Elevated expression of MeCP2 in cardiac and skeletal tissues is detrimental for normal development

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MeCP2 plays a critical role in interpreting epigenetic signatures that command chromatin conformation and regulation of gene transcription. In spite of MeCP2’s ubiquitous expression, its functions have always been considered in the context of brain physiology. In this study, we demonstrate that alterations of the normal pattern of expression of MeCP2 in cardiac and skeletal tissues are detrimental for normal development. Overexpression of MeCP2 in the mouse heart leads to embryonic lethality with cardiac septum hypertrophy and dysregulated expression of MeCP2 in skeletal tissue produces severe malformations. We further show that MeCP2’s expression in the heart is developmentally regulated; further suggesting that it plays a key role in regulating transcriptional programs in non-neural tissues.

INTRODUCTION

Cellular differentiation occurs, for the most part, without changes in the sequence of the DNA but through changes in its transcriptional activity. In mammals, the different cell- and development-specific transcriptional programs are set by the combined action of chromatin remodeling complexes and epigenetic modifications at the level of DNA and histones. Mammalian DNA is covalently modified by the addition of a methyl group to cytosines that occur predominantly in CpG dinucleotides (1). There is abundant evidence that DNA methylation is a critical process for normal mammalian development and also for the survival of differentiated cells (2,3). Mouse embryos that had severely reduced levels of cytosine methylation are unable to proceed through normal development and die at 8.5 days post coitus (dpc) (4,5). DNA methylation is also associated with phenomena such as reprogramming of transferred nuclei (11,12), carcinogenesis (13), DNA repair (14), initiation of sexual dimorphism (15) and progression through cell-division checkpoints (16).

The methyl mark is interpreted by a family of methyl-CpG binding proteins via a methyl-CpG-binding domain (MBD) (17). The founding member of the MBD family is methyl-CpG-binding protein 2 (MeCP2) (18), a multifunctional nuclear protein initially described as a methylation-dependent transcriptional repressor (19,20). In addition to the MBD, MeCP2 has a transcriptional repressor domain (TRD) that mediates its interaction with chromatin remodeling complexes including Sin3a and HDAC1/2 (19–21), the silencing mediator for retinoid and thyroid hormone receptors (SMRT) (22), the histone methyltransferase, Suv39H (23), the DNA methyltransferase I (24), c-Ski and N-CoR (25).

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The activities of MeCP2 appear to extend beyond those of an epigenetic transcriptional repressor, including gene activation (26,27) and regulation of splicing (28). The protein domains responsible for these additional activities of MeCP2 seem to reside outside the MBD and TRD. The RNA-related functions of MeCP2 involve the C terminus of the protein (28,29), while the domain that facilitates its interaction with the transcriptional activator CREB1 has not yet been characterized. The orchestration of the various activities of MeCP2 could be mediated by post-translational modifications such as phosphorylation (30–32) or SUMOylation (33).

MeCP2 is widely expressed, although with varying levels of expression and with incomplete concordance between mRNA levels and protein expression. Mecp2 mRNA transcripts are highly expressed in lung, skeletal muscle and heart, moderate in brain and low in liver and spleen (34–38), whereas the protein is abundant in brain, lung and spleen, moderately abundant in the kidney and heart, and hardly detectable in the liver, stomach and small intestine (36). Two protein isoforms are generated via alternative splicing, MeCP2-E1 and MeCP2-E2 (39,40). In spite of a differential tissue distribution (41), no functional difference has yet been identified between the two variants.

The identification of mutations in MECP2 as causative of Rett syndrome (RS), a progressive neurodevelopmental disorder, underscored the relevance of MeCP2 for neuronal function. Moreover, abnormalities in MECP2 have also been found in a variety of neuropsychiatric disorders ranging from mild learning disabilities to infantile encephalopathy (42–44).

Interestingly, variations in the number of copies of the genomic region containing MECP2 have also been associated with neurological abnormalities (45–51). Patients carrying MECP2 duplications are characterized by infantile hypotonia, severe mental retardation, progressive spasticity, language deficiencies and recurrent infections. Consistent with this, triplication of the MECP2 region results in a more severe phenotype (45,49,50), further indicating that MECP2 is a dosage sensitive gene. This sensitivity to MECP2 expression levels is supported by the demonstration that transgenic mice that over-express MeCP2 (~2-fold (52)) result in a progressive neurodevelopmental disorder.

Accumulating evidence suggests that most phenotypes associated with MeCP2 abnormalities are caused specifically by dysfunction of mature neurons (53,54) arising from misexpression of MeCP2 target genes in the brain. However, a role for MeCP2 outside of the CNS has not been ruled out and it is conceivable that certain anomalies seen in RS and related disorders may result from MeCP2 dysfunction in peripheral cells. For example, the prolonged corrected QT interval exhibited by most RS patients has been proposed to be related to MeCP2 dysfunction in the heart rather than in the CNS and their usually observed decreases in bone mineral density have been recently ascribed to abnormal osteoblast activity (55). Also, the commonly observed dysmorphic features of MeCP2 duplication patients (48,49,56–58) could stem from MeCP2 dysfunction in peripheral tissues.

