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THE *Wnt* GENE FAMILY IN TUMORIGENESIS AND IN NORMAL DEVELOPMENT

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Summary—Various members of the *Wnt* gene family have been identified as activated oncogenes in mouse mammary tumors. We show that some tumors are oligoclonal for activation of a *Wnt* gene, and clonal variation when those tumors are transplanted to become hormone-independent. The normal function of many *Wnt* genes is to control pattern formation in early embryos, as shown by expression profiles and by mutant analysis.

INTRODUCTION

Tumor cells have generally undergone mutations in genes controlling cell proliferation and/or differentiation. To identify these mutated genes (as oncogenes) can therefore be considered an approximation to a genetic analysis of eukaryotic cell regulation. Proto-oncogene products have now been implicated in many steps in signal transduction within cells; in signaling between cells; or in development of organ structures. In order to understand the mechanism of action of oncogenes, many researchers have isolated their homologs from lower eukaryotic organisms. Indeed, there are now various examples of oncogene homologs that are allelic to previously known essential genes in yeast, *Caenorhabditis elegans* and *Drosophila melanogaster*.

The *Wnt*-1 gene is one of the best examples of a gene which is essential for organizing early embryos, in both *Drosophila* and in mammals [1, 2]. Moreover, *Wnt*-1 can act as an oncogene when inadvertently activated in the mouse mammary gland. The gene appeared to encode a cysteine-rich protein with a signal peptide, extremely highly conserved in evolution [3]. By gene transfer experiments, we and others established a biological assay for *Wnt*-1; certain mammary gland-derived cell lines, but not fibroblasts, could be morphologically transformed by *Wnt*-1 [4, 5] whilst transgenic mice carrying *Wnt*-1, plus the mouse mammary tumor virus (MMTV)LTR as an enhancer, develops mammary hyperplasia and tumors [6].

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ACTIVATION OF SEVERAL *Wnt* GENES DURING PROGRESSION OF GR MOUSE MAMMARY TUMORS

The GR mouse strain develops hormone-dependent mammary tumors that regress after lactation. After several pregnancies, tumors continue to grow, even in the absence of the hormones. Hormone-dependent mammary tumors can be induced in ovariectomized, virgin GR mice by treatment with progesterone and estrogen. These tumors can then be serially transplanted in hormone treated animals. Usually, after several transplantations, hormone-independent tumors arise, which can grow in untreated mice. Development of the early hormone-dependent tumors in the GR strain is genetically controlled by a dominant locus on chromosome 18, *Mtv*-2, which contains proviral DNA of the MMTV. This locus expresses the MMTV provirus at high levels. Additional proviral integrations into the DNA of mammary gland cells are responsible for the high incidence early hormone-dependent cancers.

In a previous study [7], we examined oncogene activation during progression of GR mouse mammary tumors. We found that early, hormone-dependent tumors were oligoclonal for activation of *Wnt*-1 or int-2 rearrangements. We have now examined these tumors for possible activation of additional members of the *Wnt* gene family and found clonal emergence of *Wnt*-3 in a tumor that had lost *Wnt*-1 and int-2 positive cells. We also found that two hormone-dependent tumors contained an amplified *Wnt*-2 gene, a novel form of activation of these genes. The progression from a polyclonal

hormone-dependent to a clonal hormone-independent state upon serial transplantation of GR tumors seems to be a general phenomena as was observed by Mester *et al.* [7]. The initial polyclonal tumors can have multiple combinations of characterized and uncharacterized MMTV proviral integration events in different subclones of a polyclonal tumor. However, we have never observed any proviral integrations in the *Wnt*-2 locus.

