Loss of Nidogen-1 and -2 Results in Syndactyly and Changes in Limb Development*

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Nidogens are two ubiquitous basement membrane proteins produced mainly by mesenchymal cells. Nidogen-mediated interactions, in particular with laminin, collagen IV, and perlecan can have been considered important in the formation and maintenance of the basement membrane. However, whereas mice lacking both nidogen isoforms or carrying mutations in the high affinity nidogen-binding site upon the laminin γ1 chain have specific basement membrane defects in certain organs, particularly in the lung, characterization of these mice has also shown that basement membrane formation per se does not need nidogens or the laminin-nidogen interaction. Limb development requires the complex interplay of numerous growth factors whose expression is dependent upon the apical ectodermal ridge. Here, we show that lack of nidogen-1 and -2 results in a specific and time-limited failure in the ectodermal basement membrane of the limb bud. The absence of this basement membrane leads to aberrant apical ectodermal ridge formation. It also causes altered distribution of growth factors, such as fibroblast growth factors and leads to a fully penetrant soft tissue syndactyly caused by the dysregulation of interdigital apoptosis. Further, in certain animals more severe changes in bone formation occur, providing evidence for the interplay between growth factors and the extracellular matrix.

Basement membranes are specialized extracellular matrices found underlying all epithelia and endothelia as well as ensheathing several forms of mesenchymal cell. They have diverse structural functions in strengthening and delimiting tissues as well as in serving as selective barriers (1). Further, they play major roles in cellular differentiation, migration, proliferation, and survival by inducing signaling effects through interactions with transmembrane receptors for specific basement membrane components and by acting as reservoirs for cytokines and growth factors. All basement membranes contain members of the laminin, nidogen, proteoglycan, and collagen IV families, and basement membrane diversity is in part derived from the large numbers of differentially expressed isoforms of laminin and collagen IV (2). Both laminins and collagen IV molecules can self-assemble into extensive networks in vitro; however in vivo, although structural stability is in part dependent upon collagen IV (3), the initial development of a basement membrane requires the presence of laminin (4, 5).

To date, and with the notable exception of zebrafish, the nidogen family has been shown to contain two members in all vertebrates where comprehensive sequence data exist. Whereas both isoforms are present in all basement membranes, nidogen-2 shows a more regulated expression pattern throughout development (6). Both nidogen isoforms have been shown to interact in vitro with many other components of the basement membrane, in particular with laminin and collagen IV, and it has been proposed that nidogens act as integrating elements for basement membrane assembly (7). The binding between the laminin γ1 short arm and nidogen-1 has been especially well studied both in vitro and in vivo. This very high affinity interaction occurs at the γ1LEb3 laminin-like epidermal growth factor module (8). Incubation with antibodies directed against this module or with competing recombinant proteins results in defects in basement membrane formation (9) and a failure in epithelial branching morphogenesis in a number of tissue and organ culture systems (10). However, results from mice where this binding domain was deleted by gene targeting showed that nidogen-laminin binding, although important for the formation of certain basement membranes, is not a general prerequisite for basement membrane assembly (11). Genetic deletion of either of the nidogen genes in the mouse results in only mild changes with no alteration in tissue or basement membrane architecture (12–14). Further, the finding of a redistribution and up-regulation of nidogen-2 in nidogen-1-deficient animals suggested a partial redundancy within the family (13). Studies of mice lacking both nidogen-1 and nidogen-2 showed that this is indeed the case for the abnormalities occurring in the lung and heart, which are directly related to defects in basement membrane assembly (15). Curiously, although changes in the lung are common between the nidogen double null animals and those lacking the binding site, the latter mice do not display cardiac changes (11, 15). Further, whereas nidogen double null mice generally develop kidneys, there is a...
highly penetrant renal aplasia in mice lacking the nidogen-binding site upon the laminin γ1 chain. The finding that nidogen double null mice can in many tissues form basement membranes that appear normal by electron microscopy shows that nidogens are not essential in producing a basement membrane and that different basement membranes have different requirements for nidogens (15).

Limb formation is directed by a complex interplay of epithelial and mesenchymal factors. Original theories suggested that the apical ectodermal ridge (AER),2 a thickening of the epithelium over the distal edge of the limb bud, is maintained by sonic hedgehog expressed by mesenchyme as part of a positive feedback loop. The secretion of fibroblast growth factors (FGFs) including FGF-4 and -8 from the AER (16) directly proliferation of the underlying mesenchymal cells of the progress zone, resulting in elongation of the limb. As the limb grows, the concentration of FGFs around these cells decreases, and they leave the progress zone, stop proliferating, and differentiate into chondrocytes. FGF signaling pathways are in numerous ways strongly affected by the surrounding matrix. Not only are the growth factors retained in the extracellular matrix and so concentrated in specific regions, but they are also protected from proteolytic degradation (17). Further FGF receptor dimerization, which is required for its signaling, is dependent upon heparan sulfate side chains of either matrix or cell surface proteoglycans (18). Finally, many of the growth factor signaling pathways are shared with integrin pathways, causing extracellular matrix-modulated signaling to in part regulate growth factor responses (19). Development of digits is directed by the activity of members of the bone morphogenic protein (BMP) family, primarily BMP-2, -4, and -7. These factors, expressed in the undifferentiated limb bud mesoderm, AER, and the interdigital mesenchyme, induce, among other events, apoptosis of the interdigital webs resulting in digit separation and cartilage development in prechondrogenic blastemas leading to phalanx formation (20). Here we show that nidogen deficiency results in disturbances in basement membrane assembly at specific time points in limb development and study the role of these extracellular matrix structures in growth factor action.

