myogenic genes (Cesana et al., 2011). In the epidermis, *ANCR* represses terminal differentiation by an unknown mechanism (Kretz et al., 2012), whereas *TINCR* promotes terminal differentiation by binding and stabilizing differentiation mRNAs (Kretz et al., 2013). LncRNAs have also been shown to regulate heart development, likely via epigenetic mechanisms. The lncRNA *Bvht* interacts with the PRC2 complex and is required for cardiomyocyte differentiation *in vitro* (Klattenhoff et al., 2013), whereas the lncRNA *Fendrr* binds to both the PRC2 and MLL complexes, and it is essential for proper mouse heart development *in vivo* (Grote et al., 2013). Interestingly, recent evidence shows that several imprinted genes, including the lncRNA *H19*, are not only expressed embryonically, but are also enriched specifically in adult somatic stem cells where they may play an additional role in regulating the balance between stem cell self-renewal and differentiation in several adult tissues (Berg et al., 2011; Ferron et al., 2011; Venkatraman et al., 2013; Zacharek et al., 2011).

### 1.2. LncRNAs and cancer

Since lncRNAs regulate critical pathways in tissue development and maintenance, it might be assumed that the misregulation of lncRNAs could disrupt these delicate processes and lead to tumorigenesis. Recent transcriptional profiling of multiple human tissues, including both normal and tumor samples, have indeed begun to provide global evidence for the misexpression of lncRNAs in cancer (Brunner et al., 2012; Gibb et al., 2011b). These studies have validated the tissue-specific expression of lncRNAs in normal tissues, and have identified large sets of lncRNAs that are aberrantly expressed in either a specific cancer or multiple types of cancer. A recent large-scale study went a step further by integrating microarray data of lncRNA expression from 1,300 tumors, spanning four types of cancer, with clinical outcome and somatic copy number alterations (SCNAs) data, and identified 80–300 potential lncRNA drivers of cancer progression in each of the four cancer types (Du

**Table 1**
LncRNAs associated with mammary development and breast cancer.

| LncRNA | Expression | Function | Mechanism |
|---|---|---|---|
| **Mammary development** | | | |
| *mPINC* | Highly expressed in alveolar cells of pregnant and involuting gland | Inhibits lactogenic differentiation, alternative splice forms regulate cell cycle and survival | Interacts with PRC2 |
| *Zfas1* | Highly expressed in alveolar and ductal cells of pregnant and involuting gland | Inhibits proliferation and lactogenic differentiation | Unknown |
| **Breast cancer** | | | |
| *BC200* | Increased in invasive breast cancer and HG-DCIS | Oncogenic | Translational repression[a] |
| *GAS5* | Decreased in breast tumors and breast cancer cell lines | Tumor suppressive-induces growth arrest and apoptosis | Binds and inhibits GR from activating target genes[a] |
| *HOTAIR* | Increased in metastatic breast tumors, strong predictor of metastasis and death | Oncogenic-promotes invasion and metastasis | Silences genes in *trans* epigenetically |
| *H19* | Increased in stromal cells of breast tumors | Oncogenic-promotes proliferation and tumor growthTumor suppressive-restricts growth[a] | Unknown |
| *LncRNA-JADE* | Increased in breast tumors | Oncogenic-promotes proliferation and survival | Binds BRCA1 and enhances transcription of *Jade1* in DDR |
| *LSINCT5* | Increased in breast tumors and breast cancer cell lines | Oncogenic-promotes proliferation | Unknown |
| *MALAT1* | Increased in breast tumors, mutations in *MALAT1* associated with Luminal B subtype and poor clinical outcome | Oncogenic-promotes metastasis[a] | RNA splicing, regulation of gene expression[a] |
| *MEG3* | Expressed in mammary gland, not detected in breast cancer cell lines | Tumor suppressive-inhibits growth, induces apoptosis[a] | Unknown |
| *PTENP1* | Focally deleted in breast cancer, undergoes somatic hypermethylation in breast cancer cell lines | Tumor suppressive-represses proliferation[a] | Binds and inhibits miRNAs from targeting and repressing *PTEN* |
| *SRA* | Increased in breast tumors, associated with PR+ breast tumors | Oncogenic-promotes proliferation, metastasis Tumor suppressive-induces apoptosis | Co-activator of hormone receptors, scaffold for many transcription factors[a] |
| *treRNA* | Increased in paired breast cancer primary and lymph-node metastasis samples | Oncogenic-promotes EMT, invasion and metastasis | Enhances transcription of EMT regulators, represses translation of epithelial markers |
| *UCA1* | Increased in breast tumors, negatively correlates with p27 protein levels | Oncogenic-promotes proliferation | Binds hnRNP I, thereby preventing binding and translation of *p27* |
| *ZFAS1* | Decreased in invasive ductal carcinoma | Tumor suppressive-inhibits proliferation | Unknown |

[a] Data not shown in breast cancer cells.

et al., 2013). These global analyses provide valuable resources for the identification of novel cancer biomarkers and therapeutic targets.

Cancer is thought to arise and progress due to genetic alterations that disrupt processes required for maintaining normal cellular homeostasis, such as cell cycle, DNA damage response, survival, and migration (Hanahan and Weinberg, 2011). Misregulated lncRNAs that affect each of these processes have been identified in cancer (Gibb et al., 2011a; Martin and Chang, 2012; Prensner and Chinnaiyan, 2011; Spizzo et al., 2012). For example, the lncRNAs *PRNCR1* and *PCGEM1* are highly expressed in aggressive prostate cancer where they bind to the androgen receptor (AR) and enhance AR-mediated gene activation programs, leading to increased proliferation (Chung et al., 2011; Petrovics et al., 2004; Yang et al., 2013). Another lncRNA, called *PANDA*, is induced in response to DNA damage in a p53-dependent manner (Hung et al., 2011). *PANDA* promotes cell survival by binding the transcription factor NY-FA and inhibiting its activation of apoptotic genes. LncRNAs can also regulate cell migration, as evidenced by the lncRNA *MALAT1*, which is upregulated in metastatic lung cancer (Ji et al., 2003). Knockdown of *MALAT1* reduces lung cancer cell migration and results in the misregulation of genes associated with cell motility *in vitro*. Depletion of *MALAT1* also reduces metastasis of lung cancer cells in a pulmonary metastatic model *in vivo* (Gutschner et al., 2013b). As the functions of individual lncRNAs in cancer are beginning to be elucidated, they are being categorized and referred to as either tumor suppressor or oncogenic lncRNAs, in the same way as traditional protein-coding cancer genes (Huarte et al., 2010). They are also being discussed in relation to the well-known Hallmarks of Cancer progression, as described by Hanahan and Weinberg, thus expanding these concepts of tumorigenesis to include the noncoding genome (Gutschner and Diederichs, 2012; Hanahan and Weinberg, 2011).

In this review, we will focus on the role of lncRNAs in regulating development and differentiation of mammary epithelial cells in the normal mammary gland. Additionally, we will discuss the misregulation of lncRNAs implicated in mammary tumorigenesis, as well as how these misregulated lncRNAs might disrupt normal mammary epithelial cell development (Table 1).

## 2. LncRNAs: form and mechanism

LncRNAs are often capped, spliced, and polyadenylated, similar to their protein-coding counterparts. LncRNAs were initially defined as RNA transcripts longer than 200 nucleotides that lack protein-coding potential. However, this definition has since become blurred by accumulating evidence for multifunctional RNA molecules, such as lncRNAs that also encode proteins, and mRNAs that also function as lncRNAs (Candeias et al., 2008; Dinger et al., 2011; Poliseno et al., 2010). In addition, the length ascribed in the previous definition is somewhat arbitrary and limiting, as some RNA molecules that do not meet this requirement still function as lncRNAs, such as the lncRNA *BC200*, which will be discussed later in this review. Although the definition of lncRNAs will likely continue to evolve, the current definition is any regulatory RNA transcript that is not a small ncRNA, such as microRNAs (miRNAs), PIWI-interacting RNAs (piRNAs), or small nucleolar RNAs (snoRNAs) (Spizzo et al., 2012). However, to further clarify, some lncRNAs also contain miRNAs (Cai and Cullen, 2007) and snoRNAs (Mourtada-Maarabouni et al., 2009) embedded within them. Therefore, a further amendment to the definition should state that

classifying an RNA molecule as an lncRNA does not preclude it from possessing other coding and noncoding functions.

Thousands of lncRNAs are transcribed from the mammalian genome, and their predominant role seems to be the regulation of gene expression by a diverse spectrum of mechanisms (Rinn and Chang, 2012). Some lncRNAs are transcribed from enhancer regions (eRNAs) and are required to increase the expression of neighboring genes in *cis* (Orom et al., 2010), whereas others bind and sequester transcription factors, thus inhibiting transcription factor activity (Hung et al., 2011; Kino et al., 2010). LncRNAs can also bind to transcription factors and enhance their transcriptional activity, thus acting as co-activators (Caretti et al., 2006; Feng et al., 2006). Finally, one of the most recurrent mechanisms reported is the ability of lncRNAs to recruit chromatin-modifying complexes to target loci, thereby epigenetically activating or repressing associated genes (Lee, 2012; Mercer and Mattick, 2013). Thousands of lncRNAs have been shown to interact with the repressive chromatin-modifying complex PRC2 (Zhao et al., 2010), and many lncRNAs have been found to interact with other chromatin-modifying complexes, such as LSD1/REST/CoREST (Khalil et al., 2009; Tsai et al., 2010), PRC1 (Yap et al., 2010) and MLL1 (Bertani et al., 2011; Dinger et al., 2008; Grote et al., 2013), suggesting a substantial fraction of lncRNAs act as epigenetic regulators. The majority of lncRNAs discussed in the following sections utilize at least one of the above described mechanisms to function in mammary development and tumorigenesis.

## 3. Development of the mammary gland

The mammary gland is a unique organ from a developmental perspective, as the majority of its growth and differentiation occurs after birth (Fig. 1A) (Cowin and Wysolmerski, 2010; Gjorevski and Nelson, 2011; Watson and Khaled, 2008). At birth, the mammary fat pad contains a simple epithelial ductal structure that quiescently lies beneath the nipple until puberty. During puberty, ovarian and pituitary hormones, including estrogen, progesterone, and growth hormone, stimulate proliferation of the mammary epithelial cells to generate terminal end bud (TEB) structures at the leading tips of the ducts (Brisken and O'Malley, 2010). TEBs are dynamic structures made up of proliferative, as well as apoptotic, epithelial cells, both of which are necessary to allow ductal elongation and lumen formation. TEBs drive extension and bifurcation of the ducts toward the ends of the fat pad, resulting in a branched, ductal tree-like structure that fills the fat pad of the adult mammary gland.

In the adult, the epithelial ducts are comprised of an inner layer of luminal epithelial cells and an outer layer of basal epithelial cells, which are thought to arise from a common bi-potent stem cell during embryonic development (Fig. 1B) (Van Keymeulen et al., 2011). The luminal cells can be further subdivided into luminal progenitors and mature luminal cells, which are differentiated hormone receptor-positive cells. The outer basal layer consists of a basal stem/progenitor cell population as well as contractile myoepithelial cells, which are necessary for pushing milk out of the alveoli and toward the nipple during lactation. Luminal and basal progenitors are thought to give rise to alveolar progenitors that, during pregnancy, begin the stepwise process of differentiation to become mature alveolar cells, which are the milk-producing cells of the mammary gland (van Amerongen et al., 2012).

