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Role of Nuclear Receptor Coactivators, AIB-1 and SRC-1, in the Development of Breast Cancer

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Steroid hormones are involved in the development and growth of breast cancer. Drugs, which inhibit estrogen action, are commonly used to inhibit breast cancer growth. Unfortunately, most advanced breast cancer becomes resistant to estrogen treatment. Recently, many steroid receptor coactivators have been discovered and found to potentiate the transcriptional activity of steroid receptors and enhance the expression of hormone response genes. In the SRC-1 family of coactivators, AIB1 is found amplified and/or over-expressed in breast cancer specimens. To evaluate the potential roles of the SRC-1 family of coactivators in mammary tumorigenesis in vivo, we proposed to generate transgenic mice over-expression of AIB1 (SRC-3) in mammary glands. To target the expression of AIB1 in mammary gland, we placed the AIB1 transgene under the control of the MMTV-LTR promoter. Two lines of transgenic mice expressing AIB1 have been generated. Studies on these transgenic mice will help understand the development and progression of breast cancer and provide a molecular basis for designing novel strategies to curb and, ultimately, cure breast cancer.
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INTRODUCTION
Steroid hormones play a central role in mammary gland development. The hormonal effects are mediated by estrogen and progesterone receptors, which act as hormone dependent transcription factors. Upon ligand binding, these receptors bind to the cognate hormone responsive elements on the target promoter and recruit co-activators and general transcription factors to regulate the expression of hormone responsive genes. Several coactivators which interact with estrogen and progesterone receptors have been cloned and extensively characterized in various laboratories, including ours. One of the co-activator, Steroid Receptor Co-activator (SRC-1), has been demonstrated by us to possess intrinsic histone acetyltransferase (HAT) activity. This property may facilitate localized chromatin remodeling and enhance assembly of basal transcription factors, resulting in the increase of transcription of hormone response genes. In addition to SRC-1, two other related genes of the same family have recently been identified and characterized. These are TIF-2 (GRIP-1/SRC-2) and AIB-1 (p/CIP/ACTR/RAC-3 /TRAM/SRC-3). Like SRC-1, these two coactivators have also been demonstrated to possess HAT activity and to interact with members of the nuclear receptor superfamily to enhance their abilities to transactivate the target genes.

Two salient points strongly implicate members of the SRC-1 family of coactivators as potential oncogenes for mammary tumorigenesis. Firstly, our published results indicate that ablation of SRC-1 gene by homologous recombination in mice severely compromised mammary gland development in response to estrogen and progesterone. Secondly, AIB-1/SRC-3 is amplified in 10% of breast cancer patients and is over-expressed in 64% of primary breast cancer samples. Since these family of coactivators are essential for the maximal expression of hormone response genes, we hypothesize that ectopic over-expression of these genes may play a role in the initiation, progression or transition of tumor cell growth from a hormone dependent to a hormone independent state. To test our hypothesis, we will generate transgenic mouse lines over-expressing either SRC-1 or AIB-1/SRC-3 and assess whether over-expression of these genes will result in mammary gland hyperplasia. We expect the results obtained from these studies will address whether over-expression of this family of co-activators play a key role in breast tumor formation.
SIGNIFICANCE

AIB-1 is a member of the SRC-1 family of transcriptional coactivators which have been shown to be essential for the maximal activation of hormone responsive genes. This family of transcription factors, not only interacts with members of the large nuclear receptor superfamily, but also possesses HAT activity and interacts with other coactivators including CBP/P300 and P/CAF. Therefore, over-expression of AIB-1 in breast cancer cells may not only perturb the expression of many estrogen, progesterone and other hormonal regulated genes, but may also affect the signal transduction pathways which depend on CBP/P300 and P/CAF. Therefore, the deregulated expression of AIB-1 or SRC-1 may have a profound effect on the growth and development of mammary gland. To investigate how members of the AIB-1 family influence growth and development of mammary gland, we propose to use transgenic mice as a model system. Transgenic mouse lines over-expressing AIB-1 in the mammary gland will be generated. These lines will be used to assess whether over-expression of the AIB-1 will be sufficient to induce mammary tumorigenesis.

BODY

Expression of the AIB-1 in transgenic lines using MMTV-KCR-AIB-1 transgenic construct

Previously, we have obtained two transgenic founder lines, 2694 and 9845, expressing the AIB-1 in the mammary gland. However, the expression level of AIB-1 transgene is only 1 to 2 fold more than that of the endogenous mP/CIP (human AIB-1 homologue). In an attempt to enhance the expression level of AIB-1 in the line 9845, a homologous line was generated from this founder line. RNA samples were isolated from transgenic mice in different developmental stages and the corresponding expression levels were detected by RNase protection assays using an AIB-1 specific riboprobe and normalized by the cyclophilin expression. In the virgin / non-pregnant transgenic mice, the expression level of AIB-1 transgene of homologous line 9845 (+/+) remained low and only 1 to 2 fold more than that of the endogenous mP/CIP. However, the expression level of AIB-1 was found to be increased at a much higher level during pregnancy (P6 and P17) and early lactation (L1) stages, and it was subsequently dropped back to the original level at the late lactation.
Assessment of the effect of over-expression of AIB-1 in mammary gland growth and development

Based on the fact that the homologous transgenic line 9845 expresses AIB-1 transgene at a higher level during pregnancy and early lactation, we examined the physiological perturbation that might affect the expression of AIB-1 in these stages. Mammary gland whole-mount at different stages of development, 6-month old virgin, pregnancy (day 6, P6; and day 17, P17) and lactation (day 1, L1) were used for the assessment of the effect of over-expression of AIB-1 in mammary gland development. In the homozyous (+/+ ) transgenic mice whole-mount sections, the degree of mammary gland developments were similar to the age and stage-matched wild-type mice (Figures 1-5) Thus, no apparent perturbation was found in the transgenic mammary gland. As mentioned before, the expression of the transgene is pregnancy dependent. Thus, the multiparous transgenic glands have also been analyzed. Again, both the wild-type and transgenic gland displayed similar phenotype. Only some of the mice showed aberrant morphology of squamous nodule in the ductal tree. Since similar phenotype has also been observed in the wild-type aged mice, this might have been resulted from aging or from over-expression of AIB-1. More recent analysis showed aging by itself can generate similar phenotypes.

Alternative approach to over-express AIB-1

It is possible that mammary epithelial cell might not tolerate high levels of AIB-1 expression, so we cannot over-express the AIB-1 transgene at a high level using the traditional constitutive expression method. To overcome this problem, an inducible system developed in our laboratory will be used. This system employs a transactivator and a target. The transactivator GLp65 is a chimic protein containing a mutated human progesterone receptor ligand binding domain fused with a yeast GAL4 DNA binding domain and a partial p65 protein activation domain. To generate the transactivator line, MMTV-KCR-GLp65, a transactivator was subcloned into a modified version of MMTV-KCR vector, which can drive a mammary gland specific expression. The KCR fragment contained the partial exon II, intron II, exon III and a endogenous polyadenylation signal derived from rabbit β globin gene. For the generation of the target, the AIB-1 transgene was placed under the control of four copies of yeast transcription factor GAL4
binding sites (referred as 17X4) and a TK promoter as described previously (Ngan et al., PNAS, 99, 11187, 2002). Crossing of the transactivator line with the target line can obtain a bitransgenic mice. With the administration of RU486, the regulator can then bind to the 17X4 UAS recognition sequences upstream of the target oncogene and induce the expression of AIB-1 transgene. The expression of AIB-1 in bitransgenic mice can be turned on by RU486 at a specific window during development and the effects of this coactivator on mammary gland oncogenesis can be monitored.