**EXPERIMENTAL PROCEDURES**

*Mouse and Breeding—* The mutant Nid1 allele was generated by deleting exon 3 as described previously (13). The mutation in the Nid2 gene was introduced by insertion of a gene trap vector in intron 4 (14). Embryos lacking both nidogen isoforms were produced by breeding mice heterozygous for the nidogen-1 and homozygous for the nidogen-2 mutation. For timing of gestation, noon of the day of the vaginal plugging was considered to be E0.5. Where more precise staging was required, somites posterior to the forelimb bud were counted, with the first somite being considered somite 13. Although the Nid1−/− Nid2−/− mutant animals were generally smaller than their littermates, only mice with similar somite numbers were compared in this study.

Genotyping for the mutant Nid1 and Nid2 alleles was carried out by PCR or Southern blot hybridization from yolk sac and embryonic tissues. Because mice carrying one wild type allele of Nid1, irrespective of the Nid2 status, show no obvious postnatal phenotype, fetuses used as controls were littermates of a Nid1+/− Nid2−/− or a Nid1+/− Nid2−/− genotype.

*Antibodies and Immunofluorescence Staining of Tissues—* Polyclonal rabbit antibodies raised against the following proteins were used: perlecan, laminin-111 (5), agrin (a kind gift from Dr. Markus Ruegg, Basel), collagen XVIII (kindly given by Dr Takako Sasaki, Munich), and E-cadherin. A primary goat serum against collagen IV (Southern Biotech) and the following monoclonal antibodies were also used: rat antibodies against laminin γ1 and β1 integrin (both from Chemicon) and a mouse antibody against FGF-8 (R & D Systems). The secondary antibodies used for detection of the primary immunoglobulins were either a goat anti-rabbit or a goat anti-mouse immunoglobulin coupled to Cy3 (Jackson ImmunoResearch Laboratories) or alternatively a donkey anti-rabbit or a donkey anti-goat immunoglobulin coupled to Alexa 488 (Molecular Probes).

Mouse embryos were dissected from the uterus, fixed for 1 h in 4% (w/v) paraformaldehyde/phosphate-buffered saline and then embedded in paraffin wax. 6-μm sections were subjected to tryptic digestion or citric acid-based antigen retrieval and blocked with 5% BSA/TBS. Incubations with primary antibodies were carried out overnight in 0.8% BSA/TBS at 4 °C. Secondary antibodies were diluted in 0.8% BSA and 3% normal goat serum in TBS. After three washes in TBS, the sections were mounted in Non-Fade-Mountant (DakoCytomation). Fluorescence microscopy was performed with a Zeiss Axioskop 165 microscope or with a Leica TCS confocal laser microscope.

*Apoptosis Assays—* Staining of apoptotic cells was performed with the DeadEnd™ fluorometric TUNEL system (Promega) according to the manufacturer’s instructions. The sections were counterstained with bisbenzimide (Serva).

*Whole Mount in Situ Hybridization—* DNA fragments used to produce antisense mRNA probes for Bmp2 and 7 were gifts from Brigid Hogan (Duke University Medical Center) and for Fgf8 from Gail Martin (University of California). Whole mount in situ hybridization with a digoxigenin-labeled probe was performed by standard protocols.

*Transmission and Scanning Electron Microscopy—* Mouse embryos were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, and, after processing through ethanol, embedded in Epon. 2-μm semi-thin sections were cut with a glass knife and stained with toluidine blue for light microscopy. Ultra thin sections were cut with a diamond knife and stained with lead citrate plus uranyl acetate prior to viewing with an EM 902A Zeiss electron microscope.

For scanning electron microscopy, embryos or isolated limbs were fixed with 1% glutaraldehyde, 4% paraformaldehyde. Following washing in the same buffer, they were dehydrated by passing through an ethanol gradient to 100% ethanol and then critical point-dried and mounted upon an aluminum stub. Specimens were viewed uncoated with a FEI Quanta 200 scanning electron microscope set to the variable pressure mode.

