Regulated Expression of Wnt Family Members during Proliferation of C57mg Mammary Cells

Daniel J. Olson2 and Jackie Papkoff3
Syntex Research, Palo Alto, California 94304

Abstract
At least six members of the Wnt gene family are expressed in the murine mammary gland during growth and differentiation, whereas several other Wnt family members participate in malignant transformation of this tissue. We have used the C57mg mammary cell line, which naturally expresses the Wnt-4 and Wnt-5a genes, to examine Wnt gene expression during proliferation. The data show that the growth factors basic fibroblast growth factor, transforming growth factor β1, and epidermal growth factor are mitogenic for C57mg cells, and partial transformation by Wnt-1 can substitute for the proliferative signal provided by these factors. Several different mitogenic stimuli selectively down-modulate the levels of endogenous Wnt-4 and Wnt-5a RNA in C57mg cells. Partial transformation by either Wnt-1 or Wnt-2 is accompanied by a dramatic decrease in Wnt-4 RNA and a small decrease in Wnt-5a RNA. Mitogenic stimulation by basic fibroblast growth factor or partial transformation by Int-2, a fibroblast growth factor family member, also leads to a selective decrease in the levels of endogenous Wnt RNA. No expression of the Wnt-4 and Wnt-5a genes is detectable in C57mg cells that are fully transformed by the activated tyrosine kinase oncogene Neu. In contrast, overexpression of Wnt-5a in C57mg cells does not lead to a transformed phenotype and is not accompanied by a decrease in endogenous Wnt-4 RNA levels. Overexpression of Wnt-5a does lead to a small decrease in endogenous Wnt-5a levels, perhaps through autoregulation. These data indicate that Wnt-4 and Wnt-5a expression in mammary cells is responsive to growth regulatory signals, and the down-modulation of expression of either or both genes correlates with cell proliferation. The inverse correlation between expression of the endogenous Wnt genes and cell proliferation suggests that Wnt-4 and Wnt-5a may participate in restricting the proliferation of C57mg cells.

Introduction
The Wnt gene family encodes secreted proteins that function in cell to cell signaling during normal differentiation and development (1). The prototype member of this family, Wnt-1 (formerly Int-1), encodes a cysteine rich glycoprotein that is secreted and associated with the cell surface/extracellular matrix (2–6). The expression of Wnt-1 is tightly regulated both spatially and temporally during development. In the mouse, fetal expression is limited to the developing central nervous system, whereas adult expression is confined to postmeiotic spermatids of the testis (7, 8). Mice lacking a functional Wnt-1 gene, produced through gene targeting experiments, fail to develop a large region of the brain (9, 10). Hence, Wnt-1 provides an indispensable function in normal development of the nervous system. Studies with other organisms also implicate Wnt-1 in cell communication during differentiation. In Xenopus, Wnt-1 is expressed during neurulation, and ectopic expression of Wnt-1 in Xenopus oocytes induces the organization of a second embryonic axis (11–13). The Drosophila homologue of Wnt-1, wingless, is expressed throughout embryogenesis and is required for normal segmental pattern formation (14–16).

Although Wnt-1 normally functions during differentiation and development, it has also been implicated in the processes of mammary carcinogenesis. Wnt-1 was first characterized as a protooncogene that frequently becomes transcriptionally active upon insertion of proviral DNA in MMTV4 induced mammary tumors (17). Several experimental approaches have directly proved that the wild-type Wnt-1 protein, which is not normally present in the mammary gland, can contribute to the genesis of mammary adenocarcinomas when expressed in this tissue. The Wnt-1 gene elicits changes in cell growth and morphology upon introduction into two different mammary cell lines (18, 19). One of these lines, C57mg, exhibits a partially transformed phenotype in response to Wnt-1 exemplified by conversion from a cuboidal to elongated morphology and loss of contact inhibition (18). Several other mammary cell lines and fibroblastic cell lines fail to respond to the effects of Wnt-1 overexpression (18). The oncogenic potential of Wnt-1 has also been demonstrated using transgenic mice that express Wnt-1 in the mammary gland (20, 21). In these virgin animals, the Wnt-1 transgene induces mammary epithelial hyperplasias, similar in some respects to the response of the normal mammary gland to hormones of pregnancy. The latency and stochastic appearance of mammary carcinomas in the Wnt-1 transgenic mice suggests that Wnt-1 alone is not sufficient for malignant transformation and implies that other genetic alterations are needed. It is noteworthy that Wnt-1 as well as Int-2, a member of the FGF gene family, are both frequently activated by MMTV insertions in the same tumor, suggesting cooperation in the transformation process (22–24).