The adult mammary gland can undergo multiple rounds of pregnancy, each of which requires the expansion and terminal differentiation of alveolar cells necessary for lactation, followed by their apoptotic removal during involution (Anderson et al., 2007; Brisken and Rajaram, 2006). In early pregnancy, mammary epithelial cells proliferate and expand to generate lobuloalveolar structures throughout the mammary fat pad. During mid-to-late pregnancy, proliferation rates decline as alveolar progenitors begin to differentiate and produce low levels of milk protein. However, it is not until parturition that alveolar progenitors undergo terminal secretory differentiation to produce and secrete the large volume of milk necessary to nurse offspring throughout lactation. Finally, during involution, the majority of differentiated alveolar cells undergo rounds of programmed cell death, and the gland remodels to a pre-pregnancy-like state. It has been shown that a population of mammary epithelial cells committed to an alveolar fate persists in the regressed lobules of the involuted gland, and these cells are thought to serve as alveolar progenitors in subsequent pregnancies (Boulanger et al., 2005; Wagner et al., 2002).

## 4. LncRNAs and mammary gland development

### 4.1. Mammary gland lncRNAs of unknown function

Interestingly, two of the earliest identified regulatory lncRNAs, *H19* and *SRA* (steroid receptor RNA activator), may play a role in the developing mammary gland. The imprinted lncRNA *H19* is induced by estrogen in the mouse mammary gland, and *in situ* hybridization shows enriched expression of *H19* in mammary epithelial cells of the TEBs in pubertal mice and in the alveolar cells of pregnant mice (Adriaenssens et al., 1999). Although initially H19 was thought to only function to restrict growth during embryonic development, relatively recent data has shown an additional role for *H19* in long-term maintenance of adult hematopoietic stem cells (Venkatraman et al., 2013). If *H19* has an equivalent role in the mammary gland, then it may maintain stem and/or progenitor populations during the highly proliferative pubertal and pregnant stages of mammary development. Another lncRNA, *SRA*, has been reported to enhance proliferation, increase ductal side branching, and induce precocious alveolar differentiation when overexpressed in mammary epithelial cells of a transgenic mouse model (Lanz et al., 2003). *SRA* modulates the activity of nuclear hormone receptors (Lanz et al., 1999) and has been shown to regulate cell-type specific differentiation in multiple tissues (Caretti et al., 2006; Xu et al., 2010), suggesting a putative role for *SRA* in mammary epithelial differentiation. However, endogenous *SRA* transcript levels are barely detectable in the virgin mammary gland (Lanz et al., 2003), leading to questions regarding an actual function for *SRA* in mammary development. To validate a potential mammary function of *SRA*, further studies should include in-depth expression analysis at various mammary stages and mammary gland loss-of-function studies to complement the overexpression studies.

A relatively recent study also identified many additional lncRNAs putatively involved in postpubertal mammary gland development. As mentioned above, each pregnancy results in a dramatic cycle of hormonally regulated proliferation, differentiation, and programmed cell death of mammary epithelial cells. To better understand the molecular mechanisms that dictate these processes, microarray analysis was performed using RNA isolated from mammary epithelial cells from various stages of postpubertal mouse development (Askarian-Amiri et al., 2011). This study identified almost 100 differentially expressed lncRNAs between pregnant, lactating, and involuting mice, thus providing a useful resource for those studying lncRNAs in postpubertal stages of mammary development.

### 4.2. LncRNAs that regulate mammary epithelial differentiation

Thus far, only two lncRNAs are known to have a function in mammary epithelial cells: *mPINC* (mouse pregnancy-induced noncoding RNA) and *Zfas1* (Znfx1 antisense 1). *mPINC* and *Zfas1* were both identified in large-scale studies aimed at isolating differentially expressed genes during postpubertal mammary development

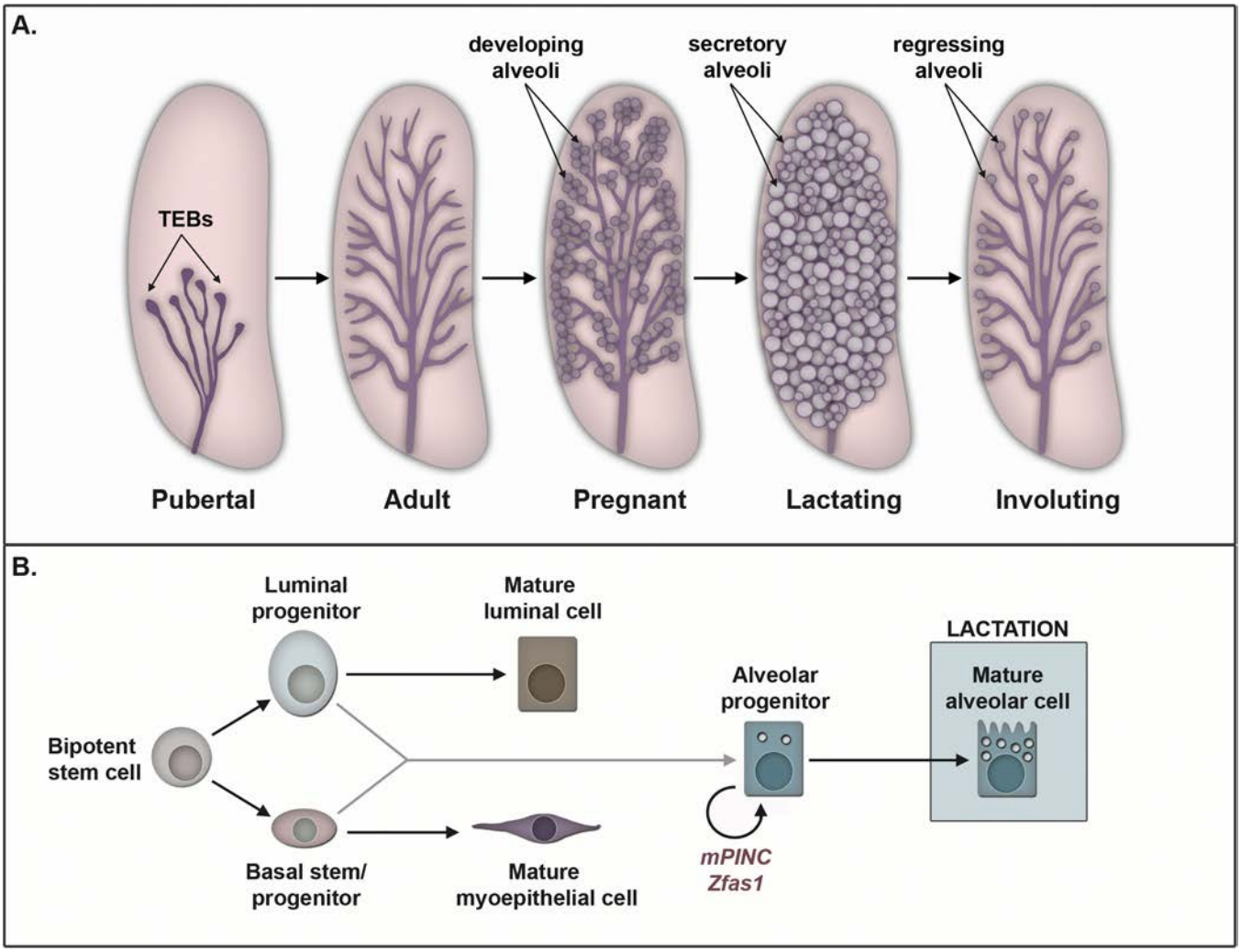


**Fig. 1.** Overview of mammary gland development. (A) A schematic diagram highlights various stages of mammary development, beginning with pubertal development, followed by each postpubertal stage. The pink shapes represent mammary fat pads, whereas the ductal epithelium is shown in purple. During pregnancy, lactation, and involution the circular objects represent lobuloalveolar units. (B) Model of mammary epithelial cell hierarchy with associated lncRNAs, *mPINC* and *Zfas1*. A bi-potent progenitor gives rise to basal and luminal progenitors during embryonic development. The luminal progenitors differentiate into mature luminal cells (hormone receptor-positive), whereas the basal progenitors differentiate into mature myoepithelial cells. Both luminal and basal progenitors may contribute to the pool of alveolar progenitors that become the milk-producing alveolar cells during lactation. Both *mPINC* and *Zfas1* are hypothesized to maintain the alveolar progenitor cells during pregnancy and involution. This is only a proposed model; therefore, these developmental relationships still need to be further validated experimentally.

(Askarian-Amiri et al., 2011; Ginger et al., 2001). Although *mPINC* and *Zfas1* were characterized independently, their functions in mammary epithelial cells are quite similar. Initial *in vitro* studies showed that knockdown of either *mPINC* or *Zfas1* in HC11 cells, a mammary epithelial cell line derived from a mid-pregnant mouse, led to increased proliferation, suggesting growth-suppressive roles (Ginger et al., 2006). However, further studies of these two lncRNAs suggest they play a more specialized role in the mammary gland.

*mPINC* and *Zfas1* both show strict spatiotemporal expression during postpubertal mammary gland development (Askarian-Amiri et al., 2011; Ginger et al., 2001; Shore et al., 2012). Their expression levels increase dramatically during pregnancy, and *in situ* hybridization of mammary glands from mid-pregnant mice shows that *mPINC* is enriched in the alveolar cells, whereas *Zfas1* localizes to ductal and alveolar cells. Furthermore, sorted populations of mammary epithelial cells show that *mPINC* is most abundantly expressed in alveolar progenitors, compared to luminal progenitors and mature luminal cells (Shore et al., 2012). In early lactation, *mPINC* and *Zfas1* levels sharply decline, as alveolar cells undergo terminal differentiation. Accordingly, *mPINC* and *Zfas1* levels also decline during lactogenic differentiation of HC11 cells. Furthermore, knockdown of either *mPINC* or *Zfas1* in HC11 cells treated with lactogenic hormones enhances lactogenic differentiation, as evidenced by the increased expression of milk protein genes and the formation of domes, *in vitro* morphological structures associated with lactogenic differentiation. Conversely, overexpression of *mPINC* inhibits lactogenic hormone-induced differentiation of HC11 cells. Taken together, these data suggest that high levels of *mPINC* and *Zfas1* during pregnancy act to repress the final step of alveologenesis, thereby maintaining an alveolar progenitor population. Additionally, the drastic reduction in their levels in early lactation may be necessary to allow terminal alveolar differentiation. Finally, *mPINC* and *Zfas1* levels both rise again in early involution where they may act to preserve alveolar progenitors for subsequent pregnancies (Fig. 1B).

