Loss of connexin43-mediated gap junctional coupling in the mesenchyme of limb buds leads to altered expression of morphogens in mice

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Received January 26, 2009; Revised March 26, 2009; Accepted May 8, 2009

Mutations in the GJA1 gene coding for connexin43 (Cx43) cause oculodentodigital dysplasia (ODDD), a pleiotropic human disorder with characteristic morphologic anomalies of face, teeth, bones and digits. Interdigital webbings, also called syndactylies, are a characteristic phenotype of this disease showing high intra- and interfamilial penetrance. Therefore, we decided to study the molecular basis of syndactylies caused by Cx43 mutations. In order to reveal the impact of Cx43-mediated gap junctional coupling, we used mice expressing the human point mutation Cx43G138R and, in addition, ‘knock-out’ mice lacking Cx43. Both conditional mouse models developed syndactylies as a consequence of disturbed interdigital apoptosis, which we show to be due to reduced expression of two key morphogens: sonic hedgehog (Shh) and bone morphogenetic protein 2 (Bmp2). Diminished levels of Bmp2 and subsequent up-regulation of fibroblast growth factors (Fgfs) lead to an insufficient induction of interdigital apoptosis. Interestingly, the reduction of Shh expression in Cx43 mutants begins on embryonic day 10.5 indicating a disturbance of the Fgf/Shh regulatory feedback loop, and confirming the recently published observation that gap junctions can relay Fgf signals to neighboring cells. Thus, Cx43-mediated gap junctional coupling in the mesenchyme of limb buds after ED11 is essential to maintain Shh expression, which regulates the downstream signaling of Bmp2. Besides diminished interdigital apoptosis, the decreased expression of Bmp2 in Cx43 mutants may also be involved in other morphological alterations in patients suffering from ODDD.

INTRODUCTION

Morphogenesis is a fundamental aspect of developmental biology which consists of cell proliferation, cellular differentiation and tissue patterning. Morphogens are diffusible molecules acting through binding to their specific receptors and carrying signals that control cell differentiation in a concentration-dependent manner.

The key molecules in the development of the limb, the craniofacial region and bone are sonic hedgehog (Shh), fibroblast growth factors (Fgfs) and bone morphogenetic proteins (Bmps) (1). During limb bud development the zone of polarizing activity (ZPA) exhibits a pattern-organizing activity via action of a morphogen gradient of Shh (2). Shh induces the apical ectodermal ridge (AER) to produce Fgf4 and Fgf8 which maintain the expression of Shh during the limb bud development through the Fgf-Shh-feedback loop (3–5). Additionally, Shh signaling from the ZPA induces the expression of Hoxd and Bmp2 genes in the mesoderm. In order to separate the digits, programmed cell death is needed which removes the tissue in-between. The signaling of Bmps induces apoptosis, while Noggin, a BMPs’ antagonist, blocks cell death in this tissue.
(6). Recently, action of FgfS on interdigital apoptosis has been described (7): Bmp2 signaling inhibits FgfS’ expression in the AER, indirectly inducing the programmed cell death of the interdigital mesenchyme. Thus, Fg4 and Fg8 act as cell survival factors in the developing limb bud.

Modulation of these signaling pathways by gap junction channels (GJC) is highly probable considering the phenotypic alterations described for Connexin43 (Cx43) mouse mutants. All Cx43 point-mutated mice described so far represent models for the rare human disorder oculodentodigital dysplasia (ODDD; ODD syndrome; OMIM #164200) (8–10). Their morphologic phenotypes like syndactylies, craniofacial or dental anomalies as well as osteopenia are developmental disorders or disturbances in tissue homeostasis. Key morphogens like Bmp2 regulate these developmental events. Nevertheless, even if not directly mediated, the proper progression of these processes seems to be affected by Cx43. Cx43 is a member of the connexin gene family which comprises 21 different proteins in human and 20 in mice (11). Connexin proteins oligomerize into hexamers, so-called connexons which form GJC after docking to each other in contacting membranes. GJC allow intercellular diffusion of sugars, amino acids, ions and other metabolites (12) or second messengers like cAMP, Ca²⁺, NAD⁺ and IP₃ (13).

During chick limb development, Cx43 is expressed in restricted domains in the AER and mesenchyme of the ZPA (14). Application of gel beads soaked with Cx43 antisense oligonucleotides at stages before limb outgrowth resulted in truncation or fragmentation of the limb bud or complete splitting into two or three branches (14). This knock down of Cx43 expression led to a drastic reduction of Shh and Bmp2 mRNA in situ. Others described a knock down of Cx43 in chick which resulted in limb patterning defects including deletion of the anterior digits (15). Shh and Bmp2 were also strongly downregulated in treated limbs. Thus, Cx43-mediated gap junctional signaling or expression is required for chick limb development. Furthermore, the modulation of chondrogenic differentiation in vitro by Bmp2 involves gap junctional coupling. The inhibition of gap junction-mediated intercellular communication by 18 alpha glycerrhetinic acid decreased chondrogenic differentiation (16). This result suggests that gap junctional coupling is not critical for the initiation, but plays an important role in the continuation of chondrogenesis, a process for which Bmp signaling is necessary. Thus, Cx43 function seems to modify Shh and Bmp2 signaling cascades in diverse tissues and developmental stages.