1 D. J. O. was supported by NIH Grant DE-07023.
2 Present address: Milton Hershey Medical Center, Division of Plastic and Reconstructive Surgery, Pennsylvania State University, P.O. Box 850, Hershey, PA 17033.
3 Present address: Sugen, Inc., 515 Galveston Drive, Redwood City, CA 94063. To whom requests for reprints should be addressed.
idea is further substantiated by the results of mating Wnt-1 and Int-2 transgenic animals. The bitransgenic offspring develop mammary tumors earlier and at a higher frequency than either single transgenic animal (25). Of interest regarding the interaction between Wnt genes and FGF family members is the observation that bFGF cooperates with Wnt-8 in the induction of dorsal mesoderm and thus neuroectoderm, where neither is capable of doing so alone (26).

How Wnt-1 contributes to transformation of the mammary gland, when it apparently plays no role in the normal growth and differentiation of this tissue, is not clear. One clue comes from the observation that at least six other members of the Wnt family exhibit regulated expression in the normal mammary gland (27, 28). In addition, C57mg cells express two Wnt family members, Wnt-4 and Wnt-5a (28). Thus, Wnt-1 may exert its transforming ability by disturbing normal growth regulation which is under the influence of other Wnt family members and growth factors. In this paper, we have used the C57mg cell line to investigate the effects of several growth factors, ectopic expression of Wnt-1 and Wnt-2 genes, and transformation by a tyrosine kinase oncogene on expression of the endogenous Wnt-4 and Wnt-5a genes. The data demonstrate that expression of Wnt-4 and Wnt-5a genes is down-regulated in response to mitogenic concentrations of bFGF, partial transformation by Wnt-1, Wnt-2, and Int-2, and complete transformation by the activated tyrosine kinase Neu. The expression of either Wnt-4 or Wnt-5a or both is down-modulated depending on the particular proliferative signal. Interestingly, overexpression of Wnt-5a in C57mg cells does not produce either a transformed phenotype or a down-modulation of endogenous Wnt-4 RNA levels. The findings reported here suggest that expression of the Wnt-4 and Wnt-5a genes must be decreased in order for C57mg cells to proliferate under the influence of growth promoting factors. These results obtained with C57mg cells support a model in which certain Wnt family members, such as Wnt-4 and Wnt-5a, play a key role in controlling proliferation and differentiation of the mammary gland. Furthermore, expression of these endogenous Wnt genes in the mammary gland is modulated in response to a variety of mitogenic and morphogenetic signals. Overexpression of several other Wnt family members, such as Wnt-1 and Wnt-3, can intervene in this growth regulatory network and thereby contribute to malignant transformation of the mammary gland. The data also imply that Wnt family members are functionally distinct from each other and therefore may interact with different receptors and/or activate different signal transduction pathways.

Results

Growth Factors Exert Mitogenic Effects on C57mg Cells but not on C57mg Cells That Overexpress Wnt-1. To determine the sensitivity of the C57mg mammary epithelial cell line to peptide growth factors, [3H]thymidine incorporation at the maximal factor concentrations used in these experiments. In the absence of growth factors, C57mg cells overexpressing Wnt-1 show a striking difference in thymidine incorporation when compared to the parental C57mg cells (Ref. 18; and data not shown). Whereas the C57mg parent cells are quiescent at confluence, the Wnt-1 expressing cells continue to synthesize DNA for at least several days. Thus, at confluence, the C57mg cells respond to Wnt-1 as if they had been stimulated with a mitogenic dose of a growth factor such as TGF-β1, bFGF, or EGF. To determine whether overexpression of Wnt-1 in C57mg cells influences the mitogenic response to bFGF, EGF, and TGF-β1, [3H]thymidine incorporation experiments were performed with C57mg cells transfected with a Wnt-1 expression vector (C57MG/Wnt-1). With this cell line, the three growth factors elicited essentially no mitogenic responses beyond that produced by the ectopic expression of Wnt-1 alone (Fig. 1, A–C, solid bars). That is, Wnt-1 mimics the effects of growth factor stimulation of C57mg cells, and growth factor treatment in the presence of Wnt-1 expression has little additional mitogenic effect.

