Table 4
Clinical profile of Arima syndrome and related diseases.

		Arima syndrome	Dekaban syndrome	Joubert syndrome	Senior-Loken syndrome	COACH syndrome	Meckel–Grüber syndrome
	Age of onset Inheritance	Birth to infancy AR	Neonate to infancy AR	Neonate to infancy AR	Neonate to infancy AR	Neonate to infancy AR	Birth AR
Psychomotor development	Severe mental retardation	+	+	+	+	+	n.d.
	Motor delay	+	+	+	+	+	n.d.
	Hypotonia	+	+	+		+	n.d.
Brain malformation	Cerebellar vermis hypo-/aplasia	+	+	+		+	+
	Brainstem malformation	+	±	±		–	+
	Cerebral malformation	+	±	±		Hydrocephalus	Encephalocele
Visual pathology and eye movement	Retinopathy	+	+	±	+	n.d.	n.d.
	Other findings	Leber congenital amaurosis,Optic atrophy,Coloboma	Leber congenital amaurosis,Chorioretinal coloboma	Coloboma	Leber congenital amaurosis	Coloboma	n.d.
	Eye movement	Horizontal nystagmus	–	Nystagmus, oculomotor apraxia	-	Nystagmus	n.d.
	Ptosis	+	–	±	-	n.d.	n.d.
Renal pathology	Nephronophthisis	+	n.d.	±	+	±	±
	Cystic kidney	+	+	±	±	±	+
Liver pathology		Cirrhosis	Hepatic fibrosis	Hepatic fibrosis	Hepatic fibrosis	Hepatic fibrosis	Hepatic fibrosis Polyductyly
Typical other symptoms		Abnormal respiration					
Causative genes		-	MKS2, NPHP1, NPHP6	AHI1, RPGRIP1L, CC2D2A, INPPSE, ARL13B	NPHP1, NPHP1, NPHP3, NPHP4, NPHP5, NPHP6, NPHP8, SDCCAG8, AHI1	CC2D2A, RPGRIP1, MKS3	MKS1, MKS3, NPHP3, NPHP6, NPHP8
References		[1,2,4–11]	[12,15]	[12,15,16]	[12,15,16]	[14,16]	[14–16]

–; No or absent, +; yes or evidenced, n.d.; not described, AR; autosomal recessive inheritance.

nephronophthisis [20]. It is necessary to describe AS clinical characteristics in detail, because it is clinically close to JS based on the present study. AS might be a more severe form than JS.

Two previous reports of AS have diagnostically described its characteristic features; brain malformation, polycystic kidney, retinitis pigmentosa and ptosis [1], and visual dysfunction, hypotonia, severe psychomotor delay, peculiar face with ptosis, abnormal eye movement and progressive renal dysfunction [2]. The symptom of ptosis in AS appears and disappears in a day, and is due to dehydration or secondary change of low intraocular pressure or small eyeball [21]. This ptosis-like peculiar facial appearance may not be a specific feature of AS, although it may be a very characteristic feature of AS. Indeed, some patients with JS obviously showed ptosis with age. Ptosis in both appeared with high frequency from the results of the present survey. The visual dysfunction of AS was identified from the early stage, and its electroretinography showed no response. AS was also sometimes accompanied with chorioretinal defect, whereas very few JS patients showed retinal dysfunction [22]. Retinal dysfunction is more common in AS than JS, although retinal dystrophy is one of major symptoms of patients with JSRD [19]. Liver signs are pathologically cirrhosis, fibrosis and fatty liver, as well as clinical hepatomegaly and hepatic dysfunction [2,5]. As a result, the clinical diagnostic criteria of AS must include the symptoms of severe psychomotor delay, renal dysfunction and visual dysfunction from the infantile period, cerebellar vermis agenesis/hypoplasia, and with or without occasional ptosis. To summarize, AS is a more severe form than JS, yet its features are very similar to those of JS. However, it has been sometimes difficult to diagnose JS with AS since the first description. From advanced technologies and several case

Please cite this article in press as: Itoh M et al. Nationwide survey of Arima syndrome: Revised diagnostic criteria from epidemiological analysis.. Brain Dev (2013), http://dx.doi.org/10.1016/j.braindev.2013.06.005

reports, we proposed a revised set of AS diagnostic criteria, including renal pathology, imagings and laboratory examinations (Table 3).

We reviewed the previously described cases of AS, JS and JSRD (Table 4). JS originally begins with signs of hypotonia, abnormal respiratory movement, oculomotor apraxia, cerebellar vermis agenesis or hypoplasia [22]. The abnormal respiratory movement of AS patients is not common. Recently, many variants of JS have become known, such as JS accompanied with visual dysfunction and/or renal dysfunction, and JS with faciodigital abnormality and status inversus [23–25]. It is considered that ciliopathies of JS are related to other diseases, such as Senior-Loken syndrome (main symptoms of congenital visual dysfunction, nephronophthisis and severe mental retardation), COACH syndrome (coloboma, hepatic fibrosis, severe mental retardation and cerebellar vermis hypoplasia) and Meckel-Grüber syndrome (encephalocele, polyductyly and multiple abnormalities in various organ systems) [15,17,27,28]. The causative genes of these diseases, JS and JSRD have been discovered in over 15 genes, contributing to cilial function [13–18,26]. Interestingly, some symptoms of AS overlap those of ciliopathies. The present survey revealed some cases of JS with visual or renal dysfunction. These functional failures may be common to AS, JS and JSRD. Although the causative genes of AS are still unknown, they provide hints to understanding the pathophysiological relationship of ciliopathies and AS.

In conclusion, we performed the first nationwide survey of AS, and determined the number of patients with the disease and their clinical characteristics. We reviewed the previously reported cases of AS and proposed a set of revised diagnostic criteria for AS.

In forthcoming study, we must pinpoint the causative gene of AS and clarify the relationship of AS and the ciliopathies, such as JS and JSRD, Senior-Loken syndrome, COACH syndrome and Dekaban syndrome.

Acknowledgements

Our heartfelt thanks to the physicians, hospitals and institutes that assisted in obtaining responses to our questionnaires.

This study was supported by Grants from the Ministry of Health, Welfare and Labor of Japan and the Japan Foundation for Pediatric Research.

References

[1] Arima M, Ono K, Hisada K, et al. A familial syndrome of maldevelopment of the brain, polycystic kidneys, congenital hapetoretinal dysplasia with coloboma and unilateral ptosis. No to Hattatsu 1971;3:330–1 [in Japanese].

[2] Matsuzaka T, Sugai K, Sakuragawa N, et al. A specific type of cerebro-oculo-hepato-renal syndrome: Arima syndrome, a distinct clinicopathological entity. Nippon Shonika Gakkai Zasshi 1986;90:1322–34 [in Japanese].

[3] Matsuzaka T, Sakuragawa N, Nakayama H, et al. Cerebro-oculo-hepato-renal syndrome (Arima's syndrome): a distinct clinicopathological entity. J. Child Neurol. 1986;1:338–46.

[4] Hisada K, Ono K, Arima M, et al. A case of familial cystic kidney, congenital brain disorder, retinopathy, unilateral ptosis. Shonika Shinryo 1972;35:486–94 [in Japanese].

[5] Koya G, Shinohara T, Morimatsu Y, et al. Congenital polycystic kidney syndrome. No to Hattatsu 1973;5:229–41 [in Japanese].

[6] Kawaguchi H, Yoshida S, Yamamoto K, et al. Three siblings associated with infantile polycystic kidney disease, tapetretinal degeneration and maldevelopment of the brain. Nippon Shonika Gakkai Zasshi 1984;88:890–9 [in Japanese].

[7] Kubota M, Shinozaki M, Ishizaki A, et al. Cerebro-oculo-hepato-renal syndrome (Arima syndrome) with slowly progressive renal insufficiency and epilepsy. No to Hattatsu 1991;23:596–600 [in Japanese].

[8] Yadoya A, Usui N, Imadate A, et al. A case of Arima syndrome, with chronic renal insufficiency, treated with CAPD. Nippon Shoni Jinfuzen Gakkai Zasshi 2000;20:112–5 [in Japanese].

[9] Kobayashi Y, Watanabe T, Morikawa A, et al. Two cases of cerebro-oculo-hepato-renal syndrome (Arima syndrome). Nippon Shoni Jinfuzen Gakkai Zasshi 2004;24:267–70 [in Japanese].

[10] Katase K, Hashizume K, Yoneda T, et al. A case of Arima syndrome (cerebro-oculo-hepato-renal syndrome) in long-term survival with hemodialysis. Nippon Jinzo Gakkai Zasshi 2006;48:731–5 [in Japanese].

[11] Takahashi K, Oka A, Mizuguchi M, et al. Interstitial deletion of 13q14.13-q32.3 presenting with Arima syndrome and bilateral retinoblastoma. Brain Dev. 2011;33:353–6.

[12] Dekaban AS. Hereditary syndrome of congenital retinal blindness (Leber), polycystic kidneys and maldevelopment of the brain. Am. J. Ophthalmol. 1969;68:1029–37.

[13] Matsuzaka T. Cerebello-oculo-renal syndrome (Arima syndrome). Shonika Rinsho 1988;41:1211–8 [in Japanese].

[14] Quinlan RJ, Tobin JL, Beales PL. Modeling ciliopathies: primary cilia in development and disease. Curr. Top. Dev. Biol. 2008;84:249–310.

[15] Sharma N, Berbari NF, Yoder BK. Ciliary dysfunction in developmental abnormalities and diseases. Curr. Top. Dev. Biol. 2008;85:371–427.

[16] Wolf MTF, Hildebrandt F. Nephronophthisis. Pediatr. Nephrol. 2011;26:181–94.

[17] Sattar S, Gleeson JG. The ciliopathies in neuronal development: a clinical approach to investigation of Joubert syndrome and Joubert syndrome-related disorders. Dev. Med. Child Neurol. 2011;53:793–8.

[18] Mykytyn K. Clinical variability in ciliary disorders. Nat. Genet. 2007;39:818–9.

[19] Parisi MA. Clinical and molecular features of Joubert syndrome and related disorders. Am. J. Med. Genet. C 2009;151C:326–40.

[20] Kumada S, Hayashi M, Arima K, et al. Renal disease in Arima syndrome is nephronophthisis as in other Joubert-related cerebello-oculo-renal syndromes. Am. J. Med. Genet. A 2004;131A:71–6.

[21] Arima M. Arima syndrome. Shoni Naika 2007;39:1257–62 [in Japanese].

[22] Joubert M, Eisenring JJ, Robb JP, et al. Familial agenesis of the cerebellar vermis. Neurology 1969;19:813–25.

[23] Gleeson JG, Keeler LC, Parisi MA, et al. Molar tooth sign of the midbrain-hindbrain junction: occurrence in multiple distinct syndromes. Am. J. Med. Genet. A 2004;125A:125–34.

[24] Valente EM, Brancati F, Dallapiccola B. Genotypes and phenotypes of Joubert syndrome and related disorders. Eur. J. Med. Genet. 2008;51:1–23.