In order to further elucidate the impact of Cx43-mediated gap junctional coupling on morphogen expression and developmental processes in mice, we used the conditional Cx43flG138R (9) and Cx43flLacZ (17) mouse lines in our studies. Cx43G138R expressing mice show a strong decrease of Cx43-mediated gap junctional coupling, whereas Cx43LacZ mice entirely lack Cx43 expression. We found that the lack of Cx43-mediated gap junctional coupling in the mesenchyme but not in the ectodermal ridge during limb bud development is causing syndactylies in these mouse mutants. The expression of Shh and Bmp2 is downregulated, whereas FgfS are upregulated in the limb buds between ED10.5 and 12.0. The altered expression of morphogens can explain the occurrence of syndactylies in these mice and in the corresponding ODDD patients.

RESULTS

Syndactylies are caused by decreased apoptosis in mice deficient in Cx43-mediated coupling

One of the characteristic phenotypic alterations in patients suffering from ODDD is syndactylies. Similarly, all mouse models of ODDD described so far develop soft tissue webbing of fingers and toes. These findings indicate insufficient apoptosis normally separating the digits by removing the tissue in-between.

To test for apoptosis in the mutated limbs embryonic paws of ED12.5, ED13.5 and ED14.5 old Cx43G138R/- mutated, Cx43-deficient and control mice were prepared for Lyso-Tracker stainings (Fig. 1). Apoptotic cells were present in mutant limbs but located only near the finger tips in the interdigital tissue (arrows in Fig. 1E–G, I–K) in comparison to controls (arrow in Fig. 1A–C). The limitation of apoptosis in embryonic limbs could be correlated with the appearance of syndactylies at later stages of embryonic development (ED15.5) (Fig. 1H and L), postnatally (after removal of the epidermis) or, in the case of Cx43G138R mutants, also in adult mice. In both Cx43-mutated mouse lines, the adhesion of the digits was prominent and the separation was already initially decreased (for the forelimb on ED12.5, see Fig. 1M) staying diminished throughout the following days during which apoptosis took place (ED13.5 and ED14.5, see Fig. 1N and O). Hence, the restricted interdigital apoptosis in Cx43 point-mutated (n = 40) or deficient limbs (n = 36), i.e. limbs defective in Cx43 coupling, led to syndactylies in these two mouse models of ODDD (ED15.5, Fig. 1H and L). The syndactyly was inherited unilaterally and affected the second, third and fourth digits. A fusion of all three digits was mostly observed (80% of all syndactylies), whereas webbings of the second with the third and the third with the fourth digit were rare (10% of all cases, respectively). Regarding the appearance and frequency of syndactylies no difference was seen between the two genotypes.

Differential inactivation of Cx43 coupling in embryonic limb buds

The developing limb, also designated initially as limb bud, consists of two major compartments, the mesenchyme and the AER. Cx43 is expressed throughout the limb bud. However, it is more strongly expressed in cells of the AER than in those of the neighboring mesenchyme (Figs 2 and 3A–C. Cx43-positive staining was also detected in limb buds of Cx43G138R-mutated mice (Fig. 2D–F) without clear difference in location or amount to controls. In Cx43−/− mice, no Cx43-positive signals were found (Fig. 2G–I). Immunoblot analyses showed a slight reduction of Cx43 expression especially in the P2 phosphorylation band in the Cx43G138R mutant. Also here, Cx43−/− limbs did not show the specific staining (Fig. 2J), indicating a disruption of Cx43-mediated gap junctional coupling.

In order to determine whether Cx43-mediated coupling in AER or mesenchyme is necessary for proper interdigital apoptosis, Keratin5 (K5)-Cre (18) and Prx1-Cre (19) mice were used to express the dominant negative mutation Cx43G138R.
in each compartment. Cx43floxLacZ mice (17) were used to visualize expression of the particular Cre recombinase used (Fig. 3). Cx43floxLacZ female mice were mated to K5-Cre or Prx1-Cre males. The resulting Cx43\(^{+/floxLacZ}\):K5-Cre or Cx43\(^{+/floxLacZ}\):Prx1-Cre mouse embryos (ED11.5) (Fig. 3F–H, K–M) showed Cx43 promoter activity in compartments as determined by beta-galactosidase activity in whole mount (Fig. 3F and G, K and L) and cryo-sections (Fig. 3H and M). LacZ was expressed after K5-Cre-mediated recombination in the epidermis and the AER of the limb (Fig. 3G and H), whereas Prx1-Cre-mediated recombination occurred in the mesenchyme (Fig. 3L and M). In order to inhibit Cx43-mediated coupling in each compartment, the same K5-Cre or Prx1-Cre expressing male mice were used for matings with Cx43floxG138R females. Resulting mutants expressing Cx43G138R in the AER (Cx43floxG138R:K5-Cre) showed normal interdigital apoptosis (Fig. 3I), however, expression of the dominant mutation in the mesenchyme led to diminished cell death (Fig. 3N) and resulted in syndactylies in adult Cx43floxG138R:Prx1-Cre mice.

