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Thee role of Rhox homeobox factors in tumorigenesis

James A. MacLean II[1]

[1]Department of Physiology, Southern Illinois University, School of Medicine, 1135 Lincoln Dr. PO Box 6652, Carbondale, IL 62901, USA.

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1. ABSTRACT

Homeobox genes encode transcription factors that have well-established roles in embryonic development. We recently discovered the *Rhox* genes, a new family of homeobox genes, which are selectively expressed in the developing embryo, postnatal and adult gonads, and accessory tissues associated with mammalian reproduction. The largest and best-characterized *Rhox* cluster is found in mouse. However, all mammals examined to date possess a set of *Rhox* genes that, while they may vary in number by species, appear relevant to reproduction and are located in the syntenic region of the X chromosome. *Rhox5*, the founding member of the family, was initially cloned from a screen to identify tumorigenic antigens from T-cell lymphomas, and was later found to be widely expressed in tumors from tissues of diverse origins that do not normally express the *Rhox* genes. This aberrant upregulation appears to be a general feature of many *Rhox* genes, but the implications of this misexpression remain largely uninvestigated. In this review, we will discuss the latest findings on the normal and abnormal roles of the *Rhox* genes and their potential contributions to the formation and progression of tumors.

2. INTRODUCTION

Homeobox genes encode transcription factors that are master regulators of developmental programs. The homeodomain is a 60-amino acid DNA-binding motif that confers the primary function of homeobox proteins, namely binding to the promoters of downstream target genes and either activating or repressing their transcription (1). However, as described in more detail later, homeodomains can be involved in specific protein-protein interaction and are typically found in the context of larger polypeptides (mostly near the C-terminus) that possess multiple functional domains that influence transcription factor recruitment or interaction with cofactors influencing non-transcription related functions (2, 3). More than 170 homeobox genes exist in higher eukaryotic genomes; they are grouped in subfamilies based on homeodomain sequence and gene structure (Figure 1). Best known is the highly conserved *Hox* subfamily, which governs diverse embryonic developmental processes, including body-axis formation, organogenesis, and limb development (4).

This review focuses on the recently discovered *Rhox* genes, a family of homeobox genes clustered on the

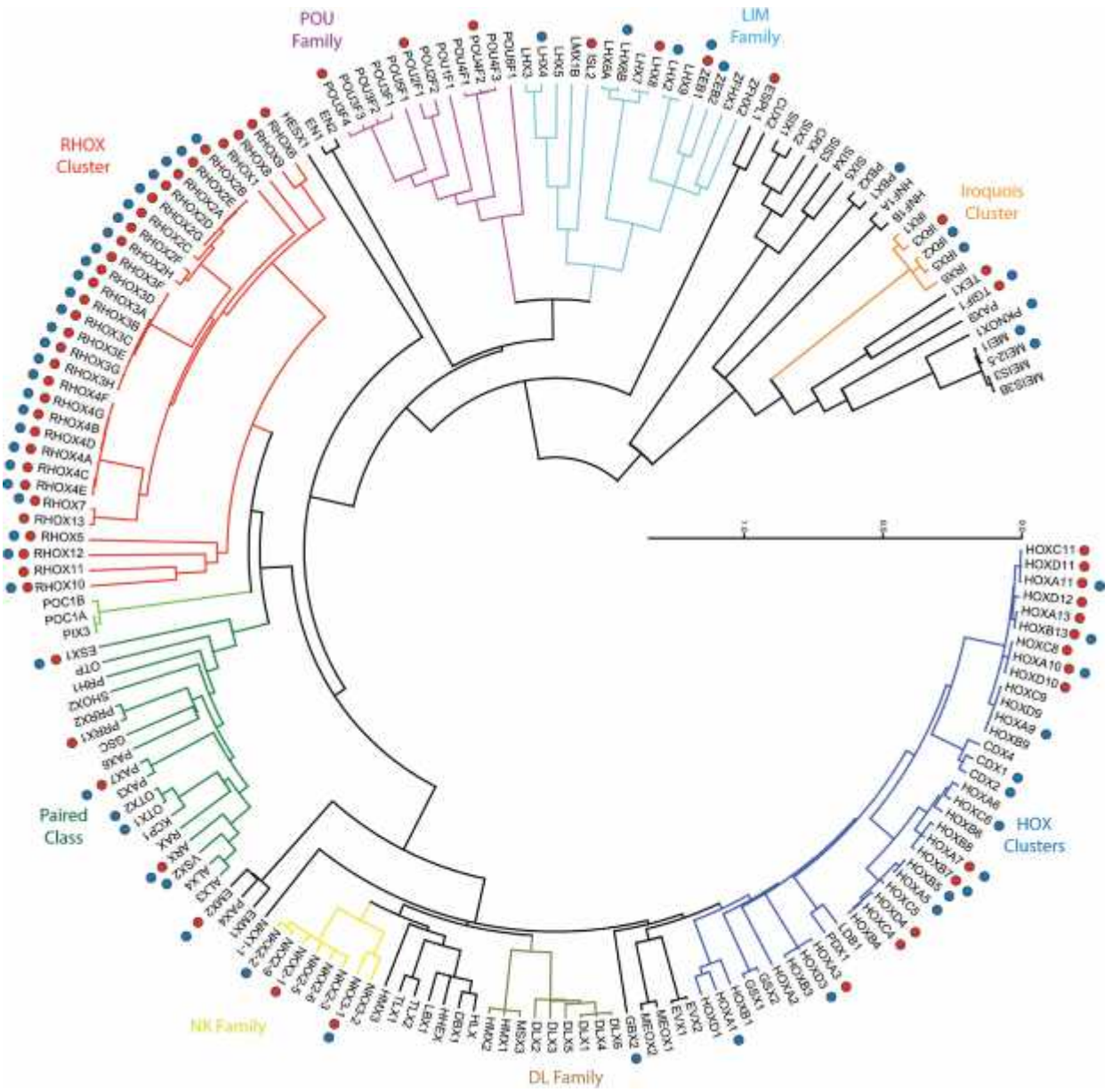

Figure 1. Phylogenetic Comparison of mouse homeodomain proteins. The 60 amino acid homeodomain portions of all described mouse homeobox genes were aligned and compared via an unrooted phylogenetic tree using the neighbor joining method. Branch lengths represent the extent of divergence. Homeobox genes with links to cancer are indicated by blue dots and genes expressed in the reproductive tract are indicated by red dots.

X chromosome, which are preferentially expressed in reproductive tissues and are thus thought to contribute to fertility (5). Similarly to other homeobox genes, the *Rhox* genes are expressed during embryonic development (5-9). However, unlike most other homeobox gene families, several members of the *Rhox* cluster remain expressed at high levels after birth and thus are candidates to control postnatal and adult developmental events, particularly those essential for male and female germ cell production (5, 10).

In contrast to their well established roles in controlling development, very little is known about the normal functions of homeobox genes in postnatal tissues, especially within the reproductive tract where the *Rhox* genes are predominantly expressed. This is somewhat surprising, as over 40 homeobox genes are expressed in postnatal and adult testes and ovary (indicated by red dots in Figure 1). Efforts to clarify the roles of some of these homeobox genes have been clouded by putative functional redundancies and embryonic lethality. Knockout mice for many homeobox genes (mainly *Hox* genes) do not exhibit any obvious defects in the reproductive tract, including spermatogenesis. It is not known whether this is because of functional redundancy (*Hox* genes are known to

compensate for each other) or because some homeobox genes have little or no role in reproduction (that is, their expression in the reproductive tract is superfluous). In other cases, targeted disruption of homeobox genes, including *Pou5f1*, *Pou3f1*, *Hoxa5*, and *Alx1*, causes embryonic lethality, thereby precluding analysis of their role in postnatal and adult events, including spermatogenesis (11, 12).

Because homeobox genes are master regulators of development, and govern the processes of cellular proliferation, differentiation, and reorganization of tissues, it's not surprising to find that many have links to cancer. The misregulation of at least 35 mouse homeobox genes has been demonstrated in cancers of blood, breast, ovary, skin, prostate, gut and others (indicated by blue dots in Figure 1). In some instances, the homeobox gene exhibits tumor suppressor activity which is lost such as *Cdx2* in the colon (13) or *PDX1* in the stomach (14). Some homeobox genes possess proliferative activity such as *Cdx1* in colorectal cancer (15) and *Irx5* in prostate (16). The role of some homeobox genes, such as *Nkx3-1*, in cancer is somewhat controversial as evidence for both of these simple cases exists (17, 18). The pleiotropic effects of homeobox genes may result from stage-dependent differences in their regulation by different hormones or shifts in the cell-type distribution within a tissue.

The majority of the published work to date centers on regulation of the *Rhox* genes and their function in fertility, which for summaries and perspectives we direct readers to prior reviews (7, 8, 10, 11, 19-22). However, historically there have been many anecdotal lines of evidence to suggest that the *Rhox* genes are relevant in cancer biology, which has been borne out by recent directed studies. Here, we update what is known about the reproductive functions, regulation, and evolution of the *Rhox* gene cluster since our last review (10), and for the first time review emerging studies that abnormal expression and actions of the *Rhox* genes may contribute to tumorigenesis.