Please cite this article in press as: Itoh M et al. Nationwide survey of Arima syndrome: Revised diagnostic criteria from epidemiological analysis.. Brain Dev (2013), http://dx.doi.org/10.1016/j.braindev.2013.06.005

[25] Chance PF, Cavalier L, Satran D, et al. Clinical nosologic and genetic aspects of Joubert and related syndromes. J. Child Neurol. 1999;14:660–6.

[26] Zaghloul NA, Katsanis N. Mechanistic insights into Bardet–Biedl syndrome, a model ciliopathy. J. Clin. Invest. 2009;119:428–37.

[27] Senior B, Friedmann AI, Braudo JL. Juvenile familial nephropathy with hapetretinal degeneration. Am. J. Ophthalmol. 1961;52:625–33.

[28] Verloes A, Lambotte C. Further delineation of a syndrome of cerebellar vermis hypo/aplasia, oligophrenia, congenital ataxia, coloboma, and hepatic fibrosis. Am. J. Med. Genet. 1989;32:227–32.

Please cite this article in press as: Itoh M et al. Nationwide survey of Arima syndrome: Revised diagnostic criteria from epidemiological analysis.. Brain Dev (2013), http://dx.doi.org/10.1016/j.braindev.2013.06.005

10 NICU後の障害児の行方 —重症心身障害児施設の立場から

岩崎 裕治　　東京都立東部療育センター

Key Word　NICU長期入院児　重症心身障害児施設　療育施設　在宅支援

はじめに

近年，周産期医療の進歩に伴い，人工呼吸器管理等の濃厚な医療が必要となり，長期にNICUでの入院を余儀なくされる患者が増加した．梶原らは，全国の新生児施設に1年以上の長期入院児が216例存在しており，今後の対応としては58%が地域の療育施設を考えているとしているが受け入れの施設がないことも多いと報告している[1,2]．楠田らはNICU長期入院児の年間発生数は全国で約220例と推定している[3]．さらに長期入院児の基礎疾患をみると先天異常，新生児仮死，神経・筋疾患等が多く，早産児は在宅に帰れる可能性が高いとしている．このように在宅に帰ることが困難な児の基礎疾患は，重症心身障害児(以下重症児)施設等で治療やリハビリテーション(以下リハ)を受けることが多い疾患であり，この児たちのQOLを考えると療育施設でみていくべきであるという声がある[1,2,4]．

一方，療育施設では，NICU長期入院児のみならず，超重症児といった濃厚な医療的ケアが必要な入所児(者)が増加し，課題も多い[4,5]．

現在，全国には，74の国立病院機構病院重症心身障害児病棟(以下国立病院機構病棟)および122の公法人立重症心身障害児施設(以下公法人立施設)がある(ここではこれらを療育施設とよぶ)．本稿ではこの現状を明らかにし，NICU後の障害児の行方を論じたい．

療育施設の現状

(1) 療育施設でのNICU長期入院児の受け入れ状況

図1は宮野前ら[6]が，国立病院機構病棟のデータベースをもとに，1967～2002年までの国立病院機構病棟への入所経路を経年的に調べたもので

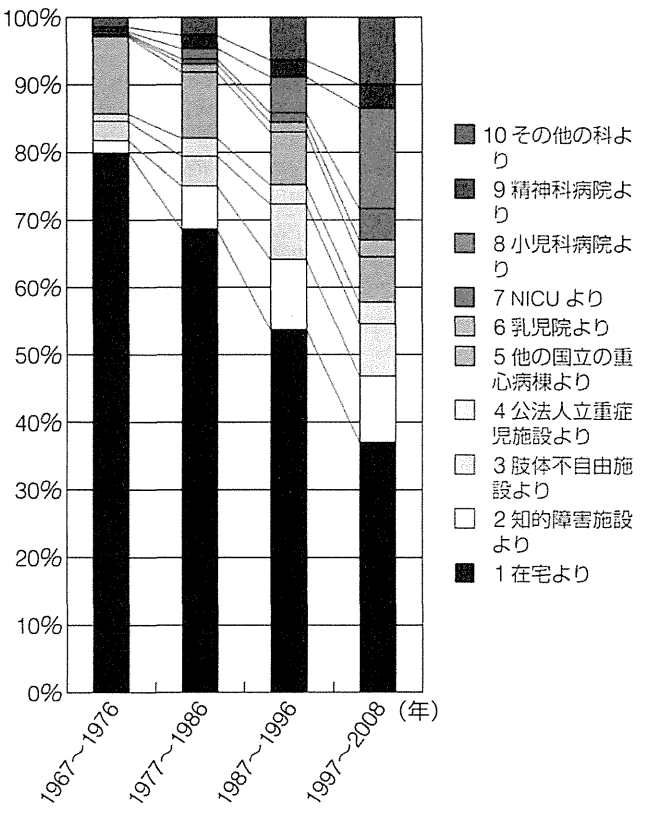

図1　公法人立重症心身障害児施設への年次別入院経路
(宮野前・他，2009)[6]

ある．これをみると年々NICUや小児科病棟からの入所が増加してきている．また，筆者ら[4]が2007～2008年度に実施した全国の療育施設を対象にしたアンケートでは，全入所児(者)に対して，NICU長期入院児は11.6%，小児科長期入院児が20.6%であり，宮野前らの調査よりさらにNICU，小児科病棟からの入所が増加してきている．この入所児の準・超重症児の割合は，NICU長期入院児で76.0%，小児科長期入院児で71.4%であり，療育施設の入所児(者)の重症化が進んでいる原因となっている．

調査時，療育施設に入所中で呼吸管理等が必要な児(者)は全体の5.9%で，SpO_2や心拍モニター等が必要なケースまで含めると17.7%となる．呼吸器が10台以上稼動している施設は国立病院機構病棟で7施設(20%)，公法人立施設で17施設(22%)，20台以上は前者で2施設(5.7%)，後者で4施設(5.3%)となる．このように入所児(者)の重度化が進んでおり，さらに医療的に重度の患者を受け入れるのが難しい現状がある．

前述のアンケートで「現在さらに呼吸器管理が必要な患者を受け入れることが可能か」という質問に，111施設中53施設が呼吸器使用でも受け入れ可能であったが，58施設では難しいということであった．それでは「NICU長期入院児の受け入れを進めていくうえで何が必要か」と聞いたところ，**図2**のように全体では，医師・看護師不足の改善が一番であった．また，医療器機不足の改善，家族の協力・理解，診療報酬改善等を望む声も多かった．地域連携関連では急変時の受け入れ施設，NICU等との連携・情報交換，中間施設の必要性等が多かった．

しかし，直接NICUから患者を受け入れることに関しては，NICUとの環境の違いが大きく危険であるとして否定的な意見も多い．重症児は環境の変化に弱く，転院等を契機に重篤な状況に陥ることがある[7,8]．

(2) 待機児(者)の状況

前述のアンケートでは，療育施設で把握している長期入所希望の待機人数は，総数971名(小児457名，成人514名)であった．この他にも重症児施設を対象に2006年に口分田[9]が，また2009

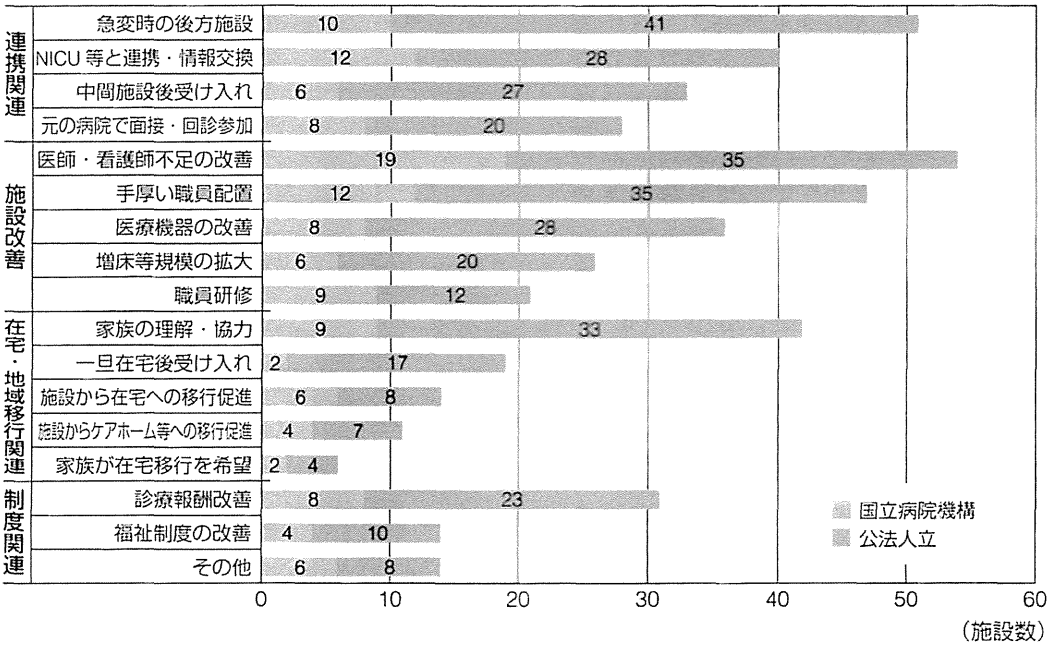

図2　NICU長期入院児受け入れに何が必要か

NICU長期入院児の受け入れを進めていくうえで，療育施設側として何が必要なのかを聞いたところ，全体では，医師･看護師不足の改善が一番であった．また医療器機不足の改善，家族の協力･理解，診療報酬改善等を望む声も多かった．地域連携関連では，急変事の後方施設，NICU等との連携・情報交換，中間施設で受け入れ後に移行等が多かった．　(岩崎・他，2010)[4]

年に澤野ら[10]が全国の公法人立施設を対象に待機児(者)の調査を行い，口分田は878名，澤野らは955名の待機児(者)が存在するという結果であった．これらの調査には，東京，大阪市，横浜市等は含まれていないことや，回答率を考えると，全国では3,000～5,000名の待機児(者)がいるのではないかと推測されている．このように待機児(者)が多く，また在宅で暮らしている重症心身障害児(者)の重症度も近年高くなっており[11]，療育施設としてNICUや小児科長期入所児も受け入れていかなくてはいけないが，在宅で暮らして療育施設への入所を待っている方たちも数多く存在するという現状がある．

NICU後の障害児の行方

(1) 連携の重要性

NICUからの療育施設への受け入れについては，お互いの状況の理解をもとにした十分な連携のなかで，その児の状況に合った施設や生活環境を考えていくことが大切である[12]．また，転院前の十分な説明や家族の理解がとても重要である．NICUと療育施設の違いや患者の状況を家族が十分に理解されないまま施設へ移行になり，トラブルになるというケースもある．超重症児等の死亡率は特に乳幼児期にとても高い．筆者らの施設の開設時に，半年の間に在宅で待機されていた6歳以下の患者30名のうち7名が亡くなっている(約20%程度)．このような厳しい現実もあるということを理解しておいてもらいたい．

