Mnt-Deficient Mammary Glands Exhibit Impaired Involution and Tumors with Characteristics of Myc Overexpression

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Abstract

The proto-oncogene c-Myc plays a central role in cell growth and the development of human tumors. c-Myc interacts with Max and Myc-Max complexes bind to E-box and related sequences to activate transcription. Max also interacts with Mnt but Mnt-Max complexes repress transcription when bound to these sequences. MMTV maps to human chromosome 17p13.3, a region frequently deleted in various human tumors, including mammary gland tumors. Consistent with the possibility that Mnt functions as a Myc antagonist, Mnt-deficient fibroblasts exhibit many of the hallmark characteristics of cells that overexpress Myc, and conditional (Cre/Lox) inactivation of Mnt in mammary gland epithelium leads to adenocarcinomas. Here, we further characterize mammary gland tissue following conditional deletion of Mnt in the mammary gland. We show that loss of Mnt severely disrupts mammary gland involution and leads to hyperplastic ducts associated with reduced numbers of apototic cells. These findings suggest that loss of Mnt in mammary tissue has similarities to Myc overexpression. We tested this directly by using promoter array analysis and mRNA expression analysis by oligonucleotide arrays. We found that Mnt and c-Myc bound to similar promoters in tumors from MMTV-c-Myc transgenic mice, and mRNA expression patterns were similar between mammary tumors from MMTV-Cre/MntKO/CKO and MMTV-c-Myc transgenic mice. These results reveal an important role for Mnt in pregnancy-associated mammary gland development and suggest that mammary gland tumorigenesis in the absence of Mnt is analogous to that caused by Myc deregulation. (Cancer Res 2006; 66(11): 5565-73)

Introduction

Myc family proteins are nuclear phosphoproteins that play a pivotal role in many cellular events, including the control of cell growth, proliferation, differentiation, and embryonic development (reviewed in ref. 1). Moreover, it is well known that the dysregulation of c-Myc is associated with tumorigenesis in a variety of contexts (reviewed in ref. 2). In many tumors and tumor-derived cell lines, myc genes are altered by gene amplification, chromosome translocations, and viral insertion, events that typically lead to deregulation of Myc with increased Myc expression (reviewed in ref. 2). The relevance of these alterations to tumorigenesis is suggested by the ability of forced overexpression of Myc in vivo to cause tumors in a wide variety of tissues, including mammary gland epithelium (3–5).

The Myc family of proteins (c-Myc, N-Myc, and L-Myc) require heterodimerization with Max through related bHLHZip motifs to bind DNA and function as transcription factors (1). As with Myc, heterodimerization with Max allows these proteins to bind the E-box consensus sequence, CACGTG, and it is hypothesized that all of these complexes can compete for binding to shared target sites in vivo (1). Recent experiments showing that Mnt-Max complexes decline when c-Myc is induced during cell cycle entry also suggest that Mnt and c-Myc compete for binding for a limiting supply of Max (6). Thus, activating Myc-Max complexes together with repressive Mnt-Max, Mad-Max, and Mga-Max complexes seem to function as an antagonistic network through the binding and transcriptional regulation of shared target genes.

The MMTV gene maps to human 17p13.3, a region that is commonly deleted in a variety of tumors, including mammary gland tumors (7–10). Although no mutations in MMTV have been described in human tumors to date (11–13), previous studies have shown that frequent loss of heterozygosity (LOH) in 17p13.3, but also in 17p25.1, 8p22, 13q12, and 22q13, correlates with postoperative recurrence in breast cancers (14). LOH involves a region telomeric to p53 at 17p13.1 (14) and clearly indicates the presence of another tumor suppressor gene(s) located in 17p13.3. A candidate tumor suppressor gene in this region is HIC1 (hypermethylated in cancer), which is aberrantly hypermethylated and transcriptionally inactivated in several types of human cancers (15–17). Heterozygous Hic1 mutant mice have no developmental phenotype but are predisposed to malignant tumors, including pulmonary carcinomas, lymphomas, and sarcomas (18). However, these mice do not display mammary gland cancer, suggesting that candidate tumor suppressor genes within 17p13.3 responsible for...
other malignant tumors, such as breast tumors, remain to be identified. A recent study suggests that MNT is a potential tumor suppressor gene in medulloblastoma (19). Chromosome 17p13.3 is also well defined as the Miller-Dieker syndrome critical region. Patients with Miller-Dieker syndrome display severe neuronal migration defects defined by lissencephaly (smooth brain) as well as dysmorphic features consistent with abnormalities in craniofacial development (reviewed in ref. 20).