Characterization of the inducible system using FGF-3 as target

Previously, we have generated four transactivator lines. To elucidate the regulatory profile of the inducible system, the MMTV-transactivator line has been crossed with the FGF-3 target line to generate the bitransgenic mice. Different dosages of RU486 and different induction times have been applied on the bitransgenic mice and the corresponding expression profiles of transgene were analyzed (Ngan et al., PNAS, 99, 11187, 2002). The expression of the FGF-3 transgene in mammary gland is tightly regulated by the administration of RU486. The kinetics analysis indicated that the FGF-3 RNA is accumulated up to a detectable level one day after administration of RU486 pellet and was maintained at a high level over weeks. In addition, the level of induced FGF-3 expression correlated with the dosage of RU486 administrated. A dose-dependent increase of FGF-3 RNA was detected when RU486 concentration was increased from 150 µg/kg to 450 µg/kg. In addition, we have also demonstrated that the long-term induction of FGF-3 results in mammary gland hyperplasia that can be reversed by RU486 withdrawal. Therefore, we believe that this inducible system is an useful animal model for our future study. The AIB-1 target line is now in preparation and will be crossed with this MMTV-transactivator line.

Generation and analysis of AIB-1 expression in bitransgenic lines

We have examined 7 AIB-1 target lines, but only one is inducible. However, the inducible expression of AIB-1 is barely detected by ribonuclease mapping assays, not by Northern blot or western analysis. Thus, this line is not very useful. Subsequently, we have generated additional
five AIB-1 target lines. Three of these lines have been crossed to the activator lines, only one line expressed AIB-1 in an inducible manor. Again, the expression is not too much higher than the endogenous AIB-1 mRNA levels (Figure 6). Nevertheless, we have followed the functional consequence of expression of this bigenic line. Unfortunately, we have not seen any perturbation in mammary gland development with the expression of low levels of AIB-1 for the last two months in virgin or lactating mammary glands (data not shown). We believed that low levels of AIB-1 expression would not elicit any phenotypic changes. Currently, we are crossing the two remaining two lines to the activator line to generate bi-transgenic animals and examine if higher levels of AIB-1 expression can be induced.

KEY RESEARCH ACCOMPLISHMENTS

1. Characterized AIB-1 expression profile of the homologous MMTV-AIB-1 transgenic line in different developmental stages.
2. Analyzed the mammary gland phenotypes of homologous transgenic mice expressing AIB-1 in different developmental stages.
3. Fully established and characterized the mammary gland specific inducible transgenic mice model using the FGF-3 target line.
4. Generation and characterization of inducible expression of AIB-1 in bi-transgenic lines

REPORTABLE OUTCOMES

1. Completed the phenotypic analysis of the mammary gland of homologous transgenic mice constitutively expressing AIB-1.
2. Established and fully characterized an inducible system for over-expression of transgene in mammary gland.

CONCLUSION

In homologous AIB-1 transgenic line, we found that AIB-1 transgene is expressed at a high level during pregnancy and early lactation. Subsequent analysis using mammary gland whole-mounts
at different developmental stages showed that there is no obvious phenotypic difference between transgenic mice and the age and stage-matched wild-type mice. The low expression of AIB-1 may account for the insignificant morphological changes. In an attempt to overexpress the AIB-1 to a high level as demonstrated in the cancer patient samples, we have generated an inducible system to express AIB-1 transgene under the control of RU486. Unfortunately, we have not been able to induce AIB-1 expression level to the same extent as FGF-3. Thus, we have not been able to demonstrate if AIB-1 can induce tumor formation by itself in transgenic mice. Nevertheless, the generation of the activator mouse line in mammary gland allows us to over-express FGF-3 and shows over-expression of FGF-3 is sufficient to induce hyperplasia in mammary gland. This line has also been sent to other laboratories, including Dr. Lewis Chodosh, for inducible expression of Ras oncogene in transgenic mice.

REFERENCES


Figure 1: Whole mount analysis of 5-month-old virgin transgenic and wild type mammary glands. The transgenic mammary glands displayed a slight but consistent increased in the number of lateral ductal branching in comparison to the wild type mice.
Figure 2. Whole mount analysis of transgenic and wild type mammary glands at pregnancy day 6. The ductal branching of transgenic and wild type mammary glands are morphological indistinguishable (A-C). D and G, E and H, and F and I are corresponding higher magnification of A, B and C, respectively.
Inducible expression of FGF-3 in mouse mammary gland

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Fibroblast growth factor-3 (FGF-3) is a crucial developmental regulator. Aberrant activation of this gene by mouse mammary tumor virus insertion results in pregnancy-responsive mammary tumorigenesis. To characterize better FGF-3 function in postnatal mammary gland development and cancer initiation/progression, we used a mifepristone (RU486)-inducible regulatory system to express conditionally FGF-3 in the mammary epithelium of transgenic mice. Ectopic overexpression of FGF-3 in pubescent mammary glands elicited severe perturbations in early mammary gland development leading to mammary hyperplasia. Ductal elongation was retarded, multiple cysts persisted in the virgin ducts, and ductal epithelium was expanded and multilayered. The altered ductal architecture and the persistence of hyperplastic multilayered epithelium reflect a defect in growth regulation, which resulted from an imbalance between mitogenic and apoptotic signals. By altering the duration of RU486 treatment, we showed that the persistence of mitogenic signal elicited by FGF-3 is crucial for the initiation, progression, and maintenance of the hyperplastic characteristic of the mammary epithelium. The manifestations elicited by FGF-3 could be reversed by RU486 withdrawal. In addition, synergism between the stimulus from estrogen and FGF-3 mitogenic pathways was evident and likely contributes to the pregnancy-dependent tumorigenesis of FGF-3. Taken together, the mifepristone-inducible regulatory system provides a powerful means for understanding the diverse roles of FGF-3 and its interactions with hormones in mammary gland tumorigenesis.

mifepristone-inducible expression | int-2 | mammary gland hyperplasia

The majority of mammary gland development takes place postnatally. Primary mammary epithelial ducts are established before birth but remain quiescent until puberty when ductal morphogenesis and patterning occurs in response to the systemic effects of estrogen and growth hormones. Through a process balanced by rapid proliferation of the cap cells and massive apoptosis of the body cells, coupled with the action of proteases, the ducts elongate, become progressively branched, and fill the fat pad at ~12 weeks of age (1). The gland then remains dormant until the hormonal stimulus of pregnancy; a cyclical phase of development is initiated in synchrony with reproductive status of the animals. The cyclic phase includes extensive growth and differentiation of the ducts for the development of lobuloalveolar structures during pregnancy, milk proteins synthesis during lactation, and massive apoptosis of the alveolar epithelium during involution. After involution, the mammary gland resembles the mature ductal tree of the virgin mouse but with increase of lateral branches.

The interplay between numerous cell-cycle regulators, ovarian hormones, growth factors, and their receptors governs pre- and postnatal mammary gland development. Ablation or misexpression of these network components causes dramatic disruption of mammary gland morphogenesis and patterning, and may also result in tumor development. In addition, the stage in development when the gene is first expressed often influences the phenotypic manifestations, suggesting that temporal regulation of gene expression is a prerequisite for proper mammary gland development. Thus, an inducible system, which is able to control the temporal expression of a gene in the mammary gland, will provide a better understanding of the role of target genes in mammary development and tumorigenesis.