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2 The abbreviations used are: AER, apical ectodermal ridge; FGF, fibroblast growth factor; BMP, bone morphogenic protein; En, embryonic day; BSA, bovine serum albumin; TBS, Tris-buffered saline; terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling.
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![Image](https://example.com/figure1.png)

**FIGURE 1. Soft tissue syndactyly in newborn Nid1−/− Nid2−/−-deficient limbs.** Upper row, control (A), variably affected Nid1−/− Nid2−/− paws (B, hindlimb; D, forelimb), and hematoxylin/eosin-stained section of a Nid1−/− Nid2−/− paw (C). Lower row, Alizarin red- and Alcian blue-stained control (E) and Nid1−/− Nid2−/− paws (F–H). Distal phalanges are frequently attached to each other, as indicated by arrows. Note that the ossification of middle phalanx is delayed in the mutant mice (arrowheads) and that the fifth digit is not included in the syndactyly.

**Alcian Blue and Alizarin Red Staining**—To stain cartilage and bone in newborn embryos, Alcian blue 8GX and Alizarin red (Sigma) were used.

**Bead Implantation**—Affi-gel blue agarose beads with a 100–200 mesh (Bio-Rad) were soaked in 100 μg/ml recombinant human BMP-4 (R & D Systems) or BSA and implanted into the interdigital tissue of E13.5 forelimbs. The limbs were placed on polycarbonate membranes with a pore size of 0.1 μm (Nucleopore) and cultured in Dulbecco’s modified Eagle’s medium: Ham’s F-12 medium (Invitrogen) supplemented with 10% fetal calf serum. After 20 h of incubation at 37 °C in 5% CO2, the limbs were processed for histology and immunofluorescence.

**RESULTS**

**Cutaneous Syndactyly Occurs in All Nid1−/− Nid2−/− Mice**—Mice lacking both nidogen forms die shortly after birth, they are usually smaller than their littermates and show defects in lung and heart development (15). Further, all Nid1−/− Nid2−/− mice have developmental defects of both fore- and hindlimbs (Fig. 1). Although the severity of this phenotype varies (see below), there was in all cases a cutaneous syndactyly with webbing of the interdigital skin. Generally, however, the fifth digit developed normally. Alizarin red and Alcian blue staining, performed to check whether skeletal involvement also occurred, showed that in most mice all of the skeletal elements were present (Fig. 1, E–H), and the bones of the digits had formed separately, a finding also seen in histological sections (Fig. 1C). However, in some animals there was fusion of the developing distal phalanges. This appeared to be the result of a defect in separation later in development rather than a failure in the formation of the cartilage anlage, because the outline of each was readily seen even when joined (Fig. 1, C, F, and G). The Nid1−/− Nid2−/− animals often showed reduced osteoid development in the middle phalanx of both fore- and hindlimbs, a finding suggestive of delayed development (Fig. 1, F and G).

**Apoptosis Is Inhibited in the Interdigital Web of Nid1−/− Nid2−/− Animals**—Digit formation and separation in amniotes occurs by two events: differential growth at the front of the developing digit and apoptosis of the intervening tissue (21). To determine whether apoptosis occurs in the webs in the absence of both nidogens, TUNEL assays were performed upon the autopods at E13.5. In wild type animals often showed reduced osteoid development in the middle phalanx of both fore- and hindlimbs, a finding suggestive of delayed development (Fig. 1, F and G).

**Figure 2. Reduced apoptosis in interdigital tissues and expression of apoptosis-inducing BMPs.** Shown are a TUNEL assay on E13.5 developing footpads in control (A and C) and Nid1−/− Nid2−/− mice (B and D) and in situ hybridization for Bmp2 (E and F) and 7 (G and H) at E13.5. The signals are decreased, and the expression domains are reduced in Nid1−/− Nid2−/− mutants (F and H) in comparison with controls (E and G). Arrowheads mark the altered Bmp7 expression domain.
the main signaling molecules inducing apoptosis in digit development (22). This is thought to occur through the regulation of pro-apoptotic signaling molecules, such as Msx family members. Three BMPs, BMP-2, -4, and -7, have been shown to be expressed during limb development and to be involved in apoptosis, digit identity, and chondrogenesis (20, 23). BMP activity, expressed during limb development and to be involved in apoptosis, is highly regulated, in large part by extracellular matrix interactions. Mutations in extracellular matrix molecules such as fibrillin-2 have been shown to curtail BMP activity and result in limb defects both in mouse (24, 25) and man (26). To determine whether a similar effect could be occurring in the absence of both nidogen isoforms, we examined Bmp expression in the developing limbs. In situ hybridization showed reduced expression of Bmp2 at E13.5 (Fig. 2, E and F), whereas Bmp7, whose signal was also decreased, had an expression domain, which did not extend so far distally in the interdigital region (Fig. 2, G and H). These results suggested either that the levels of BMP expression are not sufficient to induce cell death in the interdigital region or that BMP activity or presentation was altered by the lack of the nidogens. BMP signaling acts through a shared pathway for all isoforms. To check whether this could still occur in the Nid1−/− Nid2−/− embryos, carrier beads soaked in either BMP-4 or BSA were implanted into the presumptive interdigital regions of control and mutant limb buds (Fig. 3). The limbs were cultured for 20 h and then analyzed for apoptosis by TUNEL assay. Here unlike in the nonmanipulated limb buds, there was diffuse apoptosis observed in the autopods of control as well as mutant mice, presumably caused by the culture conditions. However, there was no increase in apoptosis at the site of the bead coated in BSA in either mutant or control animals (Fig. 3, B, C, and F). In contrast, in Nid1−/− Nid2−/− limbs the interdigital region where the BMP-4 bead had been implanted showed a robust signal when it was placed between digits 2 and 3 (Fig. 3, B, C, and D), which were usually fused. A similar increase was observed about BMP-4 beads implanted into control autopods (Fig. 3E), suggesting a graded response to BMPs and that the reduced levels of the signaling proteins were responsible for the failure in septation in the nidogen null animals.

