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Cancer is a complex multistep disease and progresses through successive accumulation of genetic mutations. Loss of tumor suppressor gene p53 and activation of oncogene Neu/ErbB2 are among the most frequent genetic alterations in human breast cancer. We performed a retroviral insertional mutagenesis screen to identify genes that may contribute to mammary tumor formation in conjunction with deregulated p53 or Neu. Multiple proviral insertions from independent tumors were identified to be located within introns of the F-box gene Fbw4, suggesting that the structural alteration at this locus may provide selective growth advantage. The viral integrations result in marked overexpression of a novel, naturally occurring Fbw4 short isoform, which is also spontaneously enriched in several mouse and human breast cancer cell lines but not in non-transformed mammary epithelial cells, thus appears to be associated with malignant transformation. Overexpression of this short isoform in the normal mouse mammary epithelial cell leads to anchorage-independent growth in soft agar. Taken together, these observations indicate that aberrant expression of the short Fbw4 isoform observed in MMTV-induced tumors and spontaneous breast cancer cell lines may contribute to mammary tumorigenesis.

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Introduction

Cancer is a complex multistep disease and progresses through accumulation and cooperation of genetic mutations. During tumor evolution, the preceding oncogenic events may dictate the need for subsequent mutations. Elucidation of collaborative tumorigenic pathways is central to understanding and treatment of cancer.

p53 is a tumor suppressor gene commonly altered in human cancers including breast cancer. Loss of p53 activity gives an advantage to tumor growth (Vogelstein et al, 2000). Mice with a reduced p53 gene dosage are also predisposed to tumorigenesis in part due to p53 haplo-insufficiency (Venkatachalam et al, 1998). Indeed, p53 haplo-insufficiency collaborated with other oncogenic events to significantly decrease tumor latency (Macleod and Jacks, 1999). On the other hand, Neu/Her2/ErbB2 is an oncogene frequently amplified and overexpressed in human breast cancer (Hynes and Stern, 1994). Transgenic mice expressing elevated levels of Neu in mammary epithelium produced mammary gland carcinomas with high efficiency (Hutchinson and Muller, 2000). However, the stochastic appearance of these tumors indicates that additional genetic lesions are required to fully transform mammary epithelial cells.

Retroviral insertional mutagenesis using mouse mammary tumor virus (MMTV) has been a powerful mammalian genetic approach to discovering cancer genes (Callahan and Smith, 2000). MMTV infects and transforms mammary epithelial cells by insertional mutation of cellular proto-oncogenes or tumor suppressor genes. Mammary cells carrying such oncogenic insertions confer a growth advantage and preferentially grow out to become a malignant tumor. Due to the essentially random viral integration into the host genome, common viral insertion sites are assumed to encode cancer-relevant genes (Mikkers et al, 2002).

We sought to identify and characterize new genetic components that participate in the development of mammary tumors in conjunction with stipulated oncogenes. In the present study we carried out a viral insertional mutagenesis screen by infecting p53 heterozygous and MMTV-Neu transgenic mice with C3H MMTV. We isolated and characterized genes that were targeted by MMTV integration in the arising mammary tumors, including previously known proto-oncogenes Wnts (Wnt-1, -3a, and -10b), and novel regulatory genes such as the F-box and WD40-repeat (fwd) gene Fbw4 and the forkhead-domain (fkh) transcription factor HFH1/FoxQ1.

The canonical Wnt signaling pathway plays critical roles in normal and malignant development (Wodarz and Nusse 1998; Moon et al, 2002; Polakis, 2000). Indeed, the first oncogene identified by MMTV is Wnt1. Transgenic mice expressing Wnt ligands or β-catenin (a key effector of the Wnt pathway) in the mammary glands develop mammary adenocarcinomas. In the absence of Wnt signals, β-catenin is recruited to a 'degradation complex' consisting of APC, Axin and GSK3, and destructed. Wnt signaling inactivates this complex and stabilizes β-catenin. Accumulated β-catenin then translocates into the nucleus where it binds to the TCF/LEF family of transcription factors to activate a transcriptional response. However, the exact mechanism of β-catenin nuclear activity is not fully understood and remains an active area of investigation (Bienz and Clevers, 2003; Tolwinski and Wieschaus, 2004).