Fibroblast growth factor-3 (FGF-3) exhibits complex expression patterns and plays a role in the proliferation and differentiation of a diverse array of developing embryonic tissues (2). In vivo expression analysis and derivation of homozygous FGF-3 null mouse mutant have suggested roles in cell migration, tissue induction, and neuronal cell differentiation (3, 4). Insertional activation of this gene by mouse mammary tumor virus (MMTV) has been correlated with the appearance of mammary tumors (5). In addition, ectopic overexpression of FGF-3 in transgenic mice resulted in pregnancy-responsive mammary hyperplasia (6, 7). Subsequently, Ornitz et al. (8) reported that the expression of FGF-3 in the early virgin mammary gland elicited abnormal development. However, the lack of temporal and reversible transgene expression in these systems nullifies attempts to assess the temporal role that FGF-3 plays in early mammary gland development and the initiation/progression events of FGF-3 tumorigenesis. Hence, to circumvent these problems, a mifepristone (RU486)-inducible system recently established in our laboratory was used to facilitate this study.

The mammary-specific inducible binary system comprises two components: (i) A chimeric transactivator, GLP65, which is responsive only to progesterone antagonists, contains the Gal4 DNA-binding domain, a mutated progesterone receptor ligand-binding domain, and an activation domain of NFκB subunit p65. (ii) The target is placed under the control of Gal4-binding sites, the upstream activating sequence (UASo). To obtain tissue-specific expression of the target gene in the mammary gland, the transactivator was placed under the control of MMTV promoter. In these binary transgenic mice, a spatiotemporal-specific expression could be demonstrated upon administration of the progesterin antagonist, RU486.

Ectopic expression of FGF-3 in the mammary glands of bigenic pubescent virgin resulted in dramatic mammary gland abnormalities. The bigenic mice displayed enlargement in duct size, retardation of ductal extension, and atypical mammary gland hyperplasia in regions highly expressing FGF-3. The extent of the induced hyperplasia depended on the length and levels of FGF-3 expression. In addition, the phenotypic alterations in mammary gland could be reversed upon withdrawal of the ligand. Finally, estrogen and progesterone further enhanced the extensive mammary epithelium proliferation elicited by FGF-3, implying the contributions of these hormones to tumor progression.

Materials and Methods

Generation of MMTV-GLP65 Transgenic Mice. The transgenic construct was created by cloning a 2.7-kb blunt-ended Asp-718–BamHI fragment of the GLP65 transactivator into the blunt-ended EcoRI site of the MMTV-KCR vector (10) to yield the MMTV-KCR-GLP65 vector. The KCR fragment was derived from the rabbit β-globin gene. To create GLP65 transgenic mice, a 5.41-kb Acc65I-

Abbreviations: FGF-3, fibroblast growth factor-3; MMTV, mouse mammary tumor virus; MAPK, mitogen-activated protein kinase; UASo, upstream activating sequence; RPA, ribonuclease protection assay.

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**BamHI** fragment was released from MMTV-KCR-GLp65 and isolated using Geneclean. The fragment (2 ng/ml) was injected into FvB/N-fertilized eggs to generate transgenic mice by using standard protocols (11). Founder mice were analyzed by PCR and Southern hybridization (12). Heterozygous MMTV-GLp65 mice were bred with homozygous UASb-FGF-3 monogenic mice obtained from Philip Leder (Harvard University, Cambridge, MA) and described by Ornitz et al. (8) to generate bigenic mice.

**Administration of RU486 to Mice.** RU486 purchased from Biomol (Plymouth Meeting, PA) was used to make pellets (Innovative Research of America) for a constant daily release of RU486 at 150, 300, and 450 μg/kg body weight.

**RNase Protection Assays.** Total RNA was isolated from mouse tissues by using the Trizol reagent (GIBCO). Expression of transgenes was detected by RNase protection assay with the RPAIII kit (Ambion, Austin, TX). To five to ten micrograms of total RNA was hybridized with 32P-labeled FGF-3 (12), GLp65, Cyclin D1 (PharMingen) and control cyclophilin or L32 antisense riboprobes and assayed as described (Ambion).

**Whole Mount, Histology, and Immunohistochemistry.** Whole-mount staining of the left fourth inguinal mammary gland was performed as described (10). For histological analyses, glands were fixed in cold 4% paraformaldehyde and embedded in paraffin. The 7-μm sections were rehydrated, microwaved for 20 min in 10 mM sodium citrate, and incubated with anti-E-Cadherin (Zymed) or p42/p44 mitogen-activated protein kinase (MAPK) (Cell Signaling, Beverly, MA) antibodies, followed by secondary antibodies (Molecular Probes) and mounting with aqueous mounting media (Vector Laboratories). For BrdUrd incorporation studies, the mice were injected i.p. with 100 μg/g body weight of BrdUrd 2 h before sacrifice to label cells in the S phase. The BrdUrd-labeled cells were detected by immunohistochemistry with monoclonal anti-BrdUrd antibody (Dako) as described (10). Analyses of 300–400 epithelial cell nuclei per section from four monogenic and bigenic glands were performed.

**Results**

**Ligand-Inducible Expression of FGF-3 in Transgenic Mice.** To achieve mammary gland-specific expression of FGF-3 in a milkstone-dependent manner, we first generated the MMTV-GLp65 line by using the transgenic construct depicted in Fig. 1.4 (MMTV-KCR-GLp65). Four transactivator mouse lines expressing GLp65 in the mammary glands were identified by ribonuclease protection assay (RPA) (data not shown). The transgenic line 5277F0 used in this study predominantly expressed GLp65 mRNA in the mammary gland and not in other tissues (Fig. 1B). By crossing this transactivator line with the UASb-FGF-3 target, we obtained bigenic mice (GLp65/UASb-FGF-3). To characterize our regulatory system, we implanted the RU486 pellets s.c. with a dosage of 150 μg/kg body weight of RU486 and persisted for the duration of the treatment. RU486 induction of GLp65 was seen only in bigenic mammary glands (Bi-Tg: GLp65/UASb-FGF-3) and not in monogenic target (UASb-FGF-3) or transactivator (MMTV-GLp65) glands given 150 μg/kg body weight of RU486 and persisted for 2 weeks. Expression of GLp65 was seen only in monogenic transactivator and bigenic glands. (A) FGF-3 expression in bigenic mice was induced after 1 day of 150 μg/kg body weight of RU486 and persisted for the duration of the treatment. (B) Dose-dependent increase in FGF-3 expression was seen only in bigenic mice. FGF-3 expression was not detected in monogenic mice given RU486.

**Phenotypic Consequences of FGF-3 Induction in Pubescent Mammary Gland.** To elucidate the role of FGF-3 in early mammary gland development, FGF-3 expression was induced in 3-week-old bigenic mice with 450 μg/kg RU486 for 2 months. Monogenic UASb-FGF-3 and wild-type controls were also given a similar dosage of RU486. The control (Fig. 2 A and B) and bigenic mice treated with placebo (Fig. 2 I and J) consistently displayed normal mammary ductal development with the fat pad completely filled with branched ducts (Fig. 2 A and I). At higher magnifications, the epithelial ducts displayed a smooth surface (Fig. 2 B and J). However, ectopic expression of the FGF-3 in bigenic mice at early puberty resulted in abnormal mammary gland development (Fig. 2 C–H). The bigenic glands showed prominent retardation in ductal elongation with the ducts displaying less side-branching and failing to penetrate beyond the lymph node proximal to the nipple (Fig. 2C). Profound differences were also visible because the bigenic ducts seemed more dilated and were punctuated with small aberrant lobular protruberances (Fig. 2D). After 4 months of induction, the bigenic gland showed extensive but aberrant lobuloalveolar development (Fig. 2 E and F). The extent of the induced lobular hyperplasia also depended on the duration of FGF-3 expression. A longer FGF-3 expression elicited a more severe form of aberrant lobular hyperplasia (compare Fig. 2 F and H for 4- and 6-month FGF-3 expression, respectively). The progression also depended on the expression level of FGF-3 and could be accelerated by increasing the concentrations of RU486 (data not shown). Therefore, this
result suggests that expression of FGF-3 can induce perturbations in the ductal elongation as well as morphogenesis. More importantly, we show that FGF-3 is not only able to initiate but is also crucial for the progression of mammary gland hyperplasia.