**Functional Cx43 coupling in the mesenchyme is crucial for interdigital apoptosis**

In order to examine the impact of the Cx43 mutations on gap junctional coupling, primary limb bud cultures were established and used for microinjection analyses (Fig. 4). Limb bud cultures were assessed and analyzed for their expression of Cx43, Shh and Bmp2. Furthermore, their molecular characteristics were ascertained using anti-vimentin and anti-Oct3/4 antibodies. A clear majority of cultured cells (up to 93 ± 5%) showed mesenchymal characteristics. The microinjection

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**Figure 1.** Decreased interdigital apoptosis in Connexin43-mutated mouse limbs results in soft-tissue syndactylies. (A–C) Normal apoptotic progression in control embryonic limbs (ED12.5–ED14.5) and disturbed apoptosis in point-mutated (E–G) and Cx43 deficient mice (I–K). Arrows point at the interdigital tissue which normally becomes apoptotic. In mutants, arrows point at regions showing apoptosis (E–G, I–K). (D, H and L) Developing limbs after apoptosis. (D) Control limbs show normal separation ofdigits, whereas webbing of digits occurs in Cx43 mutants (arrows in H + L). (M–O) Quantification of apoptotic spots at different embryonic stages reveals significantly decreased apoptosis in mutant mice already on ED12.5 (P < 0.01 on ED12.5 and ED14.5; P < 0.001 on ED13.5). (Scale bars: 500 μm.)
analyses revealed a significant decrease of gap junctional coupling in Cx43-mutated limb bud cultures to ~25% in comparison to controls. Cx43-deficient cells showed an even stronger reduction of coupling in comparison to Cx43G138R expressing cells (Fig. 4D). We speculate that the residual coupling seen in Cx43 mutants is mediated by Cx32, a second member of the connexin gene family described to be expressed in limb bud (20). Nevertheless, reduction of gap junctional intercellular communication to 25% in Cx43 mutants disturbs interdigital apoptosis in developing limb.

Expression of Shh and Bmp2 is reduced in Cx43-mutated mice

Expression of Shh and the downstream protein Bmp2, which regulates osteoblast differentiation and induces interdigital
apoptosis (21), was strongly diminished in both Cx43 mutants in comparison to controls (Fig. 5). The diffuse localization of the processed N-terminal part of Shh in the ZPA of limb buds expanded more in controls (Fig. 5B and C) as compared to the ODDD-mutated or Cx43-deficient animals (Fig. 5G and H and L and M). To distinguish whether an excessive blockade of apoptosis between the digits led to syndactylies in mutants, we analyzed histochemically the expression level of Noggin, an antagonist of apoptosis preventing the tissues from death. However, with this approach no difference in the expression of this protein could be detected between the genotypes (data not shown), indicating that a partial blockade of cell death between the digits is probably not responsible for the development of syndactylies in ODDD-mutated mice. In fact, reduction of Shh expression may lead to decreased Bmp2 levels in mutated embryos resulting in insufficient induction of apoptosis, and finally to interdigital webbing. These reductions of Shh and Bmp2 were also seen in immunoblot analyses further supporting this assumption (Fig. 5P–R).

Fibroblast growth factors 4 and 8 are up-regulated in Cx43 coupling deficient mice

Recently, Bmp2 signaling was described to control interdigital apoptosis by regulating Fgf expression. Bmp2 inhibits Fgfs’ expression by preventing survival signals targeted to the interdigital mesenchyme (7). Hence, it was important to investigate whether Fgf4 and Fgf8 expression is altered in Cx43-mutant mice. Actually, from ED11.5 on, an up-regulation of both Fgfs could be detected in these mice. Immunohistochemical analyses of Fgf8 showed more intensive staining in the AER extending towards the mesenchyme in mutants (Fig. 6B and E and C and F). The Fgf4-specific antibody staining appeared more distinct at the AER (Fig. 6G–I) and was missing at the interdigital mesenchyme in controls (arrows in Fig. 6G), but extended over this tissue in both Cx43 mutants at ED13.0 (arrows in Fig. 6H and I). The increased levels of both Fgfs could also be confirmed by immunoblot analyses of limb bud lysates (ED11.5, Fig. 6J–L). These data support the importance of inhibition of Fgf signaling during normal interdigital apoptosis in developing limb. Furthermore, it underlines that not only the down-regulation but also the resulting re-localization of Fgfs outside the interdigital mesenchyme is important in this process.