Ubiquitin-mediated protein degradation plays a fundamental role in determining the abundance of many critical regulatory proteins (Craig and Tyers, 1999). The ubiquitination pathway requires the covalent attachment of polyubiquitin to substrate proteins. The ubiquitin

transfer reactions involve the ubiquitin-activating enzyme E1, ubiquitin-conjugating enzyme E2 and ubiquitin ligase E3. F-box proteins are generally a component of the SCF (Skp1-Cullin-F-box protein) E3 ubiquitin ligase complex. The F-box is a modular domain linking F-box proteins to the core ubiquitination machinery (Bai et al, 1996), whereas the WD40 repeats serve as a substrate-binding adaptor and selectively recruit cognate ubiquitination targets.

The forkhead/winged helix transcription factors contain a conserved 110 amino acid residues encompassing DNA binding domain and are key players in development and diseases (Lehmann et al, 2003). Several members of this family (e.g. viral oncoprotein Qin, FoxO) are intimately connected to neoplasia (Accili and Arden, 2004; Kaufmann and Knochel, 1996).

Body (Results)

We infected newborn FVB p53+/- or MMTV-neu pups with MMTV by foster nursing on C3H lactating female mice which produced infectious MMTV in their milk. MMTV infected females were later bred continuously with male mice and monitored for spontaneous tumor development.

We performed genomic Southern blot analysis with MMTV env and LTR probes to verify the presence of proviral integrations in the tumors. FVB mice carry germline endogenous MMTV isotypes identified as a characteristic pattern common to all somatic samples (Lee et al, 1995). Newly acquired exogenous C3H MMTV proviral fragments were easily distinguished from the endogenous counterparts by their unique sizes. All tumors from infected wild type mice and p53 heterozygotes, and two thirds of tumors from MMTV-Neu transgenics displayed evidence of integrated exogenous proviral DNA. On average, each tumor harbored approximately 5 ectopic integrations of the exogenous C3H MMTV in the genome.

The integrated proviral DNA serves as a physically linked molecular tag to the activated oncogenes. We used an inverse PCR strategy to isolate virus-host junction DNA fragments, and thus determine the site of viral integration in the host genome (Lee et al, 1995). Initially we isolated both endogenous and exogenous MMTV sequences. Based on their genome sequence polymorphisms, we designed PCR primers that are specific for the exogenous C3H MMTV proviral DNA but divergent from the related endogenous MMTV isotypes. This allowed specific amplification of genomic DNA only flanking the exogenous provirus. Sequence homology 'BLAT' searches against the public mouse genome assembly allowed us to unambiguously map viral insertions (University of California, Santa Cruz, http://genome.ucsc.edu). Genes in the 100kb vicinity of each insertion were examined.

1. To determine the transforming activity of the Fbw4 gene (Tasks 1-3)

We identified the Fbw4 gene as a common viral integration site

A mammary tumor (#7408, derived from a p53+/- mouse) harbored an MMTV provirus integrated in the 5th intron of the F-box gene, Fbw4 (also known as Dactylin, Sidow et al, 1999). Two more independent tumors (#3456, #7153) derived from MMTV-Neu transgenics carried

viral insertions in the same Fbw4 locus (intron 4 and 5). All three insertions were located in the middle portion of the Fbw4 gene, suggesting that Fbw4 is a common viral integration site. Considering the essentially random integration of retroviral DNA and the fact that Fbw4 was targeted in virtually the same way in multiple independent tumors, alteration of the gene likely represents a selected genetic event and reflects its potential role in malignant transformation.

We identified a novel Fbw4 short isoform induced by MMTV insertion

MMTV retrovirus transforms host cells as a consequence of altered expression, structure and activity of cellular proto-oncogenes or tumor suppressor genes. We thus addressed whether the proviral insertions may alter Fbw4 normal expression pattern.

Consistent with previous report (Sidow et al, 1999), Fbw4 is a ubiquitously expressed gene and can be detected as a uniform transcript by Northern blot analysis in every adult mouse organ examined, with an estimated size around 2.8 kb. We then assessed the RNA expression of Fbw4 in mammary tumors with the Fbw4 gene interrupted by MMTV. With a cDNA probe corresponding to Fbw4 exons 6-9 that are downstream of the MMTV integration sites, we observed robust expression of a novel shortened RNA transcript (~1 kb) in the tumors, but not in normal mammary gland. On the other hand, the 2.8 kb full-length RNA message, which was readily detected in normal mammary gland, was virtually absent in these tumors. Moreover, the short Fbw4 RNA transcript did not hybridize with a probe corresponding to Fbw4 exons 1-4 that are upstream of the MMTV insertion sites, thereby representing an evidently truncated RNA species.