These dramatic morphological changes implied that prominent changes occur in the properties of the tissues. Histological analyses revealed precocious mammary hyperplasia in 100% of bigenic mice treated with RU486. Whereas wild-type ducts had a clear lumen within a monolayer of luminal epithelial cells (Fig. 3A and D), a majority of ducts in the bigenic gland were multilayered and partially filled with loosely associated epithelial cells that presumably arose by alterations in cell–cell adhesion within the ductal wall (Fig. 3B). Some ducts displayed a more severe histological abnormalities and were surrounded by dense connective tissue with an increased number of fibroblasts. In addition, the epithelial cells became completely disorganized and exhibited a nonuniform nuclear morphology (Fig. 3C). The hyperplasia at 6 months of RU486 treatment was equivalent to a moderate grade mammary intraductal neoplasia (13). Co-localization of FGF-3 mRNA with the hyperplastic mammary epithelium (Fig. 3E and F) was demonstrated by in situ hybridization with an FGF-3 antisense riboprobe (12). Thus, this finding is consistent with FGF-3 acting primarily as an autocrine or ultrashort range paracrine growth factor in the mammary epithelium (14).

Ectopic Expression of FGF-3 Results in Elevated Epithelial Cell Proliferation. To determine whether mammary hyperplasia was the result of elevated cell proliferation, a BrdUrd incorporation assay was performed. Monogenic control and bigenic females with 2 months of RU486 treatment were administrated BrdUrd, whose incorporation into DNA was detected by immunohistochemistry (Fig. 4D). The proliferation index was calculated as a percentage of BrdUrd-positive epithelial cells out of 300–400 cells per section (Fig. 4F). The proliferation rate in the bigenic mammary ductal epithelium was consistently higher than that of the monogenic control (22.3% in the bigenic gland (Bi-Tg) vs. 4.2% in the monogenic (Ctrl) control), which therefore clearly demonstrated the mitogenic nature of FGF-3, enabling it to induce hyperproliferation of the mammary epithelium.

Because cyclin D1 is the major G1 cyclin whose disruption frequently occurs in the tumor cell (15), we examined cyclin D1 expression in control and bigenic mammary glands. RPA analysis revealed that a 2- to 4-fold increase in cyclin D1 was detected in the hyperplastic bigenic glands (Fig. 4C). Monogenic mice treated with RU486 and bigenic mice treated with placebo consistently showed no significant difference from the wild-type control. Thus, ectopic expression of FGF-3 led to an elevated expression of cyclin D1, which contributes to the development of mammary neoplasia.

Altered Expression of Cell Adhesion Molecules in the Hyperplastic Mammary Epithelium. FGF-3 binding to its receptor activates the MAPK signaling pathway (16). As expected, numerous phospho-MAPK-positive cells were visible in the hyperplastic mammary epithelium as compared with the control (Fig. 5). In the hyperplastic region, the epithelium became disorganized, implying a loss of cell–cell contacts. E-cadherin, which is the adhesive component of adherent junctions, is notably absent or dysfunctional in most of the advanced, undifferentiated, and aggressive breast and other epithelial carcinomas (17, 18). Thus, it was of particular interest to examine the expression pattern of this adhesive molecule in bigenic mice. As illustrated in Fig. 5, whereas the normal epithelium exhibited a high level of luminal expression of E-cadherin, the transformed epithelium lost its polarized expression pattern with
FGF-3-elicited mammary gland hyperplasia is reversible. Whole-mount withdrawal of RU486 (OFF in E and F). Monogenic glands from mice given RU486 displayed normal morphology. (Lower) RPA analyses indicates that the FGF-3 transgene can be turned on and off in the bigenic glands in response to the administration and withdrawal of RU486. FGF-3 was undetectable in the controls.

The Phenotypic Effect of FGF-3 Expression Is Reversible. The utility of an inducible bigenic system is best illustrated by its ability to turn FGF-3 transgene expression on and off at will. To test our system, we implanted a group of 3-week-old bigenic mice with RU486 pellets at a dosage of 450 μg/kg for 2 months followed by a withdrawal of RU486 for another 2 months. One of the fourth and fifth inguinal mammary glands were removed from the mice 2 months after first induction and subjected to whole-mount and RPA analyses, respectively, to ensure the expression of the transgene. Pronounced perturbations in mammary gland development were consistently seen subsequent to the expression of FGF-3 (Fig. 2 C and D). As illustrated in Fig. 6 A and B, the control monogenic UASc.,-FGF-3 mice treated with RU486 for 4 months displayed normal ductal development. On the other hand, constitutive expression of FGF-3 in the bigenic glands for 4 months resulted in retarded ductal extension and precocious mammary hyperplasia (ON in Fig. 6 C and D). Nevertheless, nearly complete normal ductal morphology was seen in the mice withdrawn from RU486 treatment for 2 months. The ducts extended beyond the lymph node and had regularly spaced side branches similar to those of control mice (OFF in Fig. 6E). At a higher magnification, we observed only a small proportion of ducts being slightly wider and punctuated with a few small cystic vesicles (Fig. 6F). FGF-3 can be reinduced in these mice and prior exposure to FGF-3 does not alter the susceptibility of the mice to subsequent oncogenic stimuli (data not shown). The corresponding expression of FGF-3 transgene was evident by RPA as shown in Fig. 6 (Lower). With 4 months of RU486 treatment, the FGF-3 transgene was steadily expressed in bigenic glands (ON). In contrast, the FGF-3 transgene was turned off and no detectable FGF-3 was observed in the bigenic mice after 2 months withdrawal of RU486 (OFF). Taken together, the pronounced phenotypic alternations depended on FGF-3 expression, and this expression is crucial for the maintenance and progression of the mammary gland hyperplasia.

FGF-3 down-regulates extracellular matrix factors. More bigenic hyperplastic cells stain positive for phospho-MAPK than control cells. Loss of polarized expression of E-Cadherin occurred in bigenic but not in control cells. Sections were stained with anti-phospho-MAPK and E-cadherin antibodies, and positive signals visualized with Cyanine 3 (red). Nuclei were counterstained with 4',6-diamidino-2-phenylindole (blue).
I Equal amounts of sesame oil were also administrated as vehicle MAPK cascade, which has been shown to be a differentiation signal that ductal patterning and morphogenesis in mammary gland cells susceptible to further somatic mutation and successive mutation between pregnancies. To address this hormone-responsive behavior, the hormonal effects on the initiation and the progression of FGF-3-elicited mammary hyperplasia were examined. On administration of RU486 for 2 weeks, the ovariectomized monogenic control and the bigenic mice were supplemented with the (50 μg/kg) progesterone, estrogen, or both for 3 consecutive days. Equal amounts of sesame oil were also administrated as vehicle control. Comparable expression levels of FGF-3 were detected in monogenic and short-term hormonal treatment did not cause any significant alteration in the FGF-3 expression level (data not shown). Although the monogenic control exhibited an entirely normal mammary tree, overexpression of FGF-3 conferred the ability to form cystic structures in the mammary ducts of ovariectomized bigenic mice in the absence of hormone supplementation. Surprisingly, the ducts were similar in size to the control (data not shown). Treatment with estrogen but not progesterone induced expansion of the ductal epithelium in the bigenic but not in monogenic mice. The bigenic ductal size was wider and resembled those shown previously. In addition, progesterone could synergize with estrogen to elicit a more severe manifestation (data not shown).

