Dlx5-augmentation in neural crest cells reveals early development and differentiation potential of mouse apical head mesenchyme

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Neural crest cells (NCCs) give rise to various tissues including neurons, pigment cells, bone and cartilage in the head. Distal-less homeobox 5 (Dlx5) is involved in both jaw patterning and differentiation of NCC-derivatives. In this study, we investigated the differentiation potential of head mesenchyme by forcing Dlx5 to be expressed in mouse NCC (NCCDlx5). In NCCDlx5 mice, differentiation of dermis and pigment cells were enhanced with ectopic cartilage (ec) and heterotopic bone (hb) in different layers at the cranial vertex. The ec and hb were derived from the early migrating mesenchyme (EMM), the non-skeletogenic cell population located above skeletogenic supraorbital mesenchyme (SOM). The ec developed within Foxc1+ dura mater with increased PDGFRα signalling, and the hb formed with upregulation of BMP and WNT/β-catenin signalling in Dermo1+ dermal layer from E11.5. Since dermal cells express Runx2 and Msx2 in the control, osteogenic potential in dermal cells seemed to be inhibited by an anti-osteogenic function of Msx2 in normal context. We propose that, after the non-skeletogenic commitment, the EMM is divided into dermis and meninges by E11.5 in normal development. Two distinct responses of the EMM, chondrogenesis and osteogenesis, to Dlx5-augmentation in the NCCDlx5 strongly support this idea.

Neural crest cells (NCCs) are mesenchymal cells that originate from the dorsal part of neural tube by epithelial-to-mesenchymal transition. NCCs then migrate to different regions of the embryo, where they give rise to various cell types such as bone and cartilage of the skull, sensory neurons, pericytes, melanocytes and smooth muscles1. NCCs and paraxial mesodermal cells (MES) cooperatively form the craniofacial structure. NCCs contribute to the rostral craniofacial skeleton including the pharyngeal skeleton while MES give rise to caudal cranium2–4. The boundary between NCC and MES in the calvarium corresponds to the coronal suture between the frontal and parietal bones3–5.

The NCC is accurately regulated by a complex gene network from the appearance to migration and differentiation1. Distal-less homeobox 5 (Dlx5) is expressed early at the neural plate border for establishing the area of NCC delamination, but it does not control NCC specification or migration1,6. Dlx5 is required for patterning and differentiation of the NCC7. Expression of Dlx5 and its co-functional member of the Dlx gene family, Dlx6, are involved in jaw patterning8. In jaw development, Dlx5/6 works downstream of Endothelin1 (Edn1), localized in the mandibular process while Dlx5/6 are absent in the maxillary process9. Double knock-out of Dlx5/6 in mice causes mandible transformation into maxilla-like structure10. Reversely, forced Dlx5 expression in NCCs in mice (NCCDlx5) induces ectopic Dlx5 expression in the maxillary process leading to upregulation of mandibular-specific genes and appearance of several phenotypic hallmarks of the mandible in the maxilla region11.

Dlx5 is also expressed in differentiation stages of NCC-derivatives: ganglion of cranial nerves, cartilage and bone13,14. In osteoblast differentiation, Dlx5 is induced by BMP signalling, then Dlx5 enhances Runx-related transcription factor 2 (Runx2), a master transcriptional regulator for osteogenesis15–17. DLX5 directly binds to SP7, a downstream of Runx2, to promote osteoblast differentiation18. Calvarial osteoblasts isolated from Dlx5 deleted mice show reduced proliferation and differentiation19. Dlx5 is also expected to induce recruitment of fibroblasts to

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chondrogenic lineage and chondrocyte maturation in the chick.\(^{20,21}\) In mice, the calvarium and chondrocranium malformations have been shown to associate with Dlx5-downregulation.\(^{22,23}\) Meanwhile, cranial base cartilages derived from NCC are enlarged by Dlx5-overexpression, but calvarial bones have not been examined.\(^{24}\)

In calvarial development, formation of the frontal and parietal bones start with the aggregation of mesenchymal cells in the area of the supraorbital ridge at embryonic day (E) 10.5,\(^{4}\) referred to as the supraorbital ridge mesenchyme.\(^{24–26}\) or supraorbital mesenchyme (SOM).\(^{37}\) The SOM proliferates and differentiates into osteoblasts from E11.5, then intrinsically expands to the apex of the head to form the bone from E13.5.\(^{4,5}\) Importantly, due to the intimate association and mutual support of cranial bones and the dura mater, the defects in the dura mater affect calvarial bone formation and maintenance.\(^{28–31}\) Before the SOM begins apical growth, a population of head mesenchyme, termed as early migrating NCC\(^{32}\) or early migrating mesenchyme (EMM)\(^{37}\), is established above the SOM to contribute to the sutures or soft tissue layers such as the dermis and the meninges.\(^{4,25,32}\) Transcrip-tome analysis revealed that the SOM and the EMM exhibit different gene expression profiles by E12.5,\(^{13}\) and the development of the skull vault is achieved by interactions between the apical (EMM) and basal (SOM) cell populations.\(^{38}\) Although the EMM is normally non-osteogenic, previous reports demonstrated that the EMM can generate bone in genetic disorders.\(^{39,40}\)

NCC-specific Dlx5-augmentation results in a switch of the jaw identity,\(^{3,4}\) but the effect on NCC differentiation potential has not been examined. In this study, we further investigated the effect of Dlx5-overexpression in NCCs with special reference to early development and differentiation potential of the EMM.