Stage-specific Defects Are Seen in the Developing Limb Basement Membranes-Nid1−/− Nid2−/− mice show tissue-specific changes in basement membranes. To determine whether this structure is present over the limb at birth, immunostaining of the fused digits was carried out. This showed the expected linear staining patterns for perlecan and laminin, similar to that observed in the normal appearing Nid1−/− Nid2−/− littermates (not shown). We then analyzed basement membranes during limb bud development. At E10.5 Nid1−/− Nid2−/− embryos, although smaller than their littermates, showed no obvious abnormalities. However, whereas sections of the limb confirmed the presence of the AER, this structure appeared in some sections, particularly those through the median planes, to be less compacted, larger, and less organized than in controls (Fig. 4, B and E), whereas in other sections the AER appeared less well defined (Fig. 4, C and F). Immunostaining of these limb buds for basement membrane proteins revealed a linear basement membrane under the surface epithelium in the control mice, with a typical continuous staining being seen under the AER and in the developing blood vessels of the limb bud (Fig. 4, A and D). Further, the staining for the matrix molecules laminin-111 and collagen IV was restricted to these structures. In contrast, sections of limbs from Nid1−/− Nid2−/− animals showed very little or no discernable basement membrane staining under the AER. Instead, rather irregular and presumably disorganized deposits of matrix were seen. Laminin immunoreactivity was more widespread throughout the epithelium and AER (Fig. 4, B and C), its main site of production, whereas collagen IV was more generally distributed through the whole limb bud (Fig. 4, E and F). Further, in contrast to the control animals, there was no evidence for basement membrane deposition around developing blood vessels in the limb bud mesenchyme. However, distinct basement membrane staining was found elsewhere in the embryo (Fig. 4, J and K).

There are three main basement membrane-associated proteoglycans: perlecan, collagen XVIII, and agrin. Perlecan and collagen XVIII were found in the AER basement membrane of control limb buds at E10.5 (Fig. 5, A and B), whereas agrin occurred both in the basement membrane and on the epithelial cell membrane (Fig. 5C). These failed to be concentrated to the basement membrane zone under the AER in Nid1−/− Nid2−/− mutant mice (Fig. 5, D–F). Perlecan was in particular widely distributed through the limb bud, and none of the proteogly-
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FIGURE 4. Disruption of the basement membrane underneath the AER. Transverse sections of E10.5 limb buds (A–I) and neural tube (J and K) are shown. The continuous localization of basement membrane components observed in controls (A, D, and G) under the ectoderm is disrupted in the region of the AER in Nid1+/− Nid2+/− mutant mice (B, E, F, H, and I). Instead, these components are distributed irregularly throughout the mesenchyme and epithelium as seen in the insets. The Nid1+/− Nid2+/− mutant AERs (H and I) show reduced staining for β1 integrins (green) when compared with controls (G), laminin-111 costaining (red). At E10.5, laminin is deposited in basement membranes in other areas in control (J) and in Nid1+/− Nid2+/− embryos (K). The scale bars represent 100 μm.

β1 integrins were seen in the developing blood vessel basement membranes.

Semi-thin sections of control limbs at E10.5 showed a clear border between the AER and underlying mesenchyme (Fig. 6A). In the Nid1+/− Nid2+/− mutant, however, the AER was less distinctly defined, and the cells directly overlying the mesenchyme did not have the differentiated columnar phenotype seen in the control (Fig. 6B and C). In agreement with the immunohistological findings, transmission electron microscopy of the developing limb bud at E10.5 showed that the basement membrane was present as a typical intact double layer under the epithelium of control animals (Fig. 6D). However, in Nid1+/− Nid2+/− mice it was absent, although diffuse regions of matrix deposition were seen, suggesting an improper organization (Fig. 6E and F).