Although these *in vitro* data strongly suggest a role for *Zfas1* during postpubertal mammary gland development, the mechanism is unknown. The *Zfas1* transcript hosts three intronic snoRNAs. However, their levels are unaffected by *Zfas1* knockdown, suggesting that the observed phenotypes are not regulated by the snoRNAs

(Askarian-Amiri et al., 2011). Also, the lncRNA *Zfas1* is transcribed antisense to the promoter of a protein-coding gene called *Znfx1*. Although the expression levels of *Zfas1* and *Znfx1* are not correlated during postpubertal mammary development, knockdown of *Zfas1* in HC11 cells results in increased expression of *Znfx1*, suggesting that the phenotypic effects could be due, in part, to *Znfx1*. Further investigation into the relationship of these interconnected transcripts, as well as elucidating the mammary function of *Znfx1*, should lead to a better understanding of the mechanism of *Zfas1*.

The repression of alveolar differentiation by *mPINC* is likely epigenetically regulated. Like *mPINC*, a member of the chromatin-modifying PRC2 complex, *RbAp46*, is also upregulated in alveolar cells of the mammary gland during pregnancy (Ginger et al., 2001). Furthermore, RNA immunoprecipitation (RIP) assays revealed an interaction between *mPINC* and RbAp46, as well as other PRC2 complex members, in HC11 cells and in mammary epithelial cells isolated from pregnant mice (Shore et al., 2012). Finally, microarray analysis of mammary epithelial cells following modulation of *mPINC* levels identified several potential targets of the *mPINC*-PRC2 complex, including *Wnt4*. Knockdown of mPINC leads to increased *Wnt4* levels, whereas overexpression of mPINC results in decreased *Wnt4* levels. Wnt4 is a plausible mediator of the observed phenotypic effects of *mPINC*, as it is required for alveolar development *in vivo* and for lactogenic differentiation *in vitro* (Brisken et al., 2000; Zhang et al., 2006). These data suggest that a PRC2 complex that includes *mPINC* and RbAp46 confers epigenetic modifications to repress genes necessary for alveolar differentiation, thus maintaining a population of progenitor cells committed to an alveolar fate.

Initial studies showed potential growth-inhibitory roles of *mPINC* and *Zfas1* in mammary epithelial cells, implying that they may function as tumor suppressors in breast cancer. *mPINC* and *RbAp46* are both upregulated in involuted mammary glands of parous rodents compared to nulliparous mammary glands, and both have been suggested to play a role in the protective effect of pregnancy against breast cancer (Ginger et al., 2001). However, further evidence for the role of *mPINC* in breast cancer is currently lacking. On the other hand, there is evidence for a tumor suppressor role of *Zfas1* in breast cancer that will be discussed in the next section.

## 5. The current state of breast cancer research

It is estimated that approximately 233,000 women in the United States will be diagnosed with invasive breast cancer, and an additional 63,000 women will be diagnosed with breast carcinoma *in situ* in 2014 (American Cancer Society, 2014). Traditionally, breast cancer has been diagnosed, categorized, and treated based on three immunohistological markers: the estrogen receptor (ER), the progesterone receptor (PR), and the human epidermal growth factor receptor 2 (HER2) (Bertos and Park, 2011). The ER status, which generally correlates with PR status, is used to identify tumors that may respond to endocrine therapy, such as ER-antagonists and aromatase inhibitors that both function to inhibit ER-dependent signaling. Patients with HER2-positive tumors have genomic amplification and overexpression of the *HER2* oncogene and are, therefore, treated with HER2 monoclonal antibodies to disrupt HER2 signaling. Breast tumors that lack ER, PR, and HER2 expression are referred to as triple-negative (TN). At present, patients with triple-negative breast cancer (TNBC) do not have a targeted therapy option and have the worst prognosis among the subtypes. Overall, mortality rates have improved in recent years with better screening programs and treatment plans. However, it is still estimated that almost 40,000 women will die this year due to breast cancer (American Cancer Society, 2014).

In the past decade, new technologies have been employed to identify gene expression signatures and genomic alterations that may be responsible for driving breast cancer progression (Ellis and Perou, 2013). Initial genomic studies of hundreds of breast tumor samples using microarray platforms have shown that breast tumors can be divided into at least four subtypes based on their distinct gene expression patterns: Luminal A, Luminal B, HER2-enriched, and Basal-like (Perou et al., 2000; Sorlie et al., 2001, 2003). Furthermore, these molecular subtypes strongly associate with patient outcome, and these data have been used to develop improved clinical assays (Prat et al., 2012). A recent study further validated and refined the four main subtypes of breast cancer by integrating data at the RNA, DNA, and protein level (Cancer Genome Atlas, 2012). Using this approach, they identified subtype-specific somatic mutations, as well as alterations in DNA copy number, DNA methylation, and mRNA and protein expression. These data provide novel subtype-specific therapeutic targets at multiple regulatory levels and emphasize the immense level of heterogeneity between tumor subtypes.

Considerable effort has also focused on understanding the early stages of breast cancer progression. The prevailing model of breast tumorigenesis assumes a linear progression from atypical ductal hyperplasia (ADH) to ductal carcinoma in situ (DCIS) and then finally to invasive ductal carcinoma (IDC) (Fig. 2) (Polyak, 2007). ADH is a premalignant lesion of the mammary ducts characterized by multiple layers of abnormally shaped epithelial cells that have proliferated into the lumen. ADH is thought to be the precursor of DCIS, which is a pre-invasive lesion that can progress to IDC if the epithelial cells invade through the basement membrane surrounding the mammary ducts. Following basement membrane invasion, mammary epithelial cells can travel to distant organs resulting in metastatic disease, which is the predominant cause of breast cancer related death (Eckhardt et al., 2012). Throughout the sequence of events leading to IDC and metastasis, normal epithelial functions, such as proliferation, survival, differentiation, and cell adhesion, become increasingly deregulated. Thus, identifying the molecular mechanisms involved in disrupting the intact epithelium will provide a better understanding of early tumor progression. Additionally, evidence has shown that only a subset of DCIS lesions actually progress to malignancy (Erbas et al., 2006), leading to difficult treatment decisions for DCIS patients. Therefore, determining the key genetic and epigenetic alterations that govern the critical transitions from DCIS to invasive breast cancer is another area of active investigation that will likely improve the efficacy of breast cancer treatments.

## 6. LncRNAs associated with breast cancer

### 6.1. Global lncRNA expression in breast cancer

The steroid hormones estrogen and progesterone and their cognate receptors, ER and PR, play complex and pivotal roles in breast cancer progression (Brisken, 2013). Numerous studies have identified thousands of estrogen-regulated lncRNAs in breast cancer cells, using a combination of GRO-seq and RNA-seq (Hah et al., 2011; Hah and Kraus, 2013; Hah et al., 2013; Li et al., 2013). These include a large group of enhancer lncRNAs (eRNAs) that are transcribed from enhancers adjacent to estrogen upregulated genes throughout the genome. Knockdown of several of these eRNAs showed that the eRNA transcripts themselves function to increase target gene activation in *cis*, likely by strengthening enhancer-promoter looping (Li et al., 2013). In addition, other groups have identified several lncRNAs that interact with hormone receptors and modulate their activity, some of which will be discussed below. These hormone-regulated lncRNAs, as well as lncRNA that regulate

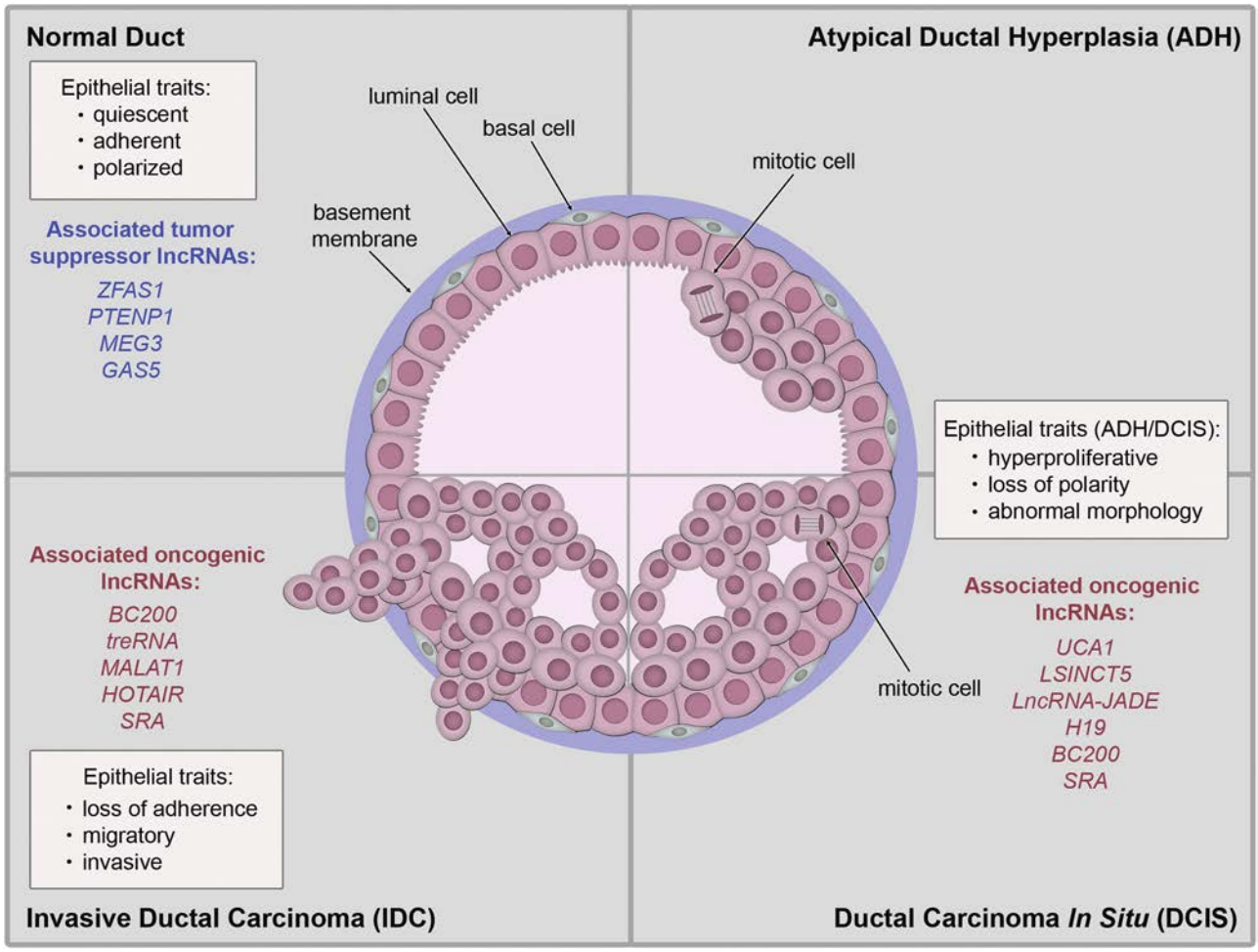


**Fig. 2.** Overview of breast cancer progression. This cartoon depicts a cross section of a mammary duct divided into quadrants that represent each stage of breast cancer progression from the normal duct to ADH, then DCIS, and finally IDC. Epithelial traits and potential lncRNAs associated with the normal duct, ADH and DCIS combined, and IDC are also shown.

hormone receptors, could serve as additional targets to modulate hormone signaling in hormone receptor-positive breast cancers.