3. CHARACTERISTICS OF THE RHOX HOMEOBOX CLUSTER

3.1. Organization and evolution of the *Rhox* genes

The largest *Rhox* gene cluster is found in mice and has been subdivided into three subclusters: alpha, beta, and gamma with each gene receiving a numeric designation based on relative position on the X chromosome with respect to the centromere (Figure 2). The alpha subcluster originally was thought to contain four genes (5), but later it was discovered that a tandem duplications of three of the alpha subcluster genes, *Rhox2*, *Rhox3*, and *Rhox4*, had occurred, resulting in 24 genes in the alpha subcluster (23-26). These paralogs are nearly identical in DNA and protein sequence (greater than 92%) and are largely coexpressed in reproductive tissues, with the first set of copies being most highly detected as determined by qPCR using paralog-specific primers (10, 26, 27). *Rhox5*, the founding member of the *Rhox* family, is the first gene in the beta subcluster, and it is followed by three genes that had appeared in the literature as independent homeobox genes prior to discovery of the cluster: *Rhox6* (*Psx1*), *Rhox8* (*Tox*), and *Rhox9* (*Psx2* and *Gpbox*) (28-30). The gamma subcluster contains four genes including the most recently discovered member of the family, *Rhox13* (5, 31). To date, the mouse *Rhox* cluster contains 33 homeobox genes, and there have been few changes in *Rhox* gene number in the past few builds of the mouse genome suggesting that the cluster is now complete.

Our lab and others have previously demonstrated that *Rhox* gene sequences are rapidly diverging, exhibiting signs of positive selection for amino acid changes (24, 26, 32). This is not surprising as it is a common feature of X-linked genes (with respect to their autosomal copies), large gene families (regardless of chromosomal location), and duplicated genes involved in reproduction (33-36). The significant divergence in sequence identity made the discovery of orthologus *Rhox* genes in different species difficult. For example, mouse *Rhox5* shares only 72% identity with its counterpart in rat, and is only 42% identical with a putative human ortholog, *RHOXF1* (37). However, recent advances in genome mapping, coupled with EST sequencing projects, have confirmed the existence of *Rhox* clusters in most mammalian species (and some fish) which have been investigated. These findings suggest that the rates of duplication (or selective pressures to maintain multiple *Rhox* gene copies) have varied between species.

After mouse, the next largest *Rhox* cluster is found in rat (Figure 2) (25, 27). While all three subclusters are present, the alpha subcluster has undergone an inversion (possibly resulting in the deletion of *Rhox1*) and there is no evidence from genomic mapping or cDNA screening to support the tandem duplication of *Rhox2*, *Rhox3*, and *Rhox4* in rat (5, 23, 25, 26). Additionally, in the beta subcluster there is only one copy of the highly similar genes, *Rhox6* and *Rhox9*, found in mice. Sequence analysis and genomic position suggests that the rat ortholog exhibits features more consistent with the designation of *Rhox9* than *Rhox6* (5, 25, 26). The gamma subcluster appears conserved in both structure and organization between rodent species. cDNA encoding *Rhox* orthologs have been identified in two other rodent species, guinea pig and hamster, but the preliminary builds of these two genomes are not refined enough to determine the size and organization of their Rhox clusters.

It appears that the majority of the other mammals have a much smaller set of *RHOX* genes with the syntenic portion of their X chromosomes (Figure 2). Examination of cDNA libraries typically yields two distinct genes, *RHOX* family 1 (*RHOXF1)* and *RHOXF2* (which may be present in multiple copies). These genes all possess the characteristic exon/intron structure of *Rhox* members (5), but as the gene number and sequences are highly variable between species, assignment of true orthologs has remained elusive. In humans, *RHOXF1* (originally called *OTEX* and *hPEPP1*), has traditionally been most associated with being the *Rhox5*-ortholog because it was first discovered, is

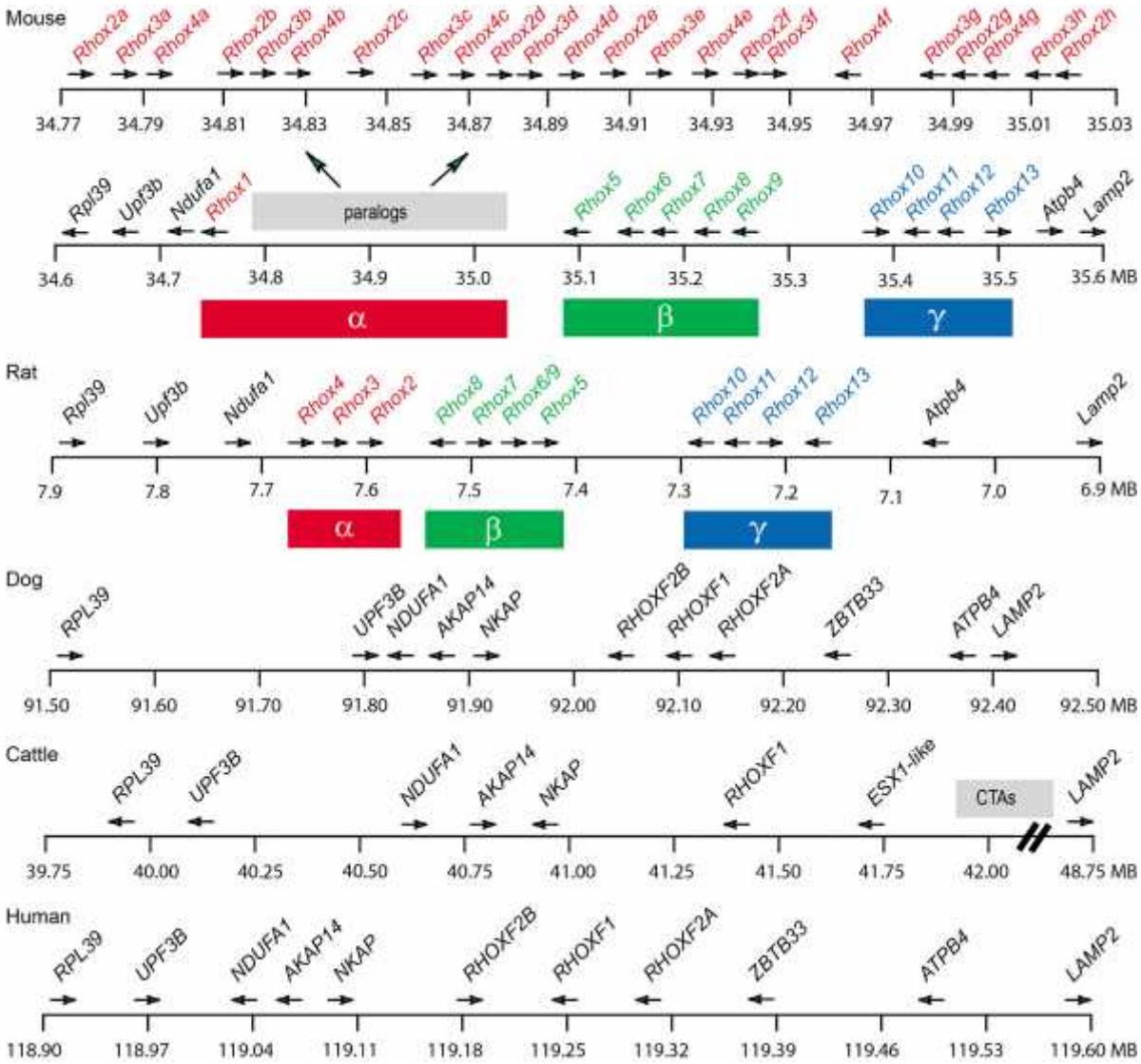

Figure 2. Organization of the *Rhox* cluster in mammalian species. The syntenic region of the X chromosome containing the *Rhox* orthologs and conserved flanking genes is shown. The rodent *Rhox* gene subclusters are indicated by red (alpha), green (beta), and blue (gamma). Established orthologus genes are indicated by dotted lines. The orthologus relationship between human *RHOX* genes and rodent *Rhox* genes cannot be clearly assigned because of the rapid evolution of *Rhox* genes. The map positions shown are according to builds 37.1 (mouse), 3.4 (rat), 3.1 (Dog), 6.1 (Cattle), and 37.1 (human).

androgen regulated, and selectively expressed in human testes (37, 38). Humans also have two recently duplicated genes, *RHOXF2A* (originally called *hPEPP2*) and *RHOXF2B*, which cannot be distinguished by qPCR analysis as they only vary in 2 positions in their 1291 nucleotide coding sequences (10, 37). Rhesus macaque, chimpanzee, and orangutans all share the precise structure of the human *RHOX* cluster and flanking genes as shown in Figure 2, only varying in MB numbering ranges (i.e. *RHOXF1* in chimpanzee is at 120.5 with respect to the centromere). Smaller primates including marmosets and gibbons possess genes encoding *RHOXF1* and *RHOXF2*, but the assembly of their genomes (build 1.1) is not yet sufficient to determine whether multiple copies of *RHOXF2* are present. Final refinement of the primate genomes will likely look much different than the scenarios presented above. While the number of unique genes in non-rodents will probably not significantly change, there is evidence that the number of copies of *RHOXF2* is under evolutionary selection (39). Niu *et al.* sequenced the *RHOXF2* genomic locus for 111 human individuals and found that 13% had either only one copy of *RHOXF2* or two identical copies, whereas the others had clear *RHOXF2A* and *RHOX2B* encoding genes. Similarly, 11 non-human primate species were found to have single or two duplicate *RHOXF2* genes. However, 4 old world monkey species all possessed at least 2 distinct *RHOXF2* genes, with cDNA evidence suggesting the number of copies in chimpanzee may be as large as 6 *RHOXF2* genes. It is not clear what reproductive advantages having multiple

RHOXF2 genes would provide from a Darwinian selection perspective, however, it appears that like the mouse *Rhox* genes they are under positive selection for amino acid changes (39). Given that RHOXF2 may have a causative role in cancer development and progression (see section 4.2), assessment of the number of copies (if truly variant in the human population) of *RHOXF2* could provide a genetic screen for the risk of developing certain cancers.

