The *Mll* partial tandem duplication: differential, tissue-specific activity in the presence or absence of the wild type allele

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Abstract

The partial tandem duplication of *MLL* (*MLL-PTD*) is found in 5-10% of patients with acute myeloid leukemia (AML) and normal cytogenetics. Its expression in leukemic blasts is coincident with a silenced wild-type (WT) *MLL* allele. We therefore generated mice expressing the *Mll*-PTD in the absence of *Mll*-WT. These *Mll*-PTD/- mice die at birth unlike the normal life expectancy of *Mll*-PTD/WT, *Mll*-WT/-, and *Mll*-WT/WT mice. Using *Mll*-WT/WT fetal liver cells (FLC) as baseline, we compared *Mll*-PTD/- with *Mll*-PTD/WT FLC and found both had increased *HoxA* gene expression and CFU-GM progenitors; in contrast, only *Mll*-PTD/WT FLC had increased CFU-GEMM progenitors. The similarities between *Mll*-PTD/WT and *Mll*-PTD/- mice suggest that the *Mll*-PTD mutation can upregulate target genes in a dominant, gain-of-function fashion. The differences between these two genotypes suggest that in select tissues the *Mll*-PTD requires cooperation with the *Mll*-WT in the genesis of the observed abnormality.
Introduction

Approximately 5-10% of patients with AML and normal cytogenetics present with rearrangement of the Mixed-Lineage Leukemia, (MLL, also known as ALL1 or HRX) gene as the result of a partial tandem duplication within a single MLL allele.\(^1,^2\) In AML blasts harboring the somatic MLL PTD mutation, the MLL wild type (WT) allele is not expressed and when re-expressed, leukemic cell death was observed.\(^3\) We previously reported on the Mll\(^{PTD/WT}\) ‘knock-in’ mice that are fully viable with modest developmental defects, have aberrant gene expression and altered hematopoiesis, but do not develop leukemia.\(^4\) These mice express both the Mll PTD and WT Mll alleles. Thus, to partially recapitulate what is observed in human primary AML blasts regarding the MLL-PTD and absence of MLL-WT expression, we generated mice that harbor a Mll-PTD but lack Mll-WT (Mll\(^{PTD/-}\)) in the germline. We then asked how loss of function of Mll-WT in the context of Mll-PTD would affect HoxA gene expression and hematological abnormalities previously observed in Mll\(^{PTD/WT}\) mice and, eventually, occurrence of leukemia.
Materials and Methods

Generation of $Mll^{PTD/-}$ mice. $Mll^{WT/-}$ mice were generously provided by the late Dr. Stanley Korsmeyer. These mice were maintained on a B6C3F1 background. To obtain $Mll^{PTD/-}$ mice, F1 offspring were obtained by crossing the $Mll^{WT/-}$ mice with the $Mll^{PTD/WT}$ mice (maintained on a pure C57Bl/6J background). This work was performed with approval of The Ohio State University institution review board and under an IACUC approved proposal.

Comparative real time RT-PCR. Total RNA was extracted from E17.5 fetal liver cells (FLC) from $Mll^{PTD/WT}$, $Mll^{PTD/-}$, $Mll^{WT/-}$, and $Mll^{WT/WT}$ embryos. Comparative real time RT-PCR on whole fetal liver, and c-kit+ and CD11b+ sorted populations was performed as previously described.

Chromatin Immunoprecipitation

H3 (Lys4) dimethylation has been shown to occur as a direct result of MLL’s SET domain methyltransferase activity. Therefore, ChIP assays were performed on 2 x $10^6$ FLC using the EZ ChIP Assay Kit with the anti-dimethyl Histone H3 (Lys4) antibody (Millipore) according to the manufacturer’s standard protocol. DNA was quantified using PCR and nested real time quantitative PCR with SYBR green incorporation (Applied Biosystems) using previously described methods.
**Colony forming unit (CFU)-progenitor assays.** Single cell suspensions were plated at a density of 50,000 cells/dish in M3434 methylcellulose (Stem Cell Technologies), and were performed according to the manufacturer’s protocol (Stem Cell Technologies) and methods as previously described.⁴

**Statistics.** To evaluate whether significant differences in CFU-GEMM, CFU-GM, CFU-BE existed between mouse genotypes as indicated in the Figure legend, paired *t*-tests were carried out using siblings.
Results and Discussion

Results of the comparative analysis between the $Mll^{WT/WT}$, $Mll^{WT/-}$, $Mll^{PTD/WT}$ and $Mll^{PTD/-}$ mice are summarized in Table 1. In terms of survival, the first three genotypes were all viable and born at expected Mendelian ratios, however, although present at normal Mendelian ratios, 100% of the pups having the $Mll^{PTD/-}$ genotype died by postpartum day 1 (P1). Interestingly, $Mll^{-/-}$ mice are also non-viable, but die around E10.5. These results indicate the $Mll$ PTD itself provides some, albeit insufficient, compensation for embryonic development in the absence of both $Mll$ WT alleles.

