Mosaic Analysis with Double Markers Reveals Cell-Type-Specific Paternal Growth Dominance

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SUMMARY

Genomic imprinting leads to preferred expression of either the maternal or paternal alleles of a subset of genes. Imprinting is essential for mammalian development, and its deregulation causes many diseases. However, the functional relevance of imprinting at the cellular level is poorly understood for most imprinted genes. We used mosaic analysis with double markers (MADM) in mice to create uniparental disomies (UPDs) and to visualize imprinting effects with single-cell resolution. Although chromosome 12 UPD did not produce detectable phenotypes, chromosome 7 UPD caused highly significant paternal growth dominance in the liver and lung, but not in the brain or heart. A single gene on chromosome 7, encoding the secreted insulin-like growth factor 2 (IGF2), accounts for most of the paternal dominance effect. Mosaic analyses implied additional imprinted loci on chromosome 7 acting cell autonomously to transmit the IGF2 signal. Our study reveals chromosome- and cell-type specificity of genomic imprinting effects.

INTRODUCTION

In diploid organisms, most genes are expressed from both parental chromosomes. However, a subset of genes in mammals and plants is subject to a unique mode of regulation called genomic imprinting (Barlow, 2011; Bartolomei and Ferguson-Smith, 2011), whereby either the maternal or paternal allele is preferentially silenced. Genomic imprinting is essential for embryonic development in mammals (Barton et al., 1984; McGrath and Solter, 1984; Surani et al., 1984). Deregulation of imprinting has been implicated in many diseases, including cancer and brain disorders such as Angelman and Prader-Willi syndromes (Feinberg, 2007; Mabb et al., 2011; Nicholls and Knepper, 2001). Despite the importance of imprinting in controlling prenatal growth, behavior, and metabolism of the whole organism (Ferguson-Smith, 2011; Wilkinson et al., 2007), the functional relevance of imprinting at the cellular level is poorly understood for most genes.

The analysis of mice carrying uniparental disomy (UPD) of whole chromosomes (somatic cells with two copies of either the maternal or paternal chromosome), as well as duplication and deficiency of defined chromosomal regions, has been fundamental for the identification and mapping of imprinted chromosomal regions and loci in the mouse genome (Cattanach and Kirk, 1985; Williamson et al., 2013). However, phenotypic analysis of mice with UPD, deletions, and duplications is limited due to the lack of assays with cellular resolution. Only very recently, the analysis of paternally or maternally inherited mutations with concurrent cell marker labeling has begun to reveal tissue-specific physiological functions for certain imprinted genes (Ferron et al., 2011; Garfield et al., 2011). Here, we use the mosaic analysis with double markers (MADM) system (Zong et al., 2005) to probe the effects of genomic imprinting at the whole-chromosome level and with single-cell resolution across many tissues and cell types in the mouse.

RESULTS AND DISCUSSION

MADM Can Assess Genomic Imprinting Phenotypes with Single-Cell Resolution

MADM can generate Cre/LoxP-dependent mitotic recombination between homologous chromosomes and at the same time label genetically defined progeny with distinct fluorescent markers. Specifically, mitotic recombination at G2 phase followed by X segregation of recombined chromosomes can produce fluorescently labeled progeny with chromosomal compositions distinct from parental cells (Zong et al., 2005). Even for chromosomes that do not harbor any mutations, G2-X events produce near-complete UPD for a particular chromosome carrying the MADM cassettes (Figures 1A and S1A). Consequently, imprinted genes located on such a chromosome will be homozygosed and either overexpressed by a factor of two or not expressed depending on their imprinting status.

Furthermore, cells with unipaternal disomy are fluorescently labeled with GFP and sister cells with unimaterial disomy with tandem dimer Tomato (tdTomato), or vice versa (Figures 1A and S1A). Thus, MADM provides in principle a unique experimental platform to systematically assay the consequences of genomic imprinting at the whole-chromosome level by visualizing the single-cell phenotypes of defined UPDs in genetic mosaic animals.

Chromosome 7, but Not Chromosome 12, UPD Leads to Drastic Paternal Growth Dominance in the Liver

To test the potential effect of imprinted genes on specific chromosomes, we produced MADM cassettes for mouse chromosome 7...
because mouse chromosome 7 is most enriched for imprinted genes and harbors several well-studied clusters of imprinted genes (Williamson et al., 2013). We inserted MADM cassettes near the centromere of chromosome 7 (Figures 1B and S1B; see Experimental Procedures for details) using a similar knockin strategy as previously described (Hippenmeyer et al., 2010). We then used a ubiquitous Cre driver (Hprt-Cre) to induce chromosome 7 UPD across the whole animal in a mosaic fashion.

