Published in final edited form as:

Ann Neurol. 2010 October ; 68(4): 454–464. doi:10.1002/ana.22103.

Primary cellular meningeal defects cause neocortical dysplasia and dyslamination

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Abstract

Objective—Cortical malformations are important causes of neurological morbidity, but in many cases their etiology is poorly understood. Mice with *Foxc1* mutations have cellular defects in meningeal development. We use hypomorphic and null alleles of *Foxc1* to study the effect of meningeal defects on neocortical organization.

Methods—Embryos with loss of *Foxc1* activity were generated using the hypomorphic *Foxc1^{hith}* allele and the null *Foxc1^{lacZ}* allele. Immunohistologic analysis was used to assess cerebral basement membrane integrity, marginal zone heterotopia formation, neuronal overmigration, meningeal defects, and changes in basement membrane composition. Dysplasia severity was quantified using two measures.

Results—Cortical dysplasia resembling cobblestone cortex, with basement membrane breakdown and lamination defects, is seen in *Foxc1* mutants. As *Foxc1* activity was reduced, abnormalities in basement membrane integrity, heterotopia formation, neuronal overmigration, and meningeal development appeared earlier in gestation and were more severe. Surprisingly, the basement membrane appeared intact at early stages of development in the face of severe deficits in meningeal development. Prominent defects in basement membrane integrity appeared as development proceeded. Molecular analysis of basement membrane laminin subunits demonstrated that loss of the meninges led to changes in basement membrane composition.

Interpretation—Cortical dysplasia can be caused by cellular defects in the meninges. The meninges are not required for basement membrane establishment but are needed for remodeling as the brain expands. Specific changes in basement membrane composition may contribute to subsequent breakdown. Our study raises the possibility that primary meningeal defects may cortical dysplasia in some cases.

Introduction

Cortical dysplasia is an important cause of neurologic morbidity. Advanced imaging techniques are increasing recognition of dysplasia and other structural brain abnormalities in patients with intellectual disability, global developmental delay, and epilepsy¹. Specific treatment, such as resective surgery for focal cortical dysplasia in epilepsy², may help some patients. However, for most there is only symptomatic treatment. Thus, understanding dysplasia pathogenesis is critical to improving diagnostic and treatment options.

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Dysplasia occurs when the exquisitely regulated process of corticogenesis is perturbed at a critical point in embryonic life. The meninges are increasingly recognized as critical regulators of cortical development due in part to the unique position of the meninges adjacent to the superficial cortex with access to the forming cortical layers as well as radial glia endfeet. The meninges comprise three distinct cellular layers covering the brain: the outer dura contacts the inner table of the skull, the inner pia adheres to the surface of the brain, and the netlike fibers of the arachnoid connect the two³. The meninges produce important regulatory factors for brain and skull development including CXCL12, regulating Cajal-Retzius cell and interneuron migration⁴⁻⁷, TGF- β and FGF-2, regulating skull development^{8, 9}, and all-trans retinoic acid, regulating cortical neurogenesis¹⁰.

We previously identified a hypomorphic mutation, *hith*, in the forkhead transcription factor, *Foxc1* with defective forebrain meningeal development and neocortical malformations characterized by cerebral basement membrane (CBM) breakdown and neuronal overmigration^{10, 11}. *Foxc1* is expressed strongly by all components of the meninges and is not expressed within the brain except for perivascular pericytes. Thus, *Foxc1* is thought to regulate brain development indirectly by controlling meningeal development.

Here, we investigate how defects in meningeal development promote CBM breakdown and dysplasia formation. Using *Foxc1* mutants, we find that more severe meningeal defects accelerate CBM breakdown, promoting formation of cortical dysplasia during development. Extensive meningeal defects in these mutants precede CBM breakdown and dysplasia formation. Our study raises the possibility that primary meningeal pathology might play a role in dysplasia formation.