We recently showed that loss of Mnt causes a hyperproliferation phenotype in mouse embryonic fibroblasts, including accelerated proliferation, increased sensitivity to apoptosis, sensitivity to transformation by H-RasV12 alone, and senescence bypass (ref. 21; also reviewed in ref. 22). In addition, conditional deletion of Mnt in mouse mammary gland epithelium led to the development of mammary adenocarcinomas after long latency. Mnt-deficient fibroblasts and mammary gland epithelium displayed decreased c-Myc, but increased cyclin E and cyclin-dependent kinase (Cdk)-4, consistent with a phenotype resulting from increased relative c-Myc activity. Here we extend these findings to show that loss of Mnt in mammary gland epithelium causes defects in mammary gland development and severely disrupts involution. We also used genome-wide location analysis (GWLA) to examine promoter binding of c-Myc and Mnt in tumors arising from overexpression of c-Myc, and oligonucleotide expression arrays to compare mRNA expression patterns between tumors resulting from loss of Mnt or overexpression of c-Myc. Our results indicate an important role for Mnt in mammary gland development and suggest that tumorigenesis caused by loss of Mnt and overexpression of c-Myc are functionally related.

Materials and Methods

Generation of Mnt mutant mice. The production of mice with a Mnt conditional knockout (Mntfloxed) allele by gene targeting is described elsewhere (23). Briefly, a loxp-flanked hygro resistance gene and third loxp were inserted into introns 3 and 6, respectively, of the Mnt gene by gene targeting. Ella-Cre transgenic mice in an FVB/n background (24) were mated with inbred 129/S6 Mnt+/+ mice to generate the Mntfloxed allele in a mixed background (129/S6 × FVB/n) mice in vivo. To produce mice with loss of Mnt in the mammary gland (MMTV-Cre/MntKO/CKO mice), MMTV-Cre mice were mated with Mntfloxed mice to produce MMTV-Cre/MntKO/CKO mice. MMTV-Cre+/MntKO/CKO mice were produced by breeding MMTV-Cre+/MntKO/CKO mice with Mntfloxed mice. We used MMTV-Cre+/MntKO/CKO mice without MMTV-Cre as control. MMTV-Cre+/MntKO/CKO mice displayed no mammary gland tumors.

Genotyping analysis. Adult mice were genotyped by PCR analysis using tail DNA. The following primers were used to detect wild-type, conditional, or deleted allele. MntWT-sense (5′-cagtcgcttgagagagaag-3′) and MntWT-rev (5′-ccgggctgccacgaccaa-3′) were employed for wild-type allele detection. MntKO3 (5′-cagtcgtttcagaaagctc-3′) and MntKO4 (5′-gagccagtggagaagaaag-3′) were used for deleted allele detection. The conditions of amplification were 35 cycles at 94°C for 30 seconds, 68°C for 60 seconds, and 72°C for 120 seconds. This PCR reaction generates a 386-bp fragment from mutant allele and a 147-bp from wild-type allele. To distinguish wild-type and conditional mutant alleles, two primers (sense, 5′-cagtttcagtcgcttgagagagaag-3′; antisense, 5′-gtctcaagtcgtgggcattg-3′) were used to amplify a 147-bp product from the wild-type allele and a 1.9-kbp product from the conditional allele. The results from this PCR were confirmed by detection of the hygro resistance gene with primers for hygro (sense, 5′-tgatgagggagggctgata-3′; antisense, 5′-gtctcaagtcgtgggcattg-3′). The MMTV-Cre allele was detected with the primers CRE1A (5′-ccgggctgccacgaccaa-3′) and CRE2A (5′-ggcgggctgccacgaccaa-3′).