When using GFP to label unipaternal disomy cells (PP), we found a massive expansion of green hepatocytes in the liver (Figures 1C and 1G) when compared with unimaternal disomy cells (MM) labeled by tdTomato. In a separate experiment in which we switched colors of UPD cells (tdTomato for PP, and GFP for MM), we found that red hepatocytes expanded as a consequence of the chromosome 7 UPD (Figure 1D). Thus, unipaternal disomy of chromosome 7 leads to a massive expansion of hepatocytes when compared with unimaternal chromosome 7 disomy.

We also created MADM cassettes near the centromere of mouse chromosome 12 for mosaic analysis of candidate genes on that chromosome (Figures 1B and S1C). Hprt-Cre together with the MADM transgenes on chromosome 12 also produced chromosome 12 UPD cells in a mosaic fashion across the whole animal and including the liver. In contrast to chromosome 7 UPD, hepatocytes carrying either paternal or maternal chromosome 12 UPD appeared similar in number (Figures 1E–1G).

### Imprinting Effects in Chromosome 7 UPD Display Cell-Type Specificity

We extended our analysis to additional tissues and cell types with mosaic chromosome 7 or chromosome 12 UPD. Qualitative (Figures 2A–2C and 2E–2G) and quantitative (Figure 2I) evaluation of hippocampal CA1 pyramidal neurons and cerebellar Purkinje cells in the brain, as well as cardiomyocytes in the heart, revealed no significant differences in cell number regardless of whether these cells carried unipaternal or unimaternal disomies for chromosome 7 or chromosome 12. By contrast, lung epithelia displayed a marked expansion of cells with unipaternal chromosome 7 but not chromosome 12 UPD (Figures 2D and 2H), similar

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**Figure 1. MADM Reveals a Chromosome-Specific Imprinting Phenotype with Single-Cell Resolution**

(A) G2-X MADM events result in near-complete uniparental chromosomal disomy labeled in green (PP) and red (MM) fluorescent colors, respectively.
(B) Chromosomal location of Hipp7 (chromosome 7) and John12 (chromosome 12) genomic loci with inserted MADM cassettes.
(C and D) P21 livers from MADM-7GT/TG; Hprt-Cre+/- mice with uniparental chromosome 7 disomy.
(E and F) P21 livers from MADM-12GT/TG; Hprt-Cre+/- mice with uniparental chromosome 12 disomy.
(G) Quantification of the PP/MM ratios of liver UPD hepatocytes in cryosections in P21 MADM-7GT/TG; HprtCre+/+ (MADM-7) and MADM-12GT/TG; HprtCre+/+ (MADM-12; n = 16 from 3 individual male mice). Values represent mean ± SEM. For detailed methods of quantifying the PP/MM ratio in MADM-7 P21 liver, see also Figures 4 and S4.

Depending on whether the GT and TG alleles were introduced from the father (blue) or the mother (pink), unipaternal disomy cells are labeled in green (C and E) or in red (D and F) as indicated. Scale bar, 200 μm. See also Figure S1.
to liver hepatocytes. We conclude that MADM-induced chromosome 7 but not chromosome 12 UPD results in cell-type-specific expansion with unipaternal disomy.

To exclude the possibility that the MADM markers on chromosome 7 themselves were selectively silenced due to imprinting, we generated mice containing constitutively expressed, reconstructed GFP (Hipp7GG/+) and tdTomato (Hipp7TT/+), respectively, and intercrossed them to generate Hipp7GG/TT animals. All cells in every organ analyzed at postnatal day (P) 21 in Hipp7GG/TT expressed both markers uniformly (Figure S2). Therefore, the unequal ratio of hepatocytes and lung epithelia with unipaternal over unimaternal chromosome 7 UPD did not result from selective silencing of one marker in putative GFP+/tdTomato− cells but reflects an imprinting phenotype whereby cells with unipaternal disomy have a growth advantage over unimaternal disomy.

**Paternal Growth Advantage in Chromosome 7 UPD Commences during Embryogenesis**

What is the developmental origin of cell number expansion in unipaternal chromosome 7 disomy? To address this question, we carried out a developmental time course analysis focusing on liver hepatocytes with MADM-induced chromosome 7 UPD. At embryonic day (E) 12, no expansion of the hepatocyte population with unipaternal disomy was apparent (Figure 3A). However, 4 days later at E16, unipaternal chromosome 7 disomy cells already outnumbered unimaternal chromosome 7 disomy cells (Figure 3B). We validated this result by clonal analysis using tamoxifen (TM)-induced CreER (Figures 3J and 3K). In liver clones induced at E12, the size of clone-harboring PP cells already exceeded that of MM by more than 2-fold by E16. These findings are consistent with previous reports showing that the embryonic liver has a remarkable capacity for growth (Stanger et al., 2007).