The presence of a *RHOXF2B* gene in dog, which diverged from a common ancestor prior to the appearance of primates (40), suggests that duplication and loss of *RHOXF2* copies is an ancient event, not restricted to primates (Figure 2). The bovine *RHOX* cluster contains a bona fide ortholog of *RHOXF1* (Figure 2). However, the current build of the *Bos Taurus* genome (6.1) does not possess an obvious second copy of *RHOXF2B* in between *NKAP* and *RHOXF1* as observed in primates and canines. At the *RHOXF2A* location, there is an *ESX1-like* gene, which likely will ultimately be determined to be a true *RHOXF2* ortholog. *ESX1* (discussed below) undoubtedly derived from the same primordial gene that gave rise to the *RHOX* genes as it shares the characteristic splicing pattern in its homeodomain (5, 37), but it resides ~20 MB outside of the *Rhox* cluster in all species examined in Figure 2. Most species share remnant *RHOX* sequences (i.e. partial exons/introns, but no full coding sequences) flanking their complete *RHOX* genes, but it is not yet clear if additional genes exist awaiting discovery, if they are leftovers from duplicated genes which were not maintained, or whether they are sequencing artifacts (JAM unpublished observations).

Evolutionarily, the RHOX family is most closely related to the paired-like subfamily of homeodomain proteins, POC1A, POC1B, and PIX3 (Figure 1). The genes encoding these proteins were probably derived from the same precursor that yielded the adjacent paired class of homeobox genes, most likely the ancient *aristaless* gene first characterized in *drosophila* (12, 41). However, it is currently believed that the *Rhox*-precursor and two paired class genes, *Esx1* and *Arx*, likely resulted from duplication of the same primordial gene around the time of the divergence of mammals (19). There are several lines of evidence to suggest a tight relationship between the *Rhox* genes, *Esx1*, and *Arx*. Namely, all are on the X chromosome, are similar in homeodomain sequence, have amino terminal domains that while highly divergent are more similar to each other than those of other paired genes, and finally all possess the hallmark exon-intron structure specifically characteristic of *Rhox* genes (i.e., introns located at two particular sites within the homeodomain-encoding region) that are not present in other paired genes (5, 26, 37). For the purposes of this discussion, *Esx1* and *Arx* are additionally similar to the *Rhox* genes in that they are normally restricted to reproductive tissues, but are misexpressed in tumors of divergent cellular origin.

3.2. Expression of the *Rhox* genes in reproductive tissues

The normal expression of RHOX transcription factors is restricted to reproductive organs and thus they are likely to control genes that modulate the production and differentiation of germ cells as well as governing the early development of the embryo (11, 25, 42). In our initial report detailing the discovery of the *Rhox* cluster (5), we demonstrated that all *Rhox* genes are selectively expressed in the ovary, testis, epididymis, and placenta in mice. Individual accounts of a few rat *Rhox* orthologs indicate that they exhibit conserved expression in reproductive tissues (25, 42-44). Recently, a formal panel screen of the rat *Rhox* cluster confirmed their selective expression in the ovary, testis, epididymis, and placenta (27). We previously reported that three mouse genes, *Rhox4*, *Rhox7*, and *Rhox8* each exhibited expression in a single non-reproductive tissue; thymus, stomach, and intestine, respectively (5). However, the isolated expression of these *Rhox* genes in non-reproductive tissues did not appear to be conserved in rats (25, 27). While expression of mouse and rat *Rhox* genes is observed in similar tissues, there are some differences of note. For example, while *Rhox5* and *Rhox8* are the most highly expressed genes in the mouse testes, in the rat they are more highly expressed in the epididymis than testes (27). Rat *Rhox2* is most highly expressed in the testis and relatively minor in other tissues, whereas in the mouse the converse is true with ovary, placental and epididymis exhibiting high expression. While rat and mouse *Rhox3* are highest in the testes, rat *Rhox3* is nearly as highly expressed in placenta, but barely detected in placenta in mouse. At present, it is not clear if differences between mouse and rat *Rhox2*, *Rhox3*, and *Rhox4* are due to different relative expressions of the 7 duplicated mouse genes which may be independently regulated, in comparison to the rat where a single gene/promoter contributed to expression.

While less information is currently available for the non-rodent *RHOX* genes, it appears that like their rodent cousins they are expressed in reproductive tissues. *RHOXF1* and *RHOXF2* are expressed in the human testis and epididymis, as assessed by Northern blot and qPCR analyses (37, 38). In our initial report, we could not detect either gene in post-delivery placental tissues (37). However, subsequent analysis of flash frozen, near term placenta suggests that *RHOXF1* may be present in trophoblast tissue. Preliminary immunohistochemical analyses with antisera generated against RHOXF1 peptides stains the same cells as androgen receptor-specific antibodies suggesting that it is produced in Sertoli cells. Furthermore, consensus androgen response elements have been identified in the putative promoters of both *RHOXF1* and *RHOXF2A* gene (37). However, AR-dependent expression has only been demonstrated for the *RHOXF1* gene (38). Thus the normal expression of *RHOXF1* is most like that of genes in the rodent beta subcluster. The relatively strong expression of *RHOXF2* in testis and epididymis, but absence in placenta and ovary, most resembles that of mouse *Rhox11*. However, absence of expression in the placenta is not consistent in general with genes of the rodent gamma subcluster suggesting that the evolution of *Rhox* cluster regulation between species is more complex that duplication of single co-regulated genes and promoter sequences. To the best of our knowledge, formal analysis of non-primate *RHOX* genes (guinea pig,

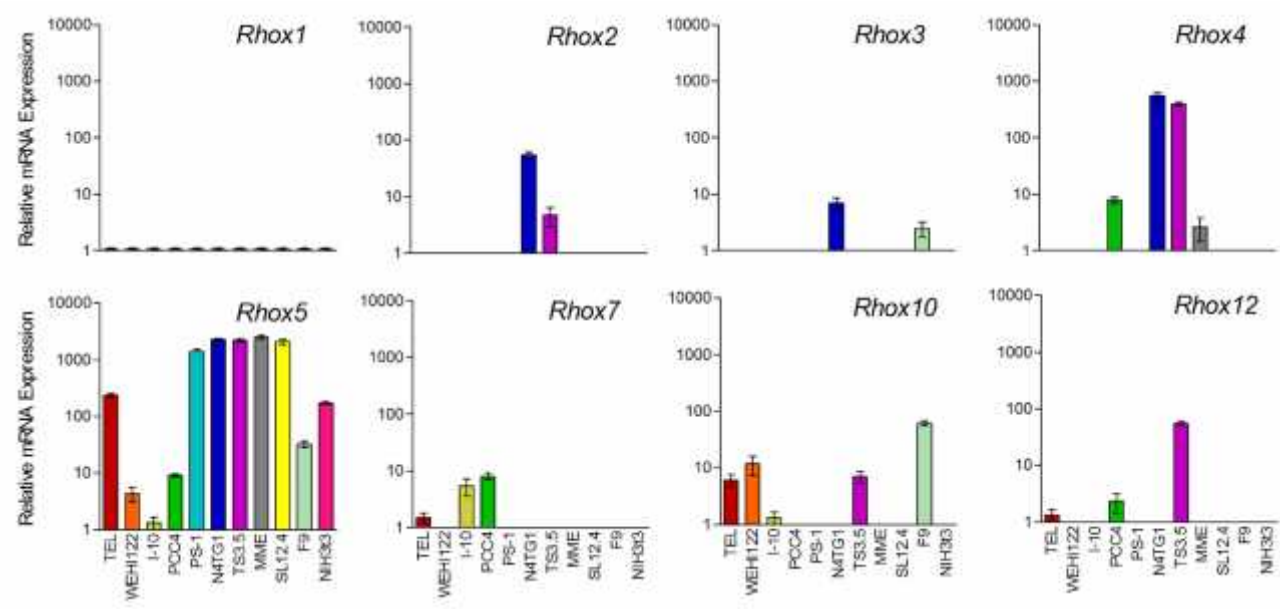

Figure 3. Expression of the mouse *Rhox* genes in tumor and immortalized cell lines. Quantitative real-time RT-PCR (qPCR) was used to determine the relative expression of each *Rhox* gene using primer pairs specific for each unique *Rhox* gene and pan-paralog-specific primers that amplify total *Rhox2*, *Rhox3*, and *Rhox4*. Data is presented as mean +/- SEM fold above background after normalization with *Rpl19* mRNA to account for differences in cDNA loading. The cell lines examined were TEL (thymic epithelial cell line), WEHI122 (lymphoma), PCC4 (embryonic carcinoma), I-10 (Leydig cell line), PS-1 (prostate mesenchymal cell line), N4TG1 (neuroblastoma), TS3.5 (a trophoblast stem cell line), MME (mouse mammary epithelial), SL12.4 (T-cell lymphoma, the original source of *Rhox5*), F9 (embryo carcinoma), and NIH3T3 (fibroblast cell line).

cattle, dog, and chicken) in tissue panels has not been performed. However, EST and cDNA sequences representing these putative orthologs have been obtained from testis and placental sources, suggesting that they may be similarly restricted to reproductive tissues.