Whole-mount analysis of mammary gland. Mammary gland tissues were dissected at various developmental stages from the two genotypes (MMTV-Cre+/MntKO/CKO and MMTV-Cre-/MntKO/CKO), spread on glass slides, and fixed immediately with Carnoy's fixative (6 volumes ethanol; 4 volumes chloroform; 1 volume acetic acid) for 5 hours at room temperature. After 3 days of acetone treatment to remove lipid, tissues were hydrated with serially diluted ethanol. Hydrated tissues were stained with 0.6% carmine solution for 1 hour at room temperature, followed by treatment with differentiating solution (36% ethanol, 18% methanol). Dehydrated tissues with ethanol were cleared with benzyl alcohol/benzyl benzoate (1/2) solution.

Histologic analyses. Mammary gland tissues (n = 4-5 for each genotype) were dissected at various stages and fixed in 4% paraformaldehyde/PBS solution for 15 hours at 4°C. Fixed tissues were embedded in paraffin, sectioned at 5 μm, and stained with H&E.

Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling analysis in the developing mammary gland. Mammary gland tissues fixed with 4% paraformaldehyde/PBS were embedded in paraffin, sectioned at 5 μm, and deparaffinized. Apoptotic cells were detected by using the ApopTag kit (Intergen Company, Purchase, NY) according to the instructions of the manufacturer. For quantification analysis, terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL)–positive cells in 100 × 150-μm area were counted in both MMTV-Cre+/MntKO/CKO mice and MMTV-Cre-/MntKO/CKO mice.

Western blot analysis. Tumors and adjacent normal tissue from MMTV-Cre+/MntKO/CKO or from wild-type C57/B6 mice were dissected and lysates were prepared as previously described (25). Antibodies against Mnt and c-myc were used as previously described (26).

Kaplan-Meier analysis. To analyze mammary gland tumor development, mice were longitudinally followed 10 MMTV-Cre+/MntKO/CKO mice and 9 MMTV-Cre-/MntKO/CKO mice as controls. All mice were checked weekly for mammary gland tumor development by palpation and mice were sacrificed when they displayed a 1-cm mammary gland tumor. The time from birth was plotted on the X axis and mammary gland tumor-free survival (as percentage of total number of mice) was plotted on the Y axis. The censored subjects are shown as solid squares or triangles in the horizontal part of the staircase and include all deaths not associated with mammary gland tumors.

Statistical analysis. H&E-stained sections (five to seven sections per mouse) of mammary gland for each developmental stage were used to determine the ductal area by using Microanalyzer software (Nihon Poladigital, Tokyo, Japan). The percentage of enlarged ducts was calculated on the basis of the measurement of ductal area. "Enlarged duct" was defined as the ducts in which the area was larger than the area of wild-type (the average area of wild-type was 1,500 μm²). Statistical significance was determined by two-sided Student's t test.

Preparation of chromatin for microarray analysis. A DNA microarray containing PCR products spanning the proximal promoters of 1,920 mouse genes (M2K, Supplementary Table S1) was chosen from the NCBI RefSeq database. These genes were selected for good gene annotation and a clear functional association. Tumors were harvested and cross-linked in formaldehyde [11% formaldehyde/0.1 mol/L NaCl/1 mmol/L Na-EDTA/0.5 mmol/L Na-EGTA/50 mmol/L HEPES (pH 8.0)] and incubated on ice for 10 minutes. The cross-linking reaction was stopped with a 1/20 volume of 2.5 mol/L glycine solution. Following cross-linking, chromatin was extracted in lysis buffer [0.2 mol/L NaCl/1 mmol/L EDTA/0.5 mmol/L EGTA/10 mmol/L Tris (pH 8)/protease inhibitor cocktail] for 10 minutes, followed by centrifugation at 2,000 × g for 10 minutes at 4°C.