Overexpression of Wnt-1 and Wnt-2 and bFGF Treatment Down-regulate Wnt-4 and Wnt-5a RNA Expression in C57mg Cells. A recent report demonstrated that C57mg cells naturally express two Wnt family members, Wnt-5a and Wnt-4 (28). We have confirmed this observation by Northern blot analysis of RNA from C57mg cells. Similar to previous results, three Wnt-5a specific RNAs and two Wnt-4 specific RNAs were detected in C57mg cells (Fig. 2). Since both overexpression of Wnt-1 and growth factor treatment are mitogenic for C57mg cells, it was of interest to determine whether the endogenous Wnt-5a and Wnt-4 gene expression was modulated by these proliferative signals. The effects of overexpression of Wnt-1 on endogenous Wnt-4 and Wnt-5a RNA levels were examined first (Fig. 2). Illustrates that Wnt-5a RNA expression was suppressed by approximately 2-fold in the C57MG/Wnt-1 cells when compared to the untransfected C57mg cells. A more striking decrease in Wnt-4 RNA expression, by approximately 10-fold in some experiments, was noted in the C57MG/Wnt-1 cells (Fig. 2). We have previously demonstrated that introduction of another Wnt family member, Wnt-2, into C57mg cells (C57MG/Wnt-2) generates a phenotype similar to that produced by Wnt-1 (29). Fig. 2 illustrates that, like C57MG/Wnt-1 cells, C57MG/Wnt-2 cells also showed a significant decrease in levels of RNA for Wnt-5a and Wnt-4. In addition, a novel transcript was detected by the Wnt-4 probe in the C57MG/Wnt-2 cells. No Wnt-4 RNA of this size has been detected previously in the C57mg cell line, its transfected derivatives, or other cell types that express Wnt-4 RNA (Refs. 28 and 30; and data not shown).

To examine the effects of growth factors on Wnt-4 and Wnt-5a expression, C57mg cells were treated for 20 h with bFGF, EGF, or TGF-β1, at mitogenic doses determined previously by [3H]thymidine incorporation. Northern blot analysis of RNA prepared from these cells revealed that both Wnt-5a and Wnt-4 RNA levels were decreased approximately 2–3-fold after bFGF treatment (Fig. 3). No significant effects on Wnt-5a and Wnt-4 RNA levels were observed after treatment with TGF-β1 or EGF (Fig. 3). Since overexpression of Wnt-1 in C57mg cells leads to a decrease in the levels of RNA for both Wnt-5a and Wnt-4, it was of interest to determine whether Wnt-4 and Wnt-5a RNA levels were further affected by treatment of these cells with bFGF, EGF, or TGF-β1. C57MG/Wnt-1 cells were treated with growth factors as
Fig. 1. [\(^{3}\)H]Thymidine incorporation of C57mg cells and C57mg/Wnt-1 cells in response to growth factors. Cultures of either C57mg cells or C57mg/Wnt-1 cells were grown and treated as described with (A) bFGF at 0, 1, 5, or 10 ng/ml; (B) EGF at 0, 10, 100, or 200 ng/ml; and (C) TGF-\(\beta\) at 0, 0.1, 1,or 5 ng/ml. Following growth factor treatment for 20 h, cells were pulse labeled with [\(^{3}\)H]thymidine for 2 h and analyzed as described in "Materials and Methods." The data are presented as a fold stimulation over untreated control cells (ratio of [\(^{3}\)H]thymidine incorporation of the growth factor treated cells to [\(^{3}\)H]thymidine incorporation of the untreated cells).
described, and RNA was subjected to Northern blot analysis. No detectable changes in the levels of either Wnt-5a or Wnt-4 RNA were found after treatment with any of the three growth factors (Fig. 3). Taken together, the data suggest that in C57mg cells both endogenous Wnt-5a and Wnt-4 RNA levels are regulated in response to mitogenic stimulation by bFGF and to the transforming effects of Wnt-1 and Wnt-2 expression.