The results presented here in conjunction with earlier work underscore that the *Wnt* genes are very potent oncogenes in mouse mammary tumorigenesis. Apart from activation by proviral insertion, they can apparently also contribute to tumorigenesis by gene amplification and resulting overexpression. The *Wnt* genes code for putative growth factors and are part of a large gene family [8]. Some members of the *Wnt* family, but not *Wnt*-1, 2 and 3, are expressed in normal mammary gland cells and their expression is regulated during pregnancies and lactation [8 and McMahon, personal communication]. Possibly, regulated expression of these genes is responsible for normal expansion and regression of mammary gland epithelium before and after lactation. The activity of these genes during normal mammary gland development may explain why the activation of the other *Wnt* genes has such a potent effect; the products of the oncogenic members of the *Wnt* family may either resemble the stimulatory effects of normal *Wnt* growth factors or interfere with their growth inhibitory effects. Because the expression of the oncogenic members, after proviral insertion or amplification, is not regulated in a normal fashion, continuous growth stimulation may ensue and a tumor can arise.

The regulated presence of *Wnt* proteins and the apparent biological activity of *Wnt* proteins in the mammary gland, implies that a *Wnt* receptor must be present on mammary epithelial cells but the identity of such a receptor remains unknown.

COOPERATION BETWEEN ONCOGENES

It has been shown previously that *Wnt*-1 and int-2 can cooperate in tumorigenesis. A significant number of tumors in the BR6 strain contain both an activated *Wnt*-1 and an activated int-2 allele. In those tumors, the two genes appeared to be rearranged in the same clone. In some GR tumors, *Wnt*-1 and int-2 are also simultaneously activated in a clone present in early passages. Upon transplantation and selection for hormone-independent cells however, these cells became extinct and another clone, positive for a *Wnt*-3 insertion, took over. The interplay between these different clones is intriguing; during the early passages it seems that the relative numbers of cells positive for *Wnt*-3 insertions is constant and that the multiplication of these cells is somehow influenced by the other cells, positive for *Wnt*-1 and int-2. This behavior raises the question of what happens when the tumor becomes hormone-independent. The *Wnt*-1/int-2 positive cells are obviously hormone-dependent and remain that way but are the *Wnt*-3 positive cells hormone-independent to start with or do they acquire an additional mutation, making them hormone-independent? Possibly, they became hormone-independent by release from the influence from the *Wnt*-1/int-2 cells. Since it is not possible to clone single cells from mammary tumors, these questions cannot be answered appropriately, but the oligoclonal nature of these tumors and the clonal switch when the tumor becomes hormone-independent suggests extensive interactions between separate tumor cells within a single tumor. Since all the oncogenes involved encode secreted proteins with a short range of action, it is likely that paracrine growth regulation by these oncogenes play an important role in oligoclonal tumorigenesis.

THE ROLE OF *Wnt*-1 IN MOUSE EMBRYOGENESIS

Recently, deliberate mutations have been made at *Wnt*-1 in the mouse germ line. Homozygous embryos at day 17 have an underdeveloped midbrain and cerebellum, but apparently a normal forebrain and spinal cord [9]. The deleted area corresponds to a site where *Wnt*-1 is normally highly expressed, a circle around the midbrain–hindbrain junction [10], but is significantly larger than that expression domain, suggesting that the *Wnt*-1 protein controls the development of surrounding tissue. Since the available antibodies to *Wnt*-1 are not suitable for detection of the protein *in situ*, the distribution of the protein cannot be tested.

On the other hand, there are also major domains of expression of *Wnt*-1, in particular the dorsal midline of the spinal cord, that are normal in the *Wnt*-1 mutant embryos. Absence

of a phenotype in the spinal cord could perhaps be explained by functional redundancy between *Wnt*-1 and related genes. In the mouse, *Wnt*-1 is part of a gene family consisting of at least 10 members [8, 11]. From the expression pattern of some of these genes it has been inferred that they play important roles during differentiation of several organs in midgestation embryos, particularly in the developing nervous system. The putative amino acid sequences of both proteins are almost 90% indentical, but *in situ* hybridization to mouse embryo sections showed highly restricted patterns of expression of *Wnt*-3 and *Wnt*-3A, largely in separate areas in the developing nervous system. In the spinal cord *Wnt*-3 was expressed at low levels in the alar laminae and in the ventral horns, whereas *Wnt*-3A expression was confined to the roof plate. In the developing brain the *Wnt*-3 was expressed broadly across the dorsal portion of the neural tube with a rostral boundary of expression at the diencephalon. In contrast *Wnt*-3A was expressed in a narrow region very close to the midline; expression extended into the bifurcating telencephalon, in a highly localized fashion. Both *Wnt*-3 and *Wnt*-3A were expressed in the ectoderm, and *Wnt*-3A was also expressed in the peri-umbilical mesenchyme. Characteristic expression patterns of these two closely related genes suggest that *Wnt*-3 and *Wnt*-3A play distinct roles in cell–cell signaling during morphogenesis of the developing neural tube.