Reduction of Shh expression in Cx43 mutants is initiated around ED10.5

As already shown in the immunohistochemical analyses, the expression of the morphogen Shh is reduced in Cx43-mutant...
Disturbed interdigital apoptosis in ODDD models investigated here leads to the observed syndactilies and is most likely due to decreased levels of Shh, subsequently leading to reduced levels of Bmp2. Bmps play an important role in a variety of developmental processes, e.g. in early limb patterning as well as in skeletogenesis. They are secreted signaling molecules which belong to the transforming growth factor β (TGFβ) superfamily (22–24). Bmp2, Bmp4 and Bmp7 are expressed in the early limb bud mesenchyme (25,26), whereas only Bmp2 and Bmp7 are active downstream of Shh signaling (27). Bmp2 and Bmp7 expression are diminished in the absence of Shh activity (28). All three Bmps induce apoptosis of the interdigital spaces (21,29,30). The involvement of Bmps in fundamental developmental processes is underlined by the early lethality of Bmp1 and Bmp4-deficient mice (31,32). Conditional deletion of Bmp2 in the limb bud mesenchyme using the Prx1-Cre mice (19) developed striking syndactilies with strongly reduced apoptosis between the digits, whereas the digits themselves developed normally (27). Application of Bmp antagonists leads to a block of apoptosis (21,30), suggesting that a threshold level of the Bmp signaling is necessary to efficiently induce programmed interdigital death. In the Cx43 mutants studied an excessive inhibition of apoptosis could be excluded because the expression of Noggin, a known Bmp2 antagonist, remained unaltered in histochemical analyses. However, the induction of interdigital apoptosis by Bmp2 was found to be disturbed in both ODDD models. The decreased level of Bmp2 in mice disturbed in Cx43 coupling was documented by whole-mount immunohistochemical (Fig. 5) and real-time PCR analyses (Fig. 8).

Recently, interplay of Fgfs (Fgf4 and Fgf8) and Bmp2 has been described in interdigital apoptosis (7). This report shows that Bmp2 inhibits Fgfs by delivering survival signals to the mesenchyme from the AER. Disturbance of Bmp2 signaling resulted in up-regulation of Fgfs leading to soft-tissue syndactilies. In our studies, Cx43 mutants show a clear up-regulation of both Fgf proteins starting on ED11.5 (Fig. 6). Notably, Fgf4 protein expression extended over the mesenchyme in Cx43 mutants, whereas it was localized only at the finger tips in controls (Fig. 6G–I). Similarly, increased Fgfs expression and signaling led to abnormal survival of mesenchymal cells causing syndactilies. Thus, in mice with disturbed Cx43-mediated coupling the decrease in Shh and Bmp2 expressions results in subsequent up-regulation of Fgf4, Fgf8 and interdigital webbing (Fig. 9A).

Cx43-mediated coupling modulates morphogen expression in developing limb buds

The immunohistochemical analyses of whole-mount embryos and in situ hybridization analyses as well as limb bud cultures revealed an alteration of major morphogens in the mutant mice, i.e. proteins with an ability to induce proliferation and regulate tissue patterning. These morphogens were Shh, Bmp2 and Fgf4 and -8, whereas other tested proteins (Bmp4 or Noggin) showed no changes between the genotypes. Furthermore, the combination of transgenic mice used here (Cx43-deficient and Cx43G138R point-mutated mice) allows to conclude that the alteration of morphogen expression must be due to a disruption of Cx43-mediated gap junctional coupling. The occurrence of syndactilies or craniofacial alterations in patients with GJA1 gene mutations, not altering
the activity of ATP releasing channels (33), exclude the action of connexin hemichannels as the reason for these symptoms. This assumption is supported by the fact that Cx43-deficient mice lack Cx43 hemichannel expression, but show the same morphogen alterations like the ODDD point-mutated mice. Furthermore, we found a clear decrease in gap junctional coupling in both Cx43 mutants investigated in cultured limb bud cells (Fig. 4).