Recent large-scale analyses have also revealed the aberrant expression of lncRNAs in breast cancer. Some groups have used whole-genome tiling arrays to identify novel lncRNAs that show altered expression in breast tumors and cell lines (Perez et al., 2008; Silva et al., 2010). Other groups have employed sequencing-based methods and reported the differential expression of 220 lncRNAs in breast carcinomas compared to normal, as well as the identification of hundreds of additional lncRNAs specifically altered in breast tumors compared to other tumor types that could serve as breast tumor biomarkers (Brunner et al., 2012; Gibb et al., 2011b). Interestingly, one study discovered 216 lncRNAs associated with cell-cycle promoters and, of those, 35 were differentially expressed in invasive ductal breast carcinomas relative to normal breast (Hung et al., 2011). Finally, recent RNA-seq analysis of normal tissue, as well as multiple tumor types, revealed normal breast- and breast cancer-specific expression of many pseudogenes, which have been shown to also function as lncRNAs (Kalyana-Sundaram et al., 2012; Poliseno, 2012). These expression data from genome-wide studies provide a tremendous resource for those interested in pursuing the functions of individual lncRNAs, as well as for the discovery of specific biomarkers and molecular targets in breast cancer.

Several individual lncRNAs have been identified that are altered in breast cancer whose functions and mechanisms are currently unknown. In addition, there are numerous lncRNAs misexpressed in other cancers whose functions are known, but their aberrant expression in breast cancer has not been reported. For the remainder of this section, we will only focus on lncRNAs that meet the following criteria: (1) there is evidence of their misexpression in breast cancer and (2) there is evidence for a potential tumorigenic function or mechanism.

### 6.2. Tumor suppressor lncRNAs

The lncRNA *Zfas1* is an inhibitor of mammary epithelial proliferation and differentiation that may also function as a tumor suppressor (Askarian-Amiri et al., 2011). As *Zfas1* was discussed in detail in the previous section, we will only mention here that human *ZFAS1* levels are reduced in the mammary epithelium of invasive ductal carcinoma samples compared to paired normal breast epithelium. This result, combined with the growth inhibitory effect of *Zfas1* in mouse mammary epithelial cells, suggests that *ZFAS1* could play a role in the suppression of mammary tumorigenesis.

*MEG3* (maternally expressed gene 3) is an imprinted lncRNA that is expressed in most adult tissues (Zhou et al., 2012). As expected of a putative tumor suppressor gene, its expression is undetectable in a variety of primary human tumors and in all tumor cell lines tested (Zhang et al., 2003). Gain-of-function studies *in vitro* have shown that *MEG3* inhibits growth, in part, by enhancing apoptosis

(Braconi et al., 2011). Moreover, *MEG3* overexpression in several cancer cell lines results in increased levels of p53 protein, as well as increased transcription of several p53 target genes, suggesting *MEG3* may suppress growth via upregulation of the p53 pathway (Zhou et al., 2007). Additionally, *in vivo* loss of *MEG3* promotes angiogenesis in the brain, suggesting that *MEG3* may inhibit tumor development via repression of angiogenesis (Gordon et al., 2010). *MEG3* is highly expressed in the mammary gland, but its expression is undetectable in MCF7 and T47D breast cancer cell lines (Zhang et al., 2003). Overexpression of *MEG3* in MCF7 cells inhibits growth in colony formation assays, indicating a potential role for *MEG3* as a tumor suppressor in breast cancer progression.

Another lncRNA with putative tumor-suppressive functions is referred to as *PTENP1* (phosphatase and tensin homolog pseudogene 1). It is a pseudogene of the tumor suppressor protein-coding gene *PTEN*. In cancer cell lines, overexpression of the *PTENP1* 3′UTR leads to increased PTEN levels and growth inhibition, whereas *PTENP1* knockdown causes decreased PTEN levels and enhanced proliferation (Poliseno et al., 2010). In addition, *PTENP1* and PTEN levels are positively correlated across normal and cancer tissue samples, further suggesting that they are co-regulated. Interestingly, *PTENP1* and *PTEN* contain common miRNA recognition elements (MREs), and it is thought that *PTENP1* may function by competing for miRNA binding with *PTEN*, thereby relieving the repressive effects of miRNAs on *PTEN*. The *PTENP1* locus is focally deleted in breast cancer (Poliseno et al., 2010) and the *PTENP1* promoter undergoes somatic hypermethylation in breast cancer cell lines, indicating that *PTENP1* loss may also contribute to breast tumor progression via epigenetic silencing (Hesson et al., 2012). Interestingly, multiple antisense lncRNAs (asRNAs) were recently identified that are also encoded from the *PTENP1* locus (Johnsson et al., 2013), highlighting the complexity of the *PTEN* regulatory network. These asRNAs regulate both *PTEN* transcription and mRNA stability and are also likely to play a role in tumorigenesis.

One of the better-characterized tumor suppressor lncRNAs in breast cancer is *GAS5* (growth arrest-specific transcript 5). *GAS5* contains two glucocorticoid response element-like sequences that bind to the glucocorticoid receptor (GR) and prevent it from interacting with its normal target genes, thus inhibiting the function of glucocorticoids through GR (Kino et al., 2010). Glucocorticoids regulate essential cellular processes, such as cell growth, metabolism, and survival. *GAS5* transcript levels are significantly reduced in breast tumor samples compared to adjacent normal tissue, and overexpression of *GAS5* in MCF7 breast cancer cells induces growth arrest and apoptosis (Mourtada-Maarabouni et al., 2009). Interestingly, *GAS5* can also suppress the ligand-dependent transcriptional activity of other steroid hormone receptors (Kino et al., 2010), such as progesterone (PR) and androgen (AR) receptors, both of which have subtype-specific roles in breast cancer (Brisken, 2013; Chang et al., 2013; Ni et al., 2011). These data imply that the *GAS5* lncRNA may have additional roles in breast cancer progression via reduced PR and AR transcriptional activity.

### 6.3. Oncogenic lncRNAs

The lncRNA *UCA1* (urothelial carcinoma-associated 1) was originally identified as being upregulated in bladder cancer, but was recently shown to have an oncogenic role in breast cancer as well (Huang et al., 2014; Wang et al., 2006). *UCA1* interacts with hnRNP I (heterogeneous nuclear ribonucleoprotein I) and inhibits its normal function of binding to, and enhancing the translation of, the mRNA of the tumor suppressor *p27*, thereby resulting in reduced levels of p27 protein. Accordingly, there is also a negative correlation between p27 and *UCA1* levels in breast tumors, suggesting that this competitive inhibition also occurs in cancer. In MCF7 breast cancer cells, overexpression of *UCA1* increases cell growth, whereas knockdown of *UCA1* reduces cell growth. Consistent with these data, knockdown of *UCA1* in MCF7 cells transplanted into mouse mammary fat pads results in reduced tumor weight and volume, and decreased tumor cell proliferation, compared to control cells. Finally, *UCA1* has also been shown to be increased in breast tumors compared to normal breast, further supporting its oncogenic function in breast cancer.

*LSINCT5* (long stress-induced noncoding transcript 5) is a nuclear-enriched lncRNA that is increased in human breast and ovarian tumors compared to normal tissue samples (Silva et al., 2010). Additionally, *LSINCT5* expression is high in several breast cancer cell lines, and knockdown of *LSINCT5* in MCF7 breast cancer cells results in decreased proliferation (Silva et al., 2011). Microarray analysis of *LSINCT5* knockdown cells showed the reduced expression of two other lncRNA transcripts, *NEAT1* and *MALAT1* (also referred to as *NEAT2*), and a protein-coding transcript, *PSPC1*. *NEAT1* and PSPC1 are both components of nuclear paraspeckles, whereas *MALAT1* is localized to nearby nuclear speckles (Bond and Fox, 2009; Mao et al., 2011). These data suggest a potential function for *LSINCT5* in the regulation of these cellular compartments, which are both thought to regulate gene expression post-transcriptionally. Interestingly, *MALAT1*, which will be discussed in more detail below, is also overexpressed in several cancers and is thought to function as an oncogenic lncRNA (Gutschner et al., 2013a). However, it is not known whether *MALAT1* mediates the oncogenic functions of *LSINCT5* or if there are alternative mechanisms.

*LncRNA-JADE* transcription is induced by DNA damage in an ATM-dependent manner (Wan et al., 2013). Upon DNA damage, *LncRNA-JADE* binds to BRCA1 and enhances transcription of the neighboring protein-coding Jade1 gene, which is essential for DNA damage-induced H4 acetylation and the subsequent DNA damage response (DDR). In MCF7 breast cancer cells, knockdown of *LncRNA-JADE* reduces proliferation, whereas its overexpression increases proliferation. Importantly, knockdown of *LncRNA-JADE* also enhances DNA damage-induced apoptosis and sensitizes MCF7 cells to DNA damaging reagents. Futhermore, *LncRNA-JADE* knockdown in breast cancer cells orthotopically transplanted into the mouse mammary fat pad reduces tumor growth compared to control cells, and in humans, *LncRNA-JADE* is expressed at higher levels in breast tumors compared to normal breast. Taken together, these studies have identified a novel DDR-associated lncRNA whose aberrant expression promotes proliferation and survival of breast cancer cells, suggesting *LncRNA-JADE* has an oncogenic role in breast cancer.

*BC200* is a primate-specific lncRNA that is normally only expressed in neurons, where it acts as a translational repressor to regulate protein synthesis at synapses (Iacoangeli and Tiedge, 2013). Interestingly, *BC200* is also upregulated in several cancers, including breast cancer (Iacoangeli et al., 2004). Thorough expression analysis by *in situ* hybridization has shown that *BC200* is increased in the epithelial cells of invasive breast cancer samples, but is undetectable in normal breast tissue. Furthermore, *BC200* is also increased in high-grade (HG) DCIS, but not in non-high-grade (NHG) DCIS, suggesting that it could be used as an indicator of invasive potential in DCIS. Whether *BC200* actually functions in breast cancer to regulate cell migration or invasion via translational repression is currently unknown.

The oncogenic lncRNA *treRNA* (translational regulatory RNA) regulates genes at both the transcriptional and translational level. In the nucleus, *treRNA* acts as an enhancer in *cis* and upregulates transcription of *Snail1*, a regulator of the epithelial-to-mesenchymal transition (EMT) (Orom et al., 2010). In the cytoplasm, *treRNA* interacts with a ribonucleoprotein complex and suppresses the translation of the epithelial marker *E-cadherin* (Gumireddy et al., 2013), which should also promote EMT.

Accordingly, overexpression of *treRNA* in MCF7 breast cancer cells results in decreased protein levels of E-cadherin and other epithelial markers, as well as increased protein levels of mesenchymal markers, suggesting that high levels of *treRNA* is able to induce a partial EMT phenotype. Overexpression of *treRNA* in MCF7 cells also increases cell migration and invasion *in vitro*, and stimulates metastasis to the lung in mouse mammary gland transplants *in vivo*. In further support of its oncogenic role, *treRNA* is significantly upregulated in paired breast cancer primary and lymph-node metastasis samples. These data suggest that *treRNA* could serve as a novel therapeutic target to suppress breast cancer metastasis.