Subsequent histological analyses showed that the control duct (Ctrl) always remained monolayered regardless of the treatment and no significant difference was found in the bigenic glands (Bi-Tg) treated with sesame oil (OIL) versus estrogen and progesterone cotreatment. In contrast, a uniform layer of epithelium was shown in all monogenic controls (Ctrl). Vehicle treatment does not alter the bigenic ductal morphology.

Interaction of Ovarian Hormones with FGF-3 in Mammary Gland Tumorigenesis. Pregnancy responsiveness is one of the characteristic features of hyperplastic lesions elicited by FGF-3 (6–8). The hyperplasia arises as a result of pregnancy and often regresses between pregnancies. To address this hormone-responsive behavior, the hormonal effects on the initiation and the progression of FGF-3-elicited mammary hyperplasia were examined. On administration of RU486 for 2 weeks, the ovariectomized monogenic control and the bigenic mice were supplemented with the (50 μg/kg) progesterone, estrogen, or both for 3 consecutive days. Equal amounts of sesame oil were also administrated as vehicle control. Comparable expression levels of FGF-3 were detected in monogenic and short-term hormonal treatment did not cause any significant alteration in the FGF-3 expression level (data not shown). Although the monogenic control exhibited an entirely normal mammary tree, overexpression of FGF-3 conferred the ability to form cystic structures in the mammary ducts of ovariectomized bigenic mice in the absence of hormone supplementation. Surprisingly, the ducts were similar in size to the control (data not shown). Treatment with estrogen but not progesterone induced expansion of the ductal epithelium in the bigenic but not in monogenic mice. The bigenic ductal size was wider and resembled those shown previously. In addition, progesterone could synergize with estrogen to elicit a more severe manifestation (data not shown).

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Fig. 7. Hormonal responsiveness of FGF-3-elicited hyperplasia. Monogenic and bigenic mice were ovariectomized and treated with RU486 (450 μg/kg) for 2 weeks. Subsequently, 50 μg/kg of estrogen or estrogen/progesterone were given for 3 consecutive days. Sesame oil was used as a vehicle control. Histological analyses showed that the bigenic (Bi-Tg) glands given estrogen manifested multilayered cells, which became more severe with estrogen and progesterone cotreatment. In contrast, a uniform layer of epithelium was shown in all monogenic controls (Ctrl). Vehicle treatment does not alter the bigenic ductal morphology.

The ductal patterning is a highly regulated process that results from end-bud bifurcations and turning maneuvers in response to local environmental signals from the stroma and nearby mammary epithelium. The FGF-3 protein contains a putative signal peptide and it may act primarily as an autocrine or as a short-range growth factor in mammary epithelium (14). The local FGF-3 mitogenic signal enhanced the proliferation rate of the bigenic epithelium as illustrated in the BrdUrd incorporation study. Thus, it is likely that the inability of ducts to elongate is caused by reduced epithelial cell proliferation. Alternatively, disruption of the synthesis and deposition of extracellular matrix material may influence branching pattern and growth of the mammary gland. Several cell-adhesion molecules emerge as the candidates that may interact with FGF-3, and we showed that the expression of E-cadherin was downregulated and became unpolarized in the transformed epithelium of the bigenic gland. In addition, Wnt-1 (21, 22) and transforming growth factor β (23, 24) have been shown to interact intimately with FGF. Thus, it is likely that no single alteration is solely responsible for the phenotypes observed, which may instead be the cumulative result of relatively minor alterations in multiple cellular functions.

Similarly, proper ductal morphogenesis also requires that cell-cell and cell-substrate adhesion systems be coordinated spatiotemporally with epithelial differentiation and apoptosis. In the bigenic gland, FGF-3 increased the proliferation rate of the mammary epithelium as demonstrated in the BrdUrd incorporation assay. On the other hand, terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling apoptosis assay did not show any significant difference between the control and bigenic glands (data not shown). Thus, overexpression of FGF-3 clearly interrupted the balance between apoptotic and proliferating signals in normal mammary epithelium. More importantly, FGF-3 activated the MAPK cascade, which has been shown to be a differentiation signal crucial for alveolar morphogenesis in mammary epithelium cell culture (20). Taken together, these alterations may result in the disorganization of the ductal cells and the persistence of aberrant lobular hyperplasia observed in the virgin bigenic ducts.

FGF-3 is Essential for the Maintenance and Progression of Mammary Gland Hyperplasia. Accumulating evidence shows that tumorigenesis is dependent on the expression of oncogenes. In the deoxy-cyclin inducible H-Ras mouse melanoma model, de-induction of H-Ras resulted in regression of primary and explanted tumors (25). Recently, mammary adenocarcinomas induced by c-myc were shown to require continued expression of c-myc oncogene (26). Similarly, we showed that continuous overexpression of FGF-3 promotes progression of mammary gland hyperplasia and that regression resulted after the withdrawal of RU486. Thus, a continued requirement for FGF-3 in promoting and sustaining non-cell-autonomous cell proliferation is essential for hyperplasia progression and maintenance.

Despite high levels and rapid presentation of phenotypes in the virgin bigenic females, very few females succumbed to mammary tumors in the absence of pregnancy. Most bigenic females developed pronounced hyperplasia after 6 months of RU486 treatment. This finding is consistent with the requirement of pregnancy for tumor development (6–8) and the occurrence of proviral MMTV-induced FGF-3 tumors at a higher frequency in pregnancy than in the virgin nulliparous state (27). Therefore, these studies suggest that a certain differentiated state and hormonal status of the mammary gland may be an important determinant for FGF-3 tumorigenesis. These studies also imply that high levels of FGF-3 expression are insufficient to evoke a full tumorigenic response. Thus, FGF-3, probably facilitates the retention and accumulation of cells susceptible to further somatic mutation and successive mutations in tumor suppressor genes and/or activation of dominant oncogenes are then required for full tumorigenesis.
Hormonal Dependence of FGF-3 Oncogenicity. Ectopic overexpression of various growth factors can confer varying degrees of increased estrogen-independent in vitro and in vivo growth to the estrogen-responsive MCF-7 human breast carcinoma cell (28–30). These findings suggest that signal transduction pathways can provide an alternative or interacting mitogenic stimulus to that supplied by estrogen. Evidently, overexpression of FGF-3 in vivo increased the ability of the mammary epithelium to confer an estrogen-independent proliferation that resulted in mammary hyperplasia in both virgin and ovariectomized bigenic mice. Conversely, participation of estrogen with the FGF-3 mitogenic signal was also shown in bigenic mice. The elevation of estrogen receptor expression by immunohistochemistry with FGF-3 overexpression in the bigenic mice implies that the bigenic epithelium retained an increased estrogen responsiveness (data not shown). Hence, administration of estrogen significantly enhanced the malignant phenotype in the ovariectomized bigenic mice. In addition, progesterone can reinforce the estrogen effect, and the combined treatment consistently conferred a more severe hyperplastic manifestation. A convergence of growth factor and steroid receptor signaling mechanisms is also evident (31–33). It was shown that signaling through growth factors might enhance the activity of the estrogen receptor through MAPK phosphorylation of a serine residue within the transactivating domain of estrogen receptor. This signaling may then activate estrogen receptor in the absence of estrogen or increase the level of estrogen-dependent activation of genes mediated by estrogen receptor. Thus, synergy between FGF-3 and hormonal mitogenic stimuli is probably required to elicit full tumorigenesis and this requirement may account for the pregnancy dependence of FGF-3-elicted mammary tumorigenesis.

In summary, we have established a useful animal model in which a dominantly acting oncoprotein can be somatically regulated in vivo. The system allowed us to show that the overexpression of FGF-3 in pube ncise mice interrupted the intrinsic genetic programs for mammary gland development. In addition, FGF-3 can confer hormonal-independent mitogenicity and plays a critical role in the initiation and maintenance of mammary hyperplasia. However, the combinatorial action from hormonal stimuli is likely a prerequisite for FGF-3 to confer full tumorigenesis.