At present, it is not known whether a local enhancer exists that drives groups of *Rhox* genes to be expressed specifically in one reproductive tissue, or if their expression is entirely dependent on their individual promoters which have also been duplicated as the cluster proliferated. In support of the latter the *Rhox*-related genes, *Arx* which is present at the opposite end of the X chromosome from the *Rhox* cluster, and *Esx1* which is located halfway between them, are both expressed in reproductive tissues. While *Arx* is highly expressed in developing male gonads, unlike the other *Rhox* genes, it is also found in several regions of the developing and adult brain (45, 46). Detailed analysis of *Arx* expression and function in fertility has been derailed by the finding that ablation of Arx results in severe neurological defects that lead to perinatal lethality (45-47). *Esx1* and its human ortholog, *ESX1*, are both expressed in testes and placenta (48-51). In the mouse testis, *Esx1* transcripts are specifically localized to spermatogonia, spermatocytes, and round spermatids in stages IV-VII of the seminiferous epithelial cycle (48). Interestingly, while *Esx1* transcripts can be detected in pre-meiotic germ cells, the ESX1 protein appears to only be translated in post-meiotic round spermatids (51). The mechanism of *Esx1* translational control has not been investigated, however, translation of *Rhox13* transcripts is inhibited in prenatal gonads by NANOS2 (52). Translation of RHOX13 occurs when the retinoic acid signaling pathway is activated in neonatal male and female gonads, and can be induced earlier by exogenous RA treatment. It is not yet certain how many other *Rhox* genes may be similarly regulated as suitable anti-sera to compare *Rhox* mRNA and *RHOX* protein levels have not been developed for all of the germ cell-specific RHOX factors.

4. ABERRANT EXPRESSION OF THE RHOX CLUSTER IN CANCER

4.1 *Rhox* genes are broadly expressed in cancerous cells of diverse tissue origin

While the founding member of the reproductive homeobox cluster, *Rhox5* (originally called *Pem*), is an important regulator of development and fertility, it actually has its roots in tumor biology. *Rhox5* was identified in a screen designed to find novel genes functioning in the development of T-cell lymphoma. This assay identified *Rhox5* as being differentially expressed between two T-cell lymphoma clones of common parental origin that exhibited different malignancy potential (53). Subsequent reports identified that the aberrant expression of *Rhox5* is not unique to cancerous T-cells, but rather that it is widely distributed in tumors derived from many different tissues and cell types (37, 53, 54). As shown in a panel screen of 11 representative tumors, *Rhox5* is highly expressed (> 100-fold above background) in 8 cell lines, and expressed in the other 3 (Figure 3). Conservative estimates suggest

that ~75% of tumor lines screened to date express *Rhox5* transcripts. The ubiquitous expression in tumors may derive, in part, from its ability to be induced by the *Ras* proto-oncogene (42, 55). Mutations that constitutively activate TP53 also lead to high expression of *Rhox5* in mouse and rat derived cell lines. In the testes, *Rhox5* mRNA and RHOX5 protein are restricted to Sertoli cells. Interestingly, *Rhox5* mRNA is not detectable in most immortalized Sertoli cell lines (TM3, TM4, and 15P-1) and is only lowly expressed in MSC1 Sertoli cells. It is probable that although these cell lines maintain both their endogenous methylation and silencing (described in detail in the next section) of the *Pd* regulatory sequence, and hypomethylation of the *Pp*, that a necessary cofactor for androgen regulation is missing or androgen receptor levels are inadequate.

The other *Rhox* genes do not appear to be as broadly or as highly expressed in tumor cells. Transcripts for *Rhox1*, *Rhox8*, and *Rhox11* were not detected in any of the cell lines examined in our panel screen (Figure 3 and data not shown). Two genes, *Rhox6* and *Rhox9*, were previously shown to be expressed in a single cell line, the TS3.5 trophoblast stem cell line (37). Since these two genes are highly expressed in normal placental tissue, we believe that this represents activation of their endogenous control mechanism and not aberrant tumor-specific activity. However, *Rhox6* and *Rhox9* are induced by the DNA-methylation inhibitor 5AzaC in mouse cell lines (56), suggesting they could potentially be expressed in tumor cells where epigenetic silencing has gone awry. The remaining *Rhox* genes exhibit expression to varying degrees in multiple cell lines. In some cases, we can speculate why a *Rhox* gene may be present, for most it's a complete mystery as regulatory elements within their 5' flanking sequences have yet to be examined. For example, the promoter of *Rhox4* contains an essential NF-Y transcription factor binding site (57). NF-Y has been shown to induce the expression of ATPase genes in N4TG1 neuroblastoma cells (58). Thus, the necessary elements for permissive transcription of *Rhox4* are present. However, why *Rhox4* would be present in one embryo carcinoma, PCC4, and not another, F9, cannot be predicted. The *Rhox* cousins, *Arx* and *Esx1*, are also expressed in cancerous cells. Mutation of ARX is linked to the formation of brain cysts (59), but few if any of the ~400 papers detailing *Arx/ARX* expression and function in developmental processes mention aberrant expression in tumor cell lines. Conversely, *Esx1* was detected by northern blot in ~40% of mouse tumor lines (37), and is a significant player in human colorectal carcinomas (60). Interestingly, expression of *Esx1* and *Rhox5* were inversely correlated in the initial tumor panel screen, but whether this is significant to tumor biology or an artifact of sample size is not known (37).

Both groups that initially cloned the human *RHOX* orthologs were initially concerned with determining the androgen regulation of these novel genes in the male reproductive tract, rather than their potential role in tumor biology. However, one showed that *RHOXF1* was not endogenously expressed the androgen-receptor negative PC-3 prostate cancer cell line (38). *RHOXF1* was abundant in HPB-ALL (acute lymphocytic leukemia), LNCaP (prostate tumor), and Hec1A (endometrial adenocarcinoma) cells, but not detected in A375 (melanoma), Hela (cervical adenocarcinoma), and SW620 (colorectal adenocarcinoma) cells as assessed by RT-PCR / Southern blot panel screening (37). A larger screen including these cell lines and 8 additional lines demonstrated expression of *RHOXF2* only in K562 (erythroleukemia) cells (37). Subsequent reports from many groups have demonstrated expression and regulation of both genes in tumors and immortalized cell lines. *RHOXF1* displays variable expression in lingual squamous carcinomas (61) and colorectal cancers (62). An RT-PCR screen of 13 colon, breast, and pancreatic cancer cell lines revealed that *RHOXF1* was broadly expressed, being absent in only the SW480 and SW620 colon cancer lines (56). Interestingly, SW480 cells were the only member of this panel to exhibit detectable expression of *RHOXF2*. However, RHOXF2 has been identified as a prominent cancer/testis antigen (63). As with *Rhox5*, *RHOXF1* and *RHOXF2B* are induced by 5AzaC in human cell lines (56). Thus, it is likely that aberrant expression of the *RHOX* genes as cells transform is dependent on permissive hypomethylation and the presence of specific transcription factors which may vary by tissue type and mutation status. In support of this, examination of publically available normal vs. cancer expression array data using stringent parameters (threshold p-value 0.0001, 4-fold change, top 10% gene rank) in the ONCOMINE database, finds a mixture of both up and down-regulated outlier datasets. However, consistent with its putative role as a tumor suppressor (discussed below) *RHOXF1* is consistently down-regulated 5-fold or greater in invasive, mucinous, and mixed lobular and ductal breast carcinomas. In contrast, *RHOXF2* is up-regulated 20-fold or more in brain, head and neck, kidney, and pancreatic cancer datasets without any counterbalancing outliers exhibiting down-regulation, making *RHOXF2*'s potential role as a cancer promoting gene clearer. Thus, directed studies to examine the reciprocal status of *RHOXF1/RHOXF2* gene expression on a tissue by tissue basis would seem in order.