Methods

Animals and tissue preparation

Foxc1^{lacZ/+} (T. Kume, Vanderbilt University) and *Foxc1^{hith/+}* animals were maintained and genotyped as described^{10, 11}. CXCR4-EGFP BAC transgenic animals (Gene Expression Nervous System Atlas (GENSAT) Project, NINDS Contracts N01NS02331 & HHSN271200723701C to The Rockefeller University (New York, NY)) were maintained on a CD-1 background. Breeding with *Foxc1^{lacZ/+}* animals allowed production of *Foxc1^{lacZ/lacZ};CXCR4-EGFP/+* embryos. Timed embryos were obtained by examining for vaginal plugs in mated females. The morning of plug detection was designated E0.5. Mouse husbandry was done using protocols approved by the University of California, San Francisco, Committee on Animal Research in facilities approved by the American Association of Laboratory Animal Care.

Timed embryos were harvested from pregnant dams after isofluorane anesthesia and cervical dislocation. Whole embryonic heads were either fixed overnight at 4°C in 4% paraformaldehyde (PFA), cryoprotected, and frozen in OCT (Sakura Finetek), or flash frozen in OCT. 12 μ m cryosections were cut and stored at -80°C until needed.

Immunostaining and Nissl staining

For antibodies using PFA fixed tissue, tissue was boiled for 10 min in citrate buffer (except for GFP staining), blocked with 10% lamb serum and 0.3% Triton X-100, then incubated with primary and appropriate secondary antibodies in 2% lamb serum and 0.3% Triton X-100. For antibodies requiring fresh frozen sections, tissue was fixed in methanol at -20°C for 5 minutes, blocked in 1% BSA for 5 minutes, and incubated with primary and appropriate secondary antibodies in 1% BSA. Nissl staining followed antibody incubation using NeuroTrace (Invitrogen) at 1:100 dilution. Isolectin B4 (Invitrogen) was used at 1:100 dilution. These antibodies and dilutions were used on PFA fixed tissue: rabbit anti-laminin,

1:75, Sigma; rabbit anti-collagen IV, 1:200, Abcam; rat anti-CTIP2, 1:800, Abcam; mouse anti-nestin, 1:500, Chemicon; chicken anti-GFP, 1:500, Aves Labs; mouse anti-TuJ1, 1:1000, Covance; mouse monoclonal IIH6 against α -dystroglycan, 1:100, Millipore; rabbit anti-Zic, 1:5000, gift of J. Aruga, RIKEN Institute (required use of the Tyramide Signal Amplification Kit (Invitrogen)). These antibodies and dilutions were used on fresh frozen tissue: rat anti-entactin, 1:200, Abcam; rat anti-laminin α 1, 1:10, Santa Cruz Biotechnology; rat anti-laminin α 2, 1:50, Santa Cruz Biotechnology; rabbit anti-laminin α 4, 1:400, gift of T. Sasaki, Oregon Health and Science University; rabbit anti-laminin α 5, 1:500, gift of J. Miner, Washington University School of Medicine. Images were acquired with a digital CCD camera and QCapturePro software (QImaging) on a Nikon epifluorescent microscope.

Quantitation of neuronal overmigration and marginal zone (MZ) heterotopias

Coronal sections of E18.5 embryos were immunostained with CTIP2 and counterstained with 4',6-diamidino-2-phenylindole (DAPI). Mosaic images for counting were generated using Photoshop (Adobe). Hemispheres without significant hemorrhagic disruption were selected. DAPI allowed identification of the cortical plate (CP) and MZ. For each hemisphere, the total number of heavily labeled CTIP2 positive cells at the superior border of the CP or in the MZ was counted.

To measure the proportion of abnormal MZ, the same tissue sections as above were analyzed. Using ImageJ (NIH), the length of the neocortex and the total length of the MZ that contained frank heterotopias or abnormal clusters of cells were measured. The proportion of the MZ that appeared abnormal was calculated as (total length of abnormal MZ)/(length of cortex) $\times$ 100.

An average of 2.25 hemispheres/embryo from three embryos from different litters were analyzed. Hemispheres were at comparable anatomic levels. Mutants were compared to age-matched controls with unpaired t-tests employing SigmaPlot (Sigmaplot Software).