**Results**

**Dlx5 expression and NCC distribution in the NCC\(^{Dlx5}\) mouse.** NCC-specific forced expression of Dlx5 was confirmed in Wnt1-Cre;R26\(^{R26\text{-Dlo5\_floox}\text{-Dlx5\_lox}\text{-Dlx5\_lox}}\) (hereafter NCC\(^{Dlx5}\)) mice at E9.5 with ectopic Dlx5 expression in the pharyngeal arch.\(^{11}\) We further examined the expression of Dlx5 in later stages by comparison with X-gal staining of Wnt1-Cre;R26\(^{R26\text{-lox}\text{-Dlx5\_lox}}\) (NCC\(^{lox}\)) (n = 3) to demonstrate the NCC distribution. NCCs of wild-type located at the maxillary process and supraorbital ridge at E10.5, but hardly detected in the surrounding of the brain at the vertex (Fig. 1a). At E11.5, NCCs made up the mandibular and maxillary processes, also the head mesenchyme surrounding the brain (Fig. 1b). Endogenous Dlx5 expression in head mesenchyme at E10.5 was found only in the mandibular process, whilst in the NCC\(^{Dlx5}\), Dlx5 was additionally expressed in the maxillary process and the SOM (n = 3) (Fig. 1c,d). At E11.5, endogenous Dlx5 expression was seen in the frontal bone primordium of the SOM (Fig. 1e). In the NCC\(^{Dlx5}\), Dlx5 was expressed in the EMM besides the SOM (n = 3) at E11.5 (Fig. 1f, arrowhead). Therefore, Dlx5 expression was successfully induced in NCCs, including EMM, in the NCC\(^{Dlx5}\).

We examined the NCC distribution in the NCC\(^{Dlx5}\) by whole-mount in situ hybridization (WISH) for Snail family transcriptional repressor 1 (Snail1), a NCC specifier,\(^{1}\) at E9.0 (n = 4). Snail1 expression was shown in a comparable pattern between the control and the NCC\(^{Dlx5}\) (Fig. 1g,h). We next examined the Snail1 expression by section ISH (n = 3). We confirmed that post-migratory NCC-derived mesenchyme at the surrounding of the forebrain and the mandibular process similarly expressed Snail1 in both of the control and the NCC\(^{Dlx5}\) (Fig. 1i,j).

In the dorsal region of the rhombencephalon, Snail1 expression was detected in migrating NCCs in the control and the NCC\(^{Dlx5}\) (Fig. 1k,l), the data revealed no difference between the two in this area. These results indicated that Dlx5-overexpression does not affect the migration and distribution of cranial NCCs.

**Predisposition of NCC differentiation in the NCC\(^{Dlx5}\) mouse.** Dlx5 is normally expressed in the trigeminal ganglion,\(^{14}\) and the size is reduced in Dlx5 knockout mice.\(^{7}\) Acetylated tubulin staining at E11.5 demonstrated that neuron localization did not show obvious difference between the control and the NCC\(^{Dlx5}\) (n = 3) (Fig. 2a–d). Reconstructed trigeminal ganglion from the serial histological sections at E17.5 illustrated the similar shape and size of the control and the NCC\(^{Dlx5}\) (n = 3) (Fig. 2g). We also examined the pigment cell, another NCC-derivative,\(^{1}\) by expression of dopachrome tautomerase (Dct)\(^{34}\) on frontal sections at E15.5 (n = 3) (Fig. 2h,i). The number of Dct-positive cells in the head dermis was significantly higher in the NCC\(^{Dlx5}\) than that of the control (p < 0.05) (Fig. 2j), suggesting that the NCC potential for pigment cell differentiation was enhanced by Dlx5-augmentation.

We next examined bone and cartilage formation in the calvarium. In the control, chondrocranial cartilages were observed at the skull base and lateral walls, while no cartilage and bone was observed at the apical part of the head at E13.5 and E14.5 (Fig. 2k,m). Interestingly, cartilage was formed at the vertex of the NCC\(^{Dlx5}\) (Fig. 2l,n). This calvarial cartilage was newly introduced to the region that usually has no cartilage, hence it is an ectopic cartilage.\(^{35}\) islands were also found in the interfrontal suture (Fig. 2p, asterisk) and the posterior part of frontal bone formation (Fig. 2p, double asterisk). In some NCC\(^{Dlx5}\) mice, “patchy” bones with holes (Supplementary Fig. S1a,b) were formed randomly in the frontal bone and interfrontal area. These irregular bones seemed to fuse to the frontal bone, therefore we called them heterotopic bones\(^{35}\) (hereafter hb). In summary, Dlx5 ectopic expression in head mesenchyme induced ec and hb formations. The development of the endogenous frontal bone was comparable between the control and the NCC\(^{Dlx5}\) at P0 (Fig. 2o,p). In the NCC\(^{Dlx5}\), Dlx5 was not augmented in MES.
but the relative position of bilateral parietal bones in the cranium was abnormal at P0, and the anterior edge of parietal bone, which comprises the coronal suture was more inclined to the posterior (Fig. 2p). We reason that the inclination was caused by the appearance of ec and hb that interfered the normal parietal bone development. Given the significant effects of Dlx5 on NCC, we attempted to analyze MES-specific Dlx5-augmented mice by crossing Mesp1-Cre mice36 and R26RCAG-flox-Dlx5/+. Four litters at E11.5–13.5 were examined, but all Mesp1-Cre;R26RCAG-flox-Dlx5/+ fetuses were lethal, making following analyses impossible.