Interestingly, the reduction of Shh expression starts during development. There is no difference in the amounts of Shh mRNA between mutated and wild-type mice when the expression of Shh is initiated, as shown on ED9.5 in Figures 7 and 8. The post-initial adjustment of Shh decrease indicates that not the initiation of expression but its maintenance is dependent on Cx43-mediated gap junctional coupling. We have described that Shh expression is maintained by Fgf signaling by the positive Fgf-Shh-feedback loop (3–5) which we conclude to be disturbed in Cx43 mutants (Fig. 9B). Probably, Cx43-mediated coupling transfers Fgf signals from the AER to Shh expressing cells in the mesenchyme. In fact, recently gap junctions have been described to relay Fgf8 signals to repress Nodal during the development of left-right asymmetry in rabbit (34). However, the precise molecular mechanism how Cx43 influences the expression of these morphogens is not known. It has been described that Cx43 containing GJCs are able to transfer Ca\(^{2+}\) or inositol-1,4,5-trisphosphate (IP\(_3\)) (35). Thus, it is possible that after binding of Fgfs to their receptor tyrosine kinases (FgfRs), increased Ca\(^{2+}\) levels are relayed through GJCs in the mesenchyme. These survival signals may lead to proliferation of Shh expressing cells sustaining proper Shh signaling. Decreased gap junctional coupling may not be sufficient to propagate the signals of these receptor tyrosine kinases to neighboring cells, thereby leading to the observed reduction of Shh amounts and alterations of downstream signaling cascades. Thus, gap junctional coupling of mesenchymal cells is likely to establish a long-range diffusion gradient of second messengers like Ca\(^{2+}\), starting with cells receiving

**Figure 5.** Reduction of sonic hedgehog (Shh) and bone morphogenic protein 2 (Bmp2) expression in Cx43 mutants. Shh expression is decreased in the zone of polarizing activity (ZPA) in mutated limb buds (G, H, L and M) compared to controls (B and C) (after ED10.5 on). No difference was seen between both Cx43 mutants. Micrographs in (A, F and K) show the overview (Shh expression pointed out by arrows), those in (B, G and L) the on-view, whereas (C, H and M) are the side-views of the limb buds. (Right column) Expression of Bmp2 proteins is reduced in Cx43 mutant embryos (I, J, N and O) in comparison to control mice (D and E) (ED11.5). Images in (E, J and O) are the enlargements of the overview pictures shown in (D, I and N). (P–R) Immunoblot analyses confirm the decrease of Shh (P) and Bmp2 (Q) in Cx43 mutants. Gel loading was tested by tubulin immunostaining (R). (Scale bars in A, F and K and D, I and N: 1 mm, in all other cases: 500 µm.)
paracrine signals like those of Fgfs. However, it cannot be excluded that other small messenger molecules are involved in this process.

**Reduction of Bmp2 levels in Cx43 mutants may explain the morphological phenotypes of ODDD**

The reduction of Bmp2 expression as observed in Cx43 mutants can explain other ODDD characteristic malformations besides syndactylies. For instance, the impact of Bmp expression on skeletogenesis is well known. The bone abnormalities observed in Cx43G138R-mutated mice could be explained by the reduced number of osteoblasts (9). Since Bmp2 is described to be necessary for the differentiation of mesenchymal precursor cells to osteoblasts (36), its decreased expression can lead to the reduced number of osteoblasts in Cx43-mutated bone.

The craniofacial malformations seen in ODDD mutants are reminiscent to those described for mouse embryos with reduced Shh expression, representing an animal model for holoprosencephaly (HPE) (37). This cephalic disorder is characterized by impaired development of the embryonic

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**Figure 6.** Expressions of Fgf4 and Fgf8 are up-regulated in mice deficient in Cx43 coupling. Starting on ED11.5 a clear up-regulation of Fgf8 could be detected in Cx43G138R expressing (B) or Cx43 deficient mice (C) in comparison to the controls (A). This increase of Fgf8 was also detected on ED13.0 in the mutants (arrows in E and F in comparison to D). (G–I) Fgf4 expression is extended over the interdigital mesenchyme in Cx43 mutants (H and I), whereas Fgf4 is missing between the digits in controls (G) (see arrows) (ED13.5). Micrographs (A’–I’) show high power magnifications of the certain region of interest. (J–L) Immunoblot analyses confirming the up-regulation of Fgfs: Fgf8 in (J), Fgf4 in (K); Gel loading was checked using anti-tubulin antibodies shown in (L). (Scale bars in A–F: 1 mm; in G–I: 500 μm.)
forebrain, prosencephalon, causing facial and sometimes brain defects. HPE is known to be mainly caused by Shh mutations or to have environmental causes with humans (38). A high intra- and interfamilial variability is reported in these patients ranging from cyclopia and perinatal lethality to hypotelorism (abnormal closeness of the eyes), microcephaly and dental malformations (39,40). Mild forms of HPE are caused by a reduction of the Shh-induced signaling and this could influence downstream pathways like that of Bmp2. Recently, it was described that mutations in the Bmp receptor genes in addition to mutant Shh can lead to HPE (41).