Results

Worsening meningeal defects are associated with more dramatic CBM breakdown

We hypothesized that disruption of meningeal function, caused by stepwise reduction of *Foxc1* activity, would promote formation of cortical dysplasia characterized by CBM breakdown and neuronal overmigration. As a first step, we examined the timing of CBM breakdown in an allelic series of *Foxc1* mutants (using combinations of the hypomorphic *Foxc1^{hith}* and the null *Foxc1^{lacZ}* alleles) from E12.5 to E18.5 by staining for laminin (Fig 1). Over the medial cortex, the CBM was apparently intact at E12.5 in all genotypes (Fig 1A-D). Areas without apparent CBM overlying the cortex appeared at E14.5 only in *Foxc1^{hith/lacZ}* and *Foxc1^{lacZ/lacZ}* mutants (Fig 1G-H) but not in the *Foxc1^{hith/hith}* mutants (Fig 1F). Defects worsened in *Foxc1^{hith/lacZ}* and *Foxc1^{lacZ/lacZ}* mutants at E16.5 and E18.5, and at both ages were more extensive in *Foxc1^{lacZ/lacZ}* mutants (Fig 1L, P) than in *Foxc1^{hith/lacZ}* mutants (Fig 1K, O). In *Foxc1^{hith/hith}* mutants, breaks in the CBM were absent at E16.5 (Fig 1J), and were uncommon even at E18.5 (Fig 1N). Dilated, abnormal blood vessels were also prominent (compare Fig 1O-P with M). Double-immunostaining with laminin and TuJ1 confirmed immature neurons had overmigrated through CBM breaks (Supplementary Fig 1A-F).

To confirm the CBM defect involved multiple CBM components, we examined expression of additional CBM proteins collagen IV and nidogen-1^{12, 13} (Supplementary Fig 1G-R). Both proteins outlined intact CBM at E12.5 in all genotypes. At E18.5, they recapitulated the pattern of CBM breakdown seen with laminin staining in the mutants. The nidogen-1 antibody required fresh frozen tissue that caused artificially fractured CBM in controls

(Supplementary Fig 1M, P), which was easily distinguished from the dramatic disorganization seen in mutants. Abnormal glycosylation of α -dystroglycan is associated with breakdown of the basement membrane and neuronal migration defects seen in congenital muscular dystrophies^{14, 15}. However, in E16.5 *Foxc1*^{lacZ/lacZ} mutants, there was no clear difference in staining with monoclonal antibody IIH6 (an antibody that does not recognize hypoglycosylated α -dystroglycan¹⁶) implying that the glycosylation of α -dystroglycan was probably intact in our mutant mice (Supplementary Fig 2). These data indicate that dosage dependent meningeal defects, caused by loss of *Foxc1* function, promote CBM breakdown and neuronal overmigration as development proceeds, through a different biochemical pathway than the congenital muscular dystrophies.

More severe meningeal defects cause more severe and extensive cortical dysplasia

In cobblestone cortex, CBM breakdown leads to neuronal overmigration forming dysplasia¹⁴. Because worsening meningeal defects promote CBM breakdown, we predicted formation of cortical dysplasia would accelerate as meningeal defects worsen. We therefore performed a detailed analysis of the timing and location of cortical dysplasia formation (Fig 2). Dysplastic cortex was most prominently seen in the dorsomedial cortex in E18.5 mutant embryos (Fig 2A-D). This was absent at E12.5 but small dysplastic areas appeared at E14.5 in the severely affected *Foxc1*^{lacZ/lacZ} mutants (data not shown). At E16.5, dysplasia was prominent in the *Foxc1*^{hith/lacZ} and *Foxc1*^{lacZ/lacZ} mutants (Fig 2G-H), but was much less apparent in the *Foxc1*^{hith/hith} mutant (Fig 2F). At E18.5, dysplasia was obvious in all mutants. Thus, at all ages, the cortical disorganization and severity of dysplasia worsened as *Foxc1* activity decreased.