Cell attachment and signaling from the basement membrane is to a large part induced through the interaction of laminins with integrins of the β1 family. To determine whether these integrins had an altered expression pattern in the absence of nidogen, we immunostained the limbs with antibodies against β1 integrin. In the control animals a strong signal was seen, which was highly concentrated at the epithelial plasma membrane, colocalizing with laminin at the contact with the basement membrane (Fig. 4G). Integrin β1 was also highly expressed on endothelial cells. In Nid1+/− Nid2+/− animals the integrin β1 signal was much reduced in the AER and was largely absent from the epithelial mesenchymal border (Fig. 4, H and I). Staining for β4 integrin, which as α6β4 forms another important laminin receptor, showed similar results (not shown).

Interestingly, although laminin, a main ligand in basement membranes for β1 integrins, appeared to be absent from forming endothelial structures (Fig. 4, B and C), integrin staining in these regions was reminiscent of that in control mice (Fig. 4, H and I).

The histological analysis had suggested that the AER was aberrantly differentiated in Nid1+/− Nid2+/− limb buds, and in some sections it appeared enlarged. Scanning electron microscopy of control limbs showed an evident ridge structure over the flattened limb bud (Fig. 6G). In mice lacking both nidogens, the AER was barely evident over much of the limb (Fig. 6, H and I), with the exception of the most distal portion, which had an enlarged extrusion with the hypercellular distal AER, giving some of the limbs an almost conical appearance (Fig. 6K). Further, the limb bud did not have the usual flattened shape seen in the controls but was thickened. To determine whether this aberrant protuberance was ectoderm or a mesenchymal extrusion, sections were stained for the epithelial cell marker E-cadherin, which was present in both control (Fig. 6I) and Nid1+/− Nid2+/− mice (Fig. 6L), showing that this is a morphological defect of the AER itself.

The limb bud begins to develop as an outgrowth from the lateral mesoderm at about E9 in the forelimb. To examine whether basement membranes were found at early stages of limb formation, we immunostained sections of mouse embryos at E9.5, prior to the development of the AER. Here a continuous basement membrane staining was observed in both control (Fig. 7, A–C) and Nid1+/− Nid2+/− mice (Fig. 7, D–F). This suggests that the basement membrane either breaks or cannot

FIGURE 5. Deposition of basement membrane proteoglycans under the AER. Transverse sections of E10.5 limb buds are shown. Continuous staining is seen in control embryos for perlecan, collagen XVII, whereas agrin staining occurs both on the epithelial cell surface and in the basement membrane (A–C). Basement membrane staining is disrupted or absent under the AER in Nid1+/− Nid2+/− embryos (D–F). The Scale bar represents 100 μm.
be efficiently remodeled under the conditions of rapid growth and cell compaction induced by the development of the AER.

Limbs buds were then examined at E13.5, when the AER has regressed, condensation of the digital mesenchyme has begun to occur, and there are initial alterations of the interdigital regions of the forelimbs (Fig. 7). Scanning electron microscopy showed the expected failure in septation in the Nid1−/−Nid2−/− mice (Fig. 7K), with a thickened mass of tissue forming a ridge over the fingers that extends as a band between the tips of the developing digits (Fig. 7, K and L). Further, it showed a less well differentiated epithelial layer over these regions. Sections taken through the developing hand of Nid1−/−Nid2−/− mutants at this stage show a continuous staining for basement membrane components such as perlecán (Fig. 7M); however, at the apex of many of the digits the epithelium becomes irregular. High power magnification showed that the basement membrane staining here displayed two linear structures separated by a thin layer of cells. To characterize this finding further, we stained parallel sections for E-cadherin (Fig. 7, N and N'). E-cadherin was present in the two layers of cells surrounded by the basement membrane and was also seen in the outer surface cells, which lacked a basement membrane. Transverse sections showed discrete breaks in the basement membrane at the very tips of the digits and in some fingers extrusion of mesenchymal cells into the epithelial layer (Fig. 7I). This is presumably caused by a weakness in the basement membrane layer, which in places may lead to the epithelium folding back as extruded mesenchyme migrates through the defect and results in the epithelial differentiation of the now exposed surface mesenchyme as shown by E-cadherin expression.

**FIGURE 6. Morphological alterations in the AER at E10.5.** Semi-thin sections of control (A) and Nid1−/−Nid2−/− mutant (B and C) forelimb buds are shown. The basal cells of the double mutant AER appear less polarized and well organized. Asterisks indicate apoptotic bodies. The junction between the AER and the underlying mesenchyme is indicated with a dashed line (A–C). The scale bars are 25 μm. Transmission electron micrographs of the corresponding ultra thin sections (D–F). The basal cells in double null AERs (E and F) are not attached to a formed basement membrane. The arrows point to intact basement membranes, and arrowheads indicate where the basement membrane is disorganized or absent. The scale bar represents 0.3 μm. Scanning electron micrographs (G, H, J, and K) show the AER, marked by arrows, as a typical ectodermal thickening in control embryos (G), whereas in Nid1−/−Nid2−/− mice the ridge is less evident, except for an irregularly formed region at the distal tip of bud (H, J, and K). The scale bar is 100 μm. E-cadherin immunostaining of this tip region shows that it is of epithelial origin (l, control; L, Nid1−/−Nid2−/−; scale bar, 50 μm).