Chromatin was fragmented using sonication on ice and immunoprecipitated using magnetic beads coupled to primary antibodies to c-Myc and Mnt as described (27). The beads were collected and the supernatant was removed. The chromatin was eluted from the beads using elution buffer [50 mmol/L Tris (pH 8)/0.1 mmol/L EDTA/1% SDS]. The cross-link was reversed and DNA was purified and prepared for labeling and microarray hybridization through random primer amplification. The samples were then hybridized to the M2K Microarray slides and analyzed using previously described protocols (28). The antibodies to c-Myc (28) and Mnt (6) have been validated previously for chromatin immunoprecipitation. To validate the array data, genomic sequence primers encompassing Bdl1, Sap18,
Cdkn1b, Rab18, and Brca1 promoter regions that bound Mnt and c-myc were used to amplify immunoprecipitated DNA. The following primers, corresponding to promoter regions of each gene, were used for the chromatin immunoprecipitation analysis: Ibd1 sense 5'-CACCTGGGGTCA-GAACATCT-3', antisense 5'-CTGTTGGAAGATTGGGCTGT-3'; Sap18 sense 5'-CTTTGCACCTGTTCCTTCTAT-3', antisense 5'-GGCCGGCTCTCTCTATCCTTAATTTCC-3'; Cdkn1b sense 5'-CTAGCCACCGAAGCTCCTAA-3', antisense 5'-TGTCCTCCTAATGGCATGGAG-3'; Rab18 sense 5'-TGATGTGGGGAGA-CACCTGA-3', antisense 5'-GGCAGAGCTGCTGTAAATCC-3'; and Brca1 sense 5'-TCTCTTGGAAGATTGGGCTGT-3', antisense 5'-GGCAGAGCTGCTGTAAATCC-3'.

Expression arrays analysis. Expression microarrays were done on the Affymetrix 430 2.0 mouse microarray chips. This chipset represents some 45,000 genes and single-nucleotide polymorphisms from the mouse genome. Total RNA was isolated from two Mnt KO/CKO, MMTV-Cre mammary tumors and two c-Myc transgenic mammary tumors. The RNA was labeled and hybridized to the array using standard protocols. Microarray data were preprocessed via the Robust Multichip Average method (29) as implemented in Bioconductor.10 a suite of programs for the R statistical programming language.11 Robust Multichip Average preprocessing consists of three steps: background adjustment, normalization, and summarization (29). Analysis of expression was done using bioweight analysis (30). Bioweight is designed to produce a higher true positive rate compared with traditionally used t test– and P value–based methods of analysis, especially for smaller sample sets. Bioweight takes into consideration the across-replicate effect of traditional fold change analysis as well as the negative decimal log of P for the gene specific t tests. This method allows the user to retain overexpressed/underexpressed genes that may be of biological significance as well as retain statistical stringency corresponding to a small P value. Therefore, the bioweight value can be described as the product of average fold change and negative log of P value, accounting for small P values and large fold changes and providing a smooth transition between the two. For a detailed explanation of bioweight analysis, see Rosenfeld et al. (30).

Results

Morphologic analysis of mammary gland development. To determine if loss of Mnt in the mammary gland was associated with developmental abnormalities, we analyzed mammary gland development during pregnancy, lactation, and involution in MMTV-Cre+/Mnt KO/CKO mice and MMTV-Cre-/Mnt KO/CKO control mice by whole-mount (Fig. 1A-L) and histologic sections (Fig. 1M-X). Virgin mammary glands (V, Fig. 1A, G and M, S) and mammary gland development during pregnancy (P17, Fig. 1B, H and N, T) and during and lactation (L8, Fig. 1C, I and O, U) were indistinguishable between genotypes. However, mammary glands from MMTV-Cre+/ Mnt KO/CKO mice displayed defects characterized by enlarged ducts during involution after weaning (arrows in Fig. 1J, K, L, W, X). In control mice, involution was advanced between 2 days postweaning (I2, 2 days after removal of pups; Fig. 1D and P) and 5 days...
postweaning (I5, 5 days after removal of pups; Fig. 1E and Q) whereas the mammary gland epithelial tree was greatly reduced by day 16 (I16, 16 days after removal of pups; Fig. 1F and R). By contrast, in MMTV-Cre+/MntKO/CKO mice, involution was delayed at I2 (Fig. 1F and V), I5 (Fig. 1K and W), and I16 (Fig. 1L and X) postweaning compared with controls. At the end of involution, the ducts of MMTV-Cre+/MntKO/CKO mice were still enlarged in comparison with those of MMTV-Cre+/MntKO/CKO mice (Fig. 1L and X, arrows). These changes were quantitated by calculating the area of ducts in histologic sections (Fig. 2), showing significant differences between genotypes at I5 and I15.