We assessed the severity of cortical dysplasia by measuring the degree of neuronal overmigration and the extent of MZ heterotopias (Fig 3). Normally, neurons expressing CTIP2 form a well-organized layer in cortical layers Vb and VI¹⁷ (Fig 3A). In the mutants, extensive neuronal overmigration occurred in areas of CBM breakdown. Many CTIP2 positive neurons were found in the upper CP and MZ, intermixed with CBM fragments (Fig 3B). In areas of apparently intact CBM, deep layer neurons either remained in place, were displaced upward mildly in the CP, or formed much smaller clusters in the MZ (data not shown). Quantification of displaced CTIP2+ neurons at the upper border of the CP or within the MZ was performed for each mutant genotype (Fig 3C-G). Displaced neurons had high expression of CTIP2 and were easily distinguished from the normal population of lightly labeled cells in the MZ (Fig 3C). In the *Foxc1*^{hith/hith} mutant, CTIP2+ neurons were displaced upward in the CP but did not enter the MZ in great numbers and the heterotopias did not contain many CTIP2+ neurons (Fig 3D). In contrast, the *Foxc1*^{hith/lacZ} and *Foxc1*^{lacZ/lacZ} mutants had large MZ heterotopias containing many CTIP2+ neurons (Fig 3E-F). The number of CTIP2+ neurons bordering on or within the MZ per hemisphere was significantly greater in mutants compared to control. Severely affected mutants had many more displaced CTIP2+ neurons than the *Foxc1*^{hith/hith} mutant (Fig 3G).

We also assessed the severity of heterotopia formation by measuring what proportion of the MZ contained heterotopic clusters of CTIP2+ neurons (Fig 3H). All mutants had a higher proportion of abnormal MZ compared to control. Paralleling results seen with CTIP2+ neuron overmigration, the severely affected mutants had higher proportions of abnormal MZ than the *Foxc1*^{hith/hith} mutant. The *Foxc1*^{lacZ/lacZ} mutant had a statistically lower extent of abnormal MZ than the *Foxc1*^{hith/lacZ} mutant ($p < 0.05$), likely due to its dramatically increased neocortical length and severe loss of post-mitotic neurons¹⁰. These results indicate that primary pathology of the meninges in *Foxc1* mutants promotes formation of cortical dysplasia.

Radial glial (RG) cell disruption is seen in areas of CBM breakdown

Disruption of RG organization is believed to be a key factor in the development of cortical dysplasia with CBM breakdown and neuronal overmigration^{13, 18-21}. Thus, we postulated that RG disorganization should also be present in *Foxc1* mutants. The glia limitans was visualized at E16.5 by double-immunostaining with nestin²² and collagen IV (Fig 4). At this age, both an extensive network of RG fibers²³ and extensive CBM breakdown occur. RG fibers were absent from CBM gaps in *Foxc1^{hihi/lacZ}* or *Foxc1^{lacZ/lacZ}* mutants, but extended to fragments of CBM bordering gaps (Fig 4C-D). In control and *Foxc1^{hihi/hihi}* embryos RG fibers extended to the CBM (Fig 4A-B). Disruption of RG fiber extension was more extensive in the *Foxc1^{lacZ/lacZ}* mutant than the *Foxc1^{hihi/lacZ}* mutant and was primarily medial, corresponding with areas of CBM breakdown.