**Overexpression of Int-2 in C57mg Cells Leads to a Partially Transformed Phenotype and Down-modulates the Expression of Endogenous Wnt-5a RNA.** The results presented above show that mitogenic concentrations of bFGF lead to a decrease in the levels of RNA for both Wnt-5a and Wnt-4 in C57mg cells. To pursue this issue further, we have introduced a cDNA encoding Int-2 into C57mg cells to analyze the effects of constitutive expression of Int-2 on cell growth, morphology, and expression of Wnt-5a and Wnt-4 RNA. The results from this experiment are of particular interest in light of previous studies which suggest that Int-2, a member of the FGF family, cooperates with Wnt-1 in the malignant transformation of the mammary gland (22–25). For our studies, C57mg cells were transfected with a retroviral expression vector containing a full length cDNA for Int-2 (31). G418 resistant colonies were selected, screened for Int-2 RNA expression, and examined for transformation specific phenotypic changes. All cell lines that were positive for Int-2 RNA expression showed phenotypic changes reminiscent of the partially transformed phenotype induced by ectopic expression of Wnt-1 or Wnt-2. The morphology of one cell line, C57MG/Int-2, which expresses the highest levels of Int-2 RNA, is shown in Fig. 4 with a comparison to the parental C57mg cells. The C57MG/Int-2 cells also exhibited enhanced [3H]thymidine incorporation at confluence and an ability, albeit weak, to grow in soft agarose (data not shown). Next, the levels of Wnt-5a and Wnt-4 RNA in the C57MG/Int-2 cells were examined and compared to the untransfected C57mg cells. Wnt-5a RNA was decreased by approximately 10-fold (Fig. 5), a more significant decrease than that observed after bFGF treatment (Fig. 3). Of note is the fact that the level of Wnt-4 RNA was relatively unaffected by the overexpression of Int-2 (Fig. 5), in contrast to the 2–3-fold decrease seen after bFGF treatment. These data show that endogenous Wnt RNA expression is down-modulated in response to a proliferative signal from two different members of the FGF family, bFGF and Int-2. However, the extent of down-modulation of either Wnt-4 or Wnt-5a ischaracteristically different for Int-2 and bFGF.

**Wnt-4 and Wnt-5a Gene Expression Is Essentially Undetectable in Fully Transformed C57mg Cells.** The results presented above show that the levels of Wnt-5a and Wnt-4 RNA are selectively decreased in C57mg cells that either are partially transformed by overexpression of Wnt-1, Wnt-2, and Int-2 or are stimulated with a mitogenic signal from bFGF. We next examined the Wnt-5a and Wnt-4 RNA levels in C57mg cells that exhibit a fully transformed phenotype, the result of overexpression of an activated tyrosine kinase oncogene. These experiments utilized a C57mg cell line infected with a retrovirus vector expressing the activated
results show that complete transformation of C57mg cells is accompanied by a loss of detectable expression of the Wnt-5a and Wnt-4 genes.

**Overexpression of Wnt-5a in C57mg Cells Does Not Induce Cell Proliferation or a Transformed Phenotype.** Together, the data presented above show that Wnt-4 and Wnt-5a expression in C57mg cells is responsive to growth regulatory signals, and down-modulation of expression of either or both genes correlates with cell proliferation. One prediction from these results is that in contrast to Wnt-1 and Wnt-2, overexpression of either Wnt-4 or Wnt-5a in C57mg cells would not lead to a transformed phenotype. To test this hypothesis, we examined the transforming potential of Wnt-5a after transfection into C57mg cells. An expression vector encoding Xenopus Wnt-5a (XWnt-5a) was introduced into C57mg cells, and stable transfected cell lines were selected. Individual cell lines were examined by Northern analysis for XWnt-5a expression, and two representative cell lines, XWnt-5a-12 and XWnt-5a-20, were selected for further analysis. Since Xenopus Wnt-5a does not cross-hybridize with murine Wnt-5a under high stringency conditions, it was possible to readily distinguish overexpressed XWnt-5a transcripts from those of the endogenous Wnt-5a
gene. None of the XWnt-5a transfectants showed morphological alterations characteristic of a transformed phenotype. In contrast to the partially transformed phenotype of C57mg cells that express ectopic Wnt-1 (Fig. 7E), the XWnt-5a transfectants show a flat, contact inhibited morphology (Fig. 7, B and C) characteristic of the parent C57mg cell line (data not shown) and control C57mg transfectants (Fig. 7D). In some cases, the XWnt-5a transfectants actually exhibited a flatter morphology than the parent cell line. Furthermore, the XWnt-5a transfectants did not show enhanced proliferation as measured by [3H]thymidine incorporation (data not shown).