Reduced apoptosis during involution of the postlactation mammary gland. As involution occurs predominantly by apoptosis, we did TUNEL analysis on sections from involution days 2, 5, and 16 (I2, I5, and I16). In MMTV-Cre−/MntKO/CKO mammary alveoli, apoptosis peaked at involution day 2 (Fig. 3A, C, and E). In contrast, apoptosis peaked at involution day 5 in MMTV-Cre+/MntKO/CKO mice (Fig. 3B, D, and F). The percentage of TUNEL+ cells was calculated in alveoli (Fig. 3G) and ducts (Fig. 3H), respectively. In alveoli, the peak level of apoptosis in MMTV-Cre+/MntKO/CKO mice was delayed compared with MMTV-Cre−/MntKO/CKO mice (Fig. 3G). In addition, a decreased number of apoptotic cells were observed in the ducts of MMTV-Cre+/MntKO/CKO mice (Fig. 3H).

These results are consistent with an important function for Mnt in the regulation of apoptosis in both alveoli and ducts during involution.

Accumulated apoptotic defects resulted in the enlargement of mammary ducts. We calculated the percentage of enlarged ducts (Fig. 4A) and average area of ducts in aged virgin female mice (Fig. 4B) and aged nonvirgin female mice that had undergone multiple (6–8) pregnancies (Fig. 4C). Nonvirgin MMTV-Cre+/MntKO/CKO mice displayed ~90% enlarged ducts in comparison with MMTV-Cre−/MntKO/CKO mice, which had only 40% enlarged ducts, and virgin MMTV-Cre+/MntKO/CKO mice and MMTV-Cre−/MntKO/CKO mice, which showed 30% and 50% enlarged ducts, respectively. The enlargement of ductal area is obvious in mammary glands after multiple pregnancies (Fig. 4C) in comparison with virgin mice (Fig. 4B). These results strongly suggest that the high percentage of enlarged ducts in nonvirgin MMTV-Cre+/MntKO/CKO mice results from accumulation of ductal material due to decreased apoptosis during successive involutions postpregnancy.

c-Myc and Mnt expression during mouse mammary gland development. To support the notion that Mnt is essential for mammary gland development during pregnancy, lactation, and involution, we analyzed Mnt and c-Myc expression by Western blotting (Fig. 5). Mnt was detectable at different stages during mammary gland development in MMTV-Cre+/MntKO/CKO mice but its expression did show small fluctuations. In MMTV-Cre−/MntKO/CKO mice, Mnt expression was strongly reduced as expected. Compared with Mnt, c-Myc expression was highly variable during mammary gland development. c-Myc increased in MMTV-Cre+/MntKO/CKO mice during pregnancy, whereas during involution, there was a reduction then an increase in c-Myc. Similar changes in c-Myc were observed in MMTV-Cre+/MntKO/CKO mice. These results suggest that reduced expression of Mnt causes an imbalance of c-Myc expression, and this may be one cause of mammary tumor development in MMTV-Cre+/MntKO/CKO mice.

Mammary gland–specific loss of Mnt results in mammary adenocarcinoma. As previously described (21), we used MMTV-Cre mice (31, 32) to produce 11 MMTV-Cre+/MntKO/CKO mice that were continuously mated to promote loss of Mnt specifically in mammary gland epithelial cells. Nine continuously mated female MMTV-Cre+/MntKO/CKO mice were used as controls. Here we
present further details of the mammary gland tumor phenotype of these mice, including Kaplan-Meier survival analysis and a more complete histologic analysis of tumors.

Seven of eleven MMTV-Cre+/MntKO/CKO mice developed mammary adenocarcinomas (Fig. 6A). Tumor latency ranged from 6 to 24 months (Fig. 6B). No tumors were observed in MMTV-Cre/C0/MntKO/CKO mice (Fig. 6A) and mammary gland morphology was normal in these mice (Fig. 6C). Some of the tumors showed lymphocyte invasion (Fig. 6D). The tumors were invasive ductal carcinoma with varying histology, including solid tubular carcinoma (Fig. 6F), tubular carcinoma (Fig. 6G), and carcinoma with sarcomatous change (Fig. 6H). In areas adjacent to the tumors, the nontumor tissue seemed to display enlarged ductal morphology although we cannot rule out that such areas were early preneoplastic lesions (Fig. 6E-H).