4.2. Potential mechanisms of *Rhox* gene misregulation

More than 30 studies have been devoted to characterization of the transcriptional regulation of *Rhox* genes using both *in vitro* cell-based and *in vivo* transgenic models. The majority of these reports center upon androgen regulation of the founding member of the cluster *Rhox5*/*Pem* in epididymis and Sertoli cells of the testis. For an in-depth summary of *Rhox* gene regulation in reproductive tissues, we direct readers to our recent review in *Reproduction* (10). In this report, we will limit our discussion to recent findings expanding the mechanism of imprinting of the *Rhox* cluster and the exciting development that RHOX factors participate in cross-regulation of other *Rhox* genes. The role of DNA methylation in the development and progression of cancer is currently one of the largest growing topics in the life sciences. The aberrant regulation of methylation may contribute in multiple tumorigenesis pathways as there is potential for both the derepression of oncogenes and silencing of anticancer genes (64). If the gene that is

aberrantly activated is a "master switch" for cancer development (65), then a single misregulation event may lead to the acceleration of cancer development. The *Rhox* genes may be relevant to such a scenario as their expression is governed by the epigenome, and one gene that is normally silenced in non-reproductive tissues has recently been shown to actively organize the expression of other members of the cluster.

Epigenetic mechanisms contribute to control of gene regulation in a wide array of developmental events including establishment of the placenta, embryonic growth, organ formation, and tissue differentiation (66, 67). Methylation is the major epigenetic modification in mammals and gene silencing, genomic imprinting, and X-chromosome inactivation all depend on its proper regulation (68, 69). Given that homeobox genes control developmental processes and that the *Rhox* genes reside on the X chromosome, it is not surprising that several reports have linked DNA methylation to control of *Rhox* gene (in particular *Rhox5* and *Esx1*) regulation (21, 50, 56, 70, 71). Recently, we formally examined the imprinting status of the entire mouse *Rhox* cluster using four independent models of imprinting and impaired DNA methylation (72). Depletion of H1 linker histone, an abundant and essential component of chromatin, in mouse embryonic stem (ES) cells was predicted to result in the global misregulation of genes silenced by methylation (73). However, microarray analysis revealed that very few genes were upregulated, and that methylation of specific CpGs within the regulatory regions of H1-dependent genes was restricted to a small subset of genes, predominantly on the X-chromosome. Interestingly, *Rhox5* was among these H1-regulated targets (73). Subsequent analysis of the *Rhox* cluster revealed that *Rhox1*, *Rhox2*, *Rhox4*, *Rhox6*, *Rhox9*, *Rhox10*, *Rhox11*, *Rhox12* and *Rhox13*were all additionally derepressed in H1-depleted ES cells (72). However, the neighboring genes upstream (*Sept6*, *Ndufa1*) and downstream (*Lamp2*, *Mcts1*, *Hprt1*, and *G6pdx*) of the *Rhox* cluster remained silenced suggesting that regulation by H1 may be directed specifically towards the *Rhox* cluster. The aberrant upregulation of *Rhox* expression was reversible as transfection of knockdown cells with H1 expression vectors resulted in the rescue of *Rhox* gene silencing. As further evidence for the role of methylation, ES cells lacking the *de novo* methylation enzymes DNMT3A and DNMT3B were found to express the same subset of *Rhox* genes upregulated upon H1-depletion (72). Conversely, the H1-indepenent *Rhox3*, *Rhox7*, *Rhox8*, and *Rhox11* were not upregulated in *Dnmt3A*/*Dnmt3B*-null ES cells.

To assess whether the *Rhox* cluster is subject to Xp imprinting, i.e. the silencing of the paternal copy of an X-linked gene, we have examined the parent of origin of *Rhox* genes expressed in the placenta. For this analysis, we identified polymorphisms between *Rhox* gene sequences obtained from *Mus musculus musculus* and *Mus musculus molossinus* mouse subspecies. Analysis of placental cDNAs from hybrid animals demonstrated that indeed the maternal copy each *Rhox* gene was the one found in trophoblast (72). Three genes were excluded from the analysis as no polymorphisms were present for *Rhox2*, and neither *Rhox7* nor *Rhox11* are expressed in the placenta. Only *Rhox3a* and *Rhox8* exhibited equivalent expression of either parent's allele, suggesting that these genes escape imprinting and exhibit random inactivation of their X chromosomes (72). Neither of these genes were upregulated in H1-depleted and *Dnmt3A/Dnmt3B*-null ES cells. As a final test, the imprinting status of the *Rhox* cluster was examined in uniparental ES cells that are manipulated to possess two copies of either parental genome (74). Microarray and qPCR analyses indicated that *Rhox1*, *Rhox2*, *Rhox4*, *Rhox5*, *Rhox6*, *Rhox9*, *Rhox10*, *Rhox12*, and *Rhox13* were all upregulated in maternally derived uniparental ES cells, consistent with expression of the maternal copy we observed in the placenta (72). In summary, the same subset of *Rhox* genes was misregulated in all four methylation/imprinting models examined. Why certain genes escape inactivation and ultimately what developmental events are affected by epigenetic regulation of RHOX factors is not yet known. However, perturbations in DNA methylation and genomic imprinting likely explain why *Rhox* genes that are normally expressed only in reproductive tissues are upregulated when cells become cancerous.

The *Rhox5* gene possesses two promoters, an androgen-dependent proximal promoter (*Pp*) and androgen-independent distal promoter (*Pd*) (10). The *Pp* directs expression of *Rhox5* in the testis and epididymis in mice and rats (43, 75-77), whereas the *Pd* is responsible for ES cell, ovarian, placental, and tumor cell expression (42, 43, 55, 72). Both of *Rhox5*'s promoters are methylated in tissues in which the gene is not expressed, i.e. the *Pd* is hypermethylated in male tissues (21). The loss of methylation is a first step to the expression of *Rhox5* as it is permissive to the recruitment of essential transcription factors which drive tissue-specific expression. This mechanism appears to be in play for both promoters. For example, in ES cells demethylation of the *Pd*, specifically at cytosine -1644, unmasks an ETS factor binding site which allows the recruitment of transcription factors that promote expression (72). The ETS factor GABP (42, 72) is sufficient to drive expression of *Rhox5* in cultured ES and ovarian granulosa cells, but the full array of endogenous ETS factors that contribute to tissue-specific expression *in vivo* remain to be determined. Transcription of *Rhox5* in the testis and epididymis depends on the recruitment of both androgen receptor and GATA transcription factors to the *Rhox5 Pp* (78). Four tandem androgen response elements contribute to *Rhox5* regulation. Interestingly three contain CpG sites that are differentially methylated in expressing and non-expressing tissues (79). The recruitment of AR and GATA is dependent upon demethylation of specific ARE and GATA binding elements that was correlated to the region-specific and temporal patterns of *Rhox5* expression in the epididymis (79). The factors that control demethylation of the *Pp* predominantly in only one region of the epididymis (the caput) and at a specific time in post-natal development (~P25) remain to be determined. However, activation of this single gene has recently been shown to have profound effects on regulation of the *Rhox* cluster in the epididymis.

As in the reproductive tract, the expression of *Rhox5* in cancer cells is dependent on both the local action of transcription factors binding its promoter and epigenetic mechanisms. It was established by ribonuclease protection assay and RT-PCR analysis that the *Rhox5 Pd* is the primary determinant of aberrant *Rhox5* expression in tumor cells (10, 43). Dissection of this promoter revealed a minimal element required for *Pd* transcription, located ~100-nt upstream of the transcription start site, which is only 22-nt in length (42, 55). Within this element are two ETS family-binding sites and one SP1 family-binding site. Mutation of any of these three significantly impaired transcription of *Rhox5* and combinations of mutations abolished activity in both tumor cell lines and primary ovarian granulosa cells (42, 55). The ETS factors GABP and ELF1 are sufficient to drive expression of *Rhox5* in SL12.4 and 10T1/2 tumor cells (55), but the full array of endogenous ETS factors that contribute to tumor-specific expression *in vivo* remain to be determined. However, upregulation of ELF1 has been associated with gynecological cancers of the cervix, uterus, and breast (80, 81). SP1 and SP3 factors are both capable of binding and activating the *Pd*, and both are widely known to participate in the regulation of genes in normal and cancerous tissues (82, 83). Because expression of these genes is thought to be ubiquitous and constitutive, it is not clear how they may differentially regulation *Rhox5* in cancer cells, although different cell types have been shown to vary greatly in their turnover rate suggesting that the kinetics of SP1/SP3 activation may be altered when cell become cancerous. A more likely explanation for the selective expression of the *Pd* is that epigenetic mechanisms preclude binding of ETS and SP1 in normal male tissues and non-cancerous cells. In support of this, *Rhox5* can be induced by the DNA-methylation inhibitor 5AzaC in mouse tumor cell lines (56). Subsequent analyses by this group discovered a correlation between low levels of active histone marks (H3ac, H4ac, and H34me2), coupled with hypermethylation and abundant repressor H3K9me2 histone marking, in tumor cell lines and primary tumors in which *Rhox5* expression is low (62). Conversely, hypomethylation and active marks were correlated to high expressing cells. Low expressing cells could be converted to active status by treatment with epigenetic drug MS-275 (62), and in agreement with previous observations, treatment of F9 embryonic carcinoma cells with retinoic acid induced *Rhox5* expression (84) and induced dynamic changes in epigenetic status (62).