Extensive meningeal defects precede CBM breakdown and dysplasia formation

Clarifying the timing of the appearance of meningeal defects with the onset of cortical dysplasia should help establish the pathologic link between the two. We assessed the extent of meningeal defects at E12.5, before dysplasia is apparent. Double-immunostaining for Zic proteins^{10, 24} and laminin identified meningeal fibroblasts and the CBM, respectively (Fig 5). A well-defined layer of meningeal fibroblasts covered the CBM over the telencephalon in controls (Fig 5A-C). Fibroblast nuclei were slightly superficial to the CBM. In addition to meningeal fibroblasts, Cajal-Retzius cells also express a Zic family member and are thus stained, but are found in the superficial MZ²⁴. Less intensely stained Zic+ cells, likely representing neural crest cells contributing to the frontal bones^{25, 26}, overlaid the brain but lay outside the layer of meningeal fibroblasts. *Foxc1^{hihi/hihi}* mutants had a small defect over the dorsomedial neocortex (Fig 5D-F) where meningeal cells were absent. Meningeal coverage remained robust and intact over the lateral cortex. *Foxc1^{hihi/lacZ}* mutants had a larger meningeal defect (Fig 5G-I) from the dorsomedial to the basolateral cortex. The *Foxc1^{lacZ/lacZ}* mutants had the most severe meningeal defect (Fig 5J-L), with no meningeal fibroblasts clearly identified over the cortex and coverage by meningeal fibroblasts restricted to the basal telencephalon. Cajal-Retzius cells, revealed by the use of a CXCR4-EGFP transgene²⁷, remained in the MZ in the *Foxc1^{lacZ/lacZ}* mutants despite the absence of meningeal fibroblasts over the cortex (Supplementary Fig 3). Importantly, and somewhat surprisingly, we did not see apparent breakdown of the CBM or dysplasia formation in the mutants at this age. Our data indicated extensive meningeal defects appeared well before CBM breakdown or dysplasia arose, intact meninges were dispensable for initial establishment of the CBM, and, at early stages of development, Cajal-Retzius cells do not require meninges to localize to the MZ.

Loss of meninges induces differential changes in CBM laminin α subunit composition

Laminin subunits $\alpha 1$ and $\alpha 2$ are known components of the embryonic CBM²⁸. In adults, there is evidence that meningeal cells and astrocytes produce laminin $\alpha 1$ and $\alpha 2$ and endothelial cells synthesize laminin $\alpha 4$ and $\alpha 5$ ²⁹. We wondered whether meningeal defects cause differential changes in laminin subunit composition during development, which might be a potential mechanism for CBM instability. Therefore, we studied the composition of laminin α subunits in the CBM using specific antibodies (Fig 6). In controls, laminin $\alpha 1$, $\alpha 2$, and $\alpha 5$ had strong expression within the CBM. As expected, laminin $\alpha 4$ appeared to be expressed mainly in blood vessels³⁰. Interestingly, we found that in mutant mice, even with large meningeal defects, the CBM had strong expression of laminin $\alpha 1$, $\alpha 1$, $\alpha 2$, and $\alpha 5$. These subunits were found in an intact CBM in all genotypes at E12.5. At E18.5, the CBM degenerated in *Foxc1^{hihi/lacZ}* and *Foxc1^{lacZ/lacZ}* mutants, consistent with our prior results (Fig 1), leaving fragments of brightly labeled CBM (arrows, Fig 6O, R, X).

Examination of controls showed laminin $\alpha 1$ and $\alpha 2$ expression in a reticulated pattern just superior to the CBM at E12.5 (arrows, Fig 6A, D) and E18.5 (Fig 6M, P) while in *Foxc1* mutants this pattern was altered with a significant decrease in laminin $\alpha 1$ expression and increase in laminin $\alpha 2$ expression (Fig 6C, F, O, R). Laminin $\alpha 2$ is expressed in brain capillaries³¹, so its increase is consistent with blood vessel overgrowth. The loss of reticulated laminin $\alpha 1$ expression paralleled the loss of meningeal cells, which are ideally located to regulate expression of laminin subunits in this area (see Fig 5B).

In parallel with the increased reticulated laminin $\alpha 2$ expression, laminin $\alpha 4$ expression was increased in the mutants at E12.5 and especially at E18.5. It became quite disorganized, consistent with formation of large, disorganized blood vessels (Fig 6H-I, T-U). Laminin $\alpha 5$ lacked the reticulated pattern superior to the CBM seen with laminin $\alpha 1$ and $\alpha 2$ (Fig 6J, V), but was expressed in some of the blood vessels within the brain and superior to the CBM in both mutants and control (data not shown and 6W).