**Mnt and Myc bind similar promoters.** The notion that Mnt may act as a counterregulator of c-Myc predicts that c-Myc and Mnt bind to similar sequences in the promoter regions of regulated genes by competitively binding to those locations. To assess this hypothesis, Genome Wide Location Analysis (GWLA) was used to determine if c-Myc and Mnt have similar promoter occupancy in MMTV-c-Myc transgenic mammary tumors (33). One Mnt and two independent c-Myc location-analysis experiments were done on a M2K microarray chip containing ~2,000 mouse gene promoters (Supplementary Table S1) as previously described (28). The P value was set at <0.001 to maintain a strict stringency for comparisons of promoter binding, and Mnt and Myc bound to a very similar set of genes by these strict criteria.
Promoter DNA sequences that coimmunoprecipitated with \(Mnt\) were rank ordered by their \(P\) value for binding affinity versus control, unenriched chromatin DNA. After this rank order was established, promoters that coprecipitated with \(c\)-Myc were aligned with the established order to determine if there was a similarity in \(P\) values for a subset of promoters. It was determined that at \(P < 0.001\) (which was considered significant for this study), there were an extremely high number of genes that bound both \(Mnt\) and \(c\)-Myc with similarly significant \(P\) values.

\(c\)-Myc bound 138 promoter sequences in one trial and 114 unique gene promoters in another trial whereas \(Mnt\) bound 147 of a possible 1,920 genes (Fig. 7A-C). When the 138 unique promoters of the first \(c\)-Myc location-analysis were compared with the 147 promoters from \(Mnt\), 111 were found to be common (Fig. 7D). Of 114 unique promoters from the second \(c\)-Myc experiment, 84 were found to be in common with the same set of \(Mnt\) bound genes (Fig. 7E). The \(c\)-Myc experiments yielded 80 common significantly bound promoters when compared with each other (\(P < 0.001\)). Of these genes, 75 were also found to bind \(Mnt\) at a significant level (Supplementary Table S2), indicating only 5 of the 80 genes were not bound by \(Mnt\) at a significant level (Fig. 7F). We validated these results with chromatin immunoprecipitation analysis of five genes that bound both \(Mnt\) and \(c\)-Myc, \(Ibd1\), \(Sap18\), \(cdkn1b\), \(Rab18\), and \(Bccl\), showing that \(Mnt\) and \(c\)-Myc bound specifically to these promoters but not sequences \(\Delta\) to the transcriptional start site of these genes (Supplementary Fig. S1). These results show a significant overlap in promoter binding of \(c\)-Myc and \(Mnt\) in tumor tissue.

Expression analysis of genes preferentially bound by \(Mnt\) and \(c\)-Myc. If \(Mnt\) acts to regulate \(c\)-Myc, and the lack of \(Mnt\) results in tumorigenesis by removing a mechanism to counter \(c\)-Myc expression, we reasoned that there may be a similarity in gene expression between the two types of tumors. To test this hypothesis, we compared the expression of 45,000 genes in \(MMTV\)-\(c\)-Myc transgenic and \(MMTV\)-\(c\)-Myc transgenic mice using GWLA at \(P \leq 0.001\) were then assessed. Many of these genes are of obvious biological relevance in tumorigenesis, cell cycle...
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regulation, proliferation, and transcriptional regulation (data not shown; Supplementary Table S2). The bioweight values for these genes varied from 0.0001 to 0.73, with the majority decreasing below 0.2 (63 of 75 were below 0.2). These low values indicate that the overall expression pattern is similar between the tumor types and these directly regulated genes are not different in expression in mammary gland tumors resulting from loss of Mnt or overexpression of c-Myc.

Discussion

Elevated c-Myc expression is a common feature of human breast cancers (reviewed in ref. 34) and overexpression of c-Myc in mammary gland tissue of transgenic mice leads to mammary gland cancer (reviewed in ref. 35). Thus, mammary gland epithelial tissue offers an excellent model system to study the function of the putative Myc antagonist Mnt. Consistent with the proposed Myc-antagonist function of Mnt, we previously showed that deletion of Mnt specifically in mammary gland tissue using a Mnt conditional knockout led to the formation of adenocarcinomas (21). Here we show that Mnt also plays an important role in mammary gland development preceding tumor formation, as loss of Mnt impairs mammary gland involution. In addition, we further characterize tumor formation caused by loss of Mnt and show that the gene expression profiles of tumors that lack Mnt are similar to those caused by Myc overexpression.