Skeletal Defects Occur in a Minority of Nid1−/−Nid2−/− Animals—Although soft tissue changes resulting in digital fusion were seen in all Nid1−/−Nid2−/− mice, a significant minority of animals (15%) showed more severe defects involving the skeleton. At birth, the feet of such mice were severely deformed with a gross soft tissue capping of the digits (Fig. 8A), and the forelimbs were twisted and held pulled under the body. Alizarin red/Alcian blue staining showed altered development not only in the autopod but also in the anterior skeletal elements of the zeugopod and stylopod. Skeletal changes were only observed in the forelimb and curiously only on the right side (Fig. 8, B and C). These exhibited a range of severity. In the least severe form there was only loss of the first digit and the deltoid tuberosity (Fig. 8E), whereas in the most severe there was also loss of more of the medial digits, the cartilage that will form the radial side of the carpus, and aplasia of the radius itself (Fig. 8, B and F). Intermediate forms with hypoplasia and absence of the distal radius were also seen (Fig. 8G); however, the ulna had developed. In the stylopod, apart from the loss of the deltoid tuberosity, a muscle attachment site with an independent ossification center, the humerus appeared normal.

FGFs expressed in the AER induce and maintain proliferation of the mesenchymal progress zone directly under the AER and so cause limb elongation (16). FGF-8 is highly significant in limb and digit formation, because its developmental stage-specific deletion in the AER results in loss of the deltoid tuberosity and/or loss of the radius in some mice as well as digital defects (27, 28). These changes are similar to those seen in the Nid1−/−Nid2−/− animals. FGF-8 mRNA expression was analyzed by in situ hybridization (Fig. 9, left panel). At E10.5 both control (Fig. 9, A and B) and Nid1−/−Nid2−/− embryos (Fig. 9, C and D) exhibited a strong expression in the AER; however, the ridge appeared more irregular in the Nid1−/−Nid2−/− mutant, and the expression domain did not extend so far anteriorly (Fig. 9, B and D). In situ hybridization of limb buds of control mice at E11.5 again showed the expected expression in the AER (Fig. 9, E and F), but in Nid1−/−Nid2−/− mice there was an obvious distortion in the AER, produced by a widening of the expression region (Fig. 9, G and H) at the tip of the limb bud and a nonresponsive center.

Previous studies have shown the importance of proteoglycans or glycosaminoglycans in FGF signaling. In part this is believed to be due to the sequestration of the growth factors, concentrating them close to their target cells and protecting them from degradation, but it has also been shown that the heparan sulfate-FGF interaction is required for FGF signaling
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per se. To determine the localization of FGF-8 protein in and around the AER, we immunostained E10.5 limb buds for FGF-8 (Fig. 9, I–N) and compared its staining with that for basement membranes (Fig. 9, J, L, and N). In the wild type animals FGF-8 was seen through the AER and part of the surface ectoderm, with a distinct border at the epithelial-mesenchymal interface (Fig. 9J). This resulted in a strong linear staining pattern, with FGF-8 colocalizing with the basement membrane separating the AER and the progress zone (Fig. 9I), presumably because of FGF-8 interacting with basement membrane proteoglycans. In the Nid1−/− Nid2−/− animals, although FGF-8 is detected in the AER, it was now colocalized with the disrupted extracellular matrix material between the cells in the ridge (Fig. 9, K–N). As a result of the absence of the basement membrane, there was no sharp border seen under the AER, and there was some signal for FGF-8 seen in the mesenchyme.

Mice with an impairment of FGF-8 function in the forelimb, either because of a reduced expression of the growth factor (29) or defects in its receptor (30), show an increase in apoptosis in the limb bud mesenchyme that results in skeletal changes similar to those described above. To verify whether loss of the two nidogen isoforms was related to altered levels of apoptosis, we then carried out TUNEL staining of the developing limb bud at E10.5. This showed a marked apoptosis at E10.5 in the aberrant AER (Fig. 8R) for many of the double mutant limbs. Further there was a region of increased cell death in the anterior-proximal regions of the limb bud not seen in the controls (Fig. 8, O, Q, and S).