We demonstrate herein that perturbations of the normal pattern of expression of MeCP2 outside of the realms of the brain, such as in the heart and the skeleton, are detrimental for normal development. Overexpression of MeCP2 in the mouse heart causes embryonic lethality with cardiac septum hypertrophy and dysregulated expression of MeCP2 in skeletal tissue produces severe malformations.

RESULTS

Conditional expression of transgenic MeCP2

We recently reported the generation of several lines of mice carrying a tetracycline-inducible transgene (hereafter referred to as TM mice) that express human MeCP2-E2 and EGFP from a bidirectional promoter (59). When crossed to transgenic mice that express the tetracycline transactivator (tTA), the bitransgenic progeny express transgenic MeCP2 in a tissue-specific and temporal pattern dictated by the promoter driving the expression of tTA (Supplementary Material, Fig. S1).

Thus, these mice could serve as tools to study the consequence of tissue-specific overexpression of MeCP2. To obtain brain-specific expression of MeCP2, we used transgenic mice that express tTA under the control of 1.8 kb of the rat enolase2 promoter (Eno2-tTA (60)). As expected, bitransgenic mice showed brain-specific expression of MeCP2. In addition, two of our four transgenic lines (TM15 and TM20) also showed high levels of transgenic MeCP2 expression in the heart, cartilage and ocular tissue and are the subject of this study.

Embryonic lethality with cardiac hypertrophy in TM20dTg mice transgenic for MeCP2

Crossing line TM20 with Eno2-tTA mice generated significantly fewer TM20;Eno2-tTA (TM20dTg from now on) offspring than anticipated (11% versus the expected 25%, n = 409, Fisher’s exact test: P < 0.0001), suggesting that overexpression of MeCP2 might result in fetal loss. A retrospective analysis showed that the majority of TM20dTg came from matings in which the TM parents were of late filial generations (F3 or higher), suggesting that MeCP2/eGFP expression was being gradually reduced by transgenerational silencing of transgene expression. To substantiate this observation and interpretation, we compared the expression levels shown by survivor offspring derived from early (F2) versus late (F5) filial generations. Notably, F2 TM20dTg displayed higher transgene expression in brain, heart and cartilage than most F5 TM20dTg (data not shown). In addition, the expression of F5 TM20dTg varied extensively between littermates and the level of expression of individual mice correlated very well with the severity of the phenotype. These results also suggest that embryonic lethality resulted from transgene expression and not from insertional effects. Due to variable transgene expression in late filial generations, all reported experiments were performed with F2 mice. Since the TM20 was indistinguishable from wild-type in every aspect analyzed, we used these single TM20 transgenic mice as controls.

To confirm that the lethality was dependent on the expression of transgenic MeCP2, we suppressed transgene activity during development by administering doxycycline (Dox) to maternal mice. Dox was added to the drinking water 2.5 days after a sperm plug was observed and mice drank ad libitum until delivery. Dox administration restored the expected Mendelian frequency (25%) of TM20dTg
As expected, GFP fluorescence was accompanied by MeCP2 expression at all ages tested (see for example (Fig.1 and data not shown). By E13.5 and E14.5, after 9.5 dpc. This early expression was observed exclusively in the TM20dTg embryos, we detected GFP fluorescence as early as 9.5 dpc. Although the Eno2 promoter is primarily regarded as a neuron-specific promoter, it has been shown to be expressed in a variety of tissues in transgenic mice (69–71). In the TM20dTg embryos, we detected GFP fluorescence as early as 9.5 dpc. This early expression was observed exclusively in the looping heart of the embryos (Fig. 1).

Table 1. Genotype of embryos from various stages of embryonic development resulting from TM20 × Eno2-tTA intercrosses (TM20 animals were of the F2 generation)

<table>
<thead>
<tr>
<th>Number of litters</th>
<th>TM20</th>
<th>Eno2-tTA</th>
<th>WT</th>
<th>TM20dTg</th>
</tr>
</thead>
<tbody>
<tr>
<td>E8.5</td>
<td>3</td>
<td>4 (16.7%)</td>
<td>9 (37.5%)</td>
<td>3 (12.5%)</td>
</tr>
<tr>
<td>E10.5</td>
<td>3</td>
<td>7 (36.8%)</td>
<td>4 (21.1%)</td>
<td>3 (15.8%)</td>
</tr>
<tr>
<td>E12.5</td>
<td>3</td>
<td>5 (23.8%)</td>
<td>8 (38.1%)</td>
<td>4 (19.0%)</td>
</tr>
<tr>
<td>E14.5</td>
<td>7</td>
<td>11 (24.4%)</td>
<td>9 (20.0%)</td>
<td>17 (37.8%)</td>
</tr>
<tr>
<td>E16.5–E19.5</td>
<td>8</td>
<td>16 (37.2%)</td>
<td>11 (25.0%)</td>
<td>13 (30.2%)</td>
</tr>
</tbody>
</table>

The genotype of absorbed embryos was not determined. 

Although several healthy mouse strains with ubiquitous high expression of GFP have been described (61–65), some reports indicate that GFP can affect cellular functions (66–68). We nevertheless ruled out the possibility that high expression of GFP was responsible for the observed lethality of the TM20dTg embryos since we also generated bitransgenic mice that overexpress Rai1 (retinoic acid inducible-1) and EGFP using the same transgenic system and observed no lethality in the highest expressing transgenic line, which showed EGFP expression in the looping heart commencing at E9.5 (data not shown).