Expansion of the MADM-labeled domains with unipaternal chromosome 7 hepatocytes became more apparent at postnatal stages (Figures 3C–3F). Nevertheless, mosaic MADM-7 livers were neither larger than wild-type nor displayed any signs of tumors in mice of up to 6 months of age, suggesting that cell expansion due to unipaternal disomy still follows organ size control mechanism as in wild-type hepatocytes (Stanger, 2008). In contrast to hepatocytes with unipaternal chromosome 7 disomy, hepatocytes with either UPD for chromosome 12 displayed equal numbers postnatally up to 6 months (Figures 3G–3I).

**Ablation of a Single Gene on Chromosome 7, Igf2, Largely Mitigates Paternal Growth Advantage of Chromosome 7 UPD**

The paternal dominance of chromosome 7 disomy cells could, in principle, reflect the consequence of homozygosing many or just a few imprinted genes on chromosome 7. Because the “imprintome” of chromosome 7 in the liver is currently unknown, we pursued a candidate gene approach to identify the causal gene. Chromosome 7 harbors several clusters of imprinted genes that either promote or antagonize growth, including paternally expressed Igf2 (insulin-like growth factor 2) (DeChiara et al., 1991), as well as maternally expressed H19 (Bartolomei et al., 1991) and Cdkn1c (cyclin-dependent kinase inhibitor 1C, aka p57kip2) (Hatada and Mukai, 1995; Matsuoka et al., 1995). The growth advantage of unipaternal disomy can be caused by overexpression of paternally expressed growth-promoting gene(s), absence of maternally expressed growth-antagonizing gene(s), or a combination of both. We began by assessing the involvement of Igf2 and introduced a null allele from the father into our
MADM analysis. Because Igf2 is paternally expressed in most cells (DeChiara et al., 1991), Igf2<sup>-/-</sup> (m stands for the wild-type allele inherited from the mother) should in principle be equivalent to homozygous Igf2<sup>+/+</sup>. Remarkably, we found a striking reduction of the large clusters of unipaternal chromosome 7 disomy cells at P21 in Igf2<sup>+/+-</sup>-MADM (Figures 4A and 4D compared with Figures 4B and 4E; Figures S3A and S3B).

To compare hepatocyte expansion with unipaternal chromosome 7 disomy produced by MADM in control mice and mice with paternal loss of Igf2, we quantified all green and red cells in entire liver sections to avoid selection bias and calculated their ratios (Figures 4G, S4A, and S4B). We found an 8.6-±0.9-fold paternal expansion in control-MADM. The PP/MM ratio was reduced to 2.2-±0.4-fold in animals with Igf2<sup>+/+-</sup>-MADM. To test whether the residual paternal dominance was due to IGF2 from a possible reactivation of the silenced maternal Igf2 allele, or in serum from distinct sources not subjected to imprinting (DeChiara et al., 1991), we also examined the liver from homozygous Igf2<sup>+-</sup> mutants with MADM labeling (Figures 4C and S3C).

We found a reduction of the PP/MM ratio (Figure 4F) to the same level (2.1±0.5) as when the Igf2 mutation was introduced only from the father. By contrast, maternal transmission of the Igf2 mutation had no effect on paternal dominance (Figures S3D, S4C, and S4D). Thus, the loss of the paternally inherited Igf2 fully accounts for the imprinting effect.

To extend our findings to other tissues, we also examined the effect of IGF2 on the paternal growth dominance in the lung epithelia. We found that paternal transmission of the Igf2 mutation also resulted in marked reduction of paternal growth dominance in the lung (Figures S4E–S4H), highlighting a general role of Igf2 imprinting in distinct cell types. Taken together, these findings indicate that Igf2 is a major factor driving unipaternal chromosome 7 growth dominance in the liver and lung.

**Evidence that Additional Imprinted Factors on Chromosome 7 Act Cell Autonomously to Receive the IGF2 Signal**

Although chromosome 7 harbors many imprinted genes controlling growth, we found that mutation of just one paternally expressed gene, Igf2, mitigates the majority of the paternal dominance effect. Our findings are in line with previous chimera studies implicating that duplication of distal chromosome 7 (where Igf2 is located) and Igf2 itself are responsible for causing overgrowth (Ferguson-Smith et al., 1991; McLaughlin et al., 1997). We further extended these studies with single-cell resolution by producing and visualizing UPD through rare mitotic recombination events enabled by MADM.