Skeletal staining analysis of the NCCDlx5 revealed that both chondrogenesis and osteogenesis were promoted simultaneously at the same region of the calvarium, which has not been reported in any other calvarial bone mutants. We thus further analysed the phenotype. Because the ec and hb were present at the NCC-MES junction, we first confirmed the cell origin of the misregulated structures. The NCC domain was visualized by enhanced yellow fluorescent protein (EYFP) in the Wnt1-Cre;R26RCAG-flox-Dlx5/EYFP (NCCDlx5/EYFP) and in the littermate control Wnt1-Cre;R26REYFP/+ (NCCEYFP) at E17.5.

In bright-field images, the baso-lateral part of the coronal suture was comparable between the NCCEYFP and the NCCDlx5/EYFP (n = 5) (Fig. 3a,b, brackets). However, the coronal suture at the vertex seemed to be shifted more posterior in the NCCDlx5/EYFP compared to the NCCEYFP (Fig. 3a,b, dashed line). Under the fluorescent microscope, we found that the frontal bone of the NCCDlx5/EYFP was highlighted while the parietal bone was not, and the “NCC tongue” protruded to the sagittal suture (n = 5) (Fig. 3a,b, arrowheads). The ec and hb were formed within the fluorescent NCC-derived domain in the NCCDlx5/EYFP (n = 5) (Fig. 3d). In sagittal sections, the ec and hb were clearly detectable by fluorescence (n = 3) (Fig. 3f). The hb was formed in line with the frontal bone and parietal bone, and the ec was always seen underneath the bone-forming layer (Fig. 3f). In the NCCDlx5/EYFP, osteogenic fronts

Figure 1. Ubiquitous Dlx5 expression in NCCs and the effect on the distribution of NCCs in the NCCDlx5. (a, b) X-gal staining of NCCLacZ counterstained by nuclear fast red to visualize NCCs at E10.5 and E11.5. (c–f) Dlx5 expression on the frontal section of the control and the NCCDlx5 at E10.5 (c, d) and E11.5 (e, f). In the NCCDlx5, Dlx5 is ectopically expressed in maxilla and supraorbital mesenchyme at E10.5 (d), and in apical head mesenchyme at E11.5 (f, arrowhead). (g, h) Snai1 expression, a marker for NCC, by WISH of the control and the NCCDlx5 at E9.0. (i–l) Snai1 expression by ISH on frontal sections of the control and the NCCDlx5 at E9.0, at planes corresponding to hatched lines in (g, b), showing the forebrain, the mandibular process (i, j), and the dorsal area (k, l). fr, frontal bone; md, mandibular process; mx, maxillary process; so, supraorbital ridge; EMM, early migrating mesenchyme; SOM, supraorbital mesenchyme; op, optic vesicle; ot, otic vesicle, rho, rhombencephalon. Scale bars; 500 μm (a, b, c, e), 200 μm (g), and 100 μm (i, k).
Figure 2. *Dlx5*-augmentation in NCCs modifies NCC-derivatives. (a–d) Immunohistochemical staining for acetylated tubulin (c, d), nuclear counterstained by Hoechst (a, b) in the trigeminal ganglion. (e, f) 3-D reconstruction of trigeminal ganglion of the control (e) and the NCC\textsuperscript{Dlx5} (f). (g) Statistical analysis of the trigeminal ganglion volume measured after reconstruction. (h, i) *Dct* localization, a pigment cell specifier, by ISH on frontal section of E15.5 heads. Insets in (h, i) are high magnified images of the boxed areas. (j) Statistical analysis of the number of *Dct*-positive cells. (k–p) Skeletal staining for cartilage (alcian blue) and bone (alizarin red). Lateral views at E13.5 (k, l), dorsal views (skull base removed) of calvaria at E14.5 (m, n) and P0 (o, p) of skeletal staining in the control and the NCC\textsuperscript{Dlx5}. Ectopic cartilage is induced in the NCC\textsuperscript{Dlx5} (arrowheads in l, n, p) from E13.5. The developing heterotopic bone overlaps with the ectopic cartilage (double asterisks in p) at P0. Dashed lines contour cranial bones. Two-tailed t-test; *p < 0.05; ns, not significant. cs, coronal suture; fr, frontal bone; pa, parietal bone; pc, pigment cell; tg, trigeminal ganglion. Scale bar; 200 μm (a–d, h, i), 500 μm (e, f) and 1 mm (k–p).
of the frontal and parietal bones normally overlap at the coronal suture (Fig. 3e), however, in the NCC