To define the exact time of embryonic lethality of TM20dTg embryos, we set up timed mating intercrossings of TM20 and Eno2-tTA heterozygous and collected embryos at different time points of development. We determined normal Mendelian inheritance of both transgenes up to 14.5 dpc. TM20dTg embryos were indistinguishable in size and morphology from WT littermates from embryonic day (E) 9 to E14.5 and we did not observe morphological differences in their vitelline vessels, yolk sac or placentae (data not shown). However, we observed several reabsorbed embryos from 14.5 dpc onwards and recovered fewer TM20dTg embryos than expected past 14.5 dpc (Table 1). Thus, we conclude that most TM20dTg embryos die at E14.5–E15.

Although the Eno2 promoter is primarily regarded as a neuron-specific promoter, it has been shown to be expressed in a variety of tissues in transgenic mice (69–71). In the TM20dTg embryos, we detected GFP fluorescence as early as 9.5 dpc. This early expression was observed exclusively in the looping heart of the embryos (Fig. 1). Transgene expression was more abundant by E11.5, predominantly in the interventricular septum (IVS), in cells of the left and right ventricles and in few cells of both atria (Fig. 1). E12.5 embryos showed high levels of transgene expression in both ventricles of the heart as well as in the outflow tract, a structure that gives rise to the pulmonary artery and aorta (Fig. 1 and data not shown). By E13.5 and E14.5, after chamber morphogenesis has taken place, embryos exhibited high levels of transgene expression in the left/right ventricles, the IVS and the outflow tract and lower levels in the atria (Fig. 1). As expected, GFP fluorescence was accompanied by MeCP2 expression at all ages tested (see for example (Fig. 1K). Immunofluorescence for MeCP2 detected colocalization of MeCP2 and EGFP fluorescence in more than 80% of observed cells.

Starting at E12.5, we also detected transgene expression in differentiating cartilage throughout the embryo. GFP fluorescence was present in the cartilaginous primordia of the head, including the primordia of the cranium, the nasal bone and septum, and the maxilla and mandible (Fig. 2 and data not shown). The condensing cartilage of the vertebral bodies in the spinal column and the cartilaginous condensations within the hindlimbs and forelimbs (Fig. 2 and Supplementary Material, Fig. S3) also showed GFP fluorescence.

Oriented by the temporal pattern of transgene expression, to determine the cause of embryonic lethality, we initially focused on the heart. We dissected hearts of TM20dTg and WT littermates at daily intervals from E9.5 to E13.5 and analyzed their morphology for heart structural abnormalities. Although we did not detect any abnormalities on E9.5–E12.5 embryos, E13.5 embryos exhibited significant septum and free ventricular wall hypertrophy (Fig. 3A), which is a common cause of intrauterine death (72).

To ascertain the immediate cause of this hypertrophy, we assessed proliferation and apoptosis in the hearts of E12.5–E14.5 embryos. Using BrdU pulse-labeling, we found markedly increased BrdU labeling in septum cardiomyocytes of E14.5 TM20dTg embryos, as compared to singly transgenic littermates (Fig. 3B). Quantification of apoptosis did not reveal any difference between TM20dTg embryos and their controls (Fig. 3C). This suggests that increased proliferation might be responsible for the observed septum hypertrophy.

**MeCP2 overexpression produces congenital malformations in a dose-dependent fashion**

In contrast to line TM20dTg, we observed that TM15:Eno2-tTA (TM15dTg) mice were born at a normal Mendelian ratio and displayed a much milder phenotype characterized by an abdominal protrusion due to a ventral hernia (not shown) that starts to be apparent at 2 months of age. To investigate the potential cause of the differential response to transgene expression in lines TM20 versus TM15, we compared the level of transgene expression between the TM15dTg and TM20dTg lines at E14.5 by western blot analysis (Fig. 4A). Results of the analysis reveal that the expression of MeCP2 in embryonic hearts of the TM15dTg and TM20dTg was 3.8 ± 0.8- and 6.9 ± 0.9-fold over the expression of endogenous
MeCP2, respectively, suggesting that overexpression of MeCP2 induces congenital abnormalities in a dose-dependent fashion. To test this hypothesis, we attempted to increase the level of transgene expression in TM15dTg by crossing heterozygous mice to transgene homozygosity and analyzed the phenotypic consequences of a potential increase in transgenic MeCP2 expression. Matings of TM15dTg heterozygous males with TM15 heterozygous females resulted in non-Mendelian inheritance of the transgenes: from 61 embryos born, only 3 were homozygous for the TM15 transgene when carrying the Eno2-tTA transgene, strongly suggesting that increased expression of MeCP2 causes embryonic lethality.