However, it is surprising that a secreted factor should be a major contributor in our experimental setting. Under the control-MADM-7 condition, all unipaternal disomy cells that overexpress Igf2 originate from mitotic recombination events in a single cell and are surrounded by a vast majority of cells with regular paternal Igf2 expression. Even with paternal growth dominance, unipaternal cells represent only a small fraction of the whole liver (see Figure S4A). We envision three possibilities to explain the paternal growth dominance that we observed in MADM-7 mice (Figure 4H). First, despite being a secreted factor, IGF2 acts predominantly cell autonomously. Furthermore, a 2-fold increase in IGF2 levels should greatly facilitate the growth of the unipaternal cells. We consider the first possibility unlikely because IGF2 is well known to bind IGF-binding proteins, mitigates the majority of the paternal dominance (Chao and D’Amore, 2008). Second, in addition to IGF2, another paternally expressed P factor acts cell autonomously to positively regulate IGF2 signal transmission. Again, this P factor stand in a dose-dependent fashion to account for the growth of unipaternal chromosome 7 disomy cells in a mostly wild-type background because it would also be present in 2-fold higher dosage levels, similar to IGF2. Third, a maternally
Figure 4. Igf2 Accounts for Most of the Paternal Dominance in Chromosome 7 UPD

(A–C) Schematic of Igf2 expression in MADM-7GT/TG;HprtCre+/−. (A) Control-MADM-7. (B) Igf2 mutation on paternal chromosome. (C) Igf2 mutation on both paternal and maternal chromosomes. Note that due to Igf2 imprinting, most cells in (B) do not express Igf2.

(D–F) Labeling of uniparental chromosome 7 disomy (PP cells are green, and MM cells are red) in P21 control, paternal Igf2 mutant, or homozygous Igf2−/− mutant MADM animals corresponding to the schemes in (A–C). Scale bar, 110 μm.

(G) Quantification of PP/MM ratio in whole-liver sections from P21 MADM animals with genotypes indicated. Values represent mean ± SEM. *p < 0.05 and **p < 0.01.

(H) Three possible models to account for expansion of cells with unipaternal disomy in sparsely generated MADM-7 clones in wild-type animal. Assuming the crossing scheme in Figure 1A, cells with unipaternal chromosome 7 disomy (pUPD7) are labeled in green and cells with unimaternal chromosome 7 disomy (mUPD7) in red upon a G2-X MADM event. Green pUPD7 cells express a double dose of Igf2 and P factor(s) but lack M factor(s), where P or M factors represent additional imprinted genes on chromosome 7 that could act cell autonomously to promote or inhibit IGF2 signaling, respectively. In Model 1, IGF2 (double dose) signals strictly autocrine to only pUPD7, but not to mUPD7. In Model 2, the double dose of additional imprinted P factor(s) acts in concert with secreted IGF2 to convey the growth advantage in pUPD7. In Model 3, the lack of M factor(s), which normally would tune down the IGF2 signal to a certain extent, promotes increased IGF2 growth signaling in pUPD7 cells. The cell-type specificity of UPD7 effects we observed may be a consequence of cell-type-specific expression of the M or P factors, or cell-type-specific interactions of these factors with IGF2.

See also Figures S3 and S4.
expressed M factor acts cell autonomously to negatively regulate IGF2 signal transmission. In unipaternal chromosome 7 disomy cells, the lack of the negative M factor results in greatly amplified IGF2 growth signaling.

In both the second and third possibilities above, the additional chromosome 7 P or M imprinted factors contribute, at most, a 2-fold difference independent of IGF2, and their major actions are manifested in the context of cell-autonomous IGF2 signal transmission. We favor the third model because it requires the least number of assumptions; indeed, Cdkn1c could represent an attractive candidate for the M factor because genetic interactions have been demonstrated between Igf2 and Cdkn1c (Casparay et al., 1999).

Although Igf2 displays a highly specific expression pattern in the brain, it is broadly expressed in peripheral embryonic tissues such as the liver, lung, and heart (Davies et al., 2002; DeChiara et al., 1991; Lehtinen et al., 2011). Because we have only detected a significant proliferation advantage in the liver and lung, but not in the heart or the brain, it is highly unlikely that the cell-type-specific effect of imprinting is accounted for by tissue-specific Igf2 expression patterns. Rather, the tissue specificity of the imprinting effect is likely caused by tissue-specific expression of the M or P factors, or their tissue-specific interactions with IGF2.