We recently demonstrated that RHOX5 is a master regulator of *Rhox* cluster expression in the epididymis (27) and related commentary (85). In the mouse, expression of *Rhox5* has previously been localized to the caput epididymis (76, 79, 86). Subsequent, *in situ* hybridization, immunohistochemistry, and qPCR analyses indicate that the other mouse *Rhox* genes are also predominantly localized to the caput epididymis (27, 87). In *Rhox5*-null animals, the expression of *Rhox1*, *Rhox3*, *Rhox4*, *Rhox8*, *Rhox10*, and *Rhox11* was diminished in the caput epididymis, suggesting that RHOX5 (or a RHOX5-dependent factor) positively regulates the expression of these genes (27). Expression of these genes was not significantly altered in the corpus and cauda epididymis, indicating caput-specific regulation. In the rat, *Rhox5* makes a switch to primarily caudal expression (27, 44). Interestingly, rat orthologs to the *Rhox* genes that were shown to be regulated by RHOX5 in *Rhox5*-null mice, also make a transition to primarily caudal localization in the rat (27). Conversely, *Rhox2*, *Rhox7*, and *Rhox9*, which were not misregulated in *Rhox5*-null animals, were equally highly expressed in all three regions of the rat epididymis. These findings support a model in which the alteration of the regional-specific regulation of a single gene could result in regional misexpression of a cadre of subordinate genes. This may be relevant to the formation and progression of tumors as *Rhox5* is widely expressed in tumors of diverse tissue of origin and its upregulation may result in the abnormal expression of other *Rhox* genes that may also have oncogenic potential.

5. CELLULAR PROCESSES GOVEREND BY RHOX FACTORS

The function of most *Rhox* genes is not yet known. However, it is likely that some or all are all involved in reproduction, as all *Rhox* genes are selectively expressed in reproductive tissues including placenta, epididymis, testis and ovary in adult mice (5, 10). It is probable that they also have roles in the fetal development of the gonad, as most *Rhox* genes are also expressed in the primordial germ cells and one (*Rhox8*) is expressed in somatic cells in the developing gonad (6). Several *Rhox* genes are induced by androgens through androgen receptor-mediated activation at their promoters indicating that they are good candidates to regulate male fertility through induction of downstream genes that support spermatogenesis (11, 37, 38, 88). These RHOX factors are likely to govern distinct subsets of genes as they display unique temporal and stage-specific expression patterns in the testes and exhibit variations in the key contact residues that interact with target promoters (5).

Transgenic mouse models have been used to examine the function of *Rhox5*, *Rhox9*, *Esx1*, and *Arx*. *Rhox5*'s region-specific expression in the epididymis and stage-specific expression in the testis suggested that *Rhox5* may be involved in regulating both spermatogenesis and sperm maturation. *Rhox5*'s role in these events is supported by our recently published studies showing that *Rhox5*-null male mice are subfertile (5). Subfertility in *Rhox5*-null animals results from a combination of insufficient germ cell output and developmental defects resulting in poor motility of spermatozoa that do survive. *Rhox9* is expressed in placenta and fetal germ cells, but testicular, ovarian and placental histology all appear normal in *Rhox9*-null animals (89). Testicular development is compromised in *Arx*-null mice and humans with *ARX* mutations, specifically failure to develop Leydig cells (46). However, unlike the *Rhox* genes, *Arx* is also expressed in the developing CNS resulting in lethality and/or mental retardation precluding further characterization of reproductive phenotype (46, 47, 90, 91). *Esx1* is an imprinted gene when ablated or misexpressed in extraembryonic tissues leads to fetal growth retardation

(50). However, the smaller pups recover after birth to match their littermates and are fertile. Thus, single gene knockouts in mice have not produced animals which are completely infertile indicating overlapping function between multiple genes. In some cases the gene responsible can be predicted. In *Rhox9*-null animals, expression of the nearly identical (in sequence and expression pattern) *Rhox6* gene is unaltered in the knockout, suggesting compensation by RHOX6 is responsible for the lack of RHOX9 phenotype. For other genes, such as the case for *Rhox5*, the redundant partner is not so obvious.

While it has been difficult to assign a definitive functions to each *Rhox* gene, data from transgenic mice has provided clues to their potential roles in governing cellular processes. To date, cell line studies have primarily been employed to investigate the regulation of *Rhox* genes, or to identify components in the downstream signaling pathways that they govern. However, recent *in vitro* studies have uncovered potential roles for *Rhox2*, *Rhox4*, *Rhox5*, *Rhox6*, *Esx1*, *RHOXF1* and *RHOXF2* in cell proliferation, differentiation, and survival.

5.1. RHOX factor regulation of proliferation

Historically, anecdotal evidence from our lab and others has suggested that RHOX5 may play a role in cellular senescence (JAM, M Wilkinson, M Rao and C Wayne, unpublished observations). This prediction was based on transfection studies in which introduction of plasmids encoding *Rhox5* seemed to increase the time required for immortalized cell lines to become confluent. However, no formal investigation into this phenomenon was undertaken as to goal of those experiments was to monitor the effect of RHOX5 on target gene promoters or to examine biochemical effects of mutant RHOX5 proteins. RHOX5 was predicted to have a causal role in tumor formation, as it induces an immune response in mouse tumors (92) and it has been shown to interact with proteins involved in malignancy (93, 94). Recently, this prediction was proven correct by Li *et al.* who demonstrated a role for RHOX5 in proliferation and migration of colon cancer cells *in vitro* and tumor growth *in vivo* (62). In their model, lentivirus-mediated knockdown of *Rhox5* in CT26 cells (a colon cancer cell line which is high in endogenous *Rhox5*) resulted in reduced proliferation compared to parental and control lentivirus-treated cells. Additionally, knockdown of *Rhox5* resulted in reduced cellular migration, implying that RHOX5 could mediate tumor proliferation and invasiveness. To test this hypothesis, control and *Rhox5*-knockdown CT26 cells were examined for tumor forming capability in nude mice where the absence of RHOX5 resulted in slower tumor growth (62). At the time of sacrifice, 19 days after inoculation, tumors derived from Rhox5-knockdown cells were 20-30% the size of control tumors. However, the *in vivo* study was not extended to examine the dissemination potential of these cells. At present, whether aberrant expression of RHOX5 functions universally to accelerate tumor growth in tumors derived from different cellular sources. For example, our prior observations were primarily based in cell lines mimicking Sertoli cells, the site of normal *Rhox5* expression. Thus, it's possible that Sertoli cells may possess a unique subset of RHOX5 interacting proteins (some of which are described in the next section) that are capable of handling high *Rhox5* expression without deleterious effects. Whereas, cell types where *Rhox5* is normally epigenetically silenced may be respond differently to the interloping homeobox factor. While we have not yet examined this hypothesis, differential expression of RHOX5-regulated genes in tumor cells has been observed suggesting that all cell types do not equivalently respond to RHOX5. For example, the RHOX5 downstream gene *Unc5c* (95), a tumor suppressor frequently silenced in colon cancer, is absent normally in CT26 cells and was not upregulated in *Rhox5*-knockdown CT26 cells (62). Thus, additional studies are required to identify novel downstream factors and events controlled by RHOX5 that lead to tumorigenesis.

Shortly after the human *RHOX* orthologs were first identified (37, 38), they were identified as cancer testis antigens (63). This heterogeneous group of genes (members encode transcription factors, structural proteins, and enzyme, etc.) are normally expressed in trophoblast and germ cells, but are aberrantly expressed in ~40% of tumors of diverse origin (96), much like had been observed for *Rhox5*. As described previously, the regulation of *RHOXF1* and *RHOXF2* in cancer cells has received some attention, but few studies have focused on the impact of RHOX misexpression in cancer cells. Current data suggests that the two human orthologs may be functionally at odds in cancer cells. A screen to discover potential oncogenes expressed in gastric, pancreatic, and glioma cell lines identified *RHOXF2* as a candidate cancer promoting gene (97). *RHOXF2* was subsequently found to be highly expressed in a variety of cancer cell lines. Knockdown of RHOXF2 in HGC27 cancer cells, which express *RHOXF2* highly, resulted in inhibition of cell growth. Conversely, overexpression of RHOXF2 in HF6 cells, which normally lack *RHOXF2*, resulted in the rapid development of leukemia in irradiated mice (97). *RHOXF2* cDNA obtained from tumors was free of mutation suggesting that the native function of RHOXF2 protein is to induce cell proliferation and cancer development.