家室は24年間にNICUから直接入所した患者と，NICUから小児科に移ってから入所した等の間接的に入所した患者の比較を報告している．NICUから直接入所した群の死亡率は間接的に入所した群に比べ高く(11/16＝69%)，その医療的重症度は想像以上であり保護者対応は困難を極めた，また保護者とのトラブルはNICUと同等の医療を望み，リハをはじめ療育に過大の期待をしていたことにあると述べている[13]．しかし，NICUとの連携会議等を開くことである程度問題は解消され，また死亡率の低下にもつながったとしている．このように連携に関連しては，急変時の受け入れ施設という点でも重要だが，療育施設の状況を理解してもらい，適切な対象児の受け入れを図ることで，医療的な予後も改善する可能性がある．

(2) 在宅支援の重要性

在宅に移行するケースではその後の在宅支援がとても重要になる．療育施設が行っている通所事業や，短期入所事業は重症児の在宅生活を支える大きな資源となる．短期入所利用児(者)数は年々増加しており，地域によってはベッド数が足りないところもある．需要に見合ったベッド数の確保が在宅生活を支えるうえで必要になる．また，医療度が高くなるにつれ短期入所中の体調不良等もよくみられる(**図3**)[14]．このような現状を踏まえたうえでスタッフの確保，医療環境の整備等を図り，在宅支援を行っていく必要がある．そのためには経済的な基盤が重要になるが，現在の短期入所の給付費では超重症児等を受け入れると経営的には赤字になってしまうという問題がある[5]．

重症心身障害児(者)の通所は，現在，A型通所が全国で61カ所，B型通所が235カ所と徐々に増加してきている．通所に通うことで，生活リズ

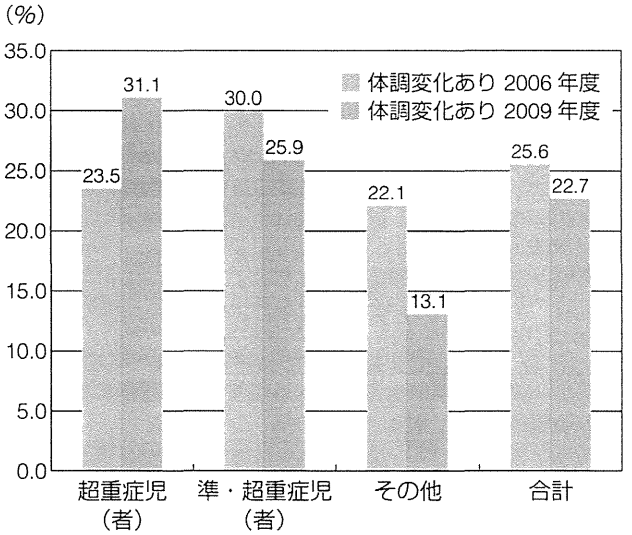

図3 短期入所中の体調変化(2006，2009年度)
都立東部療育センターに2006年，2009年度に短期入所された利用児(者)で体調変化があった割合を，重症度別に表したものである．体調変化は，準・超重症児(者)に多くみられた．また，最も多い体調変化としては発熱であり，その他，呼吸障害，嘔吐，緊張等であった．
(岩崎・他，2011)[14]

ムを整え，また，医療的ケア，入浴サービス，日中活動(レクリエーション，社会参加活動等)等の提供が受けられる．NICU長期入院児に限らず障害児には，このようなサービスを身近で受けられることが望ましい．2012年度から重症心身障害児(者)の通所施設が法制化され，市区町村が実施主体になる．市区町村が実施主体になり，より身近なところでこのようなサービスが受けられやすくなることを期待したいが，逆に地域格差が出てくることも懸念される．

(3) 中間施設について

療育施設とNICUでは医療環境(ハード，ソフト面ともに)の違いがあり，NICUから療育施設への直接の移行は難しいと考えている施設も多い．NICU側からも病院と福祉施設の中間の性格をもつ中間施設が必要ではないかという議論がある[15]．たとえば，NICUから同じ病院の小児科に一度移り，環境の違いにも慣れたころに施設への移行を考えるほうがリスクも少ない．一旦療育施設に移行した後に急変した際の受け入れを考えると，小児科の協力も必要になる．

また，重度の障害をもっている児はさまざまな合併症を併せもつことが多い．療育施設では小児科，内科，精神科の医師がほとんどであり，他科の合併症の治療は難しいことが多い．これら合併症への対応も療育施設に移行する前に，総合病院等である程度治療が済み，また何かあればそこで診てもらえる体制を確保しておきたい．地域中核病院へのアンケートでは，在宅移行後の急性増悪時の一時的な呼吸管理が可能かという問いに約1/3の病院が可能と答えている．条件付きも含めると2/3が可能ということであり，これらの病院は中間施設となり得るかもしれない[16]．

おわりに

NICU後の障害児の行方ということでは，対象となる乳幼児の状態や，家族の状況のなかで，その移行先は家族の十分な理解のもとに，関係者でよく協議をして，適切に判断することが必要である．療育施設へ移行の際には，十分な施設状況の説明や，家族の理解がなされていることが望ましい．また，直接施設への移行が難しいケースでは，小児科病棟や中間施設を経て移行する等配慮が必要である．また，今後療育施設ではさらに在宅支援を充実させていくことが求められていると考える．

文献

1) 梶原眞人・他：NICUに長期入院中の(準)超重症児の実態調査と分析：第1報．平成18年度厚生科研費 障害者自立支援法下での重症心身障害児等に対する施設サービスの効果的な在り方に関する研究，2007，pp45-53.
2) 梶原眞人・他：NICU長期入院児QOL調査．平成18-21年度厚生科研費 障害者自立支援法下での重症心身障害児等に対する施設サービスの効果的な在り方に関する研究，2009，pp43-62.
3) 楠田 聡・他：NICU長期入院児の動態調査．平成21年度厚生科研費 重症新生児に対する療養・療育環境の拡充に関する総合研究，2010，pp33-39.
4) 岩崎裕治・他：療育施設と中間施設としての地域中核小児科との連携に関する研究．平成21年厚生科研費 重症新生児に対する療養・療育環境の拡充に関する総合研究，2010，pp59-68.
5) 末光 茂：重症児(障害児)施設はどこへ向かっているか？日重障誌34：73-80，2009.
6) 宮野前健，今井雅由：入院経路の経年的変化—SMIDデータベースより．平成18年度厚生科研費 障害者自立支援法下での重症心身障害児等に対する施設サービスの効果的な在り方に関する研究，2009，pp73-78.
7) 小川昭之：重症心身障害児施設における入園後重篤反応について．小児の精と神8：134-141，1968.
8) 林 優子・他：重症心身障害児の短期入所における安全な受け入れへの取り組み．川崎医療福祉会誌11：237-244，2001.
9) 口分田政夫：重症心身障害児施設入所待機者の実態調査．平成18年独立行政法人福祉医療機構(高齢者・障害者福祉基金)助成「重症心身障害児(者)の支援体制のあり方に関する調査研究事業」報告書，2006，pp59-61.
10) 澤野邦彦，末光 茂：公法人立重症心身障害児施設における入所待機の状況．平成20年度厚生科研費 障害者自立支援法下での重症心身障害児等に対する施設サービスの効果的な在り方に関する研究，2009，pp41-54.
11) 杉本健郎・他：超重症心身障害児の医療的ケアの現状と問題点—全国8府県のアンケート調査．日児誌112：94-101，2008.
12) 北住映二：超重症児を中心とした医療ニーズの高い重症心身障害児への支援について．平成21年度 児童関連サービス調査研究等事業報告書 財団法人こどもみらい財団 重症心身障害児者の支援に関する調査研究，2010，pp61-87.
13) 家室和宏：NICUと重症児施設の連携を考える—民間の重症児施設から．日重障誌35：222，2010.
14) 岩崎裕治・他：当センター短期入所利用児(者)の体調変化について—特に発熱について．日重障誌36：355，2011.
15) 船戸正久：長期入院児の問題点と解決の方向性．NICU '93秋季増刊73：138-142，1993.
16) 田村正徳・他：中間施設としての小児救急・慢性呼吸循環管理病室のあり方の検討．平成21年度厚生科研費 重症新生児に対する療養・療育環境の拡充に関する総合研究，2010，pp49-57.

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 287, NO. 17, pp. 13859–13867, April 20, 2012

Methyl CpG-binding Protein Isoform MeCP2_e2 Is Dispensable for Rett Syndrome Phenotypes but Essential for Embryo Viability and Placenta Development*[S]

Received for publication, October 2, 2011, and in revised form, February 26, 2012 Published, JBC Papers in Press, February 28, 2012, DOI 10.1074/jbc.M111.309864

Masayuki Itoh[‡1,2], **Candice G. T. Tahimic**[§1,3], **Shuhei Ide**[‡], **Akihiro Otsuki**[§], **Toshikuni Sasaoka**[¶], **Shigeru Noguchi**[‖4], **Mitsuo Oshimura**[§**], **Yu-ichi Goto**[‡], **and Akihiro Kurimasa**[§**]

From the [‡]*Department of Mental Retardation and Birth Defect Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Kodaira 187–8502, Japan, the* [§]*Institute of Regenerative Medicine and Biofunction, Graduate School of Medical Science, and the* **21st Century Centers of Excellence Program, Research Core for Chromosome Engineering Technology, Tottori University, Yonago 683–8503, Japan, the* [¶]*Department of Laboratory Animal Science, Kitasato University School of Medicine, Sagamihara 252–0374, Japan, and the* [‖]*Signal Transduction Project, Kanagawa Academy of Science and Technology, Kawasaki 213–0012, Japan*

Background: There are two isoforms of *MeCP2*: *MeCP2_e1* and *_e2*. It is not known whether *MeCP2_e2* has specific functions *in vivo*.
Results: Deletion of *MeCP2_e2* results in no neurological phenotypes but confers a survival disadvantage to embryos and placenta defects.
Conclusion: *MeCP2_e2* functions in placenta development and embryo survival.
Significance: *MeCP2_e2* deletion results in a non-Rett syndrome phenotype but adversely affects embryo viability.

Methyl CpG-binding protein 2 gene (*MeCP2*) mutations are implicated in Rett syndrome (RTT), one of the common causes of female mental retardation. Two MeCP2 isoforms have been reported: *MeCP2_e2* (splicing of all four exons) and *MeCP2_e1* (alternative splicing of exons 1, 3, and 4). Their relative expression levels vary among tissues, with *MeCP2_e1* being more dominant in adult brain, whereas *MeCP2_e2* is expressed more abundantly in placenta, liver, and skeletal muscle. In this study, we performed specific disruption of the *MeCP2_e2*-defining exon 2 using the Cre-*loxP* system and examined the consequences of selective loss of MeCP2_e2 function *in vivo*. We performed behavior evaluation, gene expression analysis, using RT-PCR and real-time quantitative PCR, and histological analysis. We demonstrate that selective deletion of *MeCP2_e2* does not result in RTT-associated neurological phenotypes but confers a survival disadvantage to embryos carrying a *MeCP2_e2* null allele of maternal origin. In addition, we reveal a specific requirement for MeCP2_e2 function in extraembryonic tissue, where selective loss of MeCP2_e2 results in placenta defects and up-regulation of *peg-1*, as determined by the parental origin of the mutant allele. Taken together, our findings suggest a novel role for MeCP2 in normal placenta development and illustrate how paternal X chromosome inactivation in extraembryonic tissues confers a survival disadvantage for carriers of a mutant maternal *MeCP2_e2* allele. Moreover, our findings provide an explanation for the absence of reports on *MeCP2_e2*-specific exon 2 mutations in RTT. *MeCP2_e2* mutations in humans may result in a phenotype that evades a diagnosis of RTT.