Since overexpression of XWnt-5a in C57mg cells does not stimulate cell proliferation or lead to a transformed phenotype, it was of interest to examine the levels of endogenous Wnt-4 and Wnt-5a RNA in these cells. Northern blot analysis revealed that, in contrast to overexpression of Wnt-1 or Wnt-2, overexpression of XWnt-5a does not result in a decrease in the level of endogenous Wnt-4 RNA (Fig. 8). Overexpression of Wnt-5a was, however, accompanied by a small but reproducible decrease in endogenous Wnt-5a RNA levels (Fig. 8).

Discussion

Cell proliferation and differentiation in the postnatal mammary gland are under the influence of multiple hormones, growth factors, and epithelial-stromal interactions (32, 33). Six murine Wnt family members are expressed and differentially regulated in the mammary gland of the virgin animal as well as during pregnancy (27, 28). It is during these times that extensive epithelial cell proliferation and ductal branching morphogenesis occur. Interestingly, Wnt-1 and Wnt-3, two Wnt family members that are not normally expressed in the mammary gland, participate in the generation of mammary epithelial hyperplasias when activated for expression in this tissue (17, 34). The Wnt family proteins characterized to date are secreted and tightly associated with the cell surface, where they can function in localized cell to cell signaling events (2, 4, 6). Taken together, these observations implicate several members of the Wnt gene family in the regulation of normal growth and differentiation of the murine mammary gland. In support of this hypothesis, the data presented here suggest that Wnt gene expression in the mammary gland is regulated by different mitogenic signals and that some Wnt genes can regulate the expression of others, either directly or indirectly.

We have used the C57mg mammary cell line, which naturally expresses the Wnt-4 and Wnt-5a genes (28), to examine the interplay between growth factors and Wnt gene expression during proliferation. The data demonstrate that the growth factors bFGF, TGF-β1, and EGF induce a mitogenic response in C57mg cells. Except for TGF-β1, this profile of growth factor sensitivity is consistent with the known effects of these factors on cells of the mammary gland. Although the specific mammary cell type from which the C57mg cell line was derived is not known, the pattern of growth factor sensitivity of the C57mg cells most closely matches that of mammary myoepithelial cells and not epithelial cells. In particular, FGF receptors are specifically expressed on myoepithelial cells and not on ductal or alveolar epithelial cells (35). Furthermore, TGF-β1 is often inhibitory to growth of epithelial cells yet stimulatory to other cell types of fibroblastic origin (36–38). Partial transformation induced by overexpression of Wnt-1 also stimulates the proliferation of C57mg cells (18). The data shown here support the conclusion that overexpression of Wnt-1 can replace the mito-
genic signal provided by certain growth factors. Furthermore, the mitogenic signal imparted by Wnt-1 expression cannot be enhanced by additional treatment with growth factors. Although the receptor(s) that respond to Wnt proteins are as yet unknown, this may indicate that signal transduction pathways activated by Wnt-1, and perhaps other Wnt proteins, may have some elements in common with signaling pathways for growth factors such as those tested here.