The lncRNA *MALAT1* (metastasis-associated lung adenocarcinoma transcript 1, also referred to as *NEAT2*) was originally found to be associated with metastatic lung cancer (Ji et al., 2003), but it is also upregulated in several other tumor types, including breast tumors (Guffanti et al., 2009; Lin et al., 2007). *MALAT1* localizes to nuclear speckles and interacts with several splicing factors, including SRSF1, 2, and 3 (Tripathi et al., 2010). Depletion of *MALAT1* results in defective alternative splicing of a subset of transcripts, some of which are known to be involved in cancer (Lin et al., 2011), therefore, its initial ascribed function was splicing regulation. Additionally, *MALAT1* has been shown to regulate gene expression by various mechanisms. For example, *MALAT1* increases the expression level of several cell-cycle regulators by binding to unmethylated Pc2 and its associated cell cycle genes, and relocating them to nuclear speckles where they are actively transcribed (Yang et al., 2011). *MALAT1* also regulates the expression level of the oncogene and mitosis regulator, *B-MYB*, by an unknown mechanism (Tripathi et al., 2013). Finally, loss of *MALAT1 in vivo* results in the increased expression of neighboring genes, suggesting that *MALAT1* may function, in part, to repress gene transcription in *cis* (Zhang et al., 2012). Although the function of *MALAT1* in breast cancer is not understood, recent whole genome sequencing of human breast tumor biopsies identified multiple mutations and deletions in the *MALAT1* gene that are specifically associated with the Luminal B subtype of breast cancer and poor patient outcome (Ellis et al., 2012). Interestingly, these genetic alterations are in the region of *MALAT1* thought to be important for its interaction with SRSF1, implicating a splicing mechanism in the potential oncogenic role of *MALAT1* in luminal breast cancer.

*HOTAIR* (HOX antisense intergenic RNA) is one of the most extensively studied oncogenic lncRNAs in breast cancer thus far. *HOTAIR* is transcribed from the *HOXC* locus and is required for targeting the PRC2 complex to the *HOXD* locus in *trans*, resulting in chromatin compaction and transcriptional silencing of a 40 kb region of the *HOXD* locus (Rinn et al., 2007). Levels of *HOTAIR* and EZH2, a PRC2 complex member, are both increased in metastatic breast carcinomas compared to matched primary breast tumors (Chisholm et al., 2012). Furthermore, high expression of *HOTAIR* in breast tumors is a strong predictor of subsequent metastasis and death (Gupta et al., 2010). In breast cancer cell lines, overexpression of *HOTAIR* promotes invasion, whereas *HOTAIR* knockdown suppresses invasion. *HOTAIR* overexpression also promotes the metastasis of breast cancer cells orthotopically transplanted into mouse mammary fat pads to the lung in a PRC2 complex-dependent manner. *HOTAIR* is thought to promote metastasis by re-targeting the PRC complex to novel sites, thereby decreasing expression of associated genes. Several of these newly targeted genes have been implicated in breast cancer progression and metastasis, including *HOXD10* (Carrio et al., 2005), several members of the protocadherin (*PCDH*) gene family (Novak et al., 2008), and *EPHA1* (Fox and Kandpal, 2004). Interestingly, a recent study found that *HOTAIR* also competes with the tumor suppressor BRCA1 for the same binding site of EZH2, a member of the PRC2 complex (Wang et al., 2013). In breast cancer cells, expression of BRCA1 inhibits *HOTAIR* binding to EZH2, while depletion of BRCA1 enhances *HOTAIR* binding to EZH2. Depletion of BRCA1 also increases breast cancer cell migration and invasion in an EZH2-dependent manner, providing further evidence for the function of *HOTAIR* in breast cancer progression and metastasis.

### 6.4. Controversial lncRNAs in mammary tumorigenesis

*H19* is transcribed from the imprinted *H19/IGF2* locus and is one of the more controversial lncRNAs in cancer biology, as the debate continues regarding whether it acts as a tumor suppressor or an oncogene. Initial *in vitro* cancer cell line experiments, as well as more recent *in vivo* experiments using mouse tumor models, indicate that *H19* functions as a tumor suppressor in several cellular contexts (Gabory et al., 2010). Furthermore, in early development *H19* restricts embryonic growth via regulation of the nearby *IGF2* gene, as well as a cohort of other imprinted genes referred to as the imprinted gene network (IGN). Interestingly, *H19* also contains a miRNA, miR-675, embedded in its first exon that suppresses embryonic and placental growth by targeting and downregulating the IGF2 receptor, *Igfr1* (Keniry et al., 2012). Conversely, miR-675 can also promote growth and tumorigenesis by targeting and repressing the tumor suppressor *RB1* (Tsang et al., 2010). Additionally, *H19* is upregulated in several tumor types, including breast tumors, indicative of an oncogenic role for *H19* as well (Adriaenssens et al., 1998). *In situ* hybridization of breast tumor biopsies show that *H19* localizes predominantly to the stromal cells, rather than the epithelium, suggesting that *H19* does not function as an oncogene in the epithelium directly. However, further studies have shown oncogenic functions of *H19* in mammary epithelial cells. For example, one study reported that overexpression of *H19* in MDA-MB-231 breast cancer cells decreases tumor latency and increases tumor size compared to control cells when injected into cleared mouse mammary fat pads (Lottin et al., 2002). Another study found that *H19* is a direct target of the oncogenic transcription factor c-MYC in breast cancer cell lines, and that the levels of *H19* and *cMYC* transcripts are significantly correlated in breast carcinomas (Barsyte-Lovejoy et al., 2006). In addition, siRNA knockdown of *H19* reduces the clonogenicity and anchorage-independent growth of multiple breast cancer cell lines, both indicators of cellular transformation. These data provide evidence for the oncogenic role of *H19* in mammary tumorigenesis, yet the mechanisms of *H19* and its partner, miR-675, in the breast are still poorly understood. Also, *in situ* hybridization data showing increased *H19* primarily in the stroma leads to questions regarding the potential roles of *H19* in the stromal vs. epithelial compartments of breast tumors.

The *SRA* gene gives rise to both noncoding (*SRA*) and coding isoforms (steroid receptor RNA activator protein (*SRAP*)). Since the functions of *SRAP* have been reviewed previously (Cooper et al., 2011), and the focus of this review is on lncRNAs, we will only discuss the noncoding aspect of the *SRA* gene. *SRA* was originally identified as a novel RNA regulator of the progesterone receptor (PR) (Lanz et al., 1999), but has since been shown to bind multiple nuclear receptors (NRs) and NR co-regulators, as well as other non-NR transcription factors (Colley and Leedman, 2011). Thus, *SRA* is generally thought to function as a scaffolding lncRNA, often bringing together multiple transcriptional regulators at target genomic loci to modulate gene expression. *SRA* is expressed at very low levels in the normal breast and its expression is increased in breast tumors, particularly in PR-positive tumors (Cooper et al., 2009; Lanz et al., 1999; Murphy et al., 2000). To test the tumorigenic potential of *SRA in vivo*, a transgenic mouse model was created using the mouse mammary tumor virus long terminal repeat (MMTV LTR) to drive expression of human *SRA* in the mouse mammary gland (Lanz et al., 2003). Overexpression of *SRA* results in increased PR expression and proliferation of mammary epithelial cells ultimately leading to the formation of hyperplastic lesions. However,

these lesions fail to progress to malignancy. This failure is likely due to a simultaneous increase in apoptosis of epithelial cells overexpressing *SRA*. These data suggest that overexpression of *SRA* is not only insufficient to induce mammary tumor formation, but may even act to suppress tumor formation by inducing apoptosis. In the same study, *SRA* was shown to significantly reduce ras-induced mammary tumor formation in MMTV-*SRA*/MMTV-*ras* bigenic mice, providing further evidence for an unexpected tumor suppressor function of *SRA*. Conversely, a recent study showed that siRNA-induced depletion of *SRA* reduces the invasiveness and alters the expression of invasion-related genes in MDA-MB-231 breast cancer cells (Foulds et al., 2010), suggesting *SRA* could play a role in promoting metastasis. Therefore, current data support both tumor suppressive and oncogenic functions of *SRA*. Ultimately, the role of *SRA* in tumorigenesis may be context-dependent or reflective of the levels of noncoding vs. coding isoforms (Cooper et al., 2009). Thus, further investigation is warranted into the role of this modulator of hormone NR function in breast cancer.

### 6.5. Potential role of lncRNAs in breast cancer progression

Although the expression of lncRNAs in various stages of breast cancer progression has not been reported, their associations with each stage can be predicted based on their tumorigenic function (Fig. 2). The tumor suppressor lncRNAs, including *ZFAS1*, *MEG3*, *PTENP1*, and *GAS5*, are expressed in the normal mammary epithelial cells and generally act as growth inhibitors. The oncogenic lncRNAs *UCA1*, *LSINCT5*, *LncRNA-JADE*, *H19*, and *SRA* promote proliferation of mammary epithelial cells, suggesting that they may be increased in early stages of progression, such as ADH or DCIS lesions. The increased expression of *BC200* has previously been reported in both DCIS and IDC. Finally, the oncogenic lncRNAs *treRNA*, *MALAT1*, *HOTAIR*, and *SRA* promote invasion and metastasis of breast cancer cells, implicating that they play a role in later stages of progression, including IDC and metastatic disease.

## 7. Perspectives and future directions

### 7.1. In vivo function of lncRNAs

To advance our understanding of the noncoding genome and validate the relevance of lncRNAs, it is critical that we continue to elucidate their functions *in vivo*. Quite recently, 18 lncRNA mouse knockout models were generated that resulted in overt phenotypes, such as peri- and post-natal lethality, general growth defects, and developmental abnormalities in specific organs, thus demonstrating that lncRNAs do indeed play critical roles *in vivo* (Sauvageau et al., 2013). Previously, lncRNA loss-of-function mouse models were generated, but they generally lacked a notable phenotype (Lee, 2012; Mattick, 2009). This could have been due to their low abundance and strict spatiotemporal expression, thus making the phenotypes more difficult to detect. It has been suggested that more extensive phenotyping may be necessary to reveal the *in vivo* roles of some lncRNAs, especially to detect tissue-specific functions (Haerty and Ponting, 2013). In the mammary gland, this would include performing assays to measure stem and progenitor cell activity, such as limiting-dilution and serial transplantation assays (Visvader and Smith, 2011), in addition to observing the whole mount and histological effects of lncRNA loss-of-function during various stages of mammary development. To assess the function of tumor suppressive and oncogenic lncRNAs *in vivo*, lncRNA loss- and gain-of-function models should be crossed to well-characterized mouse mammary tumor models to determine effects on tumor latency, multiplicity, growth rates, and metastasis (Vargo-Gogola and Rosen, 2007). It is imperative that we have a better understanding of the *in vivo* roles of lncRNAs before they can be considered as valid therapeutic targets in cancer.

### 7.2. Identification of novel lncRNAs in mammary development and tumorigenesis

In addition to validating the functions of already known lncRNAs, current technologies, such as lncRNA microarrays and RNA-seq, should be employed to identify more cell- and stage-specific lncRNAs in mammary development and breast cancer. For example, differentially expressed lncRNAs could be identified in sorted mammary epithelial subpopulations, such as stem, basal, mature luminal, luminal progenitor, and alveolar progenitor cells. Such lineage-specific lncRNAs may act as novel regulators of stem and progenitor cell maintenance, cell fate commitment, and differentiation, and could improve our understanding of mammary gland developmental processes. Furthermore, lncRNAs are likely to be differentially expressed in the molecular subtypes of breast cancer, including Luminal A and B, Her2-enriched, and Basal-like, as well as in various stages of breast cancer progression. Identification of these lncRNAs should unveil additional subtype- and stage-specific diagnostic and therapeutic targets in breast cancer.