Furthermore, the observed enamel hypoplasia in Cx43G138R mice may also be explained by the reduction of Bmp2 expression in mutants. Bmp2, besides other members of this gene family, was found in the enamel knot, the putative signaling center regulating tooth shape, and is expressed there by dentin-forming odontoblasts and enamel-forming ameloblasts (42). However, the decrease of Bmp2 expression does not result in a dental patterning defect but leads to a reduction of the enamel thickness (43).

In conclusion, our data suggest the occurrence of a long-range diffusion gradient through Cx43 containing GJCs in the mesenchyme of the mouse limb bud. Decreased or absent gap junctional coupling in Cx43 mouse mutants leads to altered expression of the morphogens Shh, Bmp2 as well as Fgf4 and -8. These changes in the expression of morphogens result in syndactylies and most probably in other phenotypic abnormalities characteristic for ODDD.
Materials and Methods

All mice used in this study were kept under standard housing conditions with a 12/12 h dark–light cycle and with food and water ad libitum. All experiments were carried out in accordance with local and state regulations for research with animals.

Analyses of apoptosis in interdigital spaces

LysoTracker staining were performed as previously described by Grieshammer et al. (44). For these analyses developing limbs from ED10.5 to ED14.5-old embryos of all genotypes were prepared, incubated in a LysoTracker solution (5 µl/1 ml HBSS) (Sigma Aldrich, Steinheim, Germany) at 37°C for 40 min, washed twice in HBSS, twice in PBS and fixed with 4% PFA in PBS over-night at 4°C. Whole-mount specimen was collected in 100% methanol at −20°C. The staining was analyzed using a Leica MS5 stereomicroscope with fluorescence module and appropriate filter set. TUNEL assays (Roche, Mannheim, Germany) were performed on 12 µm thick cryo-slices for detection of apoptotic cells between the developing digits in paws from ED12.5 and 13.5-old limbs from at least four embryos at different stages and with DAB/NiCl2 or Histogreen reagent (Linaris, Wertheim-Bettingen, Germany) and H2O2 to a 0.03% final concentration for 2–10 min. The supplements were photographed directly after staining.

The following primary antibodies were used for immunohistochemistry studies: anti-Cx43 (1:500) (47), anti-processed N-terminal end of Shh (RnD Systems, Minneapolis, MN), goat/AF464, 1:500), anti-Bmp2 (Abcam, Cambridge, MA, rabbit/Ab14933, 1:300), anti-carboxyterminal end of Fgf4 (Abcam, ab65974, 1:500), anti-Fgf8 (RnD Systems, mouse/ MAB323, 1:500), anti-Noggin (RnD Systems, goat/AF719, 1:500). The following secondary antibodies were used for these studies: Biotin-goat anti-rabbit (Zymed, Invitrogen, Carlsbad, CA, 81-6140, 1:5000), Biotin-donkey anti-mouse (Dianova, Hamburg, Germany, 1:5000), Biotin-donkey anti-goat (Dianova 1:5000).

Transcription analyses (in situ and real-time PCR analyses)

In situ hybridization analyses using probes targeted for Shh mRNA were performed as described in detail (48). The probes were prepared from a 900 bp fragment of a mouse Bsp2 cDNA inserted into PCRII. Sense and antisense digoxigenin-UTP-labeled riboprobes were generated by standard T7 and Sp6 polymerase reactions. For real-time PCR analyses, limbs buds from at least four embryos at different stages (ED 9.5 and ED 10.5) were transferred to lysis buffer (supplied with RNA isolation kit) and homogenized (Ultra-Turrax T25basic) at full speed for 1 min. Total RNA was isolated by using NucleoSpin RNA II kit (Macherey & Nagel, includes on-column DNasel treatment). First-strand cDNA reaction was carried out with 250 or 500 ng total RNA (depending on the RNA concentration) using QuantiTect Reverse Transcription Kit (Qiagen) including DNasel treatment following the supplier’s protocol. In order to quantify the expression pattern of Shh, the positively stained areas were outlined in pseudo-color. The number of pixels in this color were prepared methanol/DMSO (4:1) at 4°C overnight. For blocking endogenous peroxidase activities, the embryos were transferred into methanol/DMSO/H2O2 (4:1:1) solution for 5–10 h at room temperature. At this stage, the supplements could be stored in 100% methanol at −20°C for several months. All following steps were carried out in siliconized microtubes. The preparations were rehydrated in 50% methanol, PBS− and twice PBS− with 2% nonfat instant milk and 0.5% Triton X-100 (PBSMT) for 30 min with rocking, respectively. The primary and secondary antibodies were applied in PBSMT at 4°C overnight consecutively and washed five times with PBSMT after their incubation as follows: once with 1 ml for 1 h at 4°C, once with 10 ml for 1 h at 4°C, three times in 10 ml for 1 h at room temperature. The supplements were rinsed in PBS− with 0.2% BSA and 0.5% Triton X-100 (PBT) twice (once with 5 ml and once with 1 ml) for 20 min, respectively. For enhancement of antibody signals specimens were incubated with the ABC-complex (included in the M.O.M. Immunodetection kit, Vector laboratories, CA). All following steps were performed in salt-free Tris–HCl buffer (0.05 M, pH 7.4). The staining is achieved with DAB/NiCl2 or Histogreen reagent (Linaris, Wertheim-Bettingen, Germany) and H2O2 to a 0.03% final concentration for 2–10 min. The supplements were photographed directly after staining.