We determined the molecular identity of the short isoform

In order to determine the identity of the short Fbw4 transcript, we performed rapid amplification of 5'-cDNA ends (5'- RACE) using template RNA prepared from tumor cells carrying MMTV integration in the Fbw4 locus. We obtained multiple RACE products, all of which contained Fbw4 exons 6-9 and were spliced identically to the Fbw4 cDNA. Surprisingly, several of these cDNAs started from sites in intron 5 which normally should have been removed during RNA maturation. Because the cDNAs were synthesized with oligo(dT) primers and the downstream introns 6-8 were correctly spliced out, these Fbw4 cDNAs were likely derived from fully processed mRNAs that were transcribed from initiation sites within intron 5, thus representing a novel isoform. We believe that transcription of the short Fbw4 isoform is initiated from an alternative, cryptic internal promoter (likely within intron 5), which is strongly activated by the transcriptional enhancer of MMTV provirus integrated upstream.

A tandem genomic duplication in the Fbw4 locus is responsible for the human split hand/split foot malformation (SHFM) disease (de Mollerat et al, 2003). Surprisingly, only a part of the Fbw4 gene is duplicated (de Mollerat et al, 2003), which also corresponds to exons 6 to 9 and conceivably encodes the short Fbw4 transcript. Together these observations imply that a mutated Fbw4 allele caused by viral insertion or genomic rearrangement encodes a shortened, biologically active product.

We determined that the Fbw4 short isoform is spontaneously enriched in some mouse and human breast cancer cell lines

Identification of a novel isoform prompted us to examine Fbw4 expression pattern by Northern blot analysis in established mouse mammary epithelial cell lines, including normal mammary cells (NMuMG) and carcinoma cell lines derived from spontaneous mammary tumors

arising from MMTV-Neu (NAF, NF324, NF980, SMF), or -Ras (SH1.1) transgenic mice. While all cell lines expressed the full-length Fbw4 RNA, several Neu-tumor lines but not the non-transformed mammary epithelial cells showed significant levels of an additional short RNA species. Oncogenes identified by retroviral insertion in mice are frequently implicated in human cancer (Callahan and Smith, 2000). In order to investigate a possible involvement of Fbw4 in human breast cancer, we examined Fbw4 expression in several commonly studied breast carcinoma cell lines (MCF7, MDA-MB-231, -435, -453, -468 and SKBR3). A short Fbw4 transcript was observed in the human breast cancer line MDA-MB-435. Given their similar size and specific hybridization only to the Fbw4 3' probe, the short Fbw4 RNA species observed in cancer cell lines is probably identical to that in MMTV-induced tumors. Therefore, rather than an artificially truncated product resulting from MMTV integration, the short transcript may represent a naturally occurring small isoform only present in breast cancer cells.

We determined that overexpression of Fbw4 short isoform confers oncogenic potential

Given the tumor association of the short isoform, we investigated if elevated levels of the short Fbw4 isoform may functionally contribute to malignant transformation of mammary cells. The non-transformed mouse mammary epithelial cells (NMuMG) exhibited no detectable short Fbw4 transcript and were unable to grow in soft agar. We generated stable clones in these cells with forced expression of the Fbw4 short isoform. These cells displayed a significantly increased frequency to form small anchorage-independent colonies indicative of a transformed phenotype (From $1x10^5$ seeding cells, Fbw4 short transcript-expressing cells were able to form 300-400 small colonies (100-200 um in size), while wild type NMuMG occasionally formed 1 or 2 colonies). However, subcutaneous inoculation of these cells into *nude* mice did not lead to the development of tumors. These results indicate that the short Fbw4 isoform may confer mild transforming activity toward mammary epithelial cells.