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Figure 3. Whole mount analysis of transgenic and wild type mammary glands at pregnancy day 17. The mammary ducts of wild type controls and the AIB-1 transgenics are morphological similar (A-C). D, E and F are corresponding higher magnification of A, B and C, respectively.

Figure 4. Whole mount histological analysis of transgenic and wild type mammary glands at lactation day 1. The mammary ducts of AIB-1 transgenics and wild type controls are morphological similar (A-C). D, E and F are corresponding higher magnification of A, B and C, respectively.
Figure 5. Whole mount histological analysis of transgenic and wild type mammary glands after lactating for 6 days and weaning for 2 days. The mammary ducts of AIB-1 transgenics and wild type controls are morphological indistinguishable (A and B). C and D are corresponding higher magnification of A and B, respectively.
Figure 6: Northern analysis of AIB-1 levels with and without mifepristone treatment. AIB-1 mRNA levels are induced upon administration of 250ug/ml of mifepristone to the lactating animals for three days (Lanes 1 and 3). AIB-1 mRNA levels of controls without administration of mifepristone(Lanes 2 and 4). In general, the induction is not very pronounced.
The mifepristone-inducible gene regulatory system in mouse models of disease and gene therapy

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The mifepristone (Mfp)-inducible gene regulatory system is designed to allow control of the spatiotemporal expression of transgenes in vivo in a ligand-dependent manner. This regulatory system is composed of two components: (1) a chimeric transactivator protein that activates transgene transcription only in the presence of the progesterone antagonist Mfp, and (2) a target transgene placed in the context of a promoter which is responsive only to the Mfp-bound chimeric transactivator. Incorporation of the components of the Mfp-inducible gene regulatory system into transgenic mice has resulted in the establishment of several novel, Mfp-dependent models of disease. Similarly, adaptation of the Mfp-inducible system for use in gene knockout models has resulted in the development of new gene ablation technology which is both tissue-specific and Mfp-dependent. Additionally, the Mfp-inducible gene regulatory system has been used in animal experiments involving somatic gene therapy, where it has shown considerable promise in the regulation of both reporter and therapeutic gene expression. This review focuses on recent application of the Mfp-inducible system to transgenic models, gene knockout models, and somatic gene therapy experiments. In so doing, it demonstrates the considerable promise that future use of this system holds for better understanding and treatment of human disease.

Key words: gene regulatory system / mifepristone-inducible / progesterone / transgenic mouse

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Introduction

The generation of transgenic mice through the introduction of foreign genetic material has been a powerful tool for elucidating the physiological and developmental roles of genes over the past two decades. However, in many instances, conventional transgenic and gene ablation models are unsatisfactory for the study of developmental and disease processes. The biggest limitations of these conventional models are the constitutive expression of transgene products or the permanent deletion of genetic material which persist throughout the developmental process. This often leads to deleterious effects that result in prenatal or postnatal death and compromise the ability to maintain transgenic lines for subsequent studies. A better animal model for study would be one in which a target gene could be expressed in a defined developmental or physiological window. Such a model could minimize the lethality or infertility caused by continuous expression or deletion of genes, and could help to identify the primary effects of gene expression in the regulation of a specific physiological process. In doing so, this spatiotemporally controlled model would be a more effective means of analyzing gene function and identifying targets for therapy. To this end, many novel transgenic models have been developed which incorporate gene regulatory systems designed to provide control over transgene expression. These regulatory systems are derived from the many ligand-inducible gene regulatory systems that have been developed over the last two decades.1-6 This review focuses solely on the mifepristone (Mfp)-inducible gene regulatory system, created by our laboratory, and recent applications of this system in transgenic and somatic gene therapy models.

The Mfp-inducible gene regulatory system

The central component of the first-generation Mfp-inducible gene regulatory system is a chimeric transactivator called GLVP (Figure 1). This fusion protein is composed of a VP16 transactivation domain, the DNA-binding domain of Gal4 (Gal4 DBD), and a truncated form of the human progesterone receptor ligand binding domain termed PRLBD-891. PRLBD-891

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differs from the normal progesterone ligand binding domain in that it possesses a deletion of 42 C-terminal amino acids, and this imparts this peptide with unique characteristics. Specifically, PRLBD-891 is capable of binding to antiprogestins such as Mfp, but is unable to bind to endogenous progesterone. When linked to components that enable transcriptional activation, such as VP16 and Gal4 DBD in GLVP, PRLBD-891 can bind to Mfp and initiate transcription at target genes appropriately flanked with specific enhancer sequences. In the case of GLVP, Mfp added to cells expressing this chimeric transactivator is quickly bound by its PRLBD-891 domain. Subsequently, the active, Mfp-bound GLVP dimerizes and is translocated to the nucleus, where its Gal4 DBD interacts with Gal4 upstream activation sequences (UAS) in the promoter of the target gene. This places the VP16 transactivation domain of GLVP in an appropriate location to activate target gene transcription, and the result is induction of target gene expression by Mfp, but NOT by endogenous progesterone.7,8 GLp65, the latest-generation Mfp-inducible gene regulatory system, functions identically to GLVP but differs structurally from GLVP in two important ways (Figure 1). First, the Mfp-responsive element of GLp65, PRLBD-914, is a truncated form of the human progesterone receptor ligand binding domain that possesses a deletion of 19 C-terminal amino acids. When compared to systems such as GLVP which use PRLBD-891, systems using PRLBD-914 demonstrate enhanced transgene transactivation in the presence of Mfp, comparably low basal levels of transgene expression, and responsiveness to concentrations of Mfp an order of magnitude lower than those used with other systems. Second, the virally-derived VP16 transactivation domain of GLVP was replaced by the transactivation domain of p65, a partner of NF-κB in the RelA transcription factor complex. As a result of these modifications, GLp65 demonstrates lower levels of transgene expression in the absence of Mfp, and superior magnitude of target gene induction upon Mfp administration.9,10

Three critical levels of specificity impart spatiotemporal control to strategies which use the Mfp-inducible system in transgenic models. First, tissue-specific promoters are used to express the Mfp-inducible system. Because target genes will only be expressed in cells expressing the Mfp-inducible system, this strategy effectively restricts target gene expression to a particular tissue. Second, because of the intrinsic nature of the Mfp-inducible system, target gene expression occurs exclusively in the presence of Mfp, and levels of expression are often dose-dependent. Third, since mammalian cells do not possess enhancer peptides capable of interacting with UAS, the placement of target genes in the context of a minimal promoter and UAS ensures minimal basal transcription of the target gene. Additionally, the Mfp-inducible system, which possesses the Gal4 DBD, has the ability to bind ONLY to copies of UAS that flank the target gene and is therefore incapable of activating the transcription of other, endogenous genes. Taken together with the excellent bioavailability and tissue distribution of Mfp in the mammalian body, these characteristics of specificity in the Mfp-inducible system make it highly amenable to the control of transgene expression and ablation in transgenic models.