Timing of expression of endogenous MeCP2 in the heart

To check whether mice expressing transgenic MeCP2 develop congenital malformations due to alterations induced by ectopic expression of MeCP2, or to a transgene-induced increase of MeCP2 in cells that endogenously express it, we collected thoracic cages (E7.5, E9.5 and E10.5) and hearts (E11.5 to P0) from wild-type embryos. We found that MeCP2 was barely detectable at the stage E9.5 by western blot analysis and that the E11.5 hearts express detectable levels of MeCP2 (Fig. 4B). Although we cannot ascribe the MeCP2 expression observed in the E9.5 and E10.5 embryos exclusively to cardiac tissue, these data are consistent with the reported presence of MeCP2 mRNA in hearts of 10.5 embryos (38,73). The amount of endogenous MeCP2 in the developing heart is appreciably higher than in the mature heart (Fig. 4C), distinct from what has been observed in the brain, in which expression of MeCP2 correlates with neuronal maturation (36). Therefore, we established that endogenous expression of MeCP2—detectable by western blot analysis or immunofluorescence—begins around E9.5.

The expression of transgenic MeCP2 in the TM20dTg line, which displays cardiac anomalies, mimics the temporal expression of endogenous MeCP2 in the heart. In contrast, transgenic MeCP2 expression in the TM15dTg mice, which do not develop cardiac symptoms and live past 12 months of age, precedes endogenous expression by 1 day. Thus, it is likely that the deleterious effects of transgenic MeCP2 in cardiac development stem from a direct effect of increased levels of the protein, rather than from the premature activation of MeCP2-related developmental programs in the heart.

MeCP2 is phosphorylated in the developing heart

MeCP2 undergoes neuronal activity-dependent phosphorylation at serine 421 (32). This phosphorylation is reported to be important for the maturation of neuronal connectivity (32). Since our
data suggest that proper interpretation of DNA methylation signals is important for normal heart development and implicates MeCP2 as a relevant component, we studied whether embryonic cardiac MeCP2 is phosphorylated. Western blot using anti-MeCP2 S421 phospho-site-specific antibody detected a band at the expected size of MeCP2 in extracts from E12.5 hearts, suggesting that cardiac embryonic MeCP2 is indeed phosphorylated (Fig. 4D). The phospho-antibody failed to recognize MeCP2 in hearts isolated from adult mice (Fig. 4D), in extracts from Mecp2−/Y null hearts (Fig. 4D), or in E12.5 heart extracts treated with alkaline phosphatase (Fig. 4E). Together, these data indicate that embryonic cardiac MeCP2 is phosphorylated at S421, that this modification is not brain specific as previously suspected, and supports a functional role for MeCP2 in heart development.

Interestingly, the increase in total MeCP2 immunoreactivity observed in transgenic heart extracts when compared with wild-type embryos was paralleled when using the anti-MeCP2 S421 phospho-site-specific antibody (Fig. 4A), indicating that transgenic MeCP2 is phosphorylated and suggesting that the MeCP2-kinase activity is not saturated at physiological conditions in heart cells.

Global abnormal gene expression in transgenic hearts overexpressing MeCP2

Normal development is directed by a delicate balance of complex dynamic transcriptional regulation. The deleterious effects of MeCP2 overexpression on the developing heart could be therefore ascribed to abnormal patterns of gene expression. To examine this possibility, we determined the expression of cardiac specific genes in developing hearts of TM20dTg mice. We measured mRNA levels of alpha-cardiac actin (α-actin), alpha-myosin heavy chain (α-MHC), beta-myosin heavy chain (β-MHC), ventricular myosin light chain 2 (Mlc2v), GATA4, atrial natriuretic factor (ANF), Tbx-5 and brain natriuretic factor (BNF) by real-time RT-PCR in E14.5 hearts. Significant downregulation of mRNA levels was observed for beta-MHC, alpha-cardiac actin and Tbx-5 when compared with expression levels of wild-type littermates (P < 0.05, Fig. 5A). We also observed a trend for upregulation of ANF expression in E14 hearts. Although this result did not reach statistical significance, when we repeated the determination with atrium-specific RNA instead of whole heart, we found significant upregulation of ANF in atrium of E14 TM20dTg embryos (Fig. 5A). This finding was supported by in situ hybridizations (ISH) of whole hearts that showed increased ANF mRNA in E14 TM20dTg atria (Fig. 5B). ISH assays in E14 TM20dTg embryos showed that the reduction in Tbx-5 expression was also predominantly atrium-specific (Fig. 5B). In addition, we detected upregulation of the transcription factor Nkx2-5 by ISH, most notably in the IVS (Fig. 5B). These results indicate that regulation of cardiac gene expression is subverted by the abnormal expression of MeCP2, resulting in unbalanced transcriptional regulation with consequences for maturation of the heart.

Skeletal abnormalities and premature death in TM20dTg survivor transgenic mice overexpressing MeCP2

We monitored the small percentage of TM20dTg mice that survived development. Notably, we observed a 2:1 skewing of the male/female ratio in the surviving TM20dTg mice. These transgenic animals appeared normal compared with

Figure 2. Skeletal transgene expression in TM20dTg embryos. Direct detection of GFP in TM20dTg E14.5 embryos (A–G) show transgene expression in appendicular (B) and axial (D and G) skeletons. (H) TM20 E14.5 control embryo showing only background fluorescence.
their WT littermates until postnatal day 7 (P7). Nevertheless, after P10, both males and females had a smaller body size and lower body weight (Supplementary Material, Fig. S2). TM20dTg male mice began to exhibit a small protrusion of the sternum and signs of kyphosis that by P16 develops into a clearly distinguishable skeletal abnormality (Supplementary Material, Fig. S2). Mice showed a curved vertebral column and a severely protruded sternum and did not live past 12 weeks of age, with most of them dying between 3 and 5 weeks of age (n = 22, data not shown).