We generated transgenic mice expressing a dominant-negative form of Fbw4 in mammary glands

Previously, a similar MMTV insertion in the *int6/eIF3* gene disrupted the structure of one allele and produce truncated molecules that possess dominant negative activities (Rasmussen et al, 2001). Full-length Fbw4 encodes a protein with an F-box motif and seven WD40 repeats (Sidow et al, 1999). The short Fbw4 isoform conceptually encodes a peptide only comprising two C-terminal WD40 repeats that might retain the determinants for association with certain substrates, and therefore might interfere with the ubiquitination reaction mediated by the full-length Fbw4. We designed an artificial dominant inhibitor of Fbw4: a construct contains all the WD40 repeats but lacks the putative F-box. The resultant protein is expected to bind potential Fbw4 substrates but fail to ubiquitinate them. We generated transgenic mice that express this construct in the mammary glands under control of the MMTV long terminal repeat (LTR) promoter/enhancer. However, no mammary tumors have been detected so far in transgenic mice.

We confirmed that Fbw4 is a component of SCF ubiquitin ligase

We performed yeast two-hybrid screen using full-length Fbw4 as a bait and found that Fbw4 interacts with Skp1 via its putative F-box motif. This result is further confirmed by co-immunoprecipitation (co-IP) assay in mammalian cells, suggesting that Fbw4 is truly a component of E3 ubiquitin ligase involved in protein degradation control.

We determined that p63 is a potential substrate for the Fbw4 ligase complex

The identity of the relevant substrate(s) for Fbw4 is unknown. Yeast two-hyb studies

using either the full length or the short isoform of Fbw4 did not lead to any putative interactors with the WD40 repeats. Fbw4 mutation in mice resulted in *dactylaplasia*, a limb defect phenotypically resembling typical human split hand/split foot malformation (SHFM) (Sidow et al, 1999). And indeed, alteration in human Fbw4 gene results in SHFM (de Mollerat et al, 2003). SHFM is genetically heterogeneous, and recently one SHFM locus has been identified as p63 (Ianakiev et al, 2000; van Bokhoven et al, 2001), a p53 family member. p63 protein is degraded in part through a ubiquitin-proteasomal pathway (Ratovitski et al, 2001). These observations raise the possibility that p63 might be a substrate for Fbw4.

We found that Fbw4 binds p63 with its putative substrate-binding region (i.e. WD40 repeats) in a co-IP assay. Moreover, in tumors harboring viral insertions in the Fbw4 locus which likely impairs Fbw4 activity, p63 protein is accumulated. The predominant isoform of p63 lacks the N-terminal transcription activation domain and acts as oncogenic antagonists to p53 (Yang and McKeon, 2000; Hibi et al, 2000). Thus aberrantly accumulated p63 may interfere with p53 tumor suppressor function and contribute to tumor progression.

2. To determine the role of the forkhead gene HFH1 in mammary tumorigenesis(Tasks 4-6)

We identified forkhead/winged helix gene HFH1/FoxQ1 as an MMTV insertion site

Tumor 3453B (derived from MMTV-neu transgenic mouse) contains a single viral insertion at the 3' UTR of HFH-1/Foxq1 (Frank and Zoll, 1998), a member of the forkhead/ winged helix family of transcription factors. This result is interesting since forkhead proteins are important in development and diseases including cancer (Accili and Arden, 2004; Lehmann et al, 2003; Kaufmann and Knochel, 1996).

However, we found that expression of FoxQ1 is toxic. While transient transfection of cells with FoxQ1 gave high level expression, we failed to obtain stable cell lines expressing FoxQ1 after extensive screen of hundreds of clones from rodent fibroblasts NIH3T3 and 10T1/2, normal mouse mammary epithelial cells NMuMG and EpH4, normal human mammary cell MCF10A and human breast cancer line MCF7. Furthermore, FoxQ1 appears to be also toxic in transgenic worm. This prevented us from assessing FoxQ1 transforming activity. In addition, FoxQ1 is not significantly upregulated in the MMTV-induced tumor, neither in established mouse and human breast cancer cell lines. On the other hand, loss of function mutations in FoxQ1 resulted in hair shaft differentiation defects and so-called satin mice with a silky, high sheen coat (Hong et al, 2001). However, no tumor development has been reported. We also examined the adjacent FoxF2 (~50kb away from the viral integration site), and no expression change was observed. Thus, despite a unique viral insertion, the role of FoxQ1 in cancer remains elusive. Further analysis of its oncogenic activity may require modified strategies (e.g. trying p53 null cell lines?).

3. To study transcriptional regulation of β-catenin in the Wnt signaling

We identified multiple Wnt family members, Wnt1, 3a, 10b from our MMTV screen. Wnt signaling is important for mammary gland development and tumorigenesis. This prompted us to further investigate molecular components and mechanisms of this pathway.