Double-staining with laminin subunits and isolectin B4, an endothelial cell marker³², in E18.5 control and *Foxc1*^{lacZ/lacZ} mutants confirmed that increased laminin $\alpha 2$ expression was associated with abnormal blood vessel proliferation (Fig 7). Mutants had increased numbers of endothelial cells, which generally expressed laminin $\alpha 2$ and not laminin $\alpha 1$ (Fig 7F, L), suggesting that meningeal cells produce laminin $\alpha 1$, and endothelial cells produce laminin $\alpha 2$. The reticulated pattern of staining superior to the CBM noted with both laminin subunits overlapped with isolectin staining, likely due to co-localization of meningeal and endothelial cells.

Discussion

We have shown that primary meningeal defects, induced by *Foxc1* mutation, cause cortical dysplasia characterized by CBM breakdown, neuronal overmigration, RG detachment, and formation of MZ heterotopias. Extensive meningeal defects are established prior to the onset of dysplasia and meningeal defects are associated with changes in the expression of laminin subunits (namely loss of $\alpha 1$ expression).

The dysplasia in the *Foxc1* mutants resembles cobblestone cortex, in which CBM breakdown is thought to initiate a process leading to neuronal overmigration and heterotopia formation¹⁴. Regulation of CBM development and stability is likely to be dynamic and complex. The CBM, like other basement membranes (BMs), consists of thin protein sheets. Major components are laminins, collagen IV, nidogens and perlecan, a heparan sulfate proteoglycan. BMs are formed from two independent networks of laminin and collagen IV, while nidogens and perlecan provide stability. Additional minor components contribute to cross-linking of the networks and interaction with the interstitial matrix. BMs have important roles in providing tissue stability and regulating access to growth factors^{30, 33}.

The *Foxc1* mutants in our study differ significantly from previously reported mutants with cobblestone cortex, which are caused either by mutations in structural CBM components^{13, 34}, CBM receptors^{18, 20, 35}, or downstream signaling molecules^{19, 21}. Importantly, similar dysplasia is characteristic of dystroglycanopathies, caused by abnormal processing of α -dystroglycan, a molecule needed for interaction of cells with the extracellular matrix. Dystroglycanopathies include Fukuyama congenital muscular dystrophy, muscle-eye-brain disease, and Walker-Warburg syndrome^{14, 15}. *Foxc1*, however, is expressed in meningeal fibroblasts and is not a known regulator of CBM structural or signaling molecules, and loss of expression does not affect α -dystroglycan glycosylation. Reduction or loss of *Foxc1* likely impairs migration of meningeal cells, which are then not in position to provide a critical function needed for CBM stability and remodeling. In addition to directly impacting

CBM development, loss of meningeal cells may remove critical secreted factors, analogous to SDF-1 and retinoic acid, needed to help migrating neurons maintain proper positioning. Interestingly, compensatory mechanisms must exist to partially maintain neuronal positioning for at least some cell types in the face of meningeal loss. We found that Cajal-Retzius cells were properly positioned early in development in the *Foxc1* mutants, even though the meninges provide an important chemoattractant for these pioneer neurons^{4, 7}.

Extensive meningeal defects are seen in the *Foxc1* mutants prior to CBM breakdown and dysplasia formation. Therefore, intact meninges are dispensable for initial formation of apparently intact CBM, and are likely more important in maintenance and stability of the CBM. An attractive idea is that the meninges regulate CBM remodeling, a process which requires controlled degradation and synthesis of extracellular matrix³⁶, as the cortex grows dramatically during embryonic life. The meninges contribute components to already established CBM^{37, 38}, suggesting they participate in a dynamic turnover of CBM components, a key feature in the development of epithelial organs³⁹. The meninges may produce enzymes implicated in BM remodeling, such as membrane-tethered matrix metalloproteinases⁴⁰, but little is known about specific proteases expressed by embryonic meninges. The decrease in laminin $\alpha 1$ expression with meningeal loss is intriguing given deficits in BM stability seen with loss of other subunits in experimental models and human disease⁴¹, and may promote CBM instability independent of alterations in remodeling. Both disturbances in CBM remodeling and alteration of CBM composition might contribute to instability and breakdown of the CBM as the underlying brain rapidly expands.