Methyl CpG-binding protein 2 gene (*MeCP2*) mutations are implicated in Rett syndrome (RTT),[5] one of the common causes of female mental retardation (1, 2). RTT patients exhibit apparently normal early psychomotor development and then gradually lose previously acquired psychomotor skills. Stereotypic hand movements and microcephaly are also clinical features of this disorder (3). MeCP2 binds to methylated CpG dinucleotides and functions as a transcriptional repressor through its interactions with the Sin3A/histone deacetylase complex and the SWI/SNF chromatin remodeling complex (4–8). To date, two MeCP2 isoforms have been characterized. The first reported MeCP2 isoform, referred to as MeCP2_e2 (translational start site in exon 2; also known as MeCP2A or MeCP2β), is generated by splicing of all four exons and has a translation start site in the middle of exon 2. The more recently discovered isoform, MeCP2_e1 (translational start site in exon 1; also known as MeCP2B or MeCP2α), results from alternative splicing of exons 1, 3, and 4 and has a translation start site in exon 1 (9, 10). Their relative expression levels vary among tissues, with MeCP2_e1 being more dominant in adult brain, whereas MeCP2_e2 is expressed more abundantly in placenta,

* This work was supported by Ministries of Health, Labor, and Welfare Grants 15B-3, 18A-3, H21-Nanchi-Ippan-110, and H22-Nanchi-Ippan-133 and by Ministries of Education, Culture, Science, Sports, and Technology of Japan Grant 18390304.
[S] This article contains supplemental Fig. 1.
[1] Both authors contributed equally to this work.
[2] To whom correspondence should be addressed: 4-1-1 Ogawahigashi, Kodaira, Tokyo 187-8502, Japan. Tel.: 81-423461713; Fax: 81-423461743; E-mail: itoh@ncnp.go.jp.
[3] Recipient of a Japanese Government Research Scholarship. Present address: Endocrine Unit, San Francisco Veterans Affairs Medical Center, San Francisco, CA 94121.
[4] Present address: Pharmaceuticals Analysis Group, Food Analysis and Safety Assessment Center, Food Technology Research Laboratories, R&D Division, Meiji Co. Ltd., Odawara 250–0862, Japan.

[5] The abbreviations used are: RTT, Rett syndrome; TRE, tetracycline-responsive promoter; tTA, tetracycline transactivator; XCI, X chromosome inactivation; PGK, phosphoglycerate kinase.

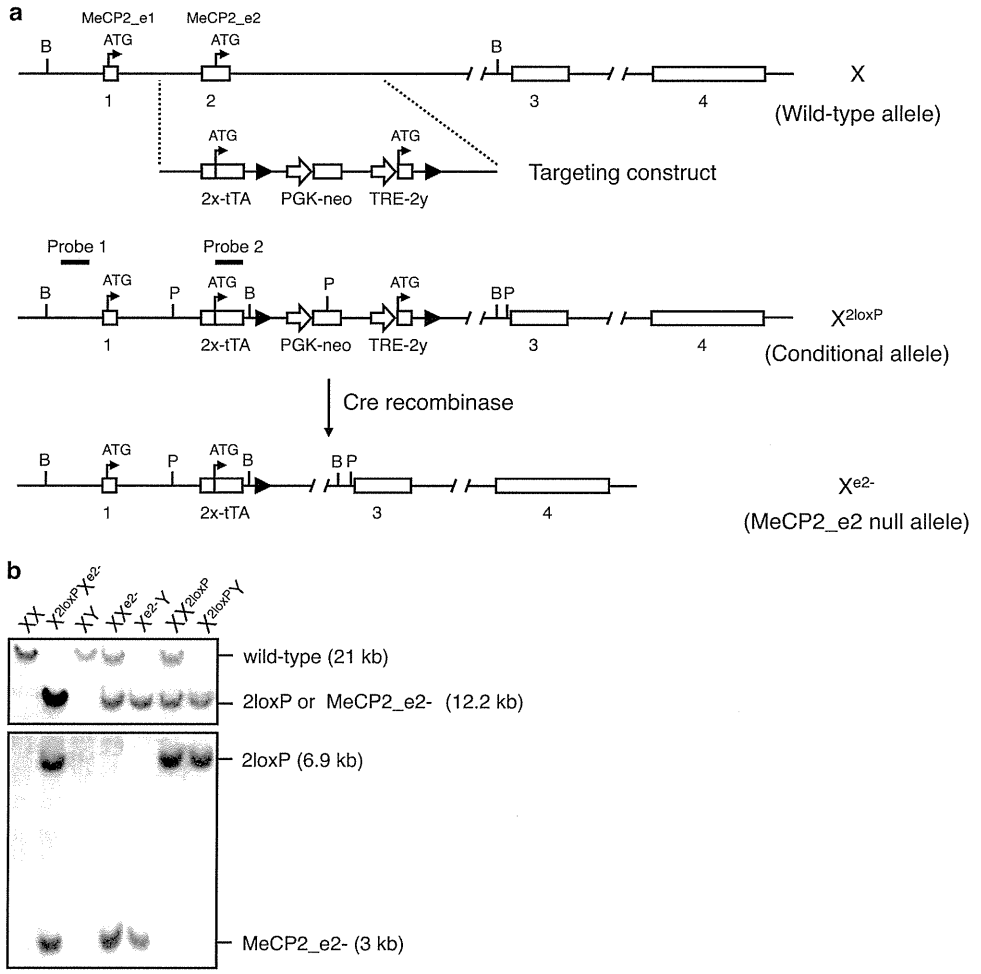

FIGURE 1. **Generation of *MeCP2_e2*-deficient mice.** *a*, strategy for selective targeting of *MeCP2_e2*. Transcription start sites for *MeCP2_e2* and *MeCP2_e1* before and after exon 2 disruption are shown. *loxP* sites are denoted as *filled triangles*. Relative location of probes for Southern hybridization, and positions of restriction enzymes BamHI (*B*) and PvuII (*P*) are indicated. Crossing of *MeCP2_e2* conditional mice with Nestin-Cre deleter mice results in the excision of the transcriptional start site of *MeCP2_e2* and the creation of the *MeCP2_e2* null allele, not only in neuronal cells but also in the germ line. Note that the transcriptional start of *MeCP2_e1* remains intact after disruption of the *MeCP2* locus. *b*, *MeCP2_e2* wild-type and mutant alleles as differentiated by two sets of Southern hybridization. For the first screening (*top*), genomic DNA was digested with BamHI and probed to visualize the presence of the targeted MeCP2 locus containing the exon 2x-tTA sequence. In the second screening (*bottom*), PvuI-digested genomic DNA was probed to differentiate between the conditional (X^{2loxP}) and null (X^{e2-}) alleles. Approximate band sizes are indicated in parentheses.

liver, and skeletal muscle (10). The most common MeCP2 mutations in RTT occur in exons shared by both isoforms (11). However, no mutation in the *MeCP2_e2*-defining exon 2 has ever been reported in RTT. In this study, we performed specific disruption of the *MeCP2_e2*-defining exon 2 using the Cre-*loxP* system and examined the consequences of selective loss of MeCP2_e2 function *in vivo*.

EXPERIMENTAL PROCEDURES

Selective Targeting of MeCP2_e2—The *MeCP2_e2* null allele was generated by Cre recombinase-mediated excision of exon 2 in *MeCP2_e2* conditional mice (Fig. 1). MeCP2 sequences were either directly derived or amplified from genomic DNA obtained from CJ7 ES cells or a BAC clone carrying the MeCP2 locus. The 5′-end of the targeting vector consisted of a 1.2-kb region possessing homology to intron 1 and was generated by high fidelity PCR. The early part of exon 2 containing the untranslated region (referred to as exon 2x) was fused to the tetracycline transactivator (tTA) gene, having a stop codon and poly(A) sequence. The latter half of exon 2 (referred to as exon 2y) beginning from the ATG start site of MeCP2_e2 was placed under the control of the tetracycline-responsive promoter, TRE. A pair of *loxP* sites flanked this TRE-exon 2y sequence. A PGK-driven neomycin selection marker was positioned between the first *loxP* site and the TRE-2y region. The 3′ arm of the targeting vector consisted of a 5.9-kb EcoRI fragment derived from intron 2.

Generation of MeCP2_e2 Null Mice—A correctly targeted ES cell clone, confirmed by Southern blot analysis, was injected into 3.5-day postconception (dpc) C57BL/6J blastocysts. Approximately 10 ES cells were injected per blastocyst, and 20 blastocysts were transferred to each pseudopregnant recipient. The resulting chimeric offspring were intercrossed mice to generate F1 progeny. For deletion of MeCP2_e2, we crossed MeCP2_e2$^{+/2loxP}$ females with deleter mice carrying a Cre recombinase transgene under the control of the Nestin promoter. However, leaky expression from Nestin promoter-driven Cre recombinase induced a deletion in the germ line, resulting in progeny that carried the *MeCP2_e2* null allele