We found that the ING family of PHD-finger proteins function as transcriptional coactivators of β-catenin. ING factors were previously identified as candidate tumor suppressors and transcriptional cofactors for the p53 tumor suppressor protein (Garkavtsev et al, 1996, 1998; Nagashima et al, 2003; Shiseki et al, 2003). Here we found that ING proteins physically associate with β-catenin and enhance its nuclear localization. ING proteins can be recruited to Wnt target gene promoters by chromatin-IP assay and augment β-catenin-mediated transcriptional activation of a responsive reporter. Conversely, RNAi-mediated depletion of certain ING factors impairs β-catenin's transcription activity. Finally, overexpression of ING factor potentiates axis-inducing activity of Wnt/β-catenin in *Xenopus* embryos. Together, these results suggest that ING proteins may function as transcriptional cofactors for β-catenin.

Given the well-established oncogenic property of Wnt signaling, the role for ING proteins in cancer may thus be context-dependent. Interestingly, ING proteins as cofactors for both tumor suppressor (p53) and oncoprotein (β-catenin) may represent an important fail-safe mechanism that protects cells from malignant transformation by aberrant Wnt signaling.

KEY RESEARCH ACCOMPLISHMENTS

Identified the Fbw4 gene as a common viral integration site.

Identified a novel Fbw4 short isoform induced by mouse mammary tumor virus insertion.

Determined the molecular identity of the short isoform

Determined that Fbw4 short isoform is a naturally occurring transcript spontaneously enriched in some breast cancer cell lines.

Determined that overexpression of Fbw4 short isoform confers oncogenic potential.

Generated transgenic mice expressing a dominant-negative form of Fbw4 in mammary glands.

Found that Fbw4 is a component of SCF E3 ubiquitin ligase.

Determined that p63 is a potential substrate for the Fbw4 ligase complex.

Identified forkhead/winged helix gene HFH1/FoxQ1 as an MMTV insertion site

Identified a cofactor of β-catenin of the Wnt signaling pathway

Reportable Outcomes

Lu J and Leder P. Growth inhibition by the F-box factor Fbw4. Poster presented at the Cold Spring Harbor Laboratory meeting on "Cancer Genetics & Tumor Suppressor Genes", Cold Spring Harbor, New York. August 2002.

Lu J and Leder P. A potential role for the F-box gene Fbw4 in mammary tumorigenesis. Poster presented at the Salk Institute/EMBL meeting on "Oncogene & Growth Control", La Jolla, California. August 2003.

Lu J and Leder P. A TBL1-containing repressor complex regulates E2F transcription activity and cell cycle. Short talk presented at the 95th Annual Meeting of American Association for Cancer Research, Florida, 2004.

Lu J and Leder P. A short isoform of the Fbw4 gene in mammary tumorigenesis. Manuscript submitted, 2004.

Lu J, Green J and Leder P. The ING proteins function as co-activators of β-catenin. Manuscript in preparation.

Conclusion

Retroviral insertional mutagenesis has been a power approach to cancer gene discovery. We identified Fbw4 as a common viral integration site for MMTV in mouse mammary tumors in the setting of predefined genetic lesions: p53 heterozygotes or MMTV-Neu transgenic mice. Viral integrations result in marked overexpression of a novel, naturally occurring Fbw4 short isoform which is also spontaneously enriched in several mouse and human breast cancer cell lines but not in non-transformed mammary epithelial cells, thus appears to be associated with malignant transformation. Ectopic expression of this short isoform in the normal mouse mammary epithelial cells leads to anchorage-independent growth in soft agar. Together, these observations indicate that aberrant expression of the short Fbw4 isoform observed in MMTV-induced tumors and spontaneous breast cancer cell lines may possess oncogenic properties that contribute to mammary tumorigenesis. We also found that the full-length Fbw4 is a component of SCF E3 ubiquitin ligase and may regulate p63 ubiquitination.

Wnt signaling plays key roles in development and cancer, and Wnt genes are common MMTV insertion sites. We provided evidence that the ING proteins function as coactivators of β-catenin, a central component of the Wnt pathway. This finding should aid in deciphering the mechanisms of Wnt signaling response. Based on the oncogenic feature of Wnt/β-catenin, identification of ING cofactors may provide a potential target to intervene the Wnt pathway.

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