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Immunohistochemistry of morphogens during mouse development

Morphogens were detected in situ by immunohistochemistry staining of whole-mount embryos following adapted protocols (45,46). Cx43+/−/PGK-Cre female mice were mated with Cx43 del/floxG138R males. Embryos were collected from pregnant females in PBS− and yolk sacs were used for DNA isolation and genotyping. Embryos were fixed in freshly prepared methanol/DMSO (4:1) at 4°C overnight. For blocking endogenous peroxidase activities, the embryos were transferred into methanol/DMSO/H2O2 (4:1:1) solution for 5–10 h at room temperature. At this stage, the supplements could be stored in 100% methanol at −20°C for several months. All following steps were carried out in siliconized microtubes. The preparations were rehydrated in 50% methanol, PBS− and twice PBS− with 2% nonfat instant milk and 0.5% Triton X-100 (PBSMT) for 30 min with rocking, respectively. The primary and secondary antibodies were applied in PBSMT at 4°C overnight consecutively and washed five times with PBSMT after their incubation as follows: once with 1 ml for 1 h at 4°C, once with 10 ml for 1 h at 4°C, three times in 10 ml for 1 h at room temperature. The supplements were rinsed in PBS− with 0.2% BSA and 0.5% Triton X-100 (PBT) twice (once with 5 ml and once with 1 ml) for 20 min, respectively. For enhancement of antibody signals specimens were incubated with the ABC-complex (included in the M.O.M. Immunodetection kit, Vector laboratories, CA). All following steps were performed in salt-free Tris–HCl buffer (0.05 M, pH 7.4). The staining is achieved with DAB/NiCl2 or Histogreen reagent (Linaris, Wertheim-Bettingen, Germany) and H2O2 to a 0.03% final concentration for 2–10 min. The supplements were photographed directly after staining.

The following primary antibodies were used for immunohistochemistry studies: anti-Cx43 (1:500) (47), anti-processed N-terminal end of Shh (RnD Systems, Minneapolis, MN), goat/AF464, 1:500), anti-Bmp2 (Abcam, Cambridge, MA, rabbit/Ab14933, 1:300), anti-carboxyterminal end of Fgf4 (Abcam, ab65974, 1:500), anti-Fgf8 (RnD Systems, mouse/ MAB323, 1:500), anti-Noggin (RnD Systems, goat/AF719, 1:500). The following secondary antibodies were used for these studies: Biotin-goat anti-rabbit (Zymed, Invitrogen, Carlsbad, CA, 81-6140, 1:5000), Biotin-donkey anti-mouse (Dianova, Hamburg, Germany, 1:5000), Biotin-donkey anti-goat (Dianova 1:5000).

Transcription analyses (in situ and real-time PCR analyses)

In situ hybridization analyses using probes targeted for Shh mRNA were performed as described in detail (48). The probes were prepared from a 900 bp fragment of a mouse Bsp2 cDNA inserted into PCRII. Sense and antisense digoxigenin-UTP-labeled riboprobes were generated by standard T7 and Sp6 polymerase reactions. For real-time PCR analyses, limbs buds from at least four embryos at different stages (ED 9.5 and ED 10.5) were transferred to lysis buffer (supplied with RNA isolation kit) and homogenized (Ultra-Turrax T25basic) at full speed for 1 min. Total RNA was isolated by using NucleoSpin RNA II kit (Macherey & Nagel, includes on-column DNasel treatment). First-strand cDNA reaction was carried out with 250 or 500 ng total RNA (depending on the RNA concentration) using QuantiTect Reverse Transcription Kit (Qiagen) including DNasel treatment following the supplier’s protocol. In order to quantify the expression pattern of Shh, the positively stained areas were outlined in pseudo-color. The number of pixels in this color were
determined using an image processing software as previously described (9).