DISCUSSION

The nidogens are one of the four protein families found ubiquitously in basement membranes. Both nidogen-1 and nidogen-2 have a particularly wide spectrum of binding partners (6), and this led to the assumption that they are crucial in basement membrane assembly. However, the genetic ablation of either nidogen-1 (12, 13) or nidogen-2 (14) appears to have no effect.
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on the production or maintenance of the basement membranes. To complement this, mice lacking nidogen-1 show an up-regulation and redistribution of nidogen-2, suggesting that intrafamily compensation occurs (13). This has recently been confirmed in mutant animals lacking both proteins, which develop to birth but die shortly after with defects in both heart and lung (15). Surprisingly, the absence of both nidogens does not alter overall organogenesis, suggesting that different basement membranes have different requirements for nidogens.

The completely penetrant syndactyly in the absence of both nidogen isoforms occurred in both fore- and hindlimbs. Alizarin red staining and histological sectioning showed that this generally involved only the soft tissues, the cartilage anlagen having formed separately and usually remaining so. In certain cases mild fusion was observed; however, this was not as severe as that seen in mice lacking the laminin α5 subunit where occasional complete fusion occurred (31). Otherwise, the skeletal elements of the digits in most Nid1−/− Nid2−/− mice appeared as in controls, with the exception of a reduced ossification in the middle phalanx, a finding previously described in other mutants (32) and considered to be related to mild developmental retardation.

The finding that nidogens are required to maintain the basement membrane underlying AER, between E9.5 and E13.5, suggests that their absence leads to a breakdown of this extracellular matrix structure under the then rapidly extending tip of the limb. The nidogens are either needed to reinforce the basement membrane or alternatively to act to speed up the correct assembly of the other components during rapid limb growth and AER compaction. As with the Nid1−/− Nid2−/− mice, mesenchymal extrusion through a basement membrane defect is also observed in the laminin α5 null mouse and in animals lacking both α3 and α6 integrins. It is interesting to note that these latter mouse strains, which develop syndactyly, also have a highly penetrant exencephalopathy (31, 33). This is caused by a failure in closure of the neural tube, because of defects either in the basement membrane at the junction of ectoderm and neuroepithelium or in the cell-matrix interaction. However, this phenotype is not seen in the mutant mice presented here, and the basement membrane about the neural tube appears normal, suggesting either that this structure is more robust in the absence of nidogens or that factors other than merely matrix stability may underlie the differences between these mouse lines.

The AER is present in the mouse between E10 and E13, its induction and maintenance being dependent upon Wnt3 and β-catenin signaling pathways (34). Wnt3 activity is in part regulated by its antagonist Dickkopf. A transgenic mouse line hypomorphic for Dickkopf leads to syndactyly, because of a grossly expanded and noncompacted AER (35, 36). In situ hybridization for AER markers in these mice showed a widened “doubleridge” structure, not dissimilar to that seen in the absence of both nidogen isoforms. Further, these mice, like the Nid1−/− Nid2−/− animals, display a lack of interdigital apoptosis (35). BMP signaling has been implicated in the programmed loss of the interdigital soft tissue in both birds and mammals. We were able to show that there is a reduced expression and an altered expression pattern of BMPs within the developing limb of Nid1−/− Nid2−/− mutants. In the limb, BMP signaling is a complex interplay with three family members, BMP-2, -4, and -7, believed to be important in joint formation as well as digit separation. However, their activity is also dependent upon the expression of their receptors and antagonists, such as noggin and gremlin. Increased expression of noggin reduces interdigital regression and induces syndactyly (22). BMPs interact with the extracellular matrix (37, 38), and their activity is altered by the surrounding matrix (24). That BMP impregnated beads could induce interdigital apoptosis in nidogen double null limbs, a finding not seen in fibrillin-2-deficient mice (24), suggests that a reduction in the overall amounts of the BMPs could be the cause of the syndactyly.
Nidogens in Limb Development

The AER, crucial in the development of the growth and patterning of the limb, is itself under tight regulation, because its cellularity and extension have direct effects on formation of the limb. A balance between BMPs, which appear negative for AER cellularity, and AER maintenance factors regulate the size and extent of the AER (39). In Bmp7−/− animals (40) or in Bmp4 haplo-insufficient mice (41), there is a longer and hypercellular AER that results in postaxial polydactyly. Similarly, ectopic expression of BMP antagonists, such as noggin, results in a larger AER and its slower regression, again causing syndactyly and polydactyly (22, 42). Although the limb defects in the Nid1−/− Nid2−/− mice are presumably caused by structural failure of the basement membrane, it is possible that nidogen isoforms have other roles in AER function. Possibly, the altered AER development observed in Nid1−/− Nid2−/− mice could be related in part to alteration in BMP expression or action. However, the length of the abnormal AER in these mice appears unaltered or reduced, and this may explain why polydactyly is not observed. Among the growth factors expressed by the murine AER, FGF-4 and -8 have been suggested to direct proliferation of the underlying mesenchymal cells of the progress zone, resulting in elongation of the limb. Mice lacking FGF-8 signaling from the AER, either through cre-lox-mediated gene deletion before E9.5 (27, 28) or a deficiency of its FGFRIIIb receptor (30), present hypoplastic/aplastic changes in the anterior skeletal elements similar to those seen in the forelimbs of some of the mice lacking both nidogens. Further, a reduction of either of the buttonhead-like zinc finger transcription factors, Sp8 or Sp9, results in reduced expression of FGF-8 and a similar phenotype (43). The possibility that a reduction in FGF-8 expression or signaling may be the underlying factor in the skeletal changes occurring in some of the nidogen double null animals is strengthened by the pattern of apoptosis in the developing anterior limb bud observed in the Nid1−/− Nid2−/− mice, which is similar to that seen in Sp8 null animals and in FGF/FGF receptor-deficient embryos. Variation in the extent of this apoptotic region could account for the difference in penetration of the skeletal phenotype. Conversely, it would be interesting to study development of the basement membrane in the FGF pathway mutants, given that FGF signaling has been shown to be important in inducing or maintaining basement membranes (44).