For example, our group has shown that *Wnt*-3A expression completely overlaps that of *Wnt*-1 in several areas [11] which may explain why mice with an inactivated *Wnt*-1 gene apparently have a normal spinal cord and hindbrain.

Wnt-1 IN *DROSOPHILA* IS IDENTICAL TO *WINGLESS*

We took advantage of the high conservation of *Wnt*-1 to clone a homolog from *Drosophila*. From its chromosomal position and its expression pattern, it became apparent that we had cloned the *wingless* segment polarity gene [12], which had independently been cloned by Baker [13].

Segment polarity genes form a subset of genes that progressively divide up the embryo into smaller compartments and set up a basic body plan of the fruitfly. Maternal coordinate genes generate axial polarity, delimiting the asymmetric domains of expression of the gap genes. This in turn generates a periodic pattern of expression of the pair-rule genes, and ultimately the segment polarity genes are expressed in every segment [14]. The signals and mechanisms leading to anterior–posterior polarity and the initial subdivision of the embryo are becoming well understood up to cellularization; many of the genes involved encode nuclear proteins that are active as transcription factors. Little is known however about the downstream pathways, after cellularization when the segments form and are subdivided into anterior and posterior compartments.

Wingless, encoding a secreted protein made in the embryo right around the time of cellularization, appears to be one of the key genes in establishing segment polarity and many of the other genes in this class are thought to interact with *wingless*. These genes may encode molecules in the *wingless* signal transduction pathway, but at the moment, the nature of these interactions is not understood. From clonal analysis of *wingless* cells, it appeared that the phenotype is non-autonomous in mosaics [15], suggesting that the gene is part of an intercellular signaling cascade. Our results on the distribution of the *wingless* protein suggested a mechanism by which *wingless* itself would be a signal; the protein was seen in between cells and even in endosome-like structures in cells adjacent to those that make it [16].

THE *Wnt*-1/*WINGLESS* PROTEINS

The characterization of the protein products of the *Wnt*/*wingless* gene family is critical to our understanding of the mechanism of action of these genes. All members of the family that have been isolated encode proteins with a signal sequence, one or several N-linked glycosylation sites and many cysteine residues. The product of the *Wnt*-1 gene has been studied most extensively. If *Wnt*-1 is overexpressed in various cell lines, the protein enters the secretory pathway [4, 17]. The protein can be detected in protease resistant structures, presumably membrane surrounded secretory organelles, and contains carbohydrate structures at several N-linked glycosylation sites. It is thus generally assumed that the *Wnt*-1 protein is secreted from cells, but extracellular forms of the protein have, nevertheless, been difficult to detect [4, 17]. In addition, most of the intracellular *Wnt*-1 protein made in transfected cells is incompletely

glycosylated, as it remains sensitive to endoglycosidase H, and has probably not been passaged through the Golgi apparatus. More recently it has been shown that *Wnt*-1 overproduction leads to small amounts of extracellular protein which has undergone more extensive glycosylations [18], and may bind to the cell surface [19] or to the extracellular matrix [20], possibly explaining its absence in free form in tissue culture medium. The cell surface associated *Wnt*-1 protein can be released by treating cells with suramin [19].

Studies underway in many laboratories will undoubtedly shed more light on the function of this intriguing gene family in embryogenesis and in tumorgenesis.

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