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Commentary

No milk today (my *Hox* have gone away)

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Vertebrate *Hox* genes are well known for their important functions during embryonic development. They are necessary for the proper organization of a variety of structures, both in term of their topology and regarding their own construction (1). In the case of the limbs, for example, *Hox* genes are not only required to organize and build up the skeletons, but also to adequately position them along the body axis such that our arms and legs are at the right places. Thirty-nine *Hox* genes have been reported in higher vertebrates so far, and the final number is not expected to be much higher. These genes are organized in four genomic loci, i.e., four linkage groups (referred to as *HoxA* to *HoxD* complexes) containing a series of contiguous genes transcribed from the same DNA strand and distributed over a distance of 100 to 150 kilobases. They encode transcription factors that are thought to act in combination with cofactors, by telling the cells which genetic program they should further implement, depending on which step these cells have reached in the course of their own developmental histories, or in response to environmental cues (1). Their status of control genes has made them attractive targets for evolutionary processes, and much evidence exists that modifications in the regulation of these genes may have played some roles in the evolution of animal body plans (e.g., ref. 2).

In the course of evolution, the emergence or improvement of functions often was accompanied by the recruitment of genes to carry out additional tasks not necessarily related to their previous roles (3). As far as *Hox* genes are concerned, little attention has been given to their potential importance during adulthood, mainly because of their title of ''developmental'' genes, a status that they have acquired because of both their orthologous relationships with *Drosophila* homeotic genes as well as the severe alterations induced in fetuses by their loss of functions. However, because many transcription factors are involved in our development, it is likely that such regulatory proteins exert additional function(s) in different physiological contexts and, subsequently, in adult life. In support of this, previous reports have shown that mice lacking the function(s) of one or several *Hox* genes could develop phenotypes not always linked to early developmental processes. For example, the solidity of hairs (4) or the size of the prostate (5) were reported to be affected by *Hox* genes inactivation, and various studies suggest that these proteins may be of importance in the hematopoietic system (6–8). Of interest, several *Hox* genes have been associated with the occurrence of leukemia, either through their over-expression (9, 10) or as a result of chromosome translocations (11, 12).

Hox genes also appear to be required in the uro-genital system of adult mammals, in particular during pregnancy. *Hoxa10*, *Hoxa11*, and *Hoxd11* are transcribed in adult uterine horns (13–15), and the level of transcripts varies in response to the estrous cycle and pregnancy (16). In this latter case, the *Hoxa11* gene may be involved in the behavior of cells during the decidual reaction. Such a physiological response nicely qualifies for an ''adult'' function and indicates that complex processes such as those required to design an efficient reproductive system in mammals made use of genes whose ancestral functions were unrelated. In this issue of the *Proceedings*, Chen and Capecchi provide another interesting example of an ''adult'' role for *Hox* genes (17). They report that the loss of function of several genes belonging to paralogous group nine impaired proper development of mammary glands during and after pregnancy, thereby leading to a strong deficit in milk production and, hence, an abnormal lactation capacity. Although female mice lacking the function of either one of the *Hoxa9*, *Hoxd9*, or *Hoxb9* genes did not exhibit any abnormal phenotype in their mammary glands, female composite mutants for the three genes were unable to properly feed their offspring. When transferred from a healthy mother to a triple mutant female, some newborn pups (with milk in their stomachs) could nevertheless develop, indicating that the milk supplied by the mutant animal, when available, was of good quality. Moreover, in the reciprocal exchange, pups delivered by a mutant female could happily survive, provided they were transferred to a wild-type lactating female. Consequently, the authors propose that early lethality was caused by the inability, for the triple mutant females, to provide the pups with sufficient amount of milk (17).

This unexpected observation stimulated the authors to carefully compare the development of mammary glands between virgin females carrying the three mutated alleles and their wild-type counterparts. Mammary glands develop mostly postnatally. At birth, only a few ducts can be observed, extending from the nipples into the underlying fat pad. Growth and extension of the glands then are arrested for a couple of weeks until the mouse enters puberty. At this stage, cells within the end buds proliferate, leading to both elongation of the ducts and production of a ductal network through branching morphogenesis. The ducts interconnect with each others until they establish a compact arborescence in the underlying fatty glandular stroma. During all of these stages of mammary glands development, glands from triple mutant virgin females were indistinguishable in all respects from those of wild-type, age-matched control females, as judged by whole mount and histological examination (17). It thus appeared that the number of glands, their embryonic induction, and gross morphologies before pregnancy were not affected in mutant specimen, suggesting that the failure to produce enough milk did not result from a major problem occurring in the course of early mammary gland development. Of interest, however, underdevelopment and hypoplasia of the mutated glands became apparent during pregnancy and after parturition.