The failure in compaction of the AER and hence the change in the FGF expression domain is shared with the Dickkopf mutant doublelimber. However, as these latter mice lack the more severe skeletal changes seen in about 15% of the Nid1−/− Nid2−/− mice, it suggests that merely a failure in AER compaction is insufficient to induce the loss of anterior skeletal elements. We could show that FGF-8 expressed by the AER in control animals is colocalized with the extracellular matrix under the AER and presumably concentrated at the basement membrane. This would then logically act to supply a high and sustained level of the growth factor to the underlying proliferating mesenchymal zone. In the Nid1−/− Nid2−/− animals, FGF-8 in part colocalizes with ectopically positioned matrix, but its contact with the distal mesenchyme is altered. Further, there is evidence from some Nid1−/− Nid2−/− animals that FGF-8 is found central within the mesenchyme, which is not usually exposed to such high levels of this growth factor, suggesting that either signaling to the progress zone of the limb is impaired or that aberrant signals, in the form of disturbed FGF concentration gradients, are delivered to the deeper mesenchyme.

FGFs have been shown to interact with heparan sulfate proteoglycans, thus protecting them from degradation and concentrating them in the vicinity of their target cells. Further, structural studies have shown that FGFs require a 1:1:1 triad of heparan sulfate proteoglycan-FGF-FGFR to induce receptor dimerization and hence growth factor signaling (17, 45). Defects in enzymes producing heparan sulfate proteoglycans have been shown to alter FGF signaling in both Drosophila (46) and mice (47). The major basement membrane proteoglycan, perlecan, has been shown to bind to FGF-2 (48) and FGF-7 (49) as well as FGF-binding protein (50) in vitro. However, mice deficient for perlecan exhibit chondrodysplasia, and syndactyly has not been reported (51, 52). It is possible that other basement membrane proteoglycans such as collagen XVIII or agrin could take over the role of FGF signaling in the absence of perlecan.

FGF signaling to and from epithelia and mesenchyme is complex, and reciprocal. FGFs expressed by mesenchyme tend to signal to epithelia utilizing FGF receptors of the IIIb splice type, whereas the converse is produced by epithelial FGFs interacting with mesenchymal FGFRIIIc splice type receptors, although cross-activation can occur. This leads to the possibility that FGFs ectopically released into the deeper mesenchyme may be involved in the defects seen in the nidogen null mice. Indeed local administration of FGFs inhibits BMP production, reduces interdigital cell death, and results in syndactyly in the chicken (53). Hence ectopic mesenchymal FGF signaling in the Nid1−/− Nid2−/− mice could be the cause of the reduced interdigital cell death.

The severity of syndactyly in the nidogen null mice varied with the limb and the digit, such that the fifth digit was almost invariably not fused. Further, as with the laminin α5-deficient mice, the forelimbs were more severely affected than hindlimbs (31). If the action of nidogens is to catalyze the formation of the newly developing basement membrane, it is possible that the turnover is slightly lower in the hindlimb than the forelimb possibly related to its later development, thus giving the tissue time to develop a nearer normal basement membrane. Curiously, all of the mice showing skeletal hypoplasia had this defect in the right limb, and even the animals with the most severe changes had normally formed skeletal structures in the left forelimb. This is in agreement with the findings of the doublelimber mice (35) and the Bmp4 haploinsufficient mice (41), which also showed a more severe defect in the right forelimb. There is no clear reason for this bias; however, one possibility is that the embryonic turning, seen in murine development between E8.5 and E9 and occurring toward the right side, leads to uneven stresses upon the embryo. Our results provide further evidence for interplay between growth factors and extracellular matrix structures and show that genetic perturbations of extracellular matrix assembly can be used to study the formation and biological effects of growth factor expression domains.
Loss of Nidogen-1 and -2 Results in Syndactyly and Changes in Limb Development
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