For real-time PCR, the reaction consisted of cDNA template (aliquot of cDNA first strand reaction), forward and reverse primers (200 nM final concentrations, primer sequences see below) and iQ SYBR Green Supermix (Bio-Rad, Munich, Germany) in a total volume of 25 µl. Three reactions per template were carried out in parallel. All experiments were repeated with independently isolated RNA samples. Each bar and error bar represents mean and SEM of independent experiments. The experiments were performed with iQ5 Real-Time PCR Detection System from Bio-Rad. GAPDH and β-actin were used as controls, standard control PCR reactions were carried out to test for contaminations. Real-time PCR was analyzed using Bio-Rad iQ5 Optical System software (version 1.1.1442.OCR), following the instruction provided by the supplier, and Microsoft Excel. The efficiency of the different primers used was determined in advance and factored into the calculations. The following oligonucleotides were used for real-time PCR analysis:

- β-actin_for: ACGGC CAGTCATCACTATTTG (81.4% efficiency); β-actin_rev: ATGGATGCCACAGGATTCCAT (49)
- GAPDH_for: TCA ACGACC-CCTTCATTGAC (80.9% efficiency); GAPDH_rev: ATGCAGGGATGATGTTCTGG (49)
- MmBmp2_for: TGCGCAGCTTCCATCACGAA (94.5% efficiency); MmBmp2_rev: GTGAGAAAC-TCGTCACTGGG (49)
- Shh_for: AATGCCTTGGCCATCTCTGT (87.5% efficiency); Shh_rev: ATGCAGGGATGATGTTCTGG (49)

Limb bud cell cultures

For assessment of limb bud cultures ED11.5 and ED14.5 embryos were dissected from pregnant female mice, the limb buds were removed, fragmented and digested with a collagenase/trypsin (3:1) solution for 20 min at 37°C. The enzyme solution was discarded and tissue fragments collected at the bottom of the tube. The primary cells were separated by pipetting of 300 µl primary cell culture medium as described for cardiomyocyte cultures (50). After incubation for 30 min at room temperature with rocking, the cells were seeded onto cover slips for immunofluorescence analyses or standard cell culture dishes for western blot lysates.

Immunofluorescence analyses of limb bud cultures

Limb bud cells from wild-type, Cx43 β/G138R and Cx43 /β embryos grown on glass cover slips to a confluence of 70–100% were fixed in 4% PFA, washed in PBS and preincubated for 1 h in blocking reagent (PBS containing 5% NGS). All slides were incubated overnight at 4°C with polyclonal anti-Cx43 (1:500) (47), anti-Oct3/4 (Santa Cruz, mouse/sc-5279, 1:1000), Vimentin (Progen Biotechnik, guinea pig/GP53, 1:100), Shh (RnD Systems, goat/AF464, 1:500) or Bmp2 (Abcam, rabbit/Ab14933, 1:300) antibodies. The localization of the proteins was visualized using the following secondary antibodies: Alexa(488)-goat anti-rabbit (Molecular Probes, A-11029, 1:1000), Cy5-donkey anti-mouse (Dianova, 115-175-166, 1:1000) or Cy3-goat anti-guinea pig (Jackson ImmunoResearch, 106-165-003, 1:1000). Cover slips were washed in PBS– and mounted with the aqueous mounting medium Permaflour (Immunotech, Marseille, France). Fluorescent signals were recorded using a Zeiss LSM510 confocal microscope. The number of mesenchymal cells (positive for vimentin and Bmp2) was estimated by counting.

Immunoblot analysis

Limb buds or cultured limb bud cells were solubilized in l × Complete (Roche) and sonicated in an ice cold bath for 30 s.
The total protein was separated in SDS–PAGE and blotted onto Hybond ECL membrane (Amersham Bioscience, Piscataway, NJ). After blocking with 5% milk powder in washing buffer (8.5 mM Tris–HCl, 1.7 mM Tris–base, 50 mM NaCl, 0.1% Tween-20) for 1 h at room temperature, a 1 h incubation was performed with Cx43 antibodies (1:1000), Shh (1:250), Bmp2 (1:250), Fgf4 (1:75) or Fgf8 (1:100). All antibodies were diluted in blocking solution, a detailed description of the antibodies is included in the immunohistochemistry section above. After three wash steps and a 45 min incubation with horseradish peroxidase-conjugated antibodies (1:25000 Dianova), with three wash steps in-between. Afterwards, the membranes were incubated with enhanced chemiluminescence reagents (Amersham Bioscience) and developed on X-ray films.

Statistical analysis

All statistic evaluations were carried out using Microsoft (Redmond, WA) Excel. Statistical significance of differences between groups was determined using the paired Student’s t-test for transfer of microinjected neurobiotin, morphogen expression in whole-mount specimen or western blot analyses, in situ hybridizations and real-time PCR analyses. A P-value <0.05 was considered statistically significant.

ACKNOWLEDGEMENTS

We thank Dr Christine Hartmann (Vienna, Austria) and Dr Malcolm Logan (London, UK) for providing Prxl-Cre transgenic mice. Furthermore, we like to thank Gaby Beine for her excellent technical assistance.

Conflict of Interest statement. None declared.

FUNDING

This work was supported by grants of the German Research Association (SFB 645, project B2 to K.W.); and Bonn Forum Biomedicine to K.W. and H.S.

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