In humans, *FOXC1* mutations cause Axenfeld-Rieger syndrome (ARS), a disorder of neural crest migration with characteristic ocular and systemic manifestations⁴². Patients with ARS and the 6p25 deletion syndrome, which includes *FOXC1* and other loci, can have psychiatric and neurologic symptoms⁴³⁻⁴⁷, although comprehensive brain imaging results are lacking. A recent study found human *FOXC1* mutations cause abnormal cerebellar development and meningeal defects⁴⁸. This study did not find cobblestone cortex on imaging, but small cortical dysplasia or abnormal organization of the neocortex cannot be excluded.

Most often cortical dysplasia is found sporadically, rather than as a part of a genetic syndrome. Since dysplasia can be acquired in association with prenatal insults, such as cytomegalovirus infection⁴⁹, based on our study we can suggest that meningeal injury at a critical point in development might be responsible for some cases of sporadic, localized cortical dysplasia. Future pathologic studies on human brain samples, especially dysplasia resected during epilepsy surgery, will be needed to test this idea.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the NIH (K12 NS01692, J.H.H., R01 DA017627, S.J.P.), Autism Speaks (J.A.S.) and the AHA/AAN (J.A.S.).

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Figure 1.

Worsening *Foxc1* mutations induce earlier and more severe CBM breakdown.

CBM breakdown in the dorsomedial neocortex revealed with laminin immunostaining in E12.5 (A-D), E14.5 (E-H), E16.5 (I-L) and E18.5 (M-P) control and *Foxc1* mutant embryos. In all panels, dorsal is to the top and medial is to the left. Embryonic age is indicated on the left and genotype on the top. Arrows indicate breaks in the BM.

Figure 2.

Progressive meningeal defects promote development of cortical dysplasia.

(A-D) Low power views of *Foxc1* mutant brains at E18.5. The red lines indicate the preferred areas of dysplasia formation in the dorsomedial cortex.

(E-L) High power views of Nissl stained dorsomedial cortex at E16.5 (E-H) and E18.5 (I-L). Locations of the MZ and CP are indicated for the controls. Black arrowheads point to heterotopic cells intruding into the MZ. In all panels medial is to the left and the pial surface is at the top. Nissl staining was used for histological analysis. MZ, marginal zone; CP, cortical plate.

Figure 3.

Quantitative analysis of the effect of meningeal defects on dysplasia formation and neuronal overmigration.

(A, B) Laminin (red) and CTIP2 (green) immunostaining in E18.5 embryos shows correlation of CBM breakdown with neuronal overmigration. (A) CTIP2 expression in deep layers of CP in control. Upper dashed line in control indicates level of CBM. The borders of the MZ, cortical layers II/III-IV, V, VI and the intermediate zone (IZ) are indicated by inferior dashed lines. (B) Area in *Foxc1^{lacZ/lacZ}* mutant showing CBM breakdown and upward displacement of CTIP2 positive cells.

(C-G) Measurement of ectopic, CTIP2 expressing deep layer neurons in the MZ. (C) In controls, deep layer neurons have high levels of CTIP2 expression and could be easily distinguished from lightly labeled MZ cells. (D) Deep layer neurons are displaced upwards in *Foxc1^{hith/hith}* mutants. Many remain in the upper CP (arrowhead). Cells that reached the superior border of the CP (arrow) or entered the MZ were included in analysis. (E, F) MZ heterotopias in the *Foxc1^{hith/lacZ}* and *Foxc1^{lacZ/lacZ}* mutants contain many deep layer neurons. Dashed line indicates upper boundary of CP. (G) Significantly more ectopic CTIP2 positive deep layer neurons were counted in the MZ per cerebral hemisphere of all *Foxc1* mutants. Data are presented as the mean $\pm$ standard error of the counts from three independent embryos. Each mutant was compared to control using the paired t-test. * $p < 0.02$. (H) Significantly more of the MZ had abnormal clusters of neurons or heterotopias in the mutants. Data and statistical analysis are as in (G). * $p < 0.05$, ** $p < 0.002$.

Figure 4.

RG detachment occurs in areas of CBM breakdown.