Several different mitogenic stimuli selectively down-modulate the levels of endogenous Wnt-4 and Wnt-5a RNA in C57mg cells. First, partial down-regulation by either Wnt-1 or Wnt-2 is accompanied by a dramatic decrease in Wnt-4 RNA and a partial decrease in Wnt-5a RNA. The mitogenic stimulation by two members of the FGF family, bFGF and Int-2, also leads to a selective decrease in the levels of endogenous Wnt RNA. This down-modulation cannot be a nonspecific response to growth promoting signals, since both EGF and TGF-β1 are also mitogenic for C57mg cells but do not significantly affect the levels of either Wnt-4 or Wnt-5a RNA. In addition, the levels of Wnt-4 and Wnt-5a RNA can be regulated independently and the effects seen are unique to each mitogenic signal. For example, Int-2 leads to a large decrease in Wnt-5a RNA levels but has no effect on Wnt-4, whereas bFGF affects both Wnt-4 and Wnt-5a to the same extent. Finally, in C57mg cells that are fully transformed by the activated tyrosine kinase oncogene Neu, expression of Wnt-4 and Wnt-5a genes is virtually absent. Taken together, these data indicate that Wnt-4 and Wnt-5a expression in mammary cells is responsive to growth regulatory signals, and the down-modulation of expression of either or both genes correlates with cell proliferation. The inverse correlation between expression of these endogenous Wnt genes and cell proliferation suggests that Wnt-4 and Wnt-5a may participate in restricting the proliferation of C57mg cells. Thus, expression of the exogenous Wnt genes may interfere with a negative growth regulatory function of Wnt-4 and Wnt-5a.

We have pursued this concept further by demonstrating that in contrast to Wnt-1 and Wnt-2, overexpression of XWnt-5a in C57mg cells does not lead to a transformed phenotype. Similar results have been obtained with murine Wnt-5a and Wnt-4. We next showed that there is no effect of overexpression of Wnt-5a on endogenous Wnt-4 RNA levels, unlike the dramatic down-modulation seen with overexpression of either Wnt-1 or Wnt-2. Overexpression of XWnt-5a does, however, lead to a slight decrease in endogenous Wnt-5a levels. One interesting explanation for this result is the idea that overexpression of Wnt-5a might decrease the levels of endogenous Wnt-5a through an autoregulatory mechanism.

The findings reported here have important implications for understanding the mechanisms by which Wnt-1, Wnt-2, and

\[^{6}\text{A. McMahon, personal communication.}\]
Wnt-3 are able to partially transform certain mammary cell types. These data suggest that the partial transformation induced in CS7mg cells by Wnt-1 and Wnt-2 is not simply the consequence of constitutive activation of a proliferation pathway that normally responds to the endogenous ligands Wnt-4 and Wnt-5a. This may reflect the fact that multiple receptors, leading to different signaling pathways, exist for the various Wnt family members. These and other previously reported data suggest that Wnt genes can be divided into two different groups with distinct biological activities in several assays. The Wnt-1 group (including Wnt-2 and Wnt-3) is capable of partially transforming CS7mg cells and contributing to murine mammary tumorigenesis (18, 29, 34). These same Wnt family members, including XWnt-8, are also capable of organizing a second embryonic axis (11, 13, 39–41) and enhancing gap junctional communication upon ectopic expression by injection of synthetic RNA into fertilized Xenopus oocytes (42, 43). Members of the second Wnt group (including Wnt-4 and Wnt-5a) do not induce a transformed phenotype when introduced into mammary cells (this report) and cannot organize a second embryonic axis or alter gap junctional communication when analyzed in Xenopus assays (40–42).

Several lines of evidence suggest that Wnt-1 and Int-2 have a cooperative effect in mammary tumorigenesis. Int-2, like Wnt-1, is a target for insertional activation by MMTV integration (44). Furthermore, a significant percentage of mammary tumors have both Int-2 and Wnt-1 genes activated (22–24). Transgenic mice with both Wnt-1 and Int-2 genes expressed in the mammary gland develop tumors with a higher frequency and shorter latency than transgenic mice that express either gene alone (25). Here, we show that overexpression of Int-2 in CS7mg cells leads to a partially transformed phenotype similar to the effects of Wnt-1 and Wnt-2 on these cells. Other studies have shown that Int-2 can transform mammary epithelial and fibroblast cell lines (31, 45). The results reported here also imply a synergistic effect of Wnt-1 and Int-2 in the regulation of endogenous Wnt-4 and Wnt-5a RNA levels in CS7mg cells. Although Wnt-1 induced transformation correlates with a decrease primarily in Wnt-4 RNA levels, Int-2 expression leads to a decrease in expression of Wnt-5a but not Wnt-4 RNA. Thus, if a decrease in both Wnt-4 and Wnt-5a RNA levels is needed for proliferation and transformation, expression of Wnt-1 and Int-2 in combination would be more effective than expression of either gene alone. A cooperative effect between FGF and Wnt family members has also been observed in Xenopus ectodermal cap assays in which bFGF and XWnt-8 together induce dorsal mesoderm, whereas neither alone can produce this phenotype (26).