The finding that loss of Mnt can lead to mammary gland tumorigenesis is consistent with our results showing that loss of Mnt causes a hyperproliferation phenotype in mouse embryonic fibroblasts, including accelerated proliferation, increased sensitivity to apoptosis, sensitivity to transformation by H-RasV12 alone, and senescence bypass (21). In both mouse embryonic fibroblasts and mammary gland tumors, loss of Mnt was found to cause up-regulation of cyclin E and Cdk4 (21), both of which have been implicated in mammary gland tumorigenesis (reviewed in ref. 36). Mnt has also been shown to preferentially bind similar promoter regions to c-Myc in mammary tumor tissues from c-Myc transgenic mice and the corresponding proteins were shown to be expressed at similar levels in both Mnt knockout and c-Myc transgenic tumors. Furthermore, because it is well established that c-Myc promotes mammary gland tumorigenesis in transgenic mice (33), these results are consistent with the proposed role of Mnt as a Myc antagonist and tumor suppressor.

Depending on the in vivo cellular context, dysregulation of c-Myc can either promote proliferation or induce apoptosis (37–39). Induction of apoptosis, and possibly induction of cell cycle arrest (40), likely provide protection against the proliferative effects of over-activity of Myc family proteins. Indeed, Myc-driven tumorigenesis is dramatically accelerated in mouse models when apoptosis is suppressed by expressing antiapoptotic proteins (39, 41–43) or by disruption of p53 pathway function (25, 43–47). Because, in general, cells are sensitized to apoptosis by Myc overexpression and we previously found apoptosis levels increased in fibroblasts lacking Mnt, it was predicted that loss of Mnt and its Myc-antagonistic activity would result in increased apoptosis in mammary gland tissue (21). However, just the opposite was observed during mammary gland involution (Fig. 3), a process characterized by high levels of apoptosis (48). It is possible that cell type differences in the response to loss of Mnt are responsible for this apparent discrepancy or that the decreased apoptosis is the result of impaired initiation of the involution process that precedes active apoptosis. Another possible explanation is that the observed decreased apoptosis is a secondary response to hyper-Myc activity caused by loss of Mnt. In the later scenario, decreased apoptosis during involution may be a manifestation of prior elimination—by apoptosis—of cells important for the involution process. Under such conditions, cells with innate or acquired resistance to apoptosis would have a selective advantage and perhaps are the precursors to tumors caused by loss of Mnt. In situations of Myc overexpression, variant cells less prone to apoptosis are selected that have disrupted ADP ribosylation factor (ARF)/murine double minute-2/p53 pathway function (25, 46, 49). Similarly, primary Mnt−/− fibroblasts seem to sustain p53 mutation or loss of ARF more rapidly than wild-type cells during serial passaging (21) and tumors arising from MMTV-Cre+/Mnt−/− mice express elevated p53, which is often indicative of disabling mutations in p53 (data not shown). Furthermore, there is a modest increase in p53 and p19ARF in the phenotypically normal mammary gland tissue of some MMTV-Cre+/Mnt−/− mice (data not shown), raising the possibility that the decrease in apoptosis during involution is associated with the evolution of cells in mammary gland tissue that have sustained mutations in the p53 gene. Interestingly, p53−/− mice on a BALB/c genetic background exhibit a delayed involution phenotype and develop mammary tumors (50). The role of the ARF/murine double minute-2/p53 pathway and suppression of apoptosis in tumor formation by MMTV-Cre+/Mnt−/− mice can be tested by determining whether mammary gland tumor formation in the absence of Mnt is accelerated by, for example, loss of ARF or p53 or by overexpression of Bcl-2 or other antiapoptotic proteins.