\[^{\text{Dlx5}}\] (Fig. 3f, arrowheads). Therefore, the extended NCC-derived area hosted the NCC-derived ec and hb, and the position of the coronal suture was shifted back-wards. Computed X-ray microtomography (μCT) data of P0 (n = 3) revealed that the NCC-derived frontal bone length at the midline of calvaria in the NCC

\[^{\text{Dlx5}}\] increased significantly by 18.5% compared to control (p < 0.001) (Fig. 3g–k). Furthermore, the NCC-derived calvarial bone volume rose by 10.2% (p < 0.05) (Fig. 3l). The volume of MES-derived parietal bone, meanwhile, did not significantly decrease (n = 3) (Supplementary Fig. S1c). Besides, the ec was found at the cranial vertex, the chondrogenic potential of the NCC

\[^{\text{Dlx5}}\] obviously increased in this area. Thus, the NCC-derived apical head mesenchyme increased chondrogenic and osteogenic potentials in response to Dlx5-overexpression.

The ec developed inside of the dura mater. Histological analysis at E15.5 showed that cranial bones had not reached to the midline, and cartilage was absent in the vertex in the control (Fig. 4a–c). In the NCC

\[^{\text{Dlx5}}\], the thickness of the ec was comparable to that of cranial base cartilages (n = 3) (Fig. 4d–f). The hb was ossified on top of the ec in the calvaria (Fig. 4d–f). As expected from the skeletal staining data (Fig. 2), the endogenous frontal bone and parietal bone of the NCC

\[^{\text{Dlx5}}\] observed on HE sections illustrated similar bone quality in terms

Figure 3. Ectopic cartilage and heterotopic bone in the NCC

\[^{\text{Dlx5}}\] formed in NCC-derived head mesenchyme. Skull vault of E17.5 NCC

\[^{\text{EYFP}}\] (a, c, e) and NCC

\[^{\text{Dlx5/EYFP}}\] (b, d, f) with brightfield (a, b) and fluorescent (c–f) images. (c) and (d) are the fluorescent images of (a) and (b), respectively. Green fluorescence implies NCC originating cells. Brackets and dashed lines in dorsal views (a, b) indicate the coronal suture at the lateral side and the prospective coronal sutures at the apex, respectively. Arrowheads in (c) point to the “NCC tongue” between the parietal bones. (e, f) Parasagittal sections of the control (NCC

\[^{\text{EYFP}}\] and mutant (NCC

\[^{\text{Dlx5/EYFP}}\] at planes indicated in (a) (a’–a”) for (e) and (b) (b’–b”) for (f). Nuclei are counterstained by Hoechst. Brackets and arrowheads in (e, f) indicate the coronal sutures of the control and the opened end-to-end junction at the coronal suture of the mutant, respectively. (g–j) μCT images of calvarial bone of the control (g, i) and the NCC

\[^{\text{Dlx5}}\] (h, j) at P0. Double-head arrows in (g–j) demonstrate the NCC-derived bone length to be measured. (k, l) Statistical analyses of NCC-derived bone length (k) and volume (l) (n = 3). Two-tailed t-test, *p < 0.05; ***p < 0.001. cs, coronal suture; ec, ectopic cartilage; fr, frontal bone; hb, heterotopic bone; ip, interparietal bone; pa, parietal bone. Scale bars; 1 mm (a–d, g–j) and 100 μm (e, f).
of thickness or degree of mineralization compared to the counterpart of the control (Supplementary Fig. S2).

We conducted a more detailed investigation by using transmission electron microscope (TEM) at E15.5 (n = 3). Using toluidine blue stained semi-thin sections, we chose the relevant area of the control and the NCC Dlx5 for TEM analysis (Fig. 4g–j). In the control, the dura mater located just underneath the bone layer, characterized by longitudinally arranged fibroblast-like cells (Fig. 4i, arrow), and collagen bundles filling intercellular spaces (Fig. 4i, arrowhead)37. The arachnoid mater was clearly seen next to the dura mater, which contains more loosely attached cells, and numerous free ribosomes28,37,38 (Fig. 4i). In the NCCDlx5, the ec occupied a large space between the bone and the brain (Fig. 4j). On its outer and inner surfaces, similar structures that had the characteristics of the dura mater were found (Fig. 4j). Besides, the arachnoid mater was recognized under the dura mater structure (Fig. 4j). Therefore, our histological analyses demonstrated that the ec developed within the dura mater.