To investigate the skeletal abnormality systemically, we stained skeletal preparations from 5- and 10-week-old TM20dTg mice with alcian blue and alizarin red that stain cartilage and ossified skeleton, respectively. We observed a striking distortion and malformation of the sternum and abnormalities in the axial skeleton (Fig. 6 and data not shown). The bones of the sternum of TM20dTg mice are significantly shorter than controls, most probably due to incomplete ossification. This lack of ossification was very obvious in the manubrium, in which the normally osseous handles are completely cartilaginous in the TM20dTg females (see arrow in Fig. 6C). Further, staining with alcian blue was also significantly reduced in the ribs, intervertebral spaces and knee and elbow cartilage in TM20dTg male mice (arrowheads in Fig. 6 and not shown). A very prominent defect in the TM20dTg vertebra is spina bifida, the failure of the two laminae of the neural arch to fuse at the dorsal midline (Fig. 7). This feature was evident from the atlas (C1) up to the 4th–5th thoracic vertebra, but exencephaly was never observed. In spite of the severity of this phenotype, TM20dTg vertebrae are normal in number and otherwise shaped appropriately according to its position along the spinal column. Ossification of the skull is also incomplete, as can be observed in Figure 7D. TM20dTg mice also have a protuberance in the base of the skull, resulting from abnormal cartilage growth at the prephenoidal synchondrosis (Fig. 7E).
Cartilage-specific dysregulation of Runx2 expression in TM20dTg mice

We presumed that the skeletal phenotype of the TM20dTg mice was also a consequence of an incorrect epigenetic control of gene expression due to overexpression of MeCP2. Thus, we compared the levels of expression of candidate genes in TM20dTg and TM20 control mice. It was reported that the Eno2 promoter in the line of Eno2-tTA transgenic mice that we used in this study was active in osteoblasts and chondrocytes (70). Since in our transgensics we observed a pattern of transgene expression consistent with that report (Fig. 2 and Supplementary Material, Fig. S3), the skeletal phenotype is coherent with incomplete or largely delayed ossification and, in a microarray-based search for MeCP2 targets in the brain we identified Runx2 as a possible MeCP2-regulated gene (unpublished results), we analyzed the expression of Runx2 in TM20dTg mice. Using real-time RT–PCR, we found that the expression of Runx2 was significantly decreased in skeletal preparations of E14.5 TM20dTg, when compared with TM20 or WT controls (Fig. 7F). This downregulation of Runx2 expression was specific for the skeleton; hearts of TM20dTg mice (E14.5 or adults, data not sown) contain similar amounts of Runx2 mRNA. No differences were detected in the level of expression of other osteogenesis factors such as Bmp1, Bmp2 or Runx3. Thus, these data suggest that Runx2 deregulation could be involved in the skeletal abnormalities by affecting the replacement of cartilage with bone.

DISCUSSION

Our studies of MeCP2 overexpression provide insights into the interface between the correct interpretation of DNA methylation patterns and normal mammalian development. We identified cardiac and skeletal abnormalities caused by embryonic localized overexpression of transgenic MeCP2.

The effects of MeCP2 overexpression were dependent on the level of transgene expression. Most TM20dTg mice, expressing high levels of MeCP2, were unable to survive embryonic development and died at E14–E15. TM15dTg mice, which express significantly less transgenic MeCP2 than TM20dTg mice, develop normally and present a milder phenotype. When the levels of transgenic MeCP2 expression were increased by breeding TM15dTg mice to homozygosity, these embryos could not survive through development.