In contrast, RHOXF1 is implicated to be a tumor suppressing factor. A microarray screen designed to identify differentially expressed homeobox genes between lingual squamous carcinomas and surrounding normal tissue showed that *RHOXF1* was downregulated in 5 of 7 samples compared (61). However, to the best of our knowledge, no active assays have been performed to examine the impact of modulation of RHOXF1 levels and tumorigenicity. This is the case, however, for the RHOX-related gene, ESX1. One study has demonstrated that a naturally produced 20-kDa C-terminal fragment of ESX1 inhibits the degradation of cyclins and leads to cell-cycle arrest (98). Another study by this group characterizing the homeodomain containing N-terminal 45-kDa region of ESX1, demonstrated that ESX1 slows tumor growth by homeodomain-dependent inhibition of *K-ras* transcription and amino-terminal domain-dependent reduction in tumorigenicity (60). The bivalent growth inhibition

properties of the ESX1 protein observed in human tumor cells contrasts with the phenotype of *Esx1*-null mice. In those mice, ablation of *Esx1* resulted in hyperplasia of the placenta and vascular abnormalities that resulted indirectly in impaired fetal growth (50). The homeodomains of mouse, rat, and human ESX1 are well conserved, in particular Helix III that primarily determines target gene specificity, so it is unlikely that the different functions observed for ESX1 are due to regulation of different sets of genes using different promoter elements. Although differences in epigenetic status of embryonic cells and cancer cells that allow permissive regulation by ESX1 cannot be ruled out. As with the majority of the molecules discussed in this review, additional experiments are required to characterize the overlapping and unique functions of the RHOX domains, in both the same cells/tumors and between tumors of different origins.

5.2. RHOX factors govern differentiation events

As previously mentioned, Jackson *et al.* showed that *Rhox4b* is expressed in ES cells and that introduction of an antisense RNA blocked RHOX4B action resulting in the inhibition of ES cell differentiation *in vitro* (99). Transfection with plasmids to overexpress RHOX4B resulted in advanced ES cell differentiation, as assessed by the appearance of hematopoietic, endothelial, and cardiac differentiation markers upon the removal of LIF. Additional assays for self-renewal and differentiation indicated that inhibition of RHOX4B results in the maintenance of the stem cell phenotype under low LIF availability. It is likely that the episomal vectors used to manipulate *Rhox4b* were equally effective in depleting all 7 paralogous copies of *Rhox4*. *Rhox4*'s function in ES cell development is consistent with a role in embryonic development where *Rhox4* transcripts exhibit a developmentally regulated pattern of expression in the early embryo (100). *In situ* hybridization initially detected *Rhox4* in the extraembryonic endoderm of E6.5 embryos, increasing between E8.5-10.5, and finally in the embryo proper, first in the anterior foregut endoderm and then in the pharyngeal pouches (100). *Rhox2* has been proposed to be most similar to *Rhox4* in structure and function, thus the function of RHOX2 was assessed in the same episomal system used to characterize *Rhox4b* (23). Depletion of *Rhox2* (presumably multiple paralogs) and overexpression of RHOX2A, resulted in the same affects as manipulation of RHOX4B. The addition of *Rhox2* antisense RNA did not deplete RHOX4 protein, indicating that alterations in ES differentiation were specific to RHOX2A. At present, it is still not clear why two large sets of homeobox genes overlapping in function would have been evolutionarily maintained. It's likely that these genes display differences in relative expression during differentiation of specific populations of cells in the developing embryo. Unfortunately, clues to what structures might be involved are speculative as their relative expression in both ES cells and post-natal reproductive associated tissues is very similar (5, 10, 23, 72).

The potential function of *Rhox6* has recently been examined using a different model of ES differentiation. *Rhox6* is highly expressed in the placenta and post-migratory primordial germ cells (PGC) (5, 6, 28, 30). To investigate the role of RHOX6 in PGC differentiation, levels of *Rhox6* were manipulated in a cell line in which EGFP under the control of the Oct3/Oct4 promoter could be used to monitor differentiation state (101). *Rhox6* and the highly related (and putatively functionally redundant (30, 89)) *Rhox9* were both detected in undifferentiated cells, with *Rhox6* being more abundant (102). However, upon initiation of an established protocol to differentiate ES cells to PGC (103), *Rhox6* levels transiently increased while *Rhox9* dropped to near undetectable levels. The expression of *Rhox6* correlated well with the transition from ES to PGC as assessed by several markers of PGC differentiation. Unfortunately, overexpression of *Rhox6* alone had little or no significant affect on PGC differentiation (102). Ablation of *Rhox6*, *Rhox9*, or both using stably transfected anti-*Rhox6/9* shRNAs (that achieved ~90% continuous knockdown) did not impair development of the epiblast. However, knockdown of *Rhox6* was found to significantly impair subsequent attempts to differentiation cultured epiblasts to PGC-like cells (102). These results and the absence of germ cell phenotype in *Rhox9*-null animals (89), suggests that RHOX6 may be uniquely necessary for the determination of the germ cell lineage. Future studies introducing *Rhox6*-knockdown cells into host blastocysts may help address this, but the lack of quality probes to differentiate localization of *Rhox6* and *Rhox9* make the proposed studies technically challenging. The authors are not currently aware whether the previously described *Rhox9*-null animals are available to use as recipients and the generation of double knockouts through combination of future *Rhox6*-null lines would be difficult as these two genes reside in close proximity on the X chromosome.

5.3. RHOX factors promote cell survival

As described previously, *Rhox5*-null mice suffered from hypofertility and had reduced numbers of round and elongated spermatids in the testis (5). This depletion was due, at least in part, to increased apoptosis of meiotic germ cells in the testis. Increased numbers of TUNEL positive cells were observed as early as day 12 post partum (P12) in *Rhox5*-null animals, but Sertoli cell numbers in the adult were normal. Significant increases in apoptotic germ cells were observed at P17, P25, and in the adult. In the testis, apoptosis is a normal process associated with spermatogenesis, but can be aberrantly triggered by gonadotropin withdrawal, heat stress, torsion, and assault by many toxic biochemical agents. Interestingly, *Rhox5*-null testes exhibited an increased frequency of apoptosis in both germ cells that normally die (stage-I to -IV spermatogonia and stage-XII spermatocytes) as well as those that do not normally die (stage-V to -XI spermatocytes) (5). This apoptosis is presumed to be due to loss of a RHOX5-dependent survival factor presented by Sertoli cells to germ cells (5, 20, 21). However, characterization of RHOX5-regulated genes in 15P-1 Sertoli cells identified *Unc5c*, a pro-apoptosis inducing factor, as a target gene repressed by RHOX5 (95). Thus, RHOX5 may function to promote cell proliferation and survival through positive mechanism, while at the same time inhibiting cell death pathways. Alternatively,

RHOX5 may normally function as a checkpoint surveillance transcription factor that, if absent, causes premature entry to the next stage and therefore an increased sensitivity to apoptosis (104). Loss of androgen signaling within the testis results in increased apoptosis beginning in mid stage VII and continuing through stage IX. Because *Rhox5* is regulated by androgen, it is tempting to speculate that RHOX5 is one mediator of apoptotic survival lost when androgen is deprived. Investigation of the mechanisms of androgen-dependent survival of gonadal cells may be relevant to survival of cancer cells in androgen-responsive tumors. In support of this, prostate-targeted androgen receptor silencing constructs eradicate xenograft tumors in mice (105). However, additional studies are required to determine whether RHOX5 is a key mediator of this process and if so, to identify the key downstream factors controlled by RHOX5 that promote cell survival.

On this front, a recent study has identified ERK signaling as a target pathway for RHOX5 in cervical cancers (106). Exogenous expression of *Rhox5* in TC-1 cells resulted in the downregulation of pro-apoptotic factors such as BCL-2 and upregulation of pro-survival factors BIM. The protective effect of RHOX5 is consistent with what we have previously observed in male germ cells (5) and Sertoli cell lines (20, 95). However, using a combination of phosphoantibody screens and chemical inhibitors, Kim *et al.* discovered that the mechanism behind the protective effects of RHOX5 lies in the regulation of the ERK1/2 signaling network and not AKT which exhibited no differences in control and *Rhox5* overexpressing cells (106). As further evidence of the role of ERK signaling, while introduction of *Rhox5* increased the tumor growth potential of TC-1 cells *in vivo*, an intra-tumor injection of ERK inhibitor stemmed tumor growth. Further insights gleaned from this study are that exogenous *Rhox5* confers resistance to chemotherapeutic medications such as Paclitaxel and blocked the ability of immune cells to kill TC-1 cells. Additional studies are necessary to determine whether these protective properties are unique to RHOX5 or a general feature of the RHOX family. The latter may be the case as in addition to a potential role in proliferation, RHOXF2 may help tumor cells evade programmed cell death, as like RHOX5, it is able to repress the expression of the pro-apoptosis factor UNC5C *in vitro* (95). If these prosurvival properties extended to RHOXF1 or RHOXF2 in general, then it may be suitable to assess tumor biopsies for RHOX levels as it could have implications for treatment prognosis or the incidence of the chemoresistant recurrence of some cancers.

6. RHOX INTERACTING PROTEINS AND LINKS TO CANCER DEVELOPMENT AND PROGRESSION

Studies to characterize the potential biochemical actions of the *Rhox* genes have primarily focused on *Rhox5*. This is likely due in part to the fact that it apparently has the highest and broadest expression in immortalized cell models (Figure 3 and prior discussion) and also the fact its discovery had a 10-15 year head start on other members of the cluster (5, 10, 53). Homeodomain transcription factors rarely act alone to stimulate or repress their target genes and in most cases coordinate with other proteins to achieve transcriptional regulation. To date, four interacting proteins have been reported for RHOX5, some were generated in a directed search to identify RHOX5 partners, some serendipitously as the "bait" of interest "selected" RHOX5 from a complex protein mixture or expression library. These factors include menin (MEN1), prosaposin (PSAP), inhibitor of MyoD family (I-MFA; also known as MDFIC), and cell division cycle 37 (CDC37) (93, 94, 107, 108). In the final section of this review we will discuss the discovery of these factors and speculate on their potential role in complexes with RHOX5 as part of their normal function in reproductive tissues and/or abnormal roles in cancerous cells.