The ec and hb were derived from the EMM. It was reported that apical mesenchyme has both osteogenic and chondrogenic potential in vitro39. Double conditional knock-out of Msh homeobox 1/2 (Msx1/2) in the mouse NCC (Msx1/2cko/cko) generated heterotopic bones from the EMM at abnormal positions including the suture area32. More recently, in vivo loss and gain of function experiments of LIM homeobox transcription factor 1 beta (Lmx1b), which is expressed in the EMM but not in the SOM, demonstrated the inhibitory function of Lmx1b on osteogenic specification in the EMM37. Lmx1b loss-of-function in head mesenchyme (Lmx1b LOFHM) induced osteogenic marker expression in the vertex mesenchyme, future interfrontal suture and expanded bone-forming area resulting in synostosis27. These previous studies suggest that the EMM has osteogenic potential, which is inhibited in normal context.

Figure 4. Histological analysis of the ectopic cartilage and heterotopic bone in the NCCDlx5 at E15.5. (a–f) Frontal sections of E15.5 control (a–c) and NCCDlx5 (d–f) stained by HE and Alcian blue. (b, c, e, f) are high magnification images of boxed areas in (a, d). Heterotopic bone is induced at the vertex of the NCCDlx5, ectopic cartilage locates under the bone forming layer (e, f). (g, h) Semi-thin sections stained by toluidine blue on frontal sections of the control (g) and the NCCDlx5 (h). (i, j) TEM analysis of the control (i) and the NCCDlx5 (j) at the white boxed areas in (g, h). Arrows and arrowheads in (i, j) point to longitudinal-arranged fibroblasts and collagen fibrils, respectively. Ectopic cartilage appears in the meninges and is flanked by dura mater (j). ar, arachnoid mater; br, brain; du, dura mater; ec, ectopic cartilage; fr, frontal bone; hb, heterotopic bone; pa, parietal bone; pi, pia mater. Scale bars; 500 μm (a, d), 100 μm (b, c, e, f), 2 μm (i, j).
We explored the gene expression change that led to ec and hb formations. At E10.5, few mesenchymal cells were detected at the apical head, and histological difference between the control and the NCCDlx5 was not noticed (Fig. 1a,b). We found that there were differences in gene expression as well as histology from E11.5. SRY-box transcription factor 9 (Sox9) and Runx-related transcription factor 2 (Runx2) were used for evaluating mesenchymal condensation of cartilage and bone, respectively. At E11.5, Sox9 and Runx2 were substantially upregulated in the EMM region of the NCCDlx5 compared to the control (Fig. 5a–d, arrowheads). Mesenchymal condensation for the ec and hb was found at E11.5, which was around the same time with the beginning of original cranial base and calvarial development. The ectopic Sox9 expression domain was not connected to any part of the future skull base domain (n = 5) (Fig. 5b). This result confirmed that the ectopic Sox9 expression was not due to the extension of skull base primordium. In contrast, Runx2 expression in the EMM seemed to be continuous with the SOM by a thin expression line in the NCCDlx5 (n = 4) (Fig. 5c,d). To test whether the developing hb was independent of the SOM, we examined expression of Sp7, an early osteoblast marker and downstream of Runx2, at E14.5 by WISH (n = 5). The development of frontal and parietal bones was visualized by Sp7 expressing domain at this stage (Fig. 5e,f, dotted line). In the EMM area of the NCCDlx5, several Sp7 expression islands were independent of the SOM (Fig. 5f, arrowheads). These results strongly suggested that the hb in the NCCDlx5 is formed in the EMM and independent of the endogenous frontal bone.

The EMM layer was thickened in the NCCDlx5 at E11.5, which contained expanded Sox9 and Runx2 expression domains (Fig. 5g,h). Our BrdU incorporation assay showed a significantly increased BrdU+ cells in the EMM of E11.5 NCCDlx5 (n = 3, p < 0.001) (Fig. 5i). Immunohistochemical (IHC) staining for cell death showed no signals in the EMM of both the control and the NCCDlx5 at E11.5 (n = 3) (Supplementary Fig. S3). Therefore, the thickened EMM in the NCCDlx5 was caused by increased cell proliferation.

**Early development of the EMM in the control and the NCCDlx5.** We examined gene expression in early development of the ec and hb (n = 4) at E11.5. In the control, expression of Forkhead Box C1 (Foxc1), transcribed in all three meningeal layers, was broadly detected in the mesenchyme, but the signal was not seen or at much lower levels just underneath the epidermis at E11.5 (Fig. 6a, arrowheads). Importantly, expression domains of Foxc1 and Dermo1, molecular markers for the meninges and the dermis, respectively, were mutually exclusive (Fig. 6a,b). Control mice showed no expression of Sox9 in the EMM (Fig. 6c), but Runx2 was expressed in the Dermo1 expressing mesenchyme as a thin layer (Fig. 6d). Msx1 expression was found in the whole head mesenchyme (Fig. 6e). Msx2 expression domain was localized to the outer layer of the EMM, including a part of the meninges and the dermis (Fig. 6f, compared to 6a,b).