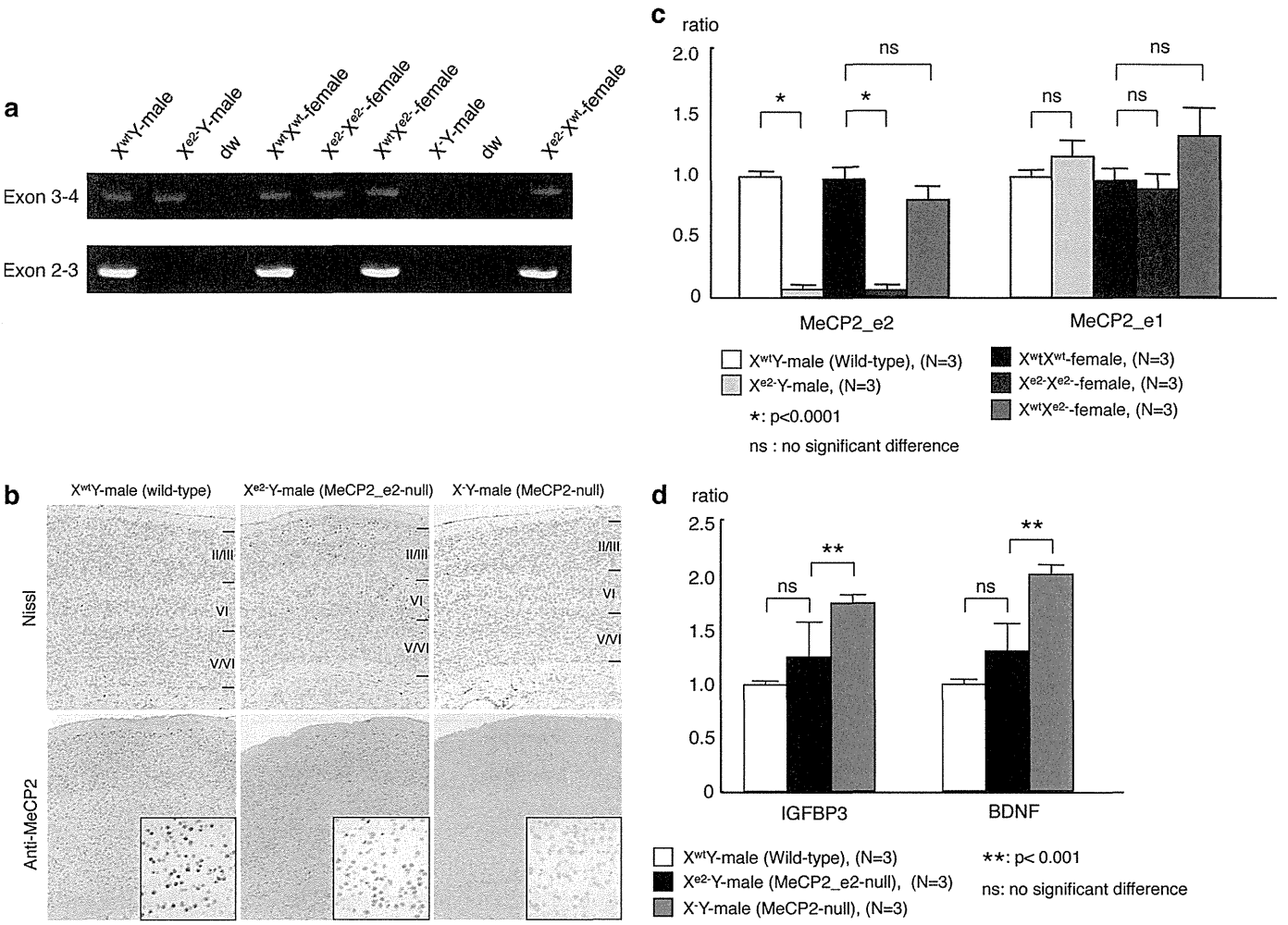

FIGURE 2. **Absence of RTT-associated phenotypes in *MeCP2_e2*-deficient mice.** *a*, reverse transcription PCR showing the selective loss of *MeCP2_e2* transcripts in brains of *MeCP2_e2* null males and females at P28. *b*, sections of P28 mouse brain were stained with cresyl violet to visualize neurons. Immunochemical staining was performed using anti-MeCP2 antibody. The *MeCP2*-deficient mouse, a previously reported *MeCP2_e2* and *MeCP2_e1* knockout (12), shows thinning of the cerebral cortex and no MeCP2-immunopositive cells. *MeCP2_e2* null mouse exhibits MeCP2-immunopositive cells in the cerebral cortex. *c*, real-time PCR analysis of *MeCP2_e2* and *MeCP2_e1* of P28 brains. The *MeCP2_e2*-deficient mouse shows *MeCP2_e1* expression but not *MeCP2_e2*, as indicated by the presence of exons 3 and 4 and the absence exons 2 and 3. *d*, quantitation of BDNF and IGFBP3 transcripts in P0 *MeCP2_e2*-deficient mice by real-time PCR. An $X^{wt}Y$ male mouse was used as a reference. Statistical analysis was performed using Student's *t* test at $p < 0.0001$ (*) and $p < 0.001$ (**). *Error bars*, S.D.

TABLE 1

Offspring distribution at 4 weeks of age; crossing of $X^{wt}X^{e2-}$ females and $X^{wt}Y$ males (maternal transmission of MeCP2_e2 null allele)

χ sum = 107.04, $p < 0.0001$. % Change = (% observed value − % expected value)/% expected value) × 100.

	$X^{wt}X^{wt}$	$X^{e2-}X^{wt}$	$X^{wt}Y$	$X^{e2-}Y$	Total
Observed	52 (27%)	27 (14%)	101 (53%)	12 (6%)	192
Estimated	48 (25%)	48 (25%)	48 (25%)	48 (25%)	192
% Change	8%	−44%	−112%	−76%	

TABLE 2

Offspring distribution at 4 weeks of age; crossing of $X^{wt}X^{e2-}$ females and $X^{e2-}Y$ males (biparental transmission of MeCP2_e2 null allele)

χ sum = 16.20, $p < 0.002$. % Change = (% observed value − % expected value)/% expected value) × 100.

	$X^{wt}X^{e2-}$	$X^{e2-}X^{e2-}$	$X^{wt}Y$	$X^{e2-}Y$	Total
Observed	11 (28%)	4 (10%)	20 (50%)	5 (12%)	40
Estimated	10 (25%)	10 (25%)	10 (25%)	10 (25%)	40
% Change	10%	−60%	100%	−50%	

(X^{e2-}). This population was expanded and used in succeeding experiments. Genotypes of the resulting progeny were assessed by an initial PCR screen followed by two sets of Southern blotting. The *MeCP2_e2* null allele was generated by Cre recombinase-mediated excision of exon 2 in *MeCP2_e2* conditional mice (Fig. 1). A previously reported MeCP2 null mouse, B6.129P2(C)-*Mecp2*$^{tm1.1Bird}$ (described as $MeCP2^{-/y}$), generated by targeted disruption of exons 3 and 4 (12), was obtained from Jackson Laboratory (Bar Harbor, ME) and used as a control for some of the experiments. All animal studies were performed with the approval of the Animal Care Committee of the National Institute of Neuroscience, National Center of Neurology and Psychiatry, Japan.

RT-PCR and Real-time Quantitative PCR—We prepared 3–8 fresh frozen brains and placentas of various genotypes at 13.5 dpc and postnatal days 0 (P0) and 28 (P28). Total RNA was isolated from mouse tissue using the RNeasy minikit (Qiagen, Valencia, CA) following the manufacturer's recommendations. We carried out reverse transcription with the First-Strand cDNA synthesis kit (Amersham Biosciences) or TaqMan

reverse transcription reagents (Applied Biosystems, Foster City, CA) using oligo(dT). Primer sequences and annealing conditions are as follows: for MECP2 exons 2 and 3, 5′-TTAGGGCTCAGGGAGGAAAA-3′ (forward) and 5′-CAAAATCATTAGGGTCCAAGG-3′ (reverse) with annealing temperature of 50 °C and expected PCR product size of 451 bp; for *MECP2* exons 3 and 4, 5′-ATTATCCGTGACCGGGGA-3′ (forward) and 5′-TGATGCTGCTGCCTTTGGT-3′ (reverse) with annealing temperature of 55 °C and an expected PCR product size of 354 bp.

For quantitative analysis, we carried out PCR amplifications using Universal PCR Master Mix (Applied Biosystems) according to the manufacturer's recommendations in a real-time ABI PRISM 7700 platform (Applied Biosystems). Relative transcript ratios were normalized to GAPDH RNA. Primers and probes for mouse *MeCP2* (common sequence of *MeCP2_e2* and *MeCP2_e1*), *MeCP2_e2*, *MAP2*, *IGFBP3*, and *BDNF* are available from Applied Biosystems. The probes 5′-CGCCGAGCGGAGGAG-3′ and 5′-CCTGGTCTTCTGACTTTTCTTCCA were designed to amplify a portion of the *MeCP2_e1* transcript, and a probe of CCTCCTCGCCTCCTCC-3′ was used. Sequence Detection System 1.7 software (Applied Biosystems) was used for analysis.

Immunohistochemical Analysis and TUNEL Assay—Tissues were fixed in 4% paraformaldehyde and then embedded in paraffin. Three-micrometer sections were prepared and stained with cresyl violet to visualize neurons. Purified MeCP2 antibody (provided by Dr. S. Kudo, Hokkaido Institute of Public Health, Sapporo, Japan), cleaved caspase-3 antibody (Chemicon International Inc., Temecula, CA), Peg-1 antibody (Atlas Antibodies AB, Stockholm, Sweden), and CRCX4 antibody (Abnova, Taipei, Taiwan) were used for immunohistological experiments. TUNEL assays were performed using terminal deoxynucleotidyltransferase (Roche Applied Science) following the manufacturer's recommendations.

Behavior Analysis—We performed tail suspension, footprinting, and open field analysis, using 4- or 5-week-old wild-type, MeCP2_e2^{-}, MeCP2_e2^{2loxP}, and MeCP2$^{-/y}$ males.

Statistical Analysis—Statistical analysis was performed using the χ^2 test. Animal crossings were performed to evaluate the effect of parent-specific transmission of the MeCP2_e2 null allele using appropriate sample sizes. Statistical significance of the expression levels was evaluated using Student's *t* test with a significance level of $p < 0.05$.

RESULTS AND DISCUSSION

MeCP2_e2-null Mouse Generation—We generated the *MeCP2_e2* mutant allele (X^{e2-}) by crossing mice carrying a tetracycline-inducible *MeCP2_e2* conditional allele (X^{2loxP}) with deleter mice carrying a Nestin-driven Cre recombinase transgene (Fig. 1). We observed germ line transmission of the *MeCP2_e2* null allele in some of the F3 generation (Fig. 1), probably resulting from leaky expression of Nestin-driven Cre recombinase in non-brain tissue. This subpopulation was expanded, and the F10 to F12 generations were used for the experiments in this study. We confirmed loss of MeCP2_e2 expression, whereas *MeCP2_e1* transcription remained intact in these animals (Fig. 2, *a* and *c*). Brain histological analysis showed no difference between *MeCP2_e2* null mouse and wild-type mice (Fig. 2*b*).

TABLE 3
Offspring distribution at 4 weeks of age; crossing of $X^{wt}X^{wt}$ females and $X^{e2-}Y$ males (paternal transmission of MeCP2_e2 null allele)
χ sum = 2.28, no significant difference. % Change = (% observed value − % expected value)/% expected value) × 100.

	$X^{wt}X^{e2-}$	$X^{wt}Y$	Total
Observed	50 (48%)	55 (52%)	105
Estimated	52.5 (50%)	52.5 (50%)	105
% Change	−4%	4%	

TABLE 4
Offspring distribution at 13.5 dpc; crossing of $X^{wt}X^{e2-}$ females and $X^{wt}Y$ males (maternal transmission of MeCP2_e2 null allele)
χ sum = 13.25, $p < 0.005$. % Change = (% observed value − % expected value)/% expected value) × 100.

	$X^{wt}X^{wt}$	$X^{e2-}X^{wt}$	$X^{wt}Y$	$X^{e2-}Y$	Total
Observed	36 (28%)	28 (22%)	46 (36%)	18 (14%)	128
Estimated	32 (25%)	32 (25%)	32 (25%)	32 (25%)	128
% Change	13%	−13%	44%	−44%	

TABLE 5
Offspring distribution at 13.5 dpc; crossing of $X^{wt}X^{wt}$ females and $X^{e2-}Y$ males (paternal transmission of MeCP2_e2 null allele)
χ sum = 2.28, no significant difference. % Change = (% observed value − % expected value)/% expected value) × 100.