Chen and Capecchi indeed report that, during pregnancy, the ductal system of triple mutant females failed to fully develop and the branching process was significantly reduced when compared with control females whose mammary glands had already spread over the entire fat pad (17). In mutant glands, although lobulo-alveolar structures appeared normal in their morphologies, they clearly were reduced in number and were restricted to the ends of the buds. After parturition, this apparent delay in gland development continued to be observed, with mammary glands of mutant females somewhat resembling those found in wild-type control animals at midg-

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estation, i.e., at an earlier stage. In addition, the morphology of lobulo-alveolar structures, after parturition, was abnormal, indicating that the triple mutation also might interfere with late stages of glandular morphogenesis, such as epithelial differentiation. Such a permanent hypoplasia resulting from abnormal growth and differentiation of the mammary epithelium certainly could account for the strong reduction in milk production (17).

Expression of *Hoxa9*, *Hoxb9*, and *Hoxd9* during mammary gland development was investigated *in situ*, and all three genes were found transcribed at day 12.5 dpc in the mesenchymal primordia surrounding primitive nipples. Further analyses revealed that these *Hox* genes (as well as *Hoxc9*, the fourth member of the same group) continued to be expressed in subsequent stages of ductal morphogenesis, mostly in mesenchyme derivatives. Mammary gland mesenchyme is known to have inductive properties, as judged by its potential to promote branching morphogenesis in organ culture *in vitro* (18), and this morphogenetic process was shown to be enhanced by hepatocyte growth factor and its receptor c-*met* (19). As hepatocyte growth factor is produced by mammary mesenchyme, it will be interesting to look for a potential decrease in hepatocyte growth factor content in glands from triple mutant females.

These nice results further demonstrate that some *Hox* genes can have a function in adult physiological mechanisms. However, hormone-dependent modifications of mammary gland morphology and function during pregnancy also could be considered as a developmental process occurring during adult life, much in the same way as the prostate, another gland produced through an epithelial branching process in adults and whose morphology is affected by mutations in *Hox* genes (5). Genetic evidence has suggested that HOX proteins may act in part through the control of cellular proliferation (see ref. 20). This may provide an explanation as to why such proteins were advantageously recruited to achieve proper glandular development in adults. As deficits in *Hox9* genes resulted in abnormal cellular proliferation and differentiation in the mammary gland, the authors emphasize the interest to look for potential gain of function of these particular genes in some mammary carcinomas in which ectopic expression of other *Hox* genes had been reported (21).

In an evolutionary context, this work tells us that, at the time when mammalian separated from other vertebrates, some *Hox* genes were recruited to help making efficient mammary glands. However, the fact that group nine paralogous genes (i.e., *Hoxa9*, *Hoxb9*, *Hoxc9*, and *Hoxd9*) all are expressed in mammary glands, in which they appear to act cooperatively, raises an important issue regarding the mechanisms underlying these functional recruitments. In the course of our phylogeny, mammary glands emerged in vertebrates in which *Hox* complexes were already present in (at least) four copies. It is therefore difficult to conceive that each one of these four genes separately acquired the regulatory potential to be transcribed in this specific subset of cells (e.g., through the design of novel control sequences). It is more likely that *Hox* function was not "recruited" in mammary gland cells *de novo* but, instead, was inherited from a previous functional status after some modifications took place. This "negative" type of recruitment (by opposition to the "positive" emergence of a novel function as a result of changes in a gene's regulatory sequences) could have occurred mainly through two alternative pathways; in the first scenario, *Hox* genes originally were expressed throughout the flank mesoderm during early development. Subsequently, their expression was maintained selectively in mammary gland mesenchyme at the time that it was switched off in the rest of flank mesoderm. In this view, induction of mammary primordia would have resulted in the maintenance of *Hox* gene transcription in mammary mesenchyme. In a second scenario, selective expression in mammary gland was acquired concomitantly for several genes through the use of a preexisting regulatory element already at work for these different genes: for example, via the design of a single specific cofactor. In this scheme, a particular regulatory sequence, present before the duplications of *Hox* complexes, originally was used by all four paralogs to mediate an ancestral function, such as, for example, establishing positions along the main body axis. The design of a novel mammary gland-specific cofactor would have made this sequence functionally available in that particular context leading to a concerted expression of all four members of a paralogy group, as reported by Chen and Capecchi (17). Further studies on the regulatory sequences present in the various *Hox* complexes as well as full DNA sequence comparisons may be informative in this respect. Analyses of *Hox* gene mutant animals also may tell us whether the number of mammary glands as well as their positions are under the control of the same genes.

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