**Transgenic models of Mfp-inducible gene regulation**

The Mfp-inducible gene regulatory system has demonstrated remarkable utility in the study of gene function
The mifepristone-inducible gene regulatory system in mouse models of disease and gene therapy

Mifepristone-Inducible Transactivator Founder Line

Tissue-Specific Promoter

GLVP

SV40 p(A)

Target Gene Founder Line

(UAS)$_4$

tk

Target

SV40 p(A)

Founder Lines crossed

Bitransgenic Mice

Simultaneously harbor both expression cassettes from founder lines

+Mfp

in vivo Target gene expression

Figure 2. Strategy for incorporation of the Mfp-inducible system into transgenic models. Separate transgenic mouse founder lines are generated which harbor expression cassettes essential to the Mfp-inducible gene regulatory system. The Mfp-inducible transactivator founder line expresses the Mfp-inducible chimeric transactivator (GLVP or GLp65) from a tissue-specific promoter. The target gene founder line contains the target transgene placed in the context of four copies of the Gal4 upstream activation sequence ((UAS)$_4$) and a minimal thymidine kinase promoter (tk). Crossing of these founder lines results in bitransgenic mice which respond to Mfp administration with dose-dependent, tissue-specific expression of the target gene.

for a variety of transgenic models. Regardless of the application, however, the same strategy is commonly employed by different investigators to generate transgenic mice expressing tissue-specific, Mfp-inducible target genes (Figure 2). Initially, constructs encoding the Mfp-inducible gene regulatory system and target genes are integrated into separate transgenic lines. Typically, as mentioned above, expression of the Mfp-inducible system is driven by a tissue-specific promoter, ensuring tissue-specific expression of the target gene. The target gene, by contrast, is placed in the context of a minimal promoter preceded by multiple copies of the UAS to ensure that the expression of the target gene is linked to activation of the Mfp-inducible system. These separate transgenic founder lines are then crossed to generate bitransgenic mice simultaneously harboring both the tissue-specific, Mfp-inducible system and the target gene. Analysis of the consequences of tissue-specific, Mfp-inducible target gene expression is then done on these bitransgenic mice.

The work of Wang et al., which represents the first application of the Mfp-inducible system to transgenic mice, was designed to demonstrate the enormous potential of using this system to drive liver-specific, Mfp-inducible transgene expression in mammalian models. The bitransgenic mice generated in this study, called TTR-GLVP/human growth hormone (hGH) mice, possessed integrated cassettes encoding GLVP in the context of a liver-specific transthyretin (TTR) promoter/enhancer, as well as the hGH gene in the context of a minimal thymidine kinase (tk) promoter and four copies of the Gal4UAS. In the absence of Mfp, minimal levels of hGH were detectable in the serum of TTR-GLVP/hGH mice. However, upon a single administration of Mfp, a 5800–33,000-fold increase in serum hGH levels was observed. Further investigations revealed that this induction was repeatable in the same mice 3 weeks later, and that the levels of hGH expression were dependent on the dose of Mfp administered. Studies of the kinetic profile of hGH gene expression after Mfp-induction revealed that, regardless of the dose of Mfp administered, hGH expression was maximal 12 h after Mfp administration and returned to baseline levels within 100 h of Mfp administration. Finally, in conjunction with these pharmacokinetic studies, preliminary analysis of the phenotypic consequences of Mfp-inducible
transgene expression was performed. While no differences in size or weight were observed between TTR-GLVP/hGH bigenic mice and controls in the absence of Mfp, TTR-GLVP/hGH bigenic mice receiving intraperitoneal injections of Mfp every second day showed a 50–57% increase in weight within 10 days, and 50–60% more weight gain with continued Mfp administration over the next 17 days.11

The above investigations represented the first in vivo pharmacokinetic studies involving transactivation of a target gene in response to administration of an exogenous ligand, and concomitant characterization of a gross phenotype in response to target gene expression. Not surprisingly, the Mfp-inducible gene regulatory system in transgenic animals was subsequently exploited in studies examining the role of ligand-inducible transgene expression in both the rescue of lethal null phenotypes as well as the pathophysiological consequences of gene overexpression in adult mice. Beginning with the TTR-GLVP founder line of Wang et al., which was engineered to express liver-specific GLVP, and crossing this line with another founder line containing an Inhibin A gene in the context of a minimal tk promoter and four copies of UAS, Pierson et al. generated bitransgenic mice, termed inh/glvp mice, which demonstrated liver-specific, Mfp-inducible expression of Inhibin A in a dose-dependent manner. Establishment of this working model of Mfp-inducible Inhibin A expression allowed investigation into the effects of overexpression of Inhibin A in inh/glvp mice. Induction of supraphysiological Inhibin A levels with Mfp led to a 2.6-fold reduction in serum FSH levels in male inh/glvp mice relative to controls. Moreover, male inh/glvp mice implanted with extended-release Mfp pellets had significantly reduced testis weight and a decrease in seminiferous tubule volume and diameter. Gonadal effects, manifested as an arrest in folliculogenesis at the early antral follicle stage and lack of copora lutea, were also seen in female inh/glvp mice expressing supraphysiological levels of Inhibin A in response to Mfp treatment. Subsequently, the inh/glvp model was applied to the rescue of the lethal Inhibin A null phenotype in male mice. By breeding the inh/glvp system into an Inhibin A null genetic background and then activating Inhibin A expression with implantation of extended-release Mfp pellets at 8 weeks of age, Pierson et al. also demonstrated that overexpression of Inhibin A with the Mfp-inducible system could prevent the sex cord stromal tumors and cachexia-like syndrome associated with the Inhibin A null phenotype in males.12

The work of Zhao et al. provides another impressive example of use of the Mfp-inducible gene regulatory system, in conjunction with transgenic mouse models, to investigate the physiological roles of overexpressed genes in adult mice. To investigate the phenotypic consequences of fibroblast growth factor receptor-2 (FGFR-2) activation in the adult mouse lung, the latest generation Mfp-inducible gene regulatory system, GLp65, was used to express fibroblast growth factor-3 (FGF-3), one of many ligands that activates FGFR-2, in a lung-specific, Mfp-inducible manner. Bi-transgenic mice, termed GLp65/UASG-FGF-3 mice, were generated in which lung-specific GLp65 expression was driven by a human surfactant protein-C (SP-C) promoter, and a copy of the FGF-3 gene was placed in the context of an elastase promoter and four copies of UAS. Four independent lines of GLp65/UASG-FGF-3 bitransgenic mice displayed the dose-dependent, Mfp-inducible gene expression characteristics of the bitransgenic mice previously reported by Wang et al. and Pierson et al. However, the GLp65/UASG-FGF-3 mice also demonstrated some additional and important pharmacokinetic and phenotypic characteristics not previously reported by either Wang et al. or Pierson et al. First, the basal levels and induced levels of FGF-3 for a given dose of Mfp were largely dependent on the relative expression levels of GLp65, which differed between the four different bitransgenic lines. Lines expressing low levels of GLp65 showed no detectable basal expression of FGF-3, and low levels of FGF-3 expression upon administration of Mfp. The mouse line expressing the highest level of GLp65 showed low basal expression of FGF-3, as well as the highest level of FGF-3 expression upon Mfp administration. Second, the phenotypic consequences of FGF-3 expression were correlated with the levels of FGF-3 expression and, consequently, with the dose of Mfp administered. Low doses of Mfp resulted in no microscopically apparent phenotype, while high doses resulted in both macrophage infiltration and type II cell proliferation in the mouse lung. Finally, the phenotypic consequences of induction of FGF-3 expression were both reversible and repeatable, as histological changes underwent nearly complete regression when Mfp was removed for 2 weeks and could be reinstated by subsequent readministration of Mfp.13 Taken together, these various inducible transgenic models demonstrate the enormous utility and versatility, in terms of both location and level of transgene expression, afforded by the incorporation of the Mfp-inducible system.
Mfp-inducible gene ablation models

Like transgenic models involving constitutive gene overexpression, transgenic models involving germline gene disruption are often limited by the facts that gene disruption occurs throughout the body of the engineered animal, and disruption of the gene of interest is often accompanied by developmental defects and compensatory regulation of other genes throughout the developmental process. Recent modification of the Mfp-inducible gene regulatory system has resulted in the development of a spatiotemporally restricted gene targeting system allowing ablation of a target gene in a specific tissue and in response to Mfp administration. To generate this system, Cre-recombinase was fused to the N-terminus of a truncated version of the progesterone receptor, termed PRI, which is bound and activated by Mfp but not endogenous progesterone. In the absence of Mfp, the Cre-recombinase-PRI fusion protein (CrePRI) is retained in the cytoplasm and remains inactive. Upon administration of Mfp, however, CrePRI translocates to the nucleus and mediates site-specific recombination between DNA sequences termed loxP sites. By flanking a given genomic region or target gene with loxP sites, and by expressing CrePRI with a tissue-specific promoter, tissue-specific, Mfp-dependent gene knockout can be accomplished. Analogously, gene sequences engineered to interfere with the expression of a particular gene can be flanked with loxP sites. Then, using tissue-specific expression of CrePRI in combination with Mfp administration, the interfering gene sequence can be removed and gene expression can resume in a given spatiotemporal window. Of course, because use of the CrePRI system relies on genomic recombination, this system differs from the GLVP and GLp65 systems in that the induction of gene expression or gene ablation by Mfp will be permanent for a given cell type expressing CrePRI and, if CrePRI is expressed in a stem cell population, has the potential to be permanent for the life of the entire animal.