Conclusions

We have used the MADM strategy to create, and concomitantly visualize with distinct fluorescent colors, cells with unipaternal and unimam paternal near whole-chromosomal disomy. Consequently, it is possible to assay the phenotype of genomic imprinting at the single-cell resolution in virtually any tissue or organ and potentially for every chromosome in the mouse. The results from MADM of chromosome 7 and chromosome 12 revealed chromosomal as well as cell-type-specific imprinting phenotypes. The cell-type specificity of genomic imprinting indicates that certain genes exploit parental control expression at the single-cell resolution. This provides a sensitive means to detect cell-type-specific effect of imprinting is accounted for by tissue-specific Igf2 expression patterns. The absence of Cre recombinase.

Heterozygote Igf2+/− (DeChiara et al., 1990), lacZ-CreERt2−/− (Guo et al., 2002), and hemizygote Hprt−/− (Tang et al., 2002) mice have been described. Mouse embryonic stem cells were targeted with a single-strand oligospecific vector to delete exon 1 of the Igf2 gene followed by a selection of Mor and Cre recombinase.

EXPERIMENTAL PROCEDURES

Generation of MADM-7 and MADM-12 Mice and Mouse Genetic Techniques

MADM-7 and MADM-12 mice were generated following a previously described strategy (Hippenmeyer et al., 2010). In brief, for MADM-7 and MADM-12-targeting constructs, we identified suitable genomic loci on chromosome 7 (Hipp7 located at 2.13cM; ~0.7 kb downstream of exon 5 of the Rps9 gene) and chromosome 12 (John12 located at 1.71cM; ~16 kb downstream of exon 1 of the Rab10 gene). The Hipp7 and John12 genomic loci were cloned, GT and TG MADM cassettes (Hippenmeyer et al., 2010; Liu et al., 2011) inserted, and targeting vectors constructed using standard molecular biology procedures (details are available upon request). Linearized targeting vectors were then electroporated into R1 ES cells, and two correctly targeted GT and TG clones for each MADM-7 and MADM-12, respectively, were injected into blastocysts to generate chimeric mice. Homozygous MADM-7T/GT, MADM-7αTGT, MADM-12αTGT, MADMM-12αTG, and transheterozygous MADM-7αTGT and MADM-12αTG were born at Mendelian frequencies, had a normal lifespan, were fertile, showed no obvious adverse phenotype, and did not exhibit GFP/tdTomato marker expression in the absence of Cre recombinase.

Analysis of Marker Expression in MADM Animals

Experimental MADM mice at various ages were perfused, and organs were processed for cryosections essentially as described (Hippenmeyer et al., 2010). Typically, we isolated the whole brain, heart, and the largest leaflets of the lung and liver from postnatal mice. Whole embryos were isolated and left intact for processing except that the body was separated from the head, and the limbs were removed. The brain, heart, and lung were cryosectioned sagittally at 60 μm (brain) and 14 μm (heart and lung); coronal sections were acquired from the liver at 14 μm. The fluorescent tdTomato and GFP signals were usually not amplified by antibody staining, but tissue sections were stained with DAPI (Invitrogen) to visualize nuclei and confocal images acquired using a LSM 510 (Zeiss). Cell Reports 3, 960–967, March 28, 2013 ©2013 The Authors 965
embedded in OCT, and sectioned in the sagittal plane at 30 μm. The frequency of MADM clone generation was extremely low and varied between cohorts with slightly different genetic background.

Computation of PP/MM Ratios
Pyramidal cells in hippocampal area CA1 were counted in confocal images from 16 (MADM-7 GT/−/Hprt-CreERT2) and 11 (MADM-12/+/Hprt-CreERT2) cryosections (derived from three animals for each genotype) encompassing the whole CA1 area. Purkinje cells in cerebella were counted in all consecutive cryosections from four (MADM-7 GT/−/Hprt-CreERT2) and three (MADM-12/+/Hprt-CreERT2) entire half-brains derived from more than two animals each. For quantification of heart cardiomyocyte and liver hepatocyte populations, the green (GFP) and red (tdTomato) MADM signals were first extracted manually from confocal images derived from cryosections to separate them from the yellow GFP/tdTomato+ signal. The total areas of green and red signals, respectively, were then computed from binary images using a custom MATLAB script. The geometric means ± SEM of the paternal/maternal ratios were calculated in Excel, and significance was determined using Student’s t test: *p < 0.05 and **p < 0.01.

SUPPLEMENTAL INFORMATION
Supplemental Information includes four figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.02.002.

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