The cause of embryonic death in TM20dTg mice appears to be abnormal heart development, since we found overexpression of ANF and Nkx2-5 and downregulation of Tbx5. Nkx2-5 is a transcription factor essential for correct cardiogenesis. Mutations of human NKX2-5 were identified in patients with a variety of congenital heart malformations (74), and mice carrying mutant forms of Nkx2-5 that accumulated in the myocardium displayed conduction defects characterized by prolonged QT intervals (75). Tbx5 is a transcription factor which plays important roles in the development of the heart and upper limbs (76). Haploinsufficiency of this gene in humans result in Holt-Oram syndrome, an autosomal-dominant disorder featuring severe heart abnormalities (77), highlighting the crucial role of Tbx5 for normal heart functioning. Tbx5 normally acts as a transcriptional activator of ANF (78). However, in the TM20dTg mice, a decrease in atrial Tbx5 is not accompanied by diminished expression of ANF, but rather an upregulation of atrial ANF, suggesting a Tbx5-independent mechanism of ANF induction in these mice and stressing the dysregulation of gene expression controls due to overexpression of MeCP2 that could ultimately result in heart failure associated with cardiac hypertrophy.
Although it is not known whether NKx2-5, Tbx5 and ANF are cardiac targets of MeCP2 regulation, our findings grant further research into this direction. We found that MeCP2 is expressed in wild-type embryonic hearts. Notably, in the developing heart, MeCP2 is phosphorylated in S421, a modification associated with functional regulation that was not detectable in adult hearts. The significance of this developmental switch in MeCP2 phosphorylation status is unknown, but suggests that MeCP2 has a functional role during heart development. Accordingly, most patients carrying genomic triplications of a region that includes MeCP2 presented heart defects [(79) 59th Annual Meeting of The American Society of Human Genetics, 2009]. The effects of deleting MeCP2 in cardiac function are not clear. Abnormalities in heart rate (80), as well as lack of disturbances in autonomic cardiovascular regulation (81), were reported for MeCP2 null mice. In any case, the lack of MeCP2 in null mice could be compensated by the presence of other MBD containing proteins in heart cells. Work is underway in our laboratory to analyze the effects of inducible deletion of Meep2 in cardiomyocytes in order to investigate the role of this gene in heart development and function. Mutations in MeCP2 in humans cause RS, whose symptomatology includes a prolongation of QT corrected interval (QTc), and diminished heart rate variability (82–86). Although the cardiac abnormalities in RS have been generally attributed to disturbances of the autonomic nervous system (87), a definitive correlation between sympathetic disturbance and QTc prolongation in Rett patients has not yet been provided (88) allowing the possibility of an intrinsic heart defect in RS. The skeletal phenotype exhibited by the TM20dTg is notable since patients with increased copies of MeCP2 present dysmorphic features such as skull asymmetry, digit abnormalities and scoliosis (45,49,50). In addition, RS patients have reduced bone formation and are prone to develop osteoporosis (89), suggesting an important role for MeCP2 in bone formation. In fact, MeCP2 was found to regulate the expression of a gene essential for the maintenance of bone homeostasis, RANKL, in mouse osteoblasts (90), further supporting MeCP2’s involvement in skeletogenesis. We, however, did not detect differences in Rankl expression in TM20dTg mice (data not shown).

RS skeletal features such as scoliosis have also been proposed to stem from motor problems. Our data suggest that
the primary cause might be abnormal ossification. We found that TM20dTg mice displayed diminished ossification resulting in severe kyphosis, a distorted sternum, spina bifida and a deformation in the base of the skull. These data suggest that the dysmorphic features seen in MeCP2 duplication and triplication patients (45,48,49) might be the result of improper ossification due to MeCP2 dysfunction in skeletal tissues. The underlying cause of abnormal ossification seems to be a deregulation of gene expression due to MeCP2 overexpression. We determined that the expression of the Runt homology domain transcription factor Runx2 is decreased in skeletal preparations of E14.5 TM20dTg mice, when compared with wild-type or single transgenic embryos.

Runx2 regulates osteoblast sequential differentiation and loss-of-function mutations in this gene cause failure to replace the cartilaginous skeleton and defective ossification, osteochondrodysplasias. Deletion of Runx2 in mice results in a complete absence of bone tissue, defective chondrocytes and undifferentiated osteoblasts (91–94). In humans, mutations in RUNX2 cause cleidocranial dysplasia (CCD), a disease characterized by open or delayed closure of calvarial sutures, hypoplastic or aplastic clavicles and supernumerary teeth (95,96). Other phenotypes caused by mutations in the RUNX2 gene include deficient bone mass and osteoporosis (97).

In summary, our data highlight the importance of an undisrupted interpretation of epigenetic control mechanisms for normal development and predict that some human developmental disorders involving cardiac and/or skeletal dysmorphogenesis might result from alterations in the normal level or pattern of MeCP2 expression.

Moreover, we envisage that mutations arising in regions of DNA involved in the regulation of MeCP2 expression levels (promoter, enhancers etc.) or in genes encoding regulatory factors of MeCP2 could cause early to mid-gestation miscarriages.

MATERIALS AND METHODS

Animal breeding

EGFP-TRE-MECP2-e2 (herein referred to as TM mice (TM = Transgenic for MECP2)) have been previously described (60).
diluted and boiled in 2X loading buffer (62.5 mM Tris-Cl, supplemented with 1% SDS and 1X protease inhibitor cocktail). We also backcrossed the FVB mice for 6 generations to a 129/SvJ background and obtained identical results. In staging the embryos, embryonic day 0.5 (E0.5) was defined as 12 noon of the day a vaginal plug was found after overnight mating. Animals were kept in an animal room under SPF conditions at a room temperature of 20°C, in a 12/12 hour light/dark cycle with water and food ad libitum. All experiments were approved by the Centro de Estudios Científicos (CECS) Animal Care and Use Committee.

**Immunoblot**

Embryonic, postnatal and adult hearts were quickly dissected in cold 0.1 M phosphate buffered saline (PBS) and homogenized in lysis buffer composed of 125 mM Tris-Cl, pH 6.8, supplemented with 1% SDS and 1X protease inhibitor cocktail (Sigma). Twenty microgram of protein extracts were then diluted and boiled in 2X loading buffer (62.5 mM Tris-Cl, pH 6.8, 25% glycerol, 2% SDS, 100 mM DTT, 0.002% Bromophenol Blue), separated in 8% SDS polyacrylamide gels and transferred onto PVDF membranes (Bio-Rad). Membranes were blocked for 1 h at RT with freshly prepared 5% non-fat dry milk diluted in TBS-Tween 0.05%, pH 7.6. Membranes were incubated for 1 h with primary antibodies rabbit anti C-terminal MeCP2 (1:1000; Upstate), rabbit anti-actin (1:10000; Sigma), rabbit anti-pMeCP2 S421 (1:5000) and anti C-terminal MeCP2 (1:1000; Upstate), rabbit anti-actin (1:10000; Sigma), rabbit anti-actin (1:10000; Sigma), rabbit anti-actin (1:10000; Sigma). Membranes were then washed five times with TBST buffer and incubated for 45 min at room temperature with anti-rabbit HRP-conjugated IgG at 1:4000 (Pierce). The bands were detected using the SuperSignal West Femto chemiluminescent substrate according to the manufacturer’s instructions (ThermoFisher Scientific). Densitometry of immunoreactive bands was quantitated with Quantity One software (Bio-Rad).