With regards to $HoxA$ gene expression, we found that the $Mll$ PTD is required for aberrant over expression of $HoxA$ genes in E17.5 FLC. $Mll^{PTD/WT}$ and $Mll^{PTD/-}$ mice showed nearly equivalent levels of $HoxA$ over-expression in unsorted FLC, while $Mll^{WT/-}$ cells expressed $HoxA$ levels that were consistently lower but not significantly different than the expression levels found in $Mll^{WT/WT}$ FLC (Figure 1A). To determine whether the over expression of $Hoxa9$ was on a per cell basis rather than an increase in a $Hox$-expressing subpopulation within the unsorted FLC, we sorted out equivalent numbers of c-kit+ (Figure 1B) and CD11b+ (Figure 1C) cells from $Mll^{PTD/WT}$, $Mll^{PTD/-}$, $Mll^{WT/WT}$ and $Mll^{WT/-}$ E17.5 fetal livers. Significantly increased levels of $Hoxa9$ were found in the $Mll^{PTD/WT}$ and $Mll^{PTD/-}$ sorted FLC but not in $Mll^{WT/WT}$ or $Mll^{WT/-}$ sorted FLC. In fact, $Mll^{WT/-}$ CD11b+ cells had no detectable levels of $Hoxa9$ transcript. These results support the notion that the over expression of $HoxA$ is occurring on a per cell basis within the fetal liver. Furthermore, ChIP assays demonstrate that the presence of the $Mll$ PTD is
associated with increased H3 (Lys4) methylation at the Hoxa9 promoter in the presence or absence of the Mll-WT allele (Figure 1D). This increased H3 (Lys4) methylation likely accounts for the observed HoxA gene overexpression in both Mll<sup>PTD/WT</sup> and Mll<sup>PTD/-</sup> FLC.

Although premature death of the Mll<sup>PTD/-</sup> mice precluded assessment of leukemia development, we did examine fetal livers for any alterations in normal hematopoiesis. We performed colony forming unit (CFU) assays to assess fetal hematopoietic liver function in vitro using E17.5 FLC obtained from each of the four genotypes. Significant increases in the CFU-GM, BFU-E and the more immature CFU-GEMM were seen in cells obtained from the Mll<sup>PTD/WT</sup> mice compared to Mll<sup>WT/WT</sup> mice (P<.01, P<.01 and P<.05, respectively), suggesting that the Mll PTD may cooperate with the Mll WT allele at an early stage of hematopoiesis such as the common myeloid progenitor cell. In contrast, FLC obtained from Mll<sup>PTD/-</sup> mice had increases in the CFU-GM and BFU-E populations compared to Mll<sup>WT/WT</sup> mice (P<.05 and P<.05, respectively), but not in the CFU-GEMM population (Figure 1E). Finally, FLC obtained from Mll<sup>PTD/-</sup> mice had a significantly lower number of BFU-E progenitors when compared to Mll<sup>PTD/WT</sup> FLC. Together these results are consistent with the notion that the Mll PTD itself is required and sufficient for abnormal expansion at some stages of progenitor cell differentiation but may not be at other stages. These results also support two recent reports that showed the role of Mll WT in hematopoietic stem cells is distinct from its role in hematopoietic progenitor cells.9,10
Similar phenotypic abnormalities observed in \( Mll^{PTD/-} \) and \( Mll^{PTD/WT} \) genotypes support the notion that the Mll PTD by itself is capable of dysregulating downstream targets and can therefore behave as a dominant gain-of-function mutation in the absence of the Mll WT. Although these studies suggest a direct role for the Mll PTD, it is important to note that a more stable (lacZ fused) Mll protein containing several important N-terminal Mll functional motifs exists in the ‘knockout’ model. Since our heterozygous animals exhibit a more severe phenotype than other (non-lacZ fused) \( Mll \) heterozygous ‘knockout’ mice\(^9,10\), we cannot exclude the possibility that the “knockout” allele acts in an interfering manner.