	$X^{wt}X^{e2-}$	$X^{wt}Y$	Total
Observed	27 (61%)	17 (39%)	44
Estimated	22 (50%)	22 (50%)	44
% Change	23%	−23%	

Phenotypes and Expression Analyses of MeCP2_e2-null Mice—At birth, mice carrying *MeCP2_e2* mutant alleles were indistinguishable from wild-type littermates. They developed into fertile adults and did not display any neurological deficits observed in murine models for RTT (12, 13), indicating that MeCP2_e1 is sufficient to carry on the functions of MeCP2 in the brain. Moreover, mice carrying MeCP2_e2 mutant alleles lived as long as their wild-type siblings, over 2 years (data not shown). Immunohistochemical staining of brain tissue from $X^{e2-}Y$ and $X^{wt}X^{e2-}$ animals at 28 days of age revealed normal morphology of neuronal layers in contrast to the denser packaging of neurons in a previously reported RTT model wherein both MeCP2 isoforms have been knocked out (Fig. 2*b*) (14, 15). Taken together, these results demonstrate that loss of MeCP2_e1 function is not sufficient to cause RTT-associated neurological phenotypes.

To examine the implications of *MeCP2_e2* deficiency on MeCP2 transcriptional silencing activity, we checked mRNA levels of two MeCP2-regulated genes, insulin like growth factor binding protein 3 (*IGFBP3*) (16, 17) and brain-derived nerve growth factor (*BDNF*) (18). The mRNA levels of these genes in brains of $X^{e2-}Y$ mice did not significantly differ from those of age-matched wild-type males (Fig. 2*d*). In contrast, IGFBP3 and BDNF transcript levels increased by 1.6- and 2-fold, respectively, in the $X^{-}Y$ total MeCP2 knockout. These findings indi-

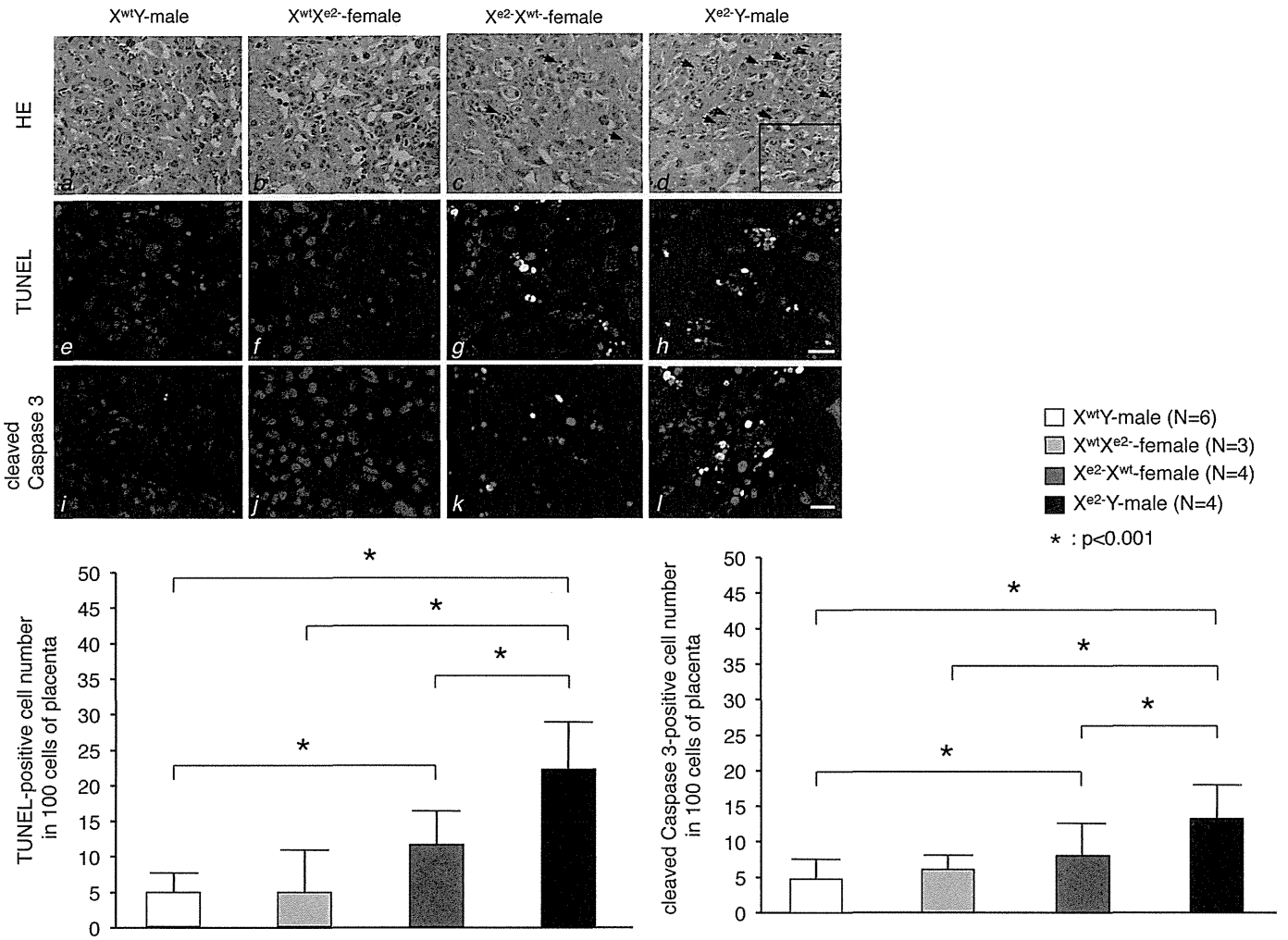

FIGURE 3. ***MeCP2_e2* deficiency results in placenta abnormalities.** The *top panels* (*a–d*) show placenta sections stained with hematoxylin and eosin. The *inset* shows the section at higher magnification. *Arrows* show apoptotic cells. The *middle panels* (*e–h*) show TUNEL staining of the same sections. Apoptotic nuclei appear as multiple spots (*yellow*), indicating DNA fragmentation. Propidium iodide was used as counterstain. The *bottom panels* (*i–l*) show cleaved caspase-3 immunostaining of the placenta. TUNEL-positive cells are indicated by *arrows*. *Scale bar*, 25 μm. An increase in the number of TUNEL-positive cells and cleaved caspase 3-positive cells was observed in the placentas of $X^{e2-}X^{wt}$ and $X^{e2-}Y$ embryos having a maternal *MeCP2_e2* null allele (refer to *bar graphs* in *lower panel* for quantitation) *, $p < 0.001$; *brackets* and *asterisks* indicate significant differences. *Error bars*, S.D.

cate that MeCP2_e2 is not essential for mediating transcriptional silencing of MeCP2 target genes in the brain.

Parent-specific Effects of MeCP2_e2 Null Allele Birth Rates—We next examined whether *MeCP2_e2* deficiency mediated any other non-neuronal phenotype. Interestingly, we observed reduced births of progeny that carried *MeCP2_e2* null allele of maternal origin. Specifically, we found a 76% reduction in $X^{e2-}Y$ males and a 44% reduction in $X^{e2-}X^{wt}$ females born to $X^{wt}X^{e2-}$ female and wild-type male pairings (Table 1). Similarly, in $X^{e2-}X^{wt}$ and $X^{e2-}Y$ pairings, $X^{e2-}Y$ and $X^{e2-}X^{e2-}$ births were reduced by 50 and 60%, respectively (Table 2). In contrast, birth rates of $X^{wt}X^{e2-}$ females (having a paternal X^{e2-}) did not deviate from the expected values (Tables 2 and 3). We exclude the possibility that these were nonspecific effects resulting from toxicity of the tTA in the targeting vector because no such decreases in births were observed in an unrelated transgenic mouse model carrying the same vector backbone.[6] Taken together, these results point to an association between reduced embryo viability and a maternally transmitted *MeCP2_e2* null allele.

To further delineate the time period at which selection against embryos carrying maternal *MeCP2_e2* null alleles occurred, we examined the genotype distribution at 13.5 dpc and observed similar trends (Tables 4 and 5). Moreover, we did not find any evidence of resorbed embryos at this time point (data not shown). We also performed morphological assessment of the uterus at preimplantation and postimplantation stages and found no abnormalities in preimplantation sites and the implantation process (data not shown). Nevertheless, these findings suggest that the reduced number of embryos carrying a mutant maternal *MeCP2_e2* allele is due neither to a failure in implantation nor to embryo lethality at postimplantation but to reduced viability of the embryo prior to implantation or early embryonic lethality after implantation.

Maternally Transmitted MeCP2_e2 Null Allele Results in Apoptosis and Altered peg-1 Expression in Placenta—During early development of the female mammal, one of the two X chromosomes becomes transcriptionally inactive to allow dos-

[6] A. Otsuki and A. Kurimasa, unpublished results.

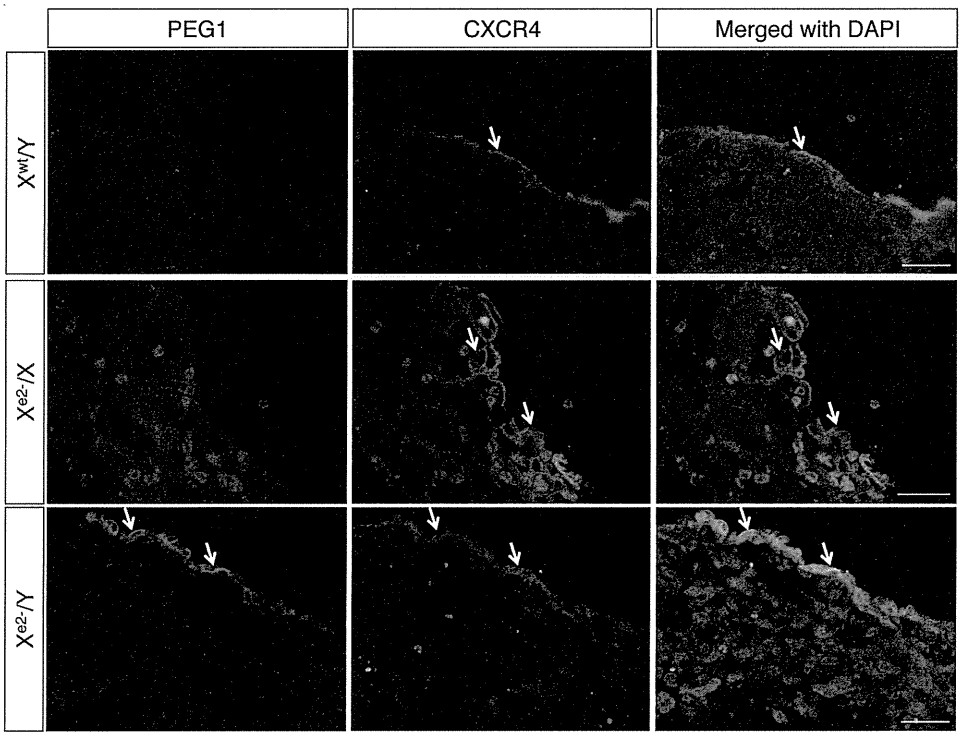

FIGURE 4. **Loss of maternal MeCP2_e2 results in failure to silence *peg-1* expression in trophoblast cells.** CXCR4 is a trophoblast cell marker. X^{wt}/Y and $X^{e2-}X^{wt}$ placenta have minimal *peg-1* expression, whereas X^{e2-}/Y placenta show elevated *peg-1* levels in trophoblast cells (*arrows*). *Scale bars*, 50 μm.

age compensation of X-linked genes (19, 20). In mouse extraembryonic lineages, such as placenta, the paternally derived X chromosome undergoes preferential inactivation, a phenomenon called imprinted paternal X chromosome inactivation (XCI) (21, 22). Hence, we examined the effect of *MeCP2_e2* deficiency in placenta tissue at 13.5 dpc. Interestingly, placentas of embryos carrying a maternal *MeCP2_e2* null allele exhibited increased apoptosis, which was more notable in placentas of males (Fig. 3). These TUNEL-positive cells expressed peg-1 (supplemental Fig. 1), an imprinted gene known to function in placenta development (23, 24). In contrast, very few apoptotic cells were observed in the placenta of $X^{wt}X^{e2-}$ embryos carrying a paternal *MeCP2_e2* null allele (Fig. 3). In addition, immunostaining revealed increased Peg-1 levels in cells expressing CXCR4, a trophoblast marker (25), in the placenta of animals carrying a maternal *MeCP2_e2* null allele (Fig. 4 and supplemental Fig. 1). Taken together, our results indicate that MeCP2_e2 is essential for the maintenance of *peg-1* silencing in trophoblast cells and that elevated expression of *peg-1* in the placenta has deleterious effects on cell survival.