**Fluorescence microscopy**

EGFP expressing double-transgenic embryos and WT littermates were immersed in cold 0.1 M PBS, pH 7.4 within 1% agarose plates and images captured with an Olympus SZX70 dissecting microscope (Japan). Photographs were taken with a Q Imaging 3.3 RTV cooled CCD camera and Q Capture Pro Software and further processed with Adobe Photoshop 7.0.

**Fluorescent immunohistochemistry**

Once dissected, heart of E14.5 embryos were briefly rinsed in cold 0.1 M PBS, pH 7.4 and fixed for 6 h at 4°C in 4% paraformaldehyde, pH 7.4 (Sigma). After overnight fixation, hearts were cryoprotected for 24 h at 4°C in 30% sucrose diluted in 0.1 M PBS, embedded in O.C.T. medium (Tissue Tek) and frozen at −80°C. Twenty micromoral coronal sections were serially collected onto pre-cleaned SuperFrost slides (Fisherbrand) and frozen at −80°C until use. For immunolabeling, sections were washed five times with 0.1 M PBS, pH 7.4 with 0.1% triton X-100 (PBS-T) and blocked at room temperature for 1 h in 0.1 M PBS-T supplemented with 5% normal goat serum (Sigma). Sections were then incubated with primary antibodies in blocking solution over night at 4°C: anti-MeCP2 C-terminal 1:100 (Upstate) and anti-GFP 1:500 (Molecular Probes). Sections were washed five times with blocking solution and incubated for 2 h at room temperature with secondary antibodies conjugated to Alexa Fluor 488 (Molecular Probes) or Cy3 (Jackson Immunoresearch) at a 1:1000 dilution. Nuclei were counterstained with a solution containing 300 nM of 4′,6-diamino-2-phenyindole (DAPI) diluted in sterile H2O for 15 min at room temperature and washed three times with 0.1 M PBS. Slides were cover slipped with fluorescent mounting medium (DAKO). Images were captured with an Olympus CX31 epifluorescence microscope or a Zeiss Axiovert 100 M confocal microscope.

**Histology**

Wild-type, single and double transgenic embryos (all experiments were conducted with littermates) were embedded in paraffin and 5 μm serial transverse sections were collected in slides treated with APES (Sigma) and stained with eosin and hematoxylin (Sigma).

**BrdU injections and immunodetection**

Timed-pregnant females were injected intraperitoneally with 100 μg/g body weight of 5-bromo-2′-deoxyuridine (BrdU; Sigma) diluted in sterile 0.1 M PBS, pH 7.4 and sacrificed via cervical dislocation 2 h later. Embryos were removed via Caesarean section, quickly rinsed in cold 0.1 M PBS, fixed overnight in 4% paraformaldehyde, pH 7.4 (Sigma) and embedded in paraffin and sectioned at 5 μm. For BrdU immunodetection, sections were deparaffinized, rehydrated and incubated in 2N HCl for 30 min at 37°C, rinsed in 0.1 M sodium borate, pH 8.3, blocked for 1 hour at RT with 0.1 M PBS-T supplemented with 5% normal goat serum and then incubated overnight at 4°C with a monoclonal anti-BrdU antibody (Sigma) at a 1:200 dilution. Secondary goat anti-mouse biotin-conjugated (Vector labs) was used at a 1:300 dilution for 2 h at RT. Sections were dehydrated, cleared in Xylene (Sigma) and mounted with Permount (Sigma).

**TUNEL assay**

Sections from paraffin-embedded tissues were examined for apoptosis by TUNEL *in situ* cell death detection kit AP (Roche Applied Science) according to the manufacturer’s instructions. The average number of positive cells was determined from five separate fields under ×40 magnification.

**Alcian blue/alizarin red co-staining of cartilage and bone**

Mice were injected intraperitoneally with a lethal dose of 2% avertine (2,2,2-tribromopropanol, 0.2 ml/g body weight) and their skin, fat and internal organs removed. Specimens were fixed for 48 h in 95% ethanol and stained with alcian blue (0.03% in 80% ethanol and 20% acetic acid) for 48 h. Specimens were then treated with 2% KOH for 24 h and counterstained with alizarin red (0.03% in 1% KOH) for 12 h. Skeletons were then cleared for 7 days in 1% KOH/20% glycerol and stored on 50% glycerol/50%...
ethanol. All incubations were performed at room temperature with gentle agitation.