In the NCCDlx5, the Foxc1 expression domain appeared to contain the ec primordium marked by Sox9 expression (Fig. 6g,i), which is consistent with the phenotype in which the ec is surrounded by the dura mater (Fig. 4j). Remarkably, Dermo1 expression was highly upregulated in the NCCDlx5 compared to the control (Fig. 6h), indicating that Dlx5-augmentation enhanced the dermis formation. Runx2 expression of the NCCDlx5 was more evident compared to the control (Fig. 6d,j). Sox9 expression domain was included in the Runx2 domain (Fig. 6i,j). Because the hb developed outside of the ec (Fig. 4f), Runx2 expressing cells outside of Sox9-positive layer were thought to differentiate into osteoblasts. Importantly, these osteoblasts also expressed Dermo1 (Fig. 6h,j), suggesting that the hb was derived from the dermal layer. In contrast, the ec shown by Sox9 expressing domain seemed not to show Dermo1 expression (Fig. 6h,i).

Moreover, Msx1 expression was present in the arachnoid and the pia mater, and was not expressed in other parts of the EMM (Fig. 6k). Msx2 was downregulated at some areas of head menenchyme, however, expressed in the ec and hb (Fig. 6l). Since Lmx1b loss-of-function induced hb formation27, we also examined Lmx1b expression. Lmx1b was expressed in both the ec and hb (Supplementary Fig. S4), suggesting that since Dlx5 is a downstream of Lmx1b27, Dlx5-overexpression does not affect Lmx1b expression.

**PDGFRe, WNT/β-catenin and Bmp2 signals are upregulated in the NCCDlx5.** Platelet-derived growth factor receptor Alpha (Pdgfra) augmented in NCCs generated ec at the coronal suture, which was similar to the ec of the NCCDlx5. We performed double immunofluorescent staining for PDGFRe and SOX9 at E11.5 (n = 3). PDGFRe was present in the outer portion of the EMM in the control, while SOX9 signal was not detected (Fig. 7a–d). In the NCCDlx5, PDGFRe expression levels were more intensive in the dermal and SOX9-positive layers (Fig. 7e–h). Semi-quantitative analysis on immunofluorescent staining showed that PDGFRe signal was intensified by Dlx5-augmentation (Fig. 7a). WNT/β-catenin signalling induces osteoblast differentiation in intramembranous ossification34,45. The conditional β-catenin loss-of-function in the dermis using Dermo1-Cre or Engrailed1-Cre driver resulted in the loss of dermis and cranial bones. Instead, cartilages were induced between the epidermis and the thinner meninges42. Reversely, the NCCDlx5 had thickened dermis (Fig. 6h) and hb (Fig. 4f). We conducted double immunofluorescent staining for β-catenin and RUNX2 signals at E11.5 (n = 3). In the control, β-catenin and RUNX2 were sparsely expressed below the epidermis (Fig. 7i–l). In the NCCDlx5, RUNX2 signal illustrated the hb (Fig. 7m,n), β-catenin signal in the dermis and hb forming area were clearly upregulated (Fig. 7m–p). Semi-quantitative analysis for β-catenin showed that the signal was significantly upregulated by Dlx5-augmentation (Fig. 7q).

Bone morphogenetic protein (Bmp) is involved in hb formation in the interfrontal suture33,46 and it regulates Runx2 expression through DLX345. Our data showed that Bmp2 was not expressed in the control head menenchyme at E11.5 (Fig. 7q,e). However, it was ectopically induced in hb forming area in the NCCDlx5 (n = 3) (Fig. 7q,t).
Figure 5. EMM developed the ectopic cartilage and heterotopic bone. (a–d) Expression of chondrocyte marker-
Sox9 (a, b) and osteoblast marker-Runx2 (c, d) on frontal sections of E11.5 control (a, c) and NCC<sup>Dlx5</sup> (b, d) by ISH. Arrowheads indicate ectopic expressions of Sox9 (b) and Runx2 (d) in the EMM of the NCC<sup>Dlx5</sup> (e, f) Lateral views of Sp7 expression, an early osteoblast marker and downstream of Runx2, by WISH on heads of E14.5 control (e) and NCC<sup>Dlx5</sup> (f). Dotted lines in (e, f) outline developing frontal and parietal bones. Arrowheads in f point bony islands independent of the frontal bone primordium in the NCC<sup>Dlx5</sup>. (g, h) BrdU incorporation assay in the EMM at E11.5 of the control (g) and the NCC<sup>Dlx5</sup> (h). BrdU positive cells were counted in the areas between the dashed lines of (g, h). (i) Statistical analysis of the percentage of BrdU positive cells over the total number of EMM cells (n = 3). Two-tailed t-test, ***p < 0.001. EMM, early migrating mesenchyme; fr, frontal bone; pa, parietal bone; SOM, supraorbital mesenchyme. Scale bars; 200 μm (a–d), 500 μm (e, f), 50 μm (g, h).
NCCDlx5 in the EMM seems to be committed to dermal and meningeal layers by E11.5 (Fig. 6). The distinct cell differentiation of Msx1/2cko/cko is attenuated, suggesting that NCCs did not fluctuate between non-skeletogenic and skeletogenic fates. 