(A-D) Nestin and collagen IV staining were used to visualize the RG and CBM, respectively at E16.5. In all panels, the pial surface is to the top. Control (A) and *Foxc1^{hith/hith}* mutants (B) show RG fibers extending to an intact CBM. RG fibers in *Foxc1^{hith/lacZ}* (C) and *Foxc1^{lacZ/lacZ}* (D) mutants fail to extend to areas where there are gaps in the CBM (arrows), but extend to CBM fragments bordering the gaps.

Figure 5.

Extensive meningeal defects precede initiation of CBM breakdown.

At E12.5, meningeal fibroblasts are identified with a pan-Zic antibody (green) and the CBM with a laminin antibody (red) in transverse sections at comparable anatomic levels.

(A-C) Meningeal fibroblasts cover the dorsal telencephalon in control embryos. In B, meningeal cells (white arrowhead) lie superior to the CBM (arrow) and Zic+ Cajal-Retzius cells are seen within the brain (blue arrowhead).

(D-F) *Foxc1^{hith/hith}* mutants have a small defect in meningeal coverage over the dorsal telencephalon (E). The lateral telencephalon is covered (F).

(G-I) *Foxc1^{hith/lacZ}* mutants have a larger defect in meningeal coverage but meningeal fibroblasts are found over both the dorsal telencephalon (H) and the lateral telencephalon (I).

(J-L) *Foxc1^{lacZ/lacZ}* mutants have extensive loss of meningeal fibroblasts over the dorsal telencephalon (K). Fibroblasts only cover the most lateral telencephalon (L).

Arrows outline the meningeal defect in D and G. In J, only the inferior border of the defect could be identified.

Figure 6.

Meningeal defects cause differential changes in laminin α -subunit expression in the CBM. Expression of laminin α -subunits in *Foxc1* mutants was assessed with specific antibodies at E12.5 (A-L) and E18.5 (M-X). Arrowheads indicate the CBM in A, D, J, M, P, and V. Arrows indicate fragments of CBM that remain in *Foxc1*^{lacZ/lacZ} mutants are shown by arrows in O, R, and X.

(A-C, M-O) Laminin α 1 expression. Arrow (A, M) shows reticular expression superficial to CBM that disappears in mutants.

(D-F, P-R) Laminin α 2 expression. Arrow (D, P) indicates reticular expression superior to CBM, which increases in mutants.

(G-I, S-U) Laminin α 4 expression is present in a vascular distribution, which increased in mutants, and not within the CBM.

(J-L, V-X) Laminin α 5 expression is expressed only in the CBM in controls as well as what appears to be a subset of blood vessels (W, left center).

Figure 7.

Foxc1^{lacZ/lacZ} mutants have excessive blood vessels that express predominantly laminin $\alpha 2$ and not laminin $\alpha 1$.

Double-labeling of laminin subunits (green) using specific antibodies and endothelial cells using isolectin B4 (red) was performed in E18.5 control and *Foxc1^{lacZ/lacZ}* mutant embryos at E18.5.

(A-F) Double-labeling of laminin $\alpha 1$ and isolectin B4 in control (A, C, E) and *Foxc1^{lacZ/lacZ}* embryos (B, D, F). Arrows indicate blood vessels expressing laminin $\alpha 1$. Little double labeling is observed in mutants but fragments of CBM remain (H and Fig 6O).

(G-H) Double-labeling of laminin $\alpha 2$ and isolectin B4 in control (G, I, K) and *Foxc1^{lacZ/lacZ}* mutant embryos (H, J, L). Arrows indicate blood vessels expressing laminin $\alpha 2$. Observe most of increased laminin $\alpha 2$ expression co-localizes with isolectin B4 expression (a few examples are noted by arrows). Note brightly labeled CBM fragments do not co-localize with isolectin B4 (red arrow, L).

Arrowheads (A, C, E, G, I, K) indicate location of reticulated pattern of laminin subunit expression noted superior to CBM (see Fig 6A, D, M, P) which overlaps with isolectin B4 labeling. The micrographs are of control and mutant littermates, were taken with the same exposure settings for both embryos, and were slightly overexposed to capture the staining of the blood vessels in sufficient detail. In all panels dorsal is to the top.