**Doxycycline administration**

In order to inhibit the tTA molecule from binding to the TRE element driving expression of the EGFP and *MECP2* genes, we treated timed-pregnant mothers starting at 2–3 dpc with freshly prepared 200 μg/ml of doxycycline (Sigma) supplemented with 1% sucrose in sterile water and changed it every other day. Transgene inhibition and drug efficacy was monitored by checking that EGFP expression in the heart and cartilage tissue was off after 8 days of treatment in TM15 double transgenic embryos.

**Real-time quantitative PCR analysis**

Total RNA was also isolated from hearts of E14.5 with TRIzol reagent according to the procedure described by the manufacturer (Invitrogen, CA, USA). Total RNA samples were all treated with DNase to remove contaminating genomic DNA (DNA-free™, Ambion, Austin, TX, USA), quantitated by measuring absorbance at 260 nm and stored at −80 °C until used. Equal amounts of total RNA (2 μg) were reverse transcribed with random hexamers (ImProm-II™ Reverse Transcription System, Promega Corporation Madison, WI, USA). PCR mixtures were prepared with Quantimix Easy Syg Kit for real-time DNA amplification and quantification (Biotools, Spain). Quantitative real-time RT–PCR amplifications were performed in triplicate in the Rotor-Gene 6000 (Corbett, Australia) in a total volume of 10 μl, each reaction containing 1 μl of diluted cDNA. The results were analyzed with the Rotor-Gene 6000 Series Software 1.7 (Corbett) and all values were normalized to the levels of the GAPDH mRNA. PCR amplification of the GAPDH RNA served as internal control. Abundance of PCR products was calculated from standard curves (correlation coefficient ≥0.98). Primers for all target genes were designed using Primer3 (Whitehead Institute for Biomedical Research) and checked for selectivity using BLAST (NCBI). Runx2 (NM_009820.2) forward 5'-GACAGAAGGATGAGGAAG-3' and reverse 5'-TTGTGTTATCTTCTGCCGGTGC-3; BMP2 (NM_007553.2) forward 5'-TGACTCTGTCCTTGTG-3' and reverse 5'-AGGCCTTCACCTTCAGCTGC-3; BMP1 (NM_009755.2) forward 5'-AGGCCGCCATCTTCTCTCAG-3 and reverse 5'-TTGTGTTCACAGCCAGCCTTC-3; BMP2 (NM_007553.2) forward 5'-TGGAAGTGCCCATTTAGAG-3 and reverse 5'-GCTTTTCTGAGAAGATGACC-3 and reverse 5'-CCAGGTTCAACGCATTTCCGAT-3 and reverse 5'-CTCTCTATCTTTCTGCGGTTGC-3; BMP1 (NM_009755.2) forward 5'-CGTTTGTGGAGC-3; forward 5'-GCCAACCCGTTAAGAGTACCC-3 and reverse 5'-GAGGTTGGAAGCAGGAGAAGT-3 and reverse 5'-AGAGCTTCTCCTCAACCAAGGC-3 and reverse 5'-GCTCTGTCGCCAGGAGGAAG-3; -MHC (NM_010856.3) forward 5'-GGCGTGAAGGCTCATGGTTGGAT-3 and reverse 5'-TGGAAGGCTCATGGTTGGAT-3; Cα-actin (NM_009608.2) forward 5'-CCAGCAGACCTTCTCAG-3 and reverse 5'-TTGTGTTCACAGCCAGCCTTC-3; BNF (NM_008726.3) forward 5'-AGGCCTTCACCTTCTCTCAG-3 and reverse 5'-TTGTGTTCACAGCCAGCCTTC-3; BNF (NM_008726.3) forward 5'-CCAGGTTCAACGCATTTCCGAT-3 and reverse 5'-CTCTCTATCTTTCTGCGGTTGC-3; -MHC (NM_010856.3) forward 5'-GGCGTGAAGGCTCATGGTTGGAT-3 and reverse 5'-TGGAAGGCTCATGGTTGGAT-3; Cα-actin (NM_009608.2) forward 5'-CCAGCAGACCTTCTCAG-3 and reverse 5'-TTGTGTTCACAGCCAGCCTTC-3; BNF (NM_008726.3) forward 5'-AGGCCTTCACCTTCTCTCAG-3 and reverse 5'-TTGTGTTCACAGCCAGCCTTC-3; ANF (NM_008725.2) forward 5'-GCCGCGTAAAGATGAGCTTCA-3 and reverse 5'-GGGCTCAATCCTGTCAATC-3; Tbx-5 (NC_000071.5) forward 5'-GAGCACAGCGAAAAATTACCA-3 and reverse 5'-CCAGGATAGAAGGTTGTCT-3; RANKL (NC_000080.5) forward 5'-TGGAAGGCTCATGGTTGGAT-3 and reverse 5'-AGGCCTTCACCTTCTCTCAG-3; BNF (NM_008726.3) forward 5'-CTGAAGGCTGTGCTCAGCAT-3 and reverse 5'-GCTTTGCTCCTCAAGAGCCTG-3; GAPDH (NM_008084) forward 5'-ACCCAGAAGACTCTGTTGATGG-3 and reverse 5'-CACATTGGGGGTAGGAACAC-3.

**Whole-mount in situ hybridization**

ISH were performed according to Wilkinson (98), using digoxigenin (Dig)-labeled probes for mouse tbx5 (99), mouse nkh2.5 and rat ANF.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

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*Conflict of Interest statement.* None declared.

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