The results presented here suggest a role for Wnt genes in the regulation of cell proliferation in the mammary gland. Depending on the environmental context in which they are expressed, Wnt genes may also function to modulate aspects of mammary gland differentiation such as epithelial-mesenchyme interactions. Thus, the responses elicited by Wnt proteins may depend upon combinations of stromal and growth factor signals that are simultaneously presented to the mammary cell. The combinatorial actions of various Wnt family proteins could provide an additional level of control. Multifunctional and combinatorial activities of TGF-β family proteins in growth and differentiation have also been described (37, 38). The specific biochemical mechanisms by which Wnt genes participate in cell growth and differentiation are still speculative. Although a receptor that transmits the Wnt signals remains unknown, several clues have emerged suggesting downstream biochemical effects of Wnt proteins on cell junctions. First, ectopic expression of Wnt-1 and XWnt-8 in Xenopus leads to an enhancement of gap junctional communication in embryos (42, 43). Other studies in Drosophila showed that wingless regulates the accumulation of armadillo protein, at the post-transcriptional level (46–48). Armadillo is homologous to the mammalian plakoglobin and β-catenin proteins that localize to desmosome and adherens cell junctions (49–51). Similarly, we have found that overexpression of Wnt-1 in several cell lines, including CS7mg, leads to increased levels of both β-catenin and plakoglobin proteins. These data suggest that Wnt-1, and perhaps other Wnt family members, functions in both proliferation and differentiation by modulation of junctional communication and adhesion.

Materials and Methods

Cell Lines. All cell lines were maintained in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal calf serum and 5% defined bovine calf serum (HyClone). Except for the CS7mg parent cell line, all cells were grown in 250 µg/ml G418 (GIBCO). The normal mouse mammary cell line, CS7mg clone D2, was derived and characterized as described (52). The CS7mg cell lines transfected with expression vectors for either Wnt-1 (clone 805) or Wnt-2 (clone R2) have been described (29). CS7mg cells expressing either the normal or activated (V659E) rat Neu gene, under the control of a mouse leukemia virus long terminal repeat, were generously provided by Dr. Arthur Bruskin (Applied Bio-Technology). These cell lines were derived by infection of CS7mg cells with retrovirus vectors expressing Neu, followed by G418 selection. Cells infected with the normal Neu expression vector are morphologically normal, do not grow in soft agar, and are nontumorigenic in nude mice. Cells infected with the activated Neu expression vector are morphologically transformed, grow in soft agar, and form tumors in nude mice. CS7mg cells were transfected with an expression vector for Int-2, pD03.2, kindly provided by Clive Dickson (31), using lipofection as described (53). Individual G418 resistant colonies were selected and expanded into cell lines. Int-2 RNA expression was verified by Northern blot analysis, and a clone expressing the highest levels of Int-2 was used for the experiments shown in Figs. 4 and 5. CS7mg cells were cotransfected with pSV2Neo and an expression vector for XWnt-5a, PECE-XWnt-5a (the XWnt-5a coding region (54) was subcloned into an expression vector and kindly provided by R. Moon), using lipofection. Individual G418 resistant colonies were selected and expanded into cell lines. XWnt-5a expression was verified by Northern blot analysis using a specific probe for Xenopus Wnt-5a that does not cross-react with endogenous mouse Wnt-5a. Two cell lines, clones 12 and 20, were selected for further analysis. For the photographs of cell morphology shown in Figs. 4 and 7, CS7mg, CS7mg/Int-2, CS7mg/XWnt-5a, CS7mg/Wnt-1, or CS7mg/Neo cell lines were plated at a density of 5 × 10³ cells/10-cm tissue culture
dish in standard growth medium. Cells were grown to confluence (approximately 3–4 days) and photographed.