Figure 8. Bioweight analysis reveals a tight clustering of expression in both the ranges of P values and fold change in expression between MMTV-c-Myc transgenic and Mnt−/− MMTV-Cre tumors. Gray-shaded points, ~45,000 genes from the Affymetrix 430 2.0 mouse microarray chip. Black-shaded points, 75 genes identified by GWLA. Horizontal dotted line, P value significance threshold corresponding to a type I error rate of 0.05. Vertical dotted lines, fold change thresholds corresponding to a binary log fold difference of 1 and −1 (which is equivalent to an absolute regular fold change of 2). Genes significantly different between tumors from MMTV-c-Myc transgenic and Mnt−/− MMTV-Cre mice are in the top left and top right areas delineated by the dotted lines, and only two genes were in those quadrants. Of the 75 genes clustered tightly by GWLA, only one was expressed at a level above 1 binary log fold difference and another gene exceeded the P value threshold.
We found that mice with multiple pregnancies had a higher percentage of enlarged ducts and larger average size of ductal lumen than MMTV-Cre+/MntKO/CKO virgin mice (Fig. 4). The accumulation of apoptotic defects in the involution of each pregnancy may be part of the reason for causing the enlargement of mammary ducts. However, virgin MMTV-Cre+/MntKO/CKO mice showed higher percentage of enlarged ducts than virgin wild-type mice. This result suggests that there are other causes than the accumulation of apoptotic defects. Hyperproliferation of the ductal epithelial cells may be a possible explanation but we could not detect any difference in proliferating cell nuclear antigen expression between MMTV-Cre+/MntKO/CKO and wild-type mice (data not shown). Although no changes were noted in levels of Mnt expression during mammary gland development, c-Myc levels varied greatly during mammary gland development. Thus, in the absence of Mnt, these changes in c-Myc will be unopposed and may lead to the developmental defects observed.

Similar promoter binding of c-Myc and Mnt in c-Myc mammary tumors (Fig. 7) supports the hypothesis that Mnt may be an important direct regulator of c-Myc in vivo by competing with c-Myc for binding to Max and shared target genes. Although the Mad protein family may be important in this regulation under certain conditions, evidence presented here, as well as data showing concurrent expression of c-Myc and Mnt in dividing cells (21), suggests that Mnt antagonism may be particularly relevant to the control of cell proliferation. In addition, our results suggest that global gene expression is virtually indistinguishable in tumors of c-Myc transgenic mice and tumors of Mnt−/−/−. MMTV-Cre conditional knockout mice (Fig. 8). Taken together with previous cell culture studies (21), these findings support the hypothesis that Mnt acts in direct functional opposition to c-Myc in regulating transcription and that loss of Mnt is, to a large extent, functionally equivalent to elevated and/or deregulated c-Myc expression.

It is known that the MMTV promoter contains a hormonal regulatory element, which can be induced by progesterone, glucocorticoids, androgens, and prolactin (51–53). We have continuously mated MMTV-Cre+/Mnt−/−/−/− mice to promote the expression of Cre transgene. There are many reports that hormones are important factors to alter mammary tumor development (54–56). Unlike female MMTV-Cre+/Mnt−/−/−/−, male mice did not develop mammary tumors (data not shown). Male MMTV-Cre+/Mnt−/−/−/− mice might develop mammary tumors after the administration of hormones and may be a good tool to analyze the effect of hormones in the development of mammary tumors in MMTV-Cre+/Mnt−/−/−/− mice. However, it remains to be determined whether hormones have a role in Myc/Mnt signaling or only affect the direct Cre transgene induction in MMTV-Cre+/Mnt−/−/−/− mice.

We also produced mice with a null mutation of Mnt using the conditional allele and germ line Cre deleter strains (21, 23). Almost all homozygous mutants (Mnt−/−) in a mixed or inbred genetic background died perinatally. Of the <2% of Mnt−/− mice in a mixed genetic background and ~3.5% Mnt−/− mice in an inbred background that survived to adulthood, all died within 1 year and most of these died within several months of birth from currently unknown causes. Therefore, it has not been possible to address whether mice completely deficient for Mnt develop mammary gland cancers and/or other tumors and we have not detected any tumors in Mnt heterozygotes. Thus, Mnt conditional knockout mice will be required to address the possibility that Mnt functions as a tumor suppressor in other cell types and to determine whether Mnt is the tumor suppressor or one of the general tumor suppressors suspected to reside at human chromosome 17p13.3 (57, 58).

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References


Mnt Is a Tumor Suppressor in Mammary Gland

In the article on how Mnt is a tumor suppressor in mammary gland in the June 1, 2006 issue of Cancer Research (1), the correct spelling of the seventh author’s name is Laure Escoubet-Lozach and the correct spelling of the eighth author’s name is Ivan Garcia-Bassets. Also, the affiliation for Christopher K. Glass should have been listed as the Department of Cellular and Molecular Medicine, Ludwig Cancer Institute, and the Department of Medicine, University of California, San Diego School of Medicine, La Jolla, California.


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