6.1. MEN1

The first and best-characterized protein identified best characterized RHOX5-interacting protein is MEN1, also known as MENIN, MEAI, and SCG2. MEN1 is a nuclear protein with established tumor suppressor activity that when mutated underlies the dominant familial cancer syndrome multiple endocrine neoplasia Type 1 (109). Two groups independently identified RHOX5 as a MEN1 interacting protein using co-immunoprecipitation (110) and GST-pulldown experiments in transfected cells (93). Transcripts for *Men1* and *Rhox5* colocalize in seminiferous tubules, suggesting that MEN1 interaction is not superfluous and could contribute to RHOX5's normal role in the testes (93). However, it is still not known whether the endogenous source of MEN1 in the testes are Sertoli cells where RHOX5 is made and functions. Although, this may be the case as upregulation of *Rhox5* occurs after mutation or conditional knockout of *Men1* (111, 112).

MEN1 is thought to play a key role in the G1-S transition, serving as a checkpoint control factor during the cell cycle (109, 113). In one study, introduction of antisense cDNA to block MEN1 expression resulted in increased IEC-17 cell proliferation (114). Thus, a potential mechanism by which RHOX5 could induce tumor cell proliferation (62) would be through the binding and sequestering of MEN1 so that it could not perform its proliferation inhibiting function. Presumably, RHOX5 could act by inhibiting interaction of MEN1 with cell cycle control factors, rather than through the use of its homeodomain to drive the transcription of growth promoting genes. It is not yet known whether there would be an advantage to RHOX5-MEN1 interaction in controlling proliferation events pertinent to germ cell development. This may be unlikely as such an interaction would probably occur in Sertoli cells which have completed division by post-natal day 12 in mice, although a role in establishment of Sertoli cells cannot be ruled out. MEN1-RHOX5 interaction is more likely to have an effect on germ cells via a signal that's translated from Sertoli cell to germ cell.

This may be the case for some of the RHOX5-regulated genes which we have begun to characterize (MacLean *et al.*, submitted and (20, 95)). For example,

MEN1 has been shown to interact with the homeobox transcription factor PDX1 in the pancreas to modulate the expression of insulin and insulin-like growth factor binding protein 2 (109, 113). Thus it is likely that RHOX5-menin interaction might also influence the expression of *Ins2* and other metabolism-related genes regulated by RHOX5. In pancreatic cells, PDX1 induces insulin transcription. Like the insulin and insulin-like growth factor genes, the *Ins2* promoter has a consensus PDX1 binding site (115). Because MEN1 inhibits PDX1 access to this site on the insulin promoter (116), by analogy in the testes, MEN1 may serve to inhibit access of RHOX5 to the *Ins2* promoter. We currently believe that disruption of local insulin signaling in the testes is responsible in part for increased germ cell apoptosis in *Rhox5*-null testes (10, 20, 21). However, insulin signaling is also known to lead to increased cellular proliferation, including germ cells, through the AKT pathway activation (117-120). Thus it is possible one of the routes of RHOX5's tumor growth promoting ability relies on overexpression that exceeds the capability for MEN1 to block the inhibition of growth promoting factors. Future studies will need to address whether RHOX5 and MEN1 compete directly the same target gene promoters, work together to stimulate a unique subset of genes that neither factor can regulate individually, or have pertinent actions other than transcriptional control in both normal cells and cancer cells.

6.2. CDC37

Using yeast two-hybrid analysis with RHOX5 as bait, CDC37 was identified as a molecular partner for RHOX5 (94). CDC37 is a cell-cycle regulator that facilitates formation of the CDC28-G1 cyclin complex (121). This suggests that RHOX5 and CDC37 might cooperate to promote cell proliferation either as part of its normal function in growing reproductive tissues or cancerous cells. CDC37 has been thought to function primarily as an accessory factor for HSP90, primarily in the shuttling of substrate kinases (122). Endogenous levels of CDC37 are typically low and targeted overexpression in tissues that lack it leads to transformation at the same rate as cyclin D1 transgenic mice (123). It is not known whether inappropriate co-regulation of both CDC37 and RHOX5 is necessary to elicit transformation or induce proliferation. If this is the case, the absence of CDC37 could explain why high expression of RHOX5 is tolerated in Sertoli cells, which do not proliferate in the testis after the second postnatal week. This may explain our anecdotal findings that transfection of Sertoli cells (and other selected cell lines) appears to result in slowing of their growth, rather than the advance in proliferation seen in colon cancer cells (62). It would be interesting to determine the time course and cellular localization of CDC37 expression in post-natal testes. Although, if CDC37 is an essential mediator of Sertoli proliferation, then it may likely interact with other RHOX factors, as ablation of *Rhox5* does not alter Sertoli cell numbers (5). Alternatively, as part of their role in the cell cycle, CDC37 and RHOX5 interaction may also function as a DNA checkpoint regulator. CDC37 may serve as a chaperone to prevent RHOX5 from coordinating the advancement of germ cells to their next step in development prior to reaching competency to do so. In support of this, transgenic mice overexpressing RHOX5 in Sertoli cells have increased DNA strand breaks in the adjacent germ cells during their maturation into elongated spermatids (104). Thus, maintaining a proper CDC37/RHOX5 ratio may be necessary for cellular health and development.

6.3. PSAP

PSAP (also known as prosaposin and SGP1), is a lysosomal enzyme activator that is necessary for the normal development of the testes and male accessory organs (124). The finding that knockouts for both *Rhox5* and *Psap* have phenotypes in the testis supports the notion that RHOX5 and PSAP protein complexes may collaborate in the testis. However, it is unlikely that the interaction found by yeast two hybrid analyses occurs frequently *in vivo*. While RHOX5 protein can occasionally be seen in the cytoplasm, it is by far a nuclear protein in Sertoli cells of the testes (76, 125). In contrast, PSAP is an almost entirely cytoplasmic and often secreted protein (126). Thus, it is unlikely that these factors meet often enough to have profound normal physiological effects. However, forced overexpression of *Rhox5* in transgenic mice does result in slightly more RHOX5 protein localized to the cytoplasm (104). Since many tumor cells express significantly higher levels of *Rhox5* than observed normally, it is possible that PSAP may have a role in these aberrantly expressing cells. In this case, PSAP may serve as a PSAP may serve as a negative regulator of RHOX5's transcriptional functions in the nucleus by sequestering RHOX5 in the cytoplasm.

6.4. MDFIC

The final protein which has been shown to directly bind RHOX5 is MDFIC. The MyoD family inhibitor domain containing protein, MDFIC, is an established repressor of transcription (127) and has been identified as a differentially expressed gene in gastric cancer following loss of heterozygosity (128). Unfortunately, subsequent analyses did not support a role for MDFIC in tumor development or suppression (128). Currently, the literature does not provide sufficient evidence to speculate about the functional link between RHOX5 and MDFIC either in reproductive tissues or cancerous cells, other than possesses putative inhibitory activity to factors that govern muscle development which may or may not have homologous actions in the gonads.

7. DISCUSSION AND FUTURE DIRECTIONS

The discovery of the *Rhox* homeobox gene cluster has opened up a new frontier in reproductive physiology and cancer biology. While the size of the *Rhox* cluster has diverged during evolution, all 33 genes in the mouse, 10 in rat, and 2-6 in other mammals including humans, are selectively expressed in the male and female reproductive tract, suggesting that native function of the RHOX homeodomain transcription factors involves regulating genes that promote male and female fertility. However, while normally restricted to reproductive tissues, many genes in the *Rhox* cluster, and their relatives *Esx1* and *Arx*, are commonly misexpressed in tumors and immortalized cells of diverse tissue origins.

Most work to date has focused on dissecting the molecular pathways that regulate RHOX factor expression, in particular androgen signaling, and their role in supporting male and female fertility in mice. While we intend to continue to investigate the redundant and unique functions of the *Rhox* genes in mice, there is a clear need to expand our functional studies to the human *RHOX* genes. The studies described in this review have likely only begun to scratch the surface on the potential events mediated by RHOX factors. However, evidence exists, perhaps as expected of homeobox genes, for a causative role in cellular processes including proliferation, differentiation, apoptosis, angiogenesis, resistance to chemotherapies, invasion, and metastasis. These qualities are typically used to define candidate factors to serve as a "Master Switch" for the development and progression of cancer (65). Whether one or more RHOX factors serve as such a switch remains to be seen. However, more studies are warranted to investigate the potential value of the *RHOX* genes as markers for tumor development (particularly for those cancers which early detection techniques are currently lacking) or as targets for therapeutic intervention.

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Send correspondence to: James A. MacLean II, Department of Physiology, School of Medicine, Southern Illinois University 1135 Lincoln Drive, Carbondale IL 62901 Tel: 618-453-1579, Fax: 618-453-1527, E-mail: jmaclean@siumed.edu