Mll has now been shown to have very different functions in different subpopulations in the hematopoietic compartment.\(^8\) Our results suggest that in some cases Mll function may have been lost and cannot be replaced by the \( Mll \) PTD allele, as in the case of \( Mll^{PTD/-} \) early lethality at P1. However, in some cases such as \( HoxA \) gene overexpression and CFU-GM expansion, the Mll PTD appears to behave more as a dominant gain-of-function mutation since the quantifications performed for these experiments were similar between the \( Mll^{PTD/WT} \) and \( Mll^{PTD/-} \) genotypes, i.e., in the presence and absence of Mll WT, respectively. In contrast, differences in the number of BFU-E and CFU-GEMM progenitors seen between the \( Mll^{PTD/WT} \) and \( Mll^{PTD/-} \) genotypes reveal the lack of dominant activity by the \( Mll \) PTD allele. In this tissue-specific context, the Mll PTD may require interaction with the Mll WT in order to achieve the maximum manifestation of the abnormality.
Acknowledgments

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Author Contributions

A.M.D. and M.A.C. designed experiments, analyzed and interpreted data and co-wrote the manuscript. A.M.D., S.L., A.C., B.R.P., D.N., M.G., W.Y and D.C. performed experiments. S.P.W. and G.M. provided intellectual expertise and careful review and editing of the manuscript. All authors agreed upon the final text version.

Conflicts of interest

The authors do not have any conflicts of interest, including financial, related to this report.

REFERENCES

Table 1. Comparison of *Mll* genotypes

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<th>Characteristic</th>
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<th><em>Mll</em>&lt;sup&gt;WT/-&lt;/sup&gt;</th>
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n.s., not significant at alpha level = 0.05.
“−” comparator genotype
* 100% mortality on P1 in > 8 liters
Figure Legend

Figure 1. Phenotypic characterization of the Mll\textsuperscript{PTD/-} mice. A.) Increased HoxA gene expression in Mll\textsuperscript{PTD/-} E17.5 FLC. Using real time RT-PCR, Hoxa7, Hoxa9, and Hoxa10 were shown to be overexpressed in E17.5 FLC from Mll\textsuperscript{PTD/-} and Mll\textsuperscript{PTD/WT} embryos compared to both Mll\textsuperscript{WT/-} and Mll\textsuperscript{WT/WT} littermate controls. Error bars show standard deviation. Equivalent numbers of B.) c-kit+ and C.) CD11b+ FLC were sorted with >95% purity from Mll\textsuperscript{WT/WT}, Mll\textsuperscript{WT/-}, Mll\textsuperscript{PTD/WT}, and Mll\textsuperscript{PTD/-} E17.5 embryos. Using real time RT-PCR, increases in Hoxa9 were measured in Mll\textsuperscript{PTD/WT} and Mll\textsuperscript{PTD/-} cells but not in Mll\textsuperscript{WT/WT} and Mll\textsuperscript{WT/-} cells. Error bars show standard deviation. D.) ChIP experiments using an anti-H3 dimethylated antibody showed increases in the levels of H3 (Lys4) methylation at the Hoxa9 promoter in both Mll\textsuperscript{PTD/WT} and Mll\textsuperscript{PTD/-} FLC compared to Mll\textsuperscript{WT/WT} and Mll\textsuperscript{WT/-} controls. Error bars show standard deviation. E.) E17.5 fetal liver hematopoietic progenitor populations were assessed using CFU assays. Mll\textsuperscript{PTD/WT} mice showed increases in CFU-GM, BFU-E and CFU-GEMM compared to Mll\textsuperscript{WT/WT}, while Mll\textsuperscript{PTD/-} mice showed increases in CFU-GM and BFU-E compared to Mll\textsuperscript{WT/WT}. Notably, Mll\textsuperscript{PTD/-} mice showed substantially lower increases in BFU-E when compared to Mll\textsuperscript{PTD/WT} mice. Error bars represent standard error of the means. *P<0.05, **P<0.01.
A

![Bar graph showing fold increase for Hoxa7, Hoxa9, and Hoxa10 in different conditions.]

- **Mll<sup>WT/WT</sup>**
- **Mll<sup>WT/-</sup>**
- **Mll<sup>PTD/WT</sup>**
- **Mll<sup>PTD/-</sup>**

Fold Increase

0 1 2 3 4 5 6

Hoxa7  Hoxa9  Hoxa10
B

**c-kit**

![Graph showing fold increase in Hoxa9 mRNA for different conditions](image_url)

- MII WT/WT
- MII WT-1
- MII PTD/WT
- MII PTDL-
C

CD11b+

Fold Increase in Hoxa9 mRNA

MII WT WT

MII NL- PT DNT

MII PT NL-
D

Hoxa9 promoter

Fold Increase in H3 (Lys4) methylation

MII WT/WT MIIWT+ MIIPTDWT MIIPTD−
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