A demonstration of complementary expression in head mesenchyme in the control (a, b) by ISH. Arrowheads in (a) point cells under the epidermis that weakly express Foxc1. Foxc1 and Dermo1 demonstrate complementary expression in head mesenchyme in the control (a, b). Dermo1 and Runx2 are co-expressed in a thin cell layer under the epidermis (b, d). Msx1 is expressed in the whole head mesenchyme (e), while Msx2 is mainly expressed at the outer layer of head mesenchyme (f). In the NCCDlx5, Msx9 expression in EMM illustrates the forming ectopic cartilage (i). Foxc1 is expressed in the meninges and faintly in the ectopic cartilage (g). Dermo1 and Runx2 are upregulated; Runx2 expression is enclosed by Dermo1 expression (h, j). Msx1 is not expressed in ectopic cartilage and heterotopic bone, whereas Msx2 is expressed in both misregulated structures. Dashed lines in (g–l) outline the ectopic cartilage forming region estimated by Sox9 expression (i). Br, brain; ec, ectopic cartilage; hb, heterotopic bone. Scale bars; 50 μm.

Discussion

Dlx5 expression in the NCC is involved in jaw patterning. Aside from that, Dlx5 is expressed in the several NCC-derived head components and is related to their differentiation, thus, we further examined predescription of the NCC affected by Dlx5-augmentation. Investigations of Snai1 expression indicated that the migration and distribution of NCCs were unaffected by Dlx5-overexpression (Fig. 1). There was little effect on trigeminal ganglion development, but the number of pigment cells was increased in the NCCDlx5 (Fig. 2), which possibly corresponds to enhanced dermal cell proliferation in the NCCDlx5 (Figs. 5h, 6h). Despite the extra skeletogenesis in the NCCDlx5, non-skeletal NCC-derivatives such as trigeminal ganglion, dermis, and pigment cells were not attenuated, suggesting that NCCs did not fluctuate between non-skeletogenic and skeletogenic fates.

Previous reports showed that modifications in molecular cascades in mouse head mesenchyme resulted in either ec or hb formation in the skull vault. In particular, Msx1/2cko/cko and Lmx1b LoFHM caused hb formation at the posterior of the frontal bone similar to the NCCDlx5, but ec formation was not reported in those mutants.

By contrast, Pdgfra upregulation in NCCs generated ec in the coronal suture, meanwhile, the frontal bone appeared unchanged. Some mutants demonstrated that cartilages replaced calvarial bones in mice, such as β-catenin knock-out and fibroblast growth factor 8 (Fgf8) gain-of-function. Therefore, chondrogenesis could be upregulated at the expense of osteogenesis. In this study, we showed that chondrogenesis and osteogenesis were promoted simultaneously in the NCCDlx5 calvaria (Fig. 2). Ec and hb formation in the NCCDlx5 occurred in the meningeal and dermal layers of the EMM, respectively (Figs. 4, 6). We also found that, in normal development, the EMM seems to be committed to dermal and meningeal layers by E11.5 (Fig. 6). The distinct cell differentiation of apical head mesenchyme in response to Dlx5-augmentation in NCCs strongly support the idea that there are different cell populations in the EMM by this stage.

The development of the ec in dura mater layer highly suggests that head mesenchyme which was originally destined to be meningeal precursor cells could be turned into chondrocytes. The ec formation within the dura mater was previously reported; residual cartilages are occasionally formed above the trigeminal ganglia in mammals, and the pila antotica near the ala temporalis in therians develops inside the dura mater as atavistic relics. In the clinical aspect, some meningeal chondrosarcomas, which are tumours containing cartilaginous islands, in the dura mater are reported. Additionally, when the dura mater is transplanted to the trunk in contact with mesodermal elements, the transplant sometimes develops cartilage. In addition, explants of mouse mesenchymal cells from the vertex area of the head (corresponding to the EMM) at E12.5–14.0 show the potential for bone and cartilage formations. These reports support our idea of dura-to-cartilage transformation.

In the NCCDlx5, the ec develops in the frontal bone area in proximity to the coronal suture, which is the NCC-MES boundary, and some small cartilages in the interfrontal suture. Loss of β-catenin is one of the causes of ec induction during calvarial development. However, β-catenin was detected in the ec forming area in the NCCDlx5 (Fig. 7l, o), suggesting that formation of ec was not attributed to change in WNT/β-catenin signalling. Pdgfra upregulation in the NCC in mice exhibits ec formation in the coronal and interfrontal suture, which is similar to the ec in the NCCDlx5. We found that PDGFRα signal was increased in the NCCDlx5. Although the layer of ec formation was not studied in the Pdgfra mutant, it is suggested that the interfrontal area, the NCC-MES boundary show potential for cartilage differentiation when stimulated by PDGFRα (Fig. 8b).