Recent use of the CrePRI system in the skin of transgenic mice has played an important role in the generation of mouse models of both the Dowling–Meara variant of epidermolysis bullosa simplex (EBS-DM) as well as epidermolytic hyperkeratosis (EHK). To generate a model mimicking EBS-DM at both the phenotypic and genetic levels, and to ensure the viability of mice with these genetic changes, Cao et al. utilized the CrePRI system to activate expression of a mutant form of keratin-14 (K14) in the keratinocytes of adult mice in a Mfp-dependent manner. Bitransgenic mice were generated which possessed an epithelial cell-specific CrePRI expression cassette, as well as a mutant K14 expression cassette with the ability to become transcriptionally active only after undergoing Cre-mediated excision of a neogene in its first intron. In bitransgenic pups exposed to topically applied Mfp, keratinocyte CrePRI was activated, and the mutant K14 gene was rendered transcriptionally active. As a result, after 2-7 days of Mfp treatment, blisters filled with fluid and histologically consistent with the EBS-DM phenotype were observed on the front legs and paws of pups. In an almost identical approach, Arin et al. established an inducible model of EHK using Mfp-mediated activation of epithelium-specific CrePRI to activate expression of a mutant form of keratin-10 (K10). Similar to what was seen with the bitransgenic EBS-DM mice of Cao et al., bitransgenic pups generated by Arin et al. developed phenotypic lesions characteristic of EHK upon topical administration of Mfp. Development of these disease models using the Mfp-inducible CrePRI system has led to a greater understanding of the pathophysiology of the respective disease processes, and has had serious implications for the development of therapeutic strategies designed to treat these diseases.

It is worthwhile to note that the feasibility of generating Mfp-inducible gene excision models using CrePRI has been investigated for both the postnatal heart and adult brain of mice. Minamino et al. engineered a transgenic mouse line expressing cardiac-specific CrePRI by placing the CrePRI gene under transcriptional control of the αMHC promoter. Bitransgenic mice resulting from a cross of this line with the ROSA26 Cre-reporter line exhibited markedly increased expression of lacZ in the heart upon systemic administration of Mfp at either 5 or 6 weeks of age. Using a similar strategy, Kellendonk et al. and Kitayama et al. generated transgenic mouse lines expressing brain-specific CrePRI through the use of the CamKIIα, Thy-1, and GluRδ2 gene promoters. Bigenic mice generated by crossing these mouse lines with Cre-reporter lines showed strong Mfp-dependent expression of lacZ in either the hippocampus and cortex when the CamKIIα or Thy-1 gene promoters were used to drive CrePRI expression, or in the Purkinje cells when the GluRδ2 gene promoter was used to drive CrePRI expression. While the above studies utilized only reporter gene expression to demonstrate the utility of the CrePRI system in generating Mfp-inducible gene ablation models, it is apparent that such spatiotemporally controlled models will have an important role in analyzing the molecular
function of genes in the postnatal heart and mature brain.

Application of the Mfp-inducible system to gene therapy

Recent advances towards clinical application of human gene therapy have made it apparent that some therapeutically relevant applications of this technology will require, among other things, a means to control the temporal expression, and therefore the levels, of therapeutic gene products. The Mfp-inducible gene regulatory system, and specifically GLp65, has become one of the more promising candidate gene regulatory systems for use in future gene therapy protocols. This is due not only to its reliable transactivation of target gene expression with sub-therapeutic dose administration of Mfp, but is also due to the extensive knowledge of Mfp pharmacokinetics and the very low side effect profile of this drug in humans. The utility of the Mfp-inducible system has been consistently demonstrated in animal experiments differing markedly in their approaches to somatic gene transfer.

The large capacity for foreign DNA that is possessed by helper-dependent adenoviral (HD-Ad) vectors has recently made the simultaneous packaging of a therapeutic gene and the Mfp-inducible gene regulatory system into a single recombinant adenovirus a reality. The work of Burcin et al. used such an HD-Ad vector engineered to simultaneously carry expression cassettes encoding both GLp65 driven by a liver-specific TTR promoter/enhancer, and the hGH gene placed in the context of four copies of the UAS and a TATA box. After introduction of this HD-Ad vector to the livers of C57BL/6 mice through tailvein injection, efficient induction of hGH expression could be accomplished by administration of Mfp. Moreover, Mfp-dependent induction of hGH expression could be repeatedly demonstrated numerous times over a period of 1 year in these mice. These results, which demonstrate both persistent and inducible transgene expression in a somatic gene therapy context, provide one of the best examples to date of the therapeutic potential of combining HD-Ad vector-mediated delivery with the Mfp-inducible gene regulatory system.

Direct muscle injection coupled to electroporation, a relatively efficient nonviral gene transfer method, has also been used in conjunction with the Mfp-inducible gene regulatory system. In a study designed to test Mfp-inducible transgene regulation after nonviral gene delivery, Abruzzese et al. simultaneously delivered two plasmids to the hind-limb muscles of adult BALB/c mice using direct DNA injection and electroporation. One plasmid encoded the Mfp-inducible gene regulatory system, while the other plasmid encoded the secreted human placental alkaline phosphatase (SEAP) gene in the context of a promoter flanked by UAS. The average magnitude of SEAP induction in response to Mfp administration was 14-19-fold, and multiple rounds of drug-dependent regulation of SEAP expression could be demonstrated over a period of approximately 3 weeks. In a similar set of experiments, Abruzzese et al. also demonstrated the ability of the Mfp-inducible system to regulate expression of vascular endothelial growth factor (VEGF) and erythropoietin (EPO) after delivery of relevant plasmids to the hind-limb muscles of adult mice using direct DNA injection and electroporation.

The overall success of these studies in animal models has shown the feasibility of combining the Mfp-inducible gene regulatory system with nonviral gene delivery methods such as electroporation.

Conclusions

In summary, GLVP and GLp65 have proven to be powerful systems which enable investigators to achieve tissue-specific, Mfp-inducible expression of transgenes in vivo. Furthermore, the CrePRI system, which is related to GLVP and GLp65, has proven to be an impressive tool in the generation of tissue-specific, Mfp-inducible gene ablation models. All three Mfp-inducible systems have demonstrated the remarkable potential that spatiotemporally controlled transgenic models have for overcoming the limitations of developmental defects and compensatory regulation that invariably occur during the developmental process using conventional transgenic and gene ablation technology. In addition, GLp65 has proven to be a feasible system for the regulation of therapeutic transgenes in the context of somatic gene therapy. It seems likely that these systems will play an important future role in both creating models that elucidate the pathophysiology of human disease and developing practical therapeutic options for the treatment of human disease.

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