**[3H]Thymidine Incorporation.** C57mg cells or C57mg/ Wnt-1 cells were plated into 5-cm dishes in standard growth medium and grown to confluence without a change of medium (approximately 4 days). The cells were rinsed twice with phosphate buffered saline, fed with 5 ml of serum-free Dulbecco's modified Eagle's medium, and treated, in triplicate, with several concentrations of bFGF, EGF, or TGF-β1 for 20 h at 37°C. Growth factor concentrations are indicated in the legend to Fig. 1. All growth factors were purchased from Collaborative Research. Next, 4 μCi of [3H]thymidine (methyl-3H, 60–90 Ci/mmol, aqueous; ICN) were added to each dish and incubated for 2 h at 37°C. The cells were subsequently washed twice with phosphate buffered saline followed by the addition of 2.0 ml ice-cold 10% trichloroacetic acid for 30 min on ice. Cells were then washed once with 10% trichloroacetic acid followed by the addition of 2.0 ml of 0.1 NaOH. The dishes were incubated at 37°C for 30 min and then neutralized with 0.2 ml of 1 M HCl. The dishes were carefully scraped, and the extract was added to 10 ml of scintillation fluid for counting. The data shown in Fig. 1 are expressed as a ratio of the [3H] incorporation of factor treated cells to the [3H] incorporation of untreated cells of the same type. Based on the results from these experiments, concentrations of 1 mg/ml TGF-β1, 100 ng/ml EGF, or 5 ng/ml bFGF were used, under conditions similar to those noted above, for the treatment of cells used in the experiment shown in Fig. 3.

**RNA Isolation and Northern Blot Analysis.** Total cellular RNA was isolated (55) from multiple dishes of confluent C57mg cells or the various transected C57mg cell lines. Since the abundance of Wnt-4 and Wnt-5a RNA is low, it was necessary to select for poly(A) RNA using an oligo-d(T) cellulose column (56). Two μg of each poly(A) RNA sample was analyzed on a 1.2% agarose formaldehyde gel (55) followed by transfer to a Hybond-N (Amersham) membrane. The membrane was baked under vacuum, prehybridized at 61°C for 3–5 h, and hybridized overnight at 61°C with 1–2 × 10⁶ cpm/ml of a riboprobe. The riboprobe vectors containing sequences specific to either Wnt-4 or Wnt-5a have been described (28) and were generously provided by Andy McMahon. The riboprobe vector specific for actin was constructed and provided by Meredith Wright (Syntex). **23P**-labeled probes were made with a Riboprobe system II kit from Promega. The prehybridization and hybridization solution consisted of 50% formamide, 4× saline-sodium phosphate-EDTA, 0.2 mg/ml sheared salmon sperm DNA, 2.5× Denhardt's solution, and 1% SDS. Membranes were washed at room temperature twice in 2× standard saline citrate-1% SDS followed by several washes, as needed, for 30 min each in 0.1× standard saline citrate-0.1% SDS at 65°C. Membranes were exposed to film at -80°C with an intensifying screen. Changes in Wnt-4 or Wnt-5a RNA levels were quantitated by scanning the films with a densitometer.

**Acknowledgments**

We thank Andy McMahon for the Wnt-4 and Wnt-5a riboprobe vectors, Meredith Wright for the actin riboprobe vector, Clive Dickson for the Int-2 expression vector, Randy Moon for the XWnt-5a expression vector, and Arthur Broxmeyer for the C6/107 cell line. We are grateful to Randy Moon, Bev Smolich, Lindsay Hinck, Paul Cannon, Charles Daniel, and Yaef Friedman for suggestions and comments on the manuscript.

**References**

26. Christian, J. L., Olson, D. J., and Moon, R. T. Wnt-8 modifies the character of mesoderm induced by bFGF in isolated Xenopus ectoderm. EMBO
Regulated Expression of Wnt Genes in C57mg Mammary Cells

206