### 7.3. LncRNAs as therapeutic targets in breast cancer

In the past ten years, our understanding of RNA molecules has been drastically altered, particularly with the emergence of lncRNAs. Overwhelming evidence shows that lncRNAs are key regulators of gene expression that guide essential processes in development and tumorigenesis, suggesting that this emerging class of molecules offers a new avenue for diagnosing and treating disease. The tissue- and cancer-specific expression of lncRNAs enhances their potential use as tumor biomarkers for disease diagnosis, compared to more ubiquitously expressed genes. For example, the lncRNA *PCA3* (prostate cancer gene 3) is upregulated in prostate tumors compared to normal prostate, and it is now routinely used in the clinic to aid prostate cancer diagnosis and reduce unnecessary biopsies (Lee et al., 2011). In addition, lncRNAs are unique from mRNAs in that they themselves function as the regulatory molecule, implying that measurement of their expression level is more directly indicative of their functional impact on the disease state (Cheetham et al., 2013; Sanchez and Huarte, 2013). As therapeutic targets, lncRNAs may also show increased efficacy and reduced side effects compared to more ubiquitously expressed proteins, due to their site-specific regulatory action in the genome and their strict spatiotemporal expression (Lee, 2012). For instance, a plasmid expressing diphtheria toxin under the control of the *H19* promoter has been successfully used to target tumor-specific *H19*-expressing cells, resulting in the reduction of tumor size in clinical trials (Mizrahi et al., 2009). These early clinical applications using lncRNAs show tremendous promise for their potential use in the diagnosis and treatment of disease. Future research efforts aimed at further elucidating the molecular mechanisms of lncRNAs will advance our understanding of their roles in both development and cancer, and will facilitate the design of novel therapeutics that can alter lncRNA function.

## Acknowledgements

We would like to thank Dr. Matthew Weston for editing the manuscript and Dr. Kevin Roarty for advice for the figures. The authors are supported by a grant from the National Cancer Institute, NCI-CA16303.

## References


Adriaenssens E, Dumont L, Lottin S, Bolle D, Lepretre A, Delobelle A, et al. H19 overexpression in breast adenocarcinoma stromal cells is associated with tumor values and steroid receptor status but independent of p53 and Ki-67 expression. Am J Pathol 1998;153:1597–607.

Adriaenssens EL, Duqimont S, Fauquette T, Coll W, Dupouy J, Boilly JP, et al. Steroid hormones modulate H19 gene expression in both mammary gland and uterus. Oncogene 1999;18:4460–73.

American Cancer Society. Estimated number of new cancer cases and deaths by sex, US, 2014; 2014.

Anderson SM, Rudolph MC, McManaman JL, Neville MC. Key stages in mammary gland development. Secretory activation in the mammary gland: it's not just about milk protein synthesis! Breast Cancer Res 2007;9:204.

Askarian-Amiri ME, Crawford J, French JD, Smart CE, Smith MA, Clark MB, et al. SNORD-host RNA Zfas1 is a regulator of mammary development and a potential marker for breast cancer. RNA 2011;17:878–91.

Barsyte-Lovejoy D, Lau SK, Boutros PC, Khosravi F, Jurisica I, Andrulis IL, et al. The c-Myc oncogene directly induces the H19 noncoding RNA by allele-specific binding to potentiate tumorigenesis. Cancer Res 2006;66:5330–7.

Bartolomei MS, Ferguson-Smith AC. Mammalian genomic imprinting. Cold Spring Harb Perspect Biol 2011;3.

Berg JS, Lin KK, Sonnet C, Boles NC, Weksberg DC, Nguyen H, et al. Imprinted genes that regulate early mammalian growth are coexpressed in somatic stem cells. PLoS ONE 2011;6:e26410.

Bertani S, Sauer S, Bolotin E, Sauer F. The noncoding RNA Mistral activates Hoxa6 and Hoxa7 expression and stem cell differentiation by recruiting MLL1 to chromatin. Mol Cell 2011;43:1040–6.

Bertos NR, Park M. Breast cancer – one term, many entities? J Clin Invest 2011;121:3789–96.

Bond AM, Vangompel MJ, Sametsky EA, Clark MF, Savage JC, Disterhoft JF, et al. Balanced gene regulation by an embryonic brain ncRNA is critical for adult hippocampal GABA circuitry. Nat Neurosci 2009;12:1020–7.

Bond CS, Fox AH. Paraspeckles: nuclear bodies built on long noncoding RNA. J Cell Biol 2009;186:637–44.

Boulanger CA, Wagner KU, Smith GH. Parity-induced mouse mammary epithelial cells are pluripotent, self-renewing and sensitive to TGF-beta1 expression. Oncogene 2005;24:552–60.

Braconi C, Kogure T, Valeri N, Huang N, Nuovo G, Costinean S, et al. microRNA-29 can regulate expression of the long non-coding RNA gene MEG3 in hepatocellular cancer. Oncogene 2011;30:4750–6.

Brisken C. Progesterone signalling in breast cancer: a neglected hormone coming into the limelight. Nat Rev Cancer 2013;13:385–96.

Brisken C, Heineman A, Chavarria T, Elenbaas B, Tan J, Dey SK, et al. Essential function of Wnt-4 in mammary gland development downstream of progesterone signaling. Genes Dev 2000;14:650–4.

Brisken C, O'Malley B. Hormone action in the mammary gland. Cold Spring Harb Perspect Biol 2010;2:a003178.

Brisken C, Rajaram RD. Alveolar and lactogenic differentiation. J Mammary Gland Biol Neoplasia 2006;11:239–48.

Brunner AL, Beck AH, Edris B, Sweeney RT, Zhu SX, Li R, et al. Transcriptional profiling of long non-coding RNAs and novel transcribed regions across a diverse panel of archived human cancers. Genome Biol 2012;13:R75.

Cabili MN, Trapnell C, Goff L, Koziol M, Tazon-Vega B, Regev A, et al. Integrative annotation of human large intergenic noncoding RNAs reveals global properties and specific subclasses. Genes Dev 2011;25:1915–27.

Cai X, Cullen BR. The imprinted H19 noncoding RNA is a primary microRNA precursor. RNA 2007;13:313–6.

Cancer Genome Atlas N. Comprehensive molecular portraits of human breast tumours. Nature 2012;490:61–70.

Candeias MM, Malbert-Colas L, Powell DJ, Daskalogianni C, Maslon MM, Naski N, et al. P53 mRNA controls p53 activity by managing Mdm2 functions. Nat Cell Biol 2008;10:1098–105.

Caretti G, Schiltz RL, Dilworth FJ, Di Padova M, Zhao P, Ogryzko V, et al. The RNA helicases p68/p72 and the noncoding RNA SRA are coregulators of MyoD and skeletal muscle differentiation. Dev Cell 2006;11:547–60.

Carrio M, Arderiu G, Myers C, Boudreau NJ. Homeobox D10 induces phenotypic reversion of breast tumor cells in a three-dimensional culture model. Cancer Res 2005;65:7177–85.

Cesana M, Cacchiarelli D, Legnini I, Santini T, Sthandier O, Chinappi M, et al. A long noncoding RNA controls muscle differentiation by functioning as a competing endogenous RNA. Cell 2011;147:358–69.

Chang C, Lee SO, Yeh S, Chang TM. Androgen receptor (AR) differential roles in hormone-related tumors including prostate, bladder, kidney, lung, breast and liver. Oncogene 2013.

Cheetham SW, Gruhl F, Mattick JS, Dinger ME. Long noncoding RNAs and the genetics of cancer. Br J Cancer 2013;108:2419–25.

Chisholm KM, Wan Y, Li R, Montgomery KD, Chang HY, West RB. Detection of long non-coding RNA in archival tissue: correlation with polycomb protein expression in primary and metastatic breast carcinoma. PLoS ONE 2012;7:e47998.

Chung S, Nakagawa H, Uemura M, Piao L, Ashikawa K, Hosono N, et al. Association of a novel long non-coding RNA in 8q24 with prostate cancer susceptibility. Cancer Sci 2011;102:245–52.

Colley SM, Leedman PJ. Steroid Receptor RNA Activator – a nuclear receptor coregulator with multiple partners: insights and challenges. Biochimie 2011;93:1966–72.

Cooper C, Guo J, Yan Y, Chooniedass-Kothari S, Hube F, Hamedani MK, et al. Increasing the relative expression of endogenous non-coding Steroid Receptor RNA Activator (SRA) in human breast cancer cells using modified oligonucleotides. Nucleic Acids Res 2009;37:4518–31.

Cooper C, Vincett D, Yan Y, Hamedani MK, Myal Y, Leygue E. Steroid receptor RNA activator bi-faceted genetic system: heads or tails? Biochimie 2011;93:1973–80.

Cowin P, Wysolmerski J. Molecular mechanisms guiding embryonic mammary gland development. Cold Spring Harb Perspect Biol 2010;2:a003251.

Derrien T, Johnson R, Bussotti G, Tanzer A, Djebali S, Tilgner H, et al. The GENCODE v7 catalog of human long noncoding RNAs: analysis of their gene structure, evolution, and expression. Genome Res 2012;22:1775–89.

Dinger ME, Amaral PP, Mercer TR, Pang KC, Bruce SJ, Gardiner BB, et al. Long noncoding RNAs in mouse embryonic stem cell pluripotency and differentiation. Genome Res 2008;18:1433–45.

Dinger ME, Gascoigne DK, Mattick JS. The evolution of RNAs with multiple functions. Biochimie 2011;93:2013–8.

Djebali S, Davis CA, Merkel A, Dobin A, Lassmann T, Mortazavi A, et al. Landscape of transcription in human cells. Nature 2012;489:101–8.

Du Z, Fei T, Verhaak RG, Su Z, Zhang Y, Brown M, et al. Integrative genomic analyses reveal clinically relevant long noncoding RNAs in human cancer. Nat Struct Mol Biol 2013;20:908–13.

Eckhardt BL, Francis PA, Parker BS, Anderson RL. Strategies for the discovery and development of therapies for metastatic breast cancer. Nat Rev Drug Discov 2012;11:479–97.

Ellis MJ, Ding L, Shen D, Luo J, Suman VJ, Wallis JW, et al. Whole-genome analysis informs breast cancer response to aromatase inhibition. Nature 2012;486:353–60.

Ellis MJ, Perou CM. The genomic landscape of breast cancer as a therapeutic roadmap. Cancer Discov 2013;3:27–34.

Erbas B, Provenzano E, Armes J, Gertig D. The natural history of ductal carcinoma in situ of the breast: a review. Breast Cancer Res Treat 2006;97:135–44.

Feng J, Bi C, Clark BS, Mady R, Shah P, Kohtz JD. The Evf-2 noncoding RNA is transcribed from the Dlx-5/6 ultraconserved region and functions as a Dlx-2 transcriptional coactivator. Genes Dev 2006;20:1470–84.