We also examined transcript levels of *peg-1* and other imprinted genes involved in placenta function, such as *peg-3*, *igf-2*, and *h19* (23). Among these four genes, *peg-1* exhibited elevated transcript levels in the placenta of embryos carrying a maternal mutant allele (Fig. 5*a*), in concordance with our immunohistological findings. The mRNA levels of the other three genes were unchanged (Fig. 5*a*). In placentas of animals carrying the *MeCP2* two-isoform knock-out allele, *peg-1* expression was also elevated (Fig. 5*b*). The *peg-1* transcript levels were not due to deregulation of imprinting in placenta because imprinted paternal XCI was found to be intact in these animals (Fig. 5*c*). Rather, elevated *peg-1* transcript levels directly correlate with the loss of *MeCP2_e2* expression effected by imprinted paternal XCI. These findings indicate that *MeCP2_e2*-specific transcriptional silencing activity is essential for the regulation of *peg-1* expression and possibly of other genes in placenta.

The imprinted gene *peg-1*, located in murine chromosome 6, has been reported to play a role in angiogenesis in extraembryonic tissue (26). Mutations in *peg-1* have also been implicated in placenta failure (24, 25) and embryonic growth retardation (27). One group has reported that paternally expressed transcripts are associated with premature placenta (28). Interestingly, paternal transmission of a *peg-1* null allele in heterozygous mice results in diminished postnatal survival rates, whereas maternal transmission does not generate any remarkable phenotype (27, 29). It is clear from these reports that deregulation of *peg-1* expression or imprinting status has deleterious consequences on embryo viability and placenta function. Our current study demonstrates that MeCP2_e2 is an essential regulator of *peg-1* expression in extraembryonic tissue. As for how increased *peg-1* expression correlates with observed placenta defects in carriers of a maternal *MeCP2_e2* null allele, we propose a scenario wherein perturbations in *peg-1* expression results in disruption of biological pathways that involve Peg-1, leading to enhanced apoptosis in placenta. Peg-1 is a membrane-bound protein that is predicted to have lipase or acyltransferase activity based on sequence homology with the α/β-hydrolase superfamily of proteins (30). Lipid metabolism is a very important biological process and is critical for the developing embryo and placenta. We propose that loss of *MeCP2_e2* results in failure to transcriptionally silence *peg-1* in extraem-

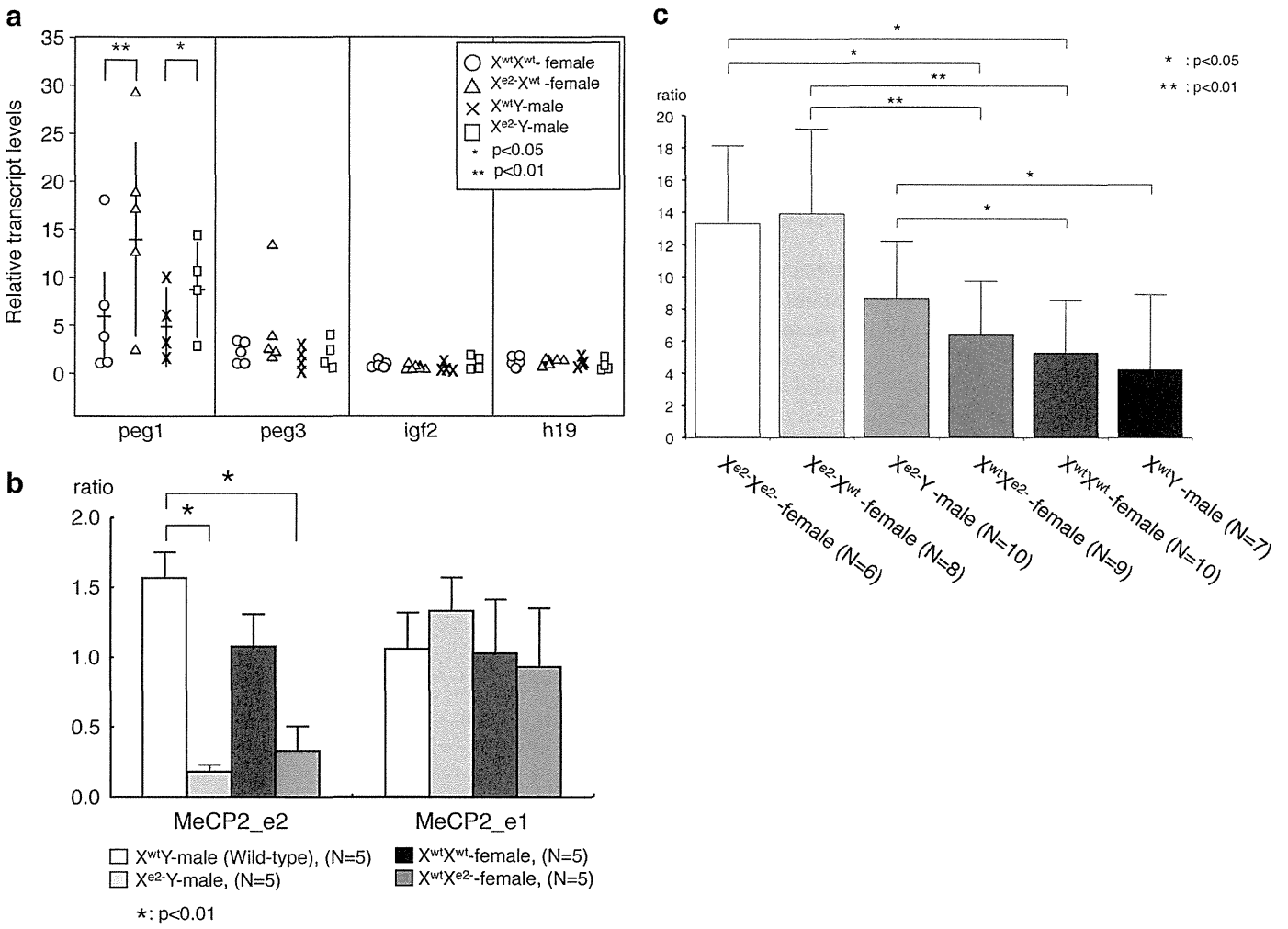

FIGURE 5. **Quantitative PCR analysis of placenta.** Shown are (*a*) placenta transcript levels of selected imprinted genes, *peg-1*, *peg-3*, *igf-2*, and *h19*, from 13.5 dpc embryos and (*b*) placenta transcript levels of peg-1 in *MeCP2_e2* and *MeCP2_e1* (two-isoform knockout) mutants. The *horizontal* and *vertical bars* of *peg-1* transcripts (*a*) show averages and S.D. of each genotype, respectively. *c*, *peg-1* expression in placentas of various genotypes. Maternally derived X^{e2-} allele up-regulated *peg-1* expression. *, $p < 0.05$; **, $p < 0.001$. *Brackets* and *asterisks* indicate significant differences. *Error bars*, S.D.

bryonic tissue, leading to increased Peg-1 enzymatic activity, aberrant regulation of Peg-1 binding partners or downstream targets, and, ultimately, apoptosis.

We have earlier stated that we found the implantation process to be normal for these animals. Moreover, at 13.5 dpc, there was no evidence of resorbed embryos, and the skewed embryo genotypes resembled that from postnatal analysis. These results, taken together with the increased number of apoptotic trophoblast cells and elevated *peg-1* expression in embryos carrying a maternal *MeCP2_e2* null allele, suggest that the loss of *MeCP2_e2* leads to trophoblast dysfunction during preimplantation through abnormal *peg-1* expression. Furthermore, we view the increase in apoptotic trophoblast cells as a persisting phenotype brought about by early perturbation of placenta gene expression. In mice, placental development begins in the blastocyst at embryonic day 3.5 when the trophectoderm layer becomes distinct from the inner cell mass (32). The trophoblast that lines the blastocyst plays an important role during attachment to the endometrium and in the formation of the placenta (31, 32). It has been reported by other groups that trophoblast dysfunction leads to disruption of placenta formation and reduction of birth number (31, 33). In our current study, we have shown that loss of *MeCP2_e2* results in a trophoblast defect that ultimately leads to reduced embryo viability.

Because some carriers of a mutant *MeCP2_e2* allele are born and develop into healthy adults, we hypothesize that the placenta abnormalities in these animals may have been overcome by *de novo MeCP2_e1* compensation or some other adaptation. In some types of extraembryonic cells, XCI can follow either a paternal or maternal pattern (34, 35). In somatic tissue, relaxation of imprinting occurs in certain pathological conditions (28, 36), and epigenetic heterogeneity at imprinted loci of autosomal chromosomes influences individual traits (37). The absence of *MeCP2_e2* correlated with up-regulation of *peg-1* expression, indicating a disturbance in regulation of downstream *MeCP2* gene targets. Although increased apoptosis in placenta could be used to explain the decreased viability of $X^{e2-}Y$ mice, this may also be interpreted as a way to eliminate functionally defective cells, thus contributing to the survival of some embryos.

The deleterious effects of *MeCP2* mutations have been viewed mostly in the context of somatic XCI patterns. A num-

ber of studies have addressed the contribution of XCI to the pathogenesis of *MeCP2* mutations (38, 39). It is suggested that XCI patterns may partly explain phenotypic variability in human RTT with *MeCP2* mutations (38) and in mouse RTT models (39). Our findings indicate that this is not the full picture and that paternal X chromosome inactivation in the extraembryonic lineage also contributes to the deleterious consequences of *MeCP2* mutations and, most likely, other X-linked gene mutations.