Ferron SR, Charalambous M, Radford E, McEwen K, Wildner H, Hind E, et al. Postnatal loss of Dlk1 imprinting in stem cells and niche astrocytes regulates neurogenesis. Nature 2011;475:381–5.

Foulds CE, Tsimelzon A, Long W, Le A, Tsai SY, Tsai MJ, et al. Research resource: expression profiling reveals unexpected targets and functions of the human steroid receptor RNA activator (SRA) gene. Mol Endocrinol 2010;24:1090–105.

Fox BP, Kandpal RP. Invasiveness of breast carcinoma cells and transcript profile: Eph receptors and ephrin ligands as molecular markers of potential diagnostic and prognostic application. Biochem Biophys Res Commun 2004;318:882–92.

Gabory A, Jammes H, Dandolo L. The H19 locus: role of an imprinted non-coding RNA in growth and development. Bioessays 2010;32:473–80.

Gibb EA, Brown CJ, Lam WL. The functional role of long non-coding RNA in human carcinomas. Mol Cancer 2011a;10:38.

Gibb EA, Vucic EA, Enfield KS, Stewart GL, Lonergan KM, Kennett JY, et al. Human cancer long non-coding RNA transcriptomes. PLoS ONE 2011b;6:e25915.

Ginger MR, Gonzalez-Rimbau MF, Gay JP, Rosen JM. Persistent changes in gene expression induced by estrogen and progesterone in the rat mammary gland. Mol Endocrinol 2001;15:1993–2009.

Ginger MR, Shore AN, Contreras A, Rijnkels M, Miller J, Gonzalez-Rimbau MF, et al. A noncoding RNA is a potential marker of cell fate during mammary gland development. Proc Natl Acad Sci USA 2006;103:5781–6.

Gjorevski N, Nelson CM. Integrated morphodynamic signalling of the mammary gland. Nat Rev Mol Cell Biol 2011;12:581–93.

Gordon FE, Nutt CL, Cheunsuchon P, Nakayama Y, Provencher KA, Rice KA, et al. Increased expression of angiogenic genes in the brains of mouse meg3-null embryos. Endocrinology 2010;151:2443–52.

Grote P, Wittler L, Hendrix D, Koch F, Wahrisch S, Beisaw A, et al. The tissue-specific lncRNA Fendrr is an essential regulator of heart and body wall development in the mouse. Dev Cell 2013;24:206–14.

Guffanti A, Iacono M, Pelucchi P, Kim N, Solda G, Croft LJ, et al. A transcriptional sketch of a primary human breast cancer by 454 deep sequencing. BMC Genomics 2009;10:163.

Gumireddy K, Li A, Yan J, Setoyama T, Johannes GJ, Orom UA, et al. Identification of a long non-coding RNA-associated RNP complex regulating metastasis at the translational step. EMBO J 2013;32:2672–84.

Gupta RA, Shah N, Wang KC, Kim J, Horlings HM, Wong DJ, et al. Long non-coding RNA HOTAIR reprograms chromatin state to promote cancer metastasis. Nature 2010;464:1071–6.

Gutschner T, Diederichs S. The hallmarks of cancer: a long non-coding RNA point of view. RNA Biol 2012;9:703–19.

Gutschner T, Hammerle M, Diederichs S. MALAT1 – a paradigm for long noncoding RNA function in cancer. J Mol Med (Berl) 2013a;91:791–801.

Gutschner T, Hammerle M, Eissmann M, Hsu J, Kim Y, Hung G, et al. The noncoding RNA MALAT1 is a critical regulator of the metastasis phenotype of lung cancer cells. Cancer Res 2013b;73:1180–9.

Guttman M, Amit I, Garber M, French C, Lin MF, Feldser D, et al. Chromatin signature reveals over a thousand highly conserved large non-coding RNAs in mammals. Nature 2009;458:223–7.

Guttman M, Donaghey J, Carey BW, Garber M, Grenier JK, Munson G, et al. lincRNAs act in the circuitry controlling pluripotency and differentiation. Nature 2011;477:295–300.

Haerty W, Ponting CP. Mutations within lncRNAs are effectively selected against in fruitfly but not in human. Genome Biol 2013;14:R49.
Hah N, Danko CG, Core L, Waterfall JJ, Siepel A, Lis JT, et al. A rapid, extensive, and transient transcriptional response to estrogen signaling in breast cancer cells. Cell 2011;145:622–34.
Hah N, Kraus WL. Hormone-regulated transcriptomes: lessons learned from estrogen signaling pathways in breast cancer cells. Mol Cell Endocrinol 2013;382:652–64.
Hah N, Murakami S, Nagari A, Danko CG, Kraus WL. Enhancer transcripts mark active estrogen receptor binding sites. Genome Res 2013;23:1210–23.
Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell 2011;144:646–74.
Hesson LB, Packham D, Pontzer E, Funchain P, Eng C, Ward RL. A reinvestigation of somatic hypermethylation at the PTEN CpG island in cancer cell lines. Biol Proced Online 2012;14:5.
Hu W, Yuan B, Flygare J, Lodish HF. Long noncoding RNA-mediated anti-apoptotic activity in murine erythroid terminal differentiation. Genes Dev 2011;25:2573–8.
Huang J, Zhou N, Watabe K, Lu Z, Wu F, Xu M, et al. Long non-coding RNA UCA1 promotes breast tumor growth by suppression of p27 (Kip1). Cell Death Dis 2014;5:e1008.
Huarte M, Guttman M, Feldser D, Garber M, Koziol MJ, Kenzelmann-Broz D, et al. A large intergenic noncoding RNA induced by p53 mediates global gene repression in the p53 response. Cell 2010;142:409–19.
Hung T, Wang Y, Lin MF, Koegel AK, Kotake Y, Grant GD, et al. Extensive and coordinated transcription of noncoding RNAs within cell-cycle promoters. Nat Genet 2011;43:621–9.
Iacoangeli A, Lin Y, Morley EJ, Muslimov IA, Bianchi R, Reilly J, et al. BC200 RNA in invasive and preinvasive breast cancer. Carcinogenesis 2004;25:2125–33.
Iacoangeli A, Tiedge H. Translational control at the synapse: role of RNA regulators. Trends Biochem Sci 2013;38:47–55.
Jeon Y, Sarma K, Lee JT. New and Xisting regulatory mechanisms of X chromosome inactivation. Curr Opin Genet Dev 2012;22:62–71.
Ji P, Diederichs S, Wang W, Boing S, Metzger R, Schneider PM, et al. MALAT-1, a novel noncoding RNA, and thymosin beta4 predict metastasis and survival in early-stage non-small cell lung cancer. Oncogene 2003;22:8031–41.
Johnsson P, Ackley A, Vidarsdottir L, Lui WO, Corcoran M, Grander D, et al. A pseudogene long-noncoding-RNA network regulates PTEN transcription and translation in human cells. Nat Struct Mol Biol 2013;20:440–6.
Kalyana-Sundaram S, Kumar-Sinha C, Shankar S, Robinson DR, Wu YM, Cao X, et al. Expressed pseudogenes in the transcriptional landscape of human cancers. Cell 2012;149:1622–34.
Keniry A, Oxley D, Monnier P, Kyba M, Dandolo L, Smits G, et al. The H19 lincRNA is a developmental reservoir of miR-675 that suppresses growth and Igf1r. Nat Cell Biol 2012;14:659–65.
Khalil AM, Guttman M, Huarte M, Garber M, Raj A, Rivea Morales D, et al. Many human large intergenic noncoding RNAs associate with chromatin-modifying complexes and affect gene expression. Proc Natl Acad Sci USA 2009;106:11667–72.
Kino T, Hurt DE, Ichijo T, Nader N, Chrousos GP. Noncoding RNA gas5 is a growth arrest- and starvation-associated repressor of the glucocorticoid receptor. Sci Signal 2010;3:ra8.
Klattenhoff CA, Scheuermann JC, Surface LE, Bradley RK, Fields PA, Steinhauser ML, et al. Braveheart, a long noncoding RNA required for cardiovascular lineage commitment. Cell 2013;152:570–83.
Kretz M, Siprashvili Z, Chu C, Webster DE, Zehnder A, Qu K, et al. Control of somatic tissue differentiation by the long non-coding RNA TINCR. Nature 2013;493:231–5.
Kretz M, Webster DE, Flockhart RJ, Lee CS, Zehnder A, Lopez-Pajares V, et al. Suppression of progenitor differentiation requires the long noncoding RNA ANCR. Genes Dev 2012.
Lanz RB, Chua SS, Barron N, Soder BM, DeMayo F, O'Malley BW. Steroid receptor RNA activator stimulates proliferation as well as apoptosis in vivo. Mol Cell Biol 2003;23:7163–76.
Lanz RB, McKenna NJ, Onate SA, Albrecht U, Wong J, Tsai SY, et al. A steroid receptor coactivator, SRA, functions as an RNA and is present in an SRC-1 complex. Cell 1999;97:17–27.
Lee GL, Dobi A, Srivastava S. Prostate cancer: diagnostic performance of the PCA3 urine test. Nat Rev Urol 2011;8:123–4.
Lee JT. Epigenetic regulation by long noncoding RNAs. Science 2012;338:1435–9.
Lee JT, Bartolomei MS. X-inactivation, imprinting, and long noncoding RNAs in health and disease. Cell 2013;152:1308–23.
Li W, Notani D, Ma Q, Tanasa B, Nunez E, Chen AY, et al. Functional roles of enhancer RNAs for oestrogen-dependent transcriptional activation. Nature 2013;498:516–20.
Lin R, Maeda S, Liu C, Karin M, Edgington TS. A large noncoding RNA is a marker for murine hepatocellular carcinomas and a spectrum of human carcinomas. Oncogene 2007;26:851–8.
Lin R, Roychowdhury-Saha M, Black C, Watt AT, Marcusson EG, Freier SM, et al. Control of RNA processing by a large non-coding RNA over-expressed in carcinomas. FEBS Lett 2011;585:671–6.
Lottin S, Adriaenssens E, Dupressoir T, Berteaux N, Montpellier C, Coll J, et al. Overexpression of an ectopic H19 gene enhances the tumorigenic properties of breast cancer cells. Carcinogenesis 2002;23:1885–95.
Mao YS, Zhang B, Spector DL. Biogenesis and function of nuclear bodies. Trends Genet 2011;27:295–306.
Martin L, Chang HY. Uncovering the role of genomic "dark matter" in human disease. J Clin Invest 2012;122:1589–95.
Mattick JS. The genetic signatures of noncoding RNAs. PLoS Genet 2009;5:e1000459.
Mercer TR, Dinger ME, Sunkin SM, Mehler MF, Mattick JS. Specific expression of long noncoding RNAs in the mouse brain. Proc Natl Acad Sci USA 2008;105:716–21.
Mercer TR, Mattick JS. Structure and function of long noncoding RNAs in epigenetic regulation. Nat Struct Mol Biol 2013;20:300–7.
Mizrahi A, Czerniak A, Levy T, Amiur S, Gallula J, Matouk I, et al. Development of targeted therapy for ovarian cancer mediated by a plasmid expressing diphtheria toxin under the control of H19 regulatory sequences. J Transl Med 2009;7:69.
Mourtada-Maarabouni M, Pickard MR, Hedge VL, Farzaneh F, Williams GT. GAS5, a non-protein-coding RNA, controls apoptosis and is downregulated in breast cancer. Oncogene 2009;28:195–208.
Murphy LC, Simon SL, Parkes A, Leygue E, Dotzlaw H, Snell L, et al. Altered expression of estrogen receptor coregulators during human breast tumorigenesis. Cancer Res 2000;60:6266–71.
Ng SY, Johnson R, Stanton LW. Human long non-coding RNAs promote pluripotency and neuronal differentiation by association with chromatin modifiers and transcription factors. EMBO J 2012;31:522–33.
Ni M, Chen Y, Lim E, Wimberly H, Bailey ST, Imai Y, et al. Targeting androgen receptor in estrogen receptor-negative breast cancer. Cancer Cell 2011;20:119–31.
Novak P, Jensen T, Oshiro MM, Watts GS, Kim CJ, Futscher BW. Agglomerative epigenetic aberrations are a common event in human breast cancer. Cancer Res 2008;68:8616–25.
Orom UA, Derrien T, Beringer M, Gumireddy K, Gardini A, Bussotti G, et al. Long noncoding RNAs with enhancer-like function in human cells. Cell 2010;143:46–58.
Perez DS, Hoage TR, Pritchett JR, Ducharme-Smith AL, Halling ML, Ganapathiraju SC, et al. Long, abundantly expressed non-coding transcripts are altered in cancer. Hum Mol Genet 2008;17:642–55.
Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, et al. Molecular portraits of human breast tumours. Nature 2000;406:747–52.
Petrovics G, Zhang W, Makarem M, Street JP, Connelly R, Sun L, et al. Elevated expression of PCGEM1, a prostate-specific gene with cell growth-promoting function, is associated with high-risk prostate cancer patients. Oncogene 2004;23:605–11.
Poliseno L. Pseudogenes, newly discovered players in human cancer. Science Signal 2012;5:re5.
Poliseno L, Salmena L, Zhang J, Carver B, Haveman WJ, Pandolfi PP. A coding-independent function of gene and pseudogene mRNAs regulates tumour biology. Nature 2010;465:1033–8.
Polyak K. Breast cancer: origins and evolution. J Clin Invest 2007;117:3155–63.
Ponjavic J, Oliver PL, Lunter G, Ponting CP. Genomic and transcriptional co-localization of protein-coding and long non-coding RNA pairs in the developing brain. PLoS Genet 2009;5:e1000617.
Prat A, Ellis MJ, Perou CM. Practical implications of gene-expression-based assays for breast oncologists. Nat Rev Clin Oncol 2012;9:48–57.
Prensner JR, Chinnaiyan AM. The emergence of lncRNAs in cancer biology. Cancer Discov 2011;1:391–407.
Qureshi IA, Mattick JS, Mehler MF. Long non-coding RNAs in nervous system function and disease. Brain Res 2010;1338:20–35.
Ramos AD, Diaz A, Nellore A, Delgado RN, Park KY, Gonzales-Roybal G, et al. Integration of genome-wide approaches identifies lncRNAs of adult neural stem cells and their progeny in vivo. Cell Stem Cell 2013;12:616–28.
Ravasi T, Suzuki H, Pang KC, Katayama S, Furuno M, Okunishi R, et al. Experimental validation of the regulated expression of large numbers of non-coding RNAs from the mouse genome. Genome Res 2006;16:11–9.
Rinn JL, Chang HY. Genome regulation by long noncoding RNAs. Annu Rev Biochem 2012;81:145–66.
Rinn JL, Kertesz M, Wang JK, Squazzo SL, Xu X, Brugmann SA, et al. Functional demarcation of active and silent chromatin domains in human HOX loci by noncoding RNAs. Cell 2007;129:1311–23.
Sanchez Y, Huarte M. Long non-coding RNAs: challenges for diagnosis and therapies. Nucleic Acid Ther 2013;23:15–20.
Sauvageau M, Goff LA, Lodato S, Bonev B, Groff AF, Gerhardinger C, et al. Multiple knockout mouse models reveal lincRNAs are required for life and brain development. eLife 2013;2:e01749.
Shore AN, Kabotyanski EB, Roarty K, Smith MA, Zhang Y, Creighton CJ, et al. Pregnancy-induced noncoding RNA (PINC) associates with polycomb repressive complex 2 and regulates mammary epithelial differentiation. PLoS Genet 2012;8:e1002840.
Silva JM, Boczek NJ, Berres MW, Ma X, Smith DI. LSINCT5 is over expressed in breast and ovarian cancer and affects cellular proliferation. RNA Biol 2011;8:496–505.
Silva JM, Perez DS, Pritchett JR, Halling ML, Tang H, Smith DI. Identification of long stress-induced non-coding transcripts that have altered expression in cancer. Genomics 2010;95:355–62.
Sorlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci USA 2001;98:10869–74.
Sorlie T, Tibshirani R, Parker J, Hastie T, Marron JS, Nobel A, et al. Repeated observation of breast tumor subtypes in independent gene expression data sets. Proc Natl Acad Sci USA 2003;100:8418–23.
Spizzo R, Almeida MI, Colombatti A, Calin GA. Long non-coding RNAs and cancer: a new frontier of translational research? Oncogene 2012;31:4577–87.
Tripathi V, Ellis JD, Shen Z, Song DY, Pan Q, Watt AT, et al. The nuclear-retained noncoding RNA MALAT1 regulates alternative splicing by modulating SR splicing factor phosphorylation. Mol Cell 2010;39:925–38.

Tripathi V, Shen Z, Chakraborty A, Giri S, Freier SM, Wu X, et al. Long noncoding RNA MALAT1 controls cell cycle progression by regulating the expression of oncogenic transcription factor B-MYB. PLoS Genet 2013;9:e1003368.
Tsai MC, Manor O, Wan Y, Mosammaparast N, Wang JK, Lan F, et al. Long noncoding RNA as modular scaffold of histone modification complexes. Science 2010;329:689–93.
Tsang WP, Ng EK, Ng SS, Jin H, Yu J, Sung JJ, et al. Oncofetal H19-derived miR-675 regulates tumor suppressor RB in human colorectal cancer. Carcinogenesis 2010;31:350–8.
van Amerongen R, Bowman AN, Nusse R. Developmental stage and time dictate the fate of Wnt/beta-catenin-responsive stem cells in the mammary gland. Cell Stem Cell 2012;11:387–400.
Van Keymeulen A, Rocha AS, Ousset M, Beck B, Bouvencourt G, Rock J, et al. Distinct stem cells contribute to mammary gland development and maintenance. Nature 2011;479:189–93.
Vargo-Gogola T, Rosen JM. Modelling breast cancer: one size does not fit all. Nature Rev Cancer 2007;7:659–72.
Venkatraman A, He XC, Thorvaldsen JL, Sugimura R, Perry JM, Tao F, et al. Maternal imprinting at the H19-Igf2 locus maintains adult haematopoietic stem cell quiescence. Nature 2013;500:345–9.
Visvader JE, Smith GH. Murine mammary epithelial stem cells: discovery, function, and current status. Cold Spring Harb Perspect Biol 2011;3.
Wagner KU, Boulanger CA, Henry MD, Sgagias M, Hennighausen L, Smith GH. An adjunct mammary epithelial cell population in parous females: its role in functional adaptation and tissue renewal. Development 2002;129:1377–86.
Wagner LA, Christensen CJ, Dunn DM, Spangrude GJ, Georgelas A, Kelley L, et al. EGO, a novel, noncoding RNA gene, regulates eosinophil granule protein transcript expression. Blood 2007;109:5191–8.
Wan G, Hu X, Liu Y, Han C, Sood AK, Calin GA, et al. A novel non-coding RNA lncRNA-JADE connects DNA damage signalling to histone H4 acetylation. EMBO J 2013;32:2833–47.
Wang L, Zeng X, Chen S, Ding L, Zhong J, Zhao JC, et al. BRCA1 is a negative modulator of the PRC2 complex. EMBO J 2013;32:1584–97.
Wang XS, Zhang Z, Wang HC, Cai JL, Xu QW, Li MQ, et al. Rapid identification of UCA1 as a very sensitive and specific unique marker for human bladder carcinoma. Clin Cancer Res 2006;12:4851–8.
Watson CJ, Khaled WT. Mammary development in the embryo and adult: a journey of morphogenesis and commitment. Development 2008;135:995–1003.
Xu B, Gerin I, Miao H, Vu-Phan D, Johnson CN, Xu R, et al. Multiple roles for the non-coding RNA SRA in regulation of adipogenesis and insulin sensitivity. PLoS ONE 2010;5:e14199.
Yang L, Lin C, Jin C, Yang JC, Tanasa B, Li W, et al. lncRNA-dependent mechanisms of androgen-receptor-regulated gene activation programs. Nature 2013;500:598–602.
Yang L, Lin C, Rosenfeld MG. A lincRNA switch for embryonic stem cell fate. Cell Res 2011;21:1646–8.
Yap KL, Li S, Munoz-Cabello AM, Raguz S, Zeng L, Mujtaba S, et al. Molecular interplay of the noncoding RNA ANRIL and methylated histone H3 lysine 27 by polycomb CBX7 in transcriptional silencing of INK4a. Mol Cell 2010;38:662–74.
Zacharek SJ, Fillmore CM, Lau AN, Gludish DW, Chou A, Ho JW, et al. Lung stem cell self-renewal relies on BMI1-dependent control of expression at imprinted loci. Cell Stem Cell 2011;9:272–81.
Zhang B, Arun G, Mao YS, Lazar Z, Hung G, Bhattacharjee G, et al. The lncRNA Malat1 is dispensable for mouse development but its transcription plays a cis-regulatory role in the adult. Cell Rep 2012;2:111–23.
Zhang H, Singh RR, Talukder AH, Kumar R. Metastatic tumor antigen 3 is a direct corepressor of the Wnt4 pathway. Genes Dev 2006;20:2943–8.
Zhang X, Lian Z, Padden C, Gerstein MB, Rozowsky J, Snyder M, et al. A myelopoiesis-associated regulatory intergenic noncoding RNA transcript within the human HOXA cluster. Blood 2009;113:2526–34.
Zhang X, Zhou Y, Mehta KR, Danila DC, Scolavino S, Johnson SR, et al. A pituitary-derived MEG3 isoform functions as a growth suppressor in tumor cells. J Clin Endocrinol Metab 2003;88:5119–26.
Zhao J, Ohsumi TK, Kung JT, Ogawa Y, Grau DJ, Sarma K, et al. Genome-wide identification of polycomb-associated RNAs by RIP-seq. Mol Cell 2010;40:939–53.
Zhou Y, Zhang X, Klibanski A. MEG3 noncoding RNA: a tumor suppressor. J Mol Endocrinol 2012;48:R45–53.
Zhou Y, Zhong Y, Wang Y, Zhang X, Batista DL, Gejman R, et al. Activation of p53 by MEG3 non-coding RNA. J Biol Chem 2007;282:24731–42.