Recently, it has been reported that transgenic expression of either the *MeCP2_e1* or *MeCP2_e2* splice variant prevents the development of RTT-like neuronal phenotypic manifestations in a mouse model lacking *MeCP2*. This finding indicates that either *MeCP2* splice variant is sufficient to fulfill MeCP2 function in the mouse brain (40). Our findings reveal a novel mechanism for the pathogenesis of *MeCP2* mutations in extraembryonic tissue, wherein maternally inherited *MeCP2_e2* mutations result in placenta abnormalities that ultimately lead to a survival disadvantage for carriers of this mutant allele. Our study also provides an explanation for the absence of reports on *MeCP2_e2*-specific exon 2 mutations in RTT. It is conceivable that *MeCP2_e2* mutations in humans may result in a phenotype that evades a diagnosis of RTT. Moreover, the possible link between a novel genetic disorder characterized by reduced embryo viability and MeCP2 exon 2 mutations is a concept that merits further exploration. In summary, we have demonstrated that MeCP2_e2 is dispensable for RTT-associated neurological phenotypes. We have also discovered a novel requirement for MeCP2_e2 in placenta and embryo viability and have provided proof of existence of isoform-specific functions for two MeCP2 splicing variants.

Acknowledgments—We thank Dr. S. Kudo for the MeCP2 antibody and helpful suggestions and S. Kumagai and N. Tomimatsu for help with some of the experiments.

REFERENCES

1. Amir, R. E., Van den Veyver, I. B., Wan, M., Tran, C. Q., Francke, U., and Zoghbi, H. Y. (1999) Rett syndrome is caused by mutations in X-linked MECP2, encoding methyl-CpG-binding protein 2. *Nat. Genet.* **23,** 185–188
2. Rett, A. (1966) [On an unusual brain atrophy syndrome in hyperammonemia in childhood]. *Wien Med. Wochenschr.* **116,** 723–726
3. Hagberg, B., Aicardi, J., Dias, K., and Ramos, O. (1983) A progressive syndrome of autism, dementia, ataxia, and loss of purposeful hand use in girls. Rett's syndrome. Report of 35 cases. *Ann. Neurol.* **14,** 471–479
4. Lewis, J. D., Meehan, R. R., Henzel, W. J., Maurer-Fogy, I., Jeppesen, P., Klein, F., and Bird, A. (1992) Purification, sequence, and cellular localization of a novel chromosomal protein that binds to methylated DNA. *Cell* **69,** 905–914
5. Meehan, R. R., Lewis, J. D., and Bird, A. P. (1992) Characterization of MeCP2, a vertebrate DNA-binding protein with affinity for methylated DNA. *Nucleic Acids Res.* **20,** 5085–5092
6. Nan, X., Ng, H. H., Johnson, C. A., Laherty, C. D., Turner, B. M., Eisenman, R. N., and Bird, A. (1998) Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. *Nature* **393,** 386–389
7. Jones, P. L., Veenstra, G. J., Wade, P. A., Vermaak, D., Kass, S. U., Landsberger, N., Strouboulis, J., and Wolffe, A.P. (1998) Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. *Nat. Genet.* **2,** 187–191
8. Harikrishnan, K. N., Chow, M. Z., Baker, E. K., Pal, S., Bassal, S., Brasacchio, D., Wang, L., Craig, J. M., Jones, P. L., Sif, S., and El-Osta, A. (2005) Brahma links the SWI/SNF chromatin-remodeling complex with MeCP2-dependent transcriptional silencing. *Nat. Genet.* **37,** 254–264
9. Kriaucionis, S., and Bird, A. (2004) The major form of MeCP2 has a novel N terminus generated by alternative splicing. *Nucleic Acids Res.* **32,** 1818–1823
10. Mnatzakanian, G. N., Lohi, H., Munteanu, I., Alfred, S. E., Yamada, T., MacLeod, P. J., Jones, J. R., Scherer, S. W., Schanen, N. C., Friez, M. J., Vincent, J. B., and Minassian, B. A. (2004) A previously unidentified MECP2 open reading frame defines a new protein isoform relevant to Rett syndrome. *Nat. Genet.* **36,** 339–341
11. Bienvenu, T., and Chelly, J. (2006) Molecular genetics of Rett syndrome. When DNA methylation goes unrecognized. *Nat. Rev. Genet.* **7,** 415–426
12. Guy, J., Hendrich, B., Holmes, M., Martin, J. E., and Bird, A. (2001) A mouse Mecp2-null mutation causes neurological symptoms that mimic Rett syndrome. *Nat. Genet.* **27,** 322–326
13. Chen, R. Z., Akbarian, S., Tudor, M., and Jaenisch, R. (2001) Deficiency of methyl-CpG binding protein-2 in CNS neurons results in a Rett-like phenotype in mice. *Nat. Genet.* **27,** 327–331
14. Fukuda, T., Itoh, M., Ichikawa, T., Washiyama, K., and Goto, Y. (2005) Delayed maturation of neuronal architecture and synaptogenesis in cerebral cortex of Mecp2-deficient mice. *J. Neuropathol. Exp. Neurol.* **64,** 537–544
15. Dragich, J. M., Kim, Y. H., Arnold, A. P., and Schanen, N. C. (2007) Differential distribution of the MeCP2 splice variants in the postnatal mouse brain. *J. Comp. Neurol.* **501,** 526–542
16. Chang, Y. S., Wang, L., Suh, Y. A., Mao, L., Karpen, S. J., Khuri, F. R., Hong, W. K., and Lee, H. Y. (2004) Mechanisms underlying lack of insulin-like growth factor-binding protein-3 expression in non-small-cell lung cancer. *Oncogene* **23,** 6569–6580
17. Itoh, M., Ide, S., Takashima, S., Kudo, S., Nomura, Y., Segawa, M., Kubota, T., Mori, H., Tanaka, S., Horie, H., Tanabe, Y., and Goto, Y. (2007) Methyl CpG-binding protein 2 (a mutation of which causes Rett syndrome) directly regulates insulin-like growth factor binding protein 3 in mouse and human brains. *J. Neuropathol. Exp. Neurol.* **66,** 117–123
18. Chen, W. G., Chang, Q., Lin, Y., Meissner, A., West, A. E., Griffith, E. C., Jaenisch, R., and Greenberg, M. E. (2003) Derepression of BDNF transcription involves calcium-dependent phosphorylation of MeCP2. *Science* **302,** 885–889
19. Mak, W., Nesterova, T. B., de Napoles, M., Appanah, R., Yamanaka, S., Otte, A. P., and Brockdorff, N. (2004) Reactivation of the paternal X chromosome in early mouse embryos. *Science* **303,** 666–669
20. Sado, T., and Ferguson-Smith, A. C. (2005) Imprinted X inactivation and reprogramming in the preimplantation mouse embryo. *Hum. Mol. Genet.* **14,** R59–64
21. Takagi, N., and Sasaki, M. (1975) Preferential inactivation of the paternally derived X chromosome in the extraembryonic membranes of the mouse. *Nature* **256,** 640–642
22. Harper, M. I., Fosten, M., and Monk, M. (1982) Preferential paternal X inactivation in extraembryonic tissues of early mouse embryos. *J. Embryol. Exp. Morphol.* **67,** 127–135
23. Obata, Y., Kaneko-Ishino, T., Koide, T., Takai, Y., Ueda, T., Domeki, I., Shiroishi, T., Ishino, F., and Kono, T. (1998) Disruption of primary imprinting during oocyte growth leads to the modified expression of imprinted genes during embryogenesis. *Development* **125,** 1553–1560
24. Coan, P. M., Burton, G. J., and Ferguson-Smith, A. C. (2005) Imprinted genes in the placenta. A review. *Placenta* **26,** S10–S20
25. Wu, X., Li, D. J., Yuan, M. M., Zhu, Y., and Wang, M. Y. (2004) The expression of CXCR4/CXCL12 in first-trimester human trophoblast cells. *Biol. Reprod.* **70,** 1877–1885
26. Mayer, W., Hemberger, M., Frank, H. G., Grümmer, R., Winterhager, E., Kaufmann, P., and Fundele, R. (2000) Expression of the imprinted genes MEST/Mest in human and murine placenta suggests a role in angiogenesis. *Dev. Dyn.* **217,** 1–10
27. Lefebvre, L., Viville, S., Barton, S. C., Ishino, F., Keverne, E. B., and Surani, M. A. (1998) Abnormal maternal behavior and growth retardation associated with loss of the imprinted gene Mest. *Nat. Genet.* **20,** 163–169

28. Looijenga, L. H., Gillis, A. J., Verkerk, A. J., van Putten, W. L., and Oosterhuis, J. W. (1999) Heterogeneous X inactivation in trophoblastic cells of human full-term female placentas. *Am. J. Hum. Genet.* **64,** 1445–1452
29. Beechey, C. V. (2000) Peg1/Mest locates distal to the currently defined imprinting region on mouse proximal chromosome 6 and identifies a new imprinting region affecting growth. *Cytogenet. Cell Genet.* **90,** 309–314
30. Nikonova, L., Koza, R. A., Mendoza, T., Chao, P. M., Curley, J. P., Kozak, L. P. (2008) Mesoderm-specific transcript is associated with fat mass expansion in response to a positive energy balance. *FASEB J.* **22,** 3925–3937
31. Lee, K. Y., Jeong, J. W., Tsai, S. Y., Lydon, J. P., and DeMayo, F. J. (2007) Mouse models of implantation. *Trends Endcrinol. Metab.* **18,** 234–239
32. Watson, E. D., and Cross, J. C. (2005) Development of structures and transport functions in the mouse placenta. *Physiology* **20,** 180–193
33. Chaddha, V., Viero, S., Huppertz, B., and Kingdom, J. (2004) Developmental biology of the placenta and the origins of placental insufficiency. *Semin. Fetal Neonatal Med.* **9,** 357–369
34. Migeon, B. R., Wolf, S. F., Axelman, J., Kaslow, D. C., and Schmidt, M. (1985) Incomplete X chromosome dosage compensation in chorionic villi of human placenta. *Proc. Natl. Acad. Sci. U.S.A.* **82,** 3390–3394
35. Coutinho-Camillo, C. M., Brentani, M. M., Butugan, O., Torloni, H., and Nagai, M. A. (2003) Relaxation of imprinting of IGFII gene in juvenile nasopharyngeal angiofibromas. *Diagn. Mol. Pathol.* **12,** 57–62
36. Sakatani, T., Wei, M., Katoh, M., Okita, C., Wada, D., Mitsuya, K., Meguro, M., Ikeguchi, M., Ito, H., Tycko, B., and Oshimura, M. (2001) Epigenetic heterogeneity at imprinted loci in normal populations. *Biochem. Biophys. Res. Commun.* **283,** 1124–1130
37. Bourdon, V., Philippe, C., Martin, D., Verloès, A., Grandemenge, A., and Jonveaux, P. (2003) MECP2 mutations or polymorphisms in mentally retarded boys. Diagnostic implications. *Mol. Diagn.* **7,** 3–7
38. Shahbazian, M. D., Sun, Y., and Zoghbi, H. Y. (2002) Balanced X chromosome inactivation patterns in the Rett syndrome brain. *Am. J. Med. Genet.* **111,** 164–168
39. Young, J. I., and Zoghbi, H. Y. (2004) X-chromosome inactivation patterns are unbalanced and affect the phenotypic outcome in a mouse model of rett syndrome. *Am. J. Hum. Genet.* **74,** 511–520
40. Kerr, B., Soto, C. J., Saez, M., Abrams, A., Walz, K., and Young, J. I. (2012) Transgenic complementation of MeCP2 deficiency. Phenotypic rescue of Mecp2-null mice by isoform-specific transgenes. *Eur. J. Hum. Genet.* **20,** 69–76