Our gene expression analyses suggest that, in the control, Runx2-expressing layer just below the epidermis in the EMM does not associate with the meninges at E11.5. The Runx2-expressing layer is present within the
Figure 7. PDGFRα, β-catenin and Bmp2 signals in the EMM at E11.5. (a–h) Double immunofluorescent staining for SOX9 and PDGFRα on frontal sections at E11.5, nuclear counterstained by Hoechst of the control (a–d) and the NCC<sup>Dlx5</sup> (e–h). Arrows in f, h point to the ectopic cartilage. PDGFRα signal is shown in the SOX9-positive area (h). (i–p) Double immunofluorescent staining for RUNX2 and β-catenin on frontal sections at E11.5, nuclear counterstained by Hoechst of the control (i–l) and the NCC<sup>Dlx5</sup> (m–p). Arrows in (n, p) point to the heterotopic bone. β-catenin signal is shown in the RUNX2-positive area (p). (q–t) Bmp2 expression by ISH on frontal sections at E11.5 of the control (q, r) and the NCC<sup>Dlx5</sup> (s, t). (r, t) are high magnified images of the boxed area in (q, s). (u, v) Semi-quantification analysis on immunofluorescent staining for PDGFRα and β-catenin. Two-tailed t-test;**p < 0.01;***p < 0.001. ec, ectopic cartilage; hb, heterotopic bone. Scale bars; 50 μm (a, e, i, m, r, t); 200 μm (q, s).
expression domain of *Dermo1*, a dermal cell marker, and this expression domain does not express *Frox1*, a meningeal marker. Based on these results, we propose that the cells expressing both *Runx2* and *Dermo1* in the EMM are dermal progenitors (Fig. 8a). Furthermore, the *Runx2* expression in mesenchymal cells suggests the intrinsic osteogenic potential of the dermis. Anti-osteogenic functions of *Msx2* and *Lmx1b* are likely to suppress the osteogenic potential in the EMM.32

*Msx2* can act as either osteogenic inhibitor or activator (Fig. 8, *Msx2* (in), *Msx2* (ac))33. *Msx2* inhibits *Runx2* transcriptional activity34,35, and competes with Runx2 in binding to a regulatory sequence of Osteocalcin (Ocn), an osteogenic induction gene56 (*Msx2* (in)). The anti-osteogenic activity of *Msx1* and *Msx2* in the EMM explained the hb in the *Msx1*/*Msx2*LoF mutants27,28. Altogether, the hb formation in the EMM is consistent with *Msx1*/*Msx2*LoF (Fig. 8b). By contrast, *Dlx5* appears to promote the pro-osteogenic function of *Msx2* in concert with other osteoblast activators such as *Bmp2* and β-catenin (Fig. 8b). *Dlx5* is a downstream target of Bmp247, and we observed increased *Bmp2* expression in head mesenchyme of the NCC56,57 mutants27,32. These results suggest that *Dlx5* activates *Bmp2* through positive feedback. Since *Msx2* is also a downstream target of *Bmp2*26, maintenance of *Msx2* expression in the NCC56,57 could be caused by *Bmp2* induction. Wnt/β-catenin promotes intramembranous bone formation and dermal layer differentiation42. We demonstrated that β-catenin levels were increased in the forming hb and probably associated with the enhanced dermis differentiation (Fig. 7m–p). Our data are consistent with previous reports that *Bmp2* and β-catenin synergistically induce *Msx2*26,63. *Bmp2* upregulation in the NCC56,57 is consistent with *Msx1*/*Msx2*LoF and *Lmx1b* LoF mutants27,32. Altogether, the hb formation in the NCC56,57 was caused by enhanced osteogenic induction of *Bmp2* and β-catenin signalling pathways that involve *Msx2* (Fig. 8b). It will be intriguing to clarify in more detail about the molecular mechanism for the phenotype, involved in the dual function of *Msx2* in the future.

It was reported that *Lmx1b* prevents ossification of EMM from E9.527. In this study, we found that expression patterns of *Dermo1* and *Frox1* are mutually complementary at E11.5 in the control (Fig. 6a,b). Taken together, we propose that after non-skeletal commitment, the EMM is divided into two populations, dermal and meningeal layers by E11.5 in normal development (Fig. 8a). However, this commitment is not irreversible because the cell fate can be altered to cartilage and bone by responding to pro-skeletal signals such as augmentation of *Dlx5*. Given that the EMM has differentiation potentials to both cartilage and bone, it should be carefully evaluated which mesenchyme (EMM or SOM) contributes to ectopic and heterotopic skeletogenesis in the calvarium when mutant mice are examined.

Apical-basal patterning in cranial development by the interaction between the EMM and SOM has yet to be fully elucidated. Although the molecular basis of EMM differentiation in its early development is still unknown, our findings provide a more detailed picture of the EMM sublayers together with their potentials, shedding light on developmental mechanisms of cranial development.
Materials and methods

Mice. Mice with Dlx5 conditional expression reporter allele by utilizing Cre-LoxP recombination system, R26R-CAG-flu-Dlo5+/+, were described previously11. R26R-CAG-flu-Dlo5+/+ mice were maintained on ICR genetic background. Wnt1-Cre (#022501) mice were obtained from the Jackson Laboratory (#022501, MA, USA) and maintained on ICR genetic background. Wnt1-Cre driver targets the NCCDlx5+. R26R-CAG-flu-Dlo5+/+ mice were crossed with Wnt1-Cre mice to constitutively activate Dlx5 expression in NCC. Wnt1-Cre;R26R-CAG-flu-Dlo5+/+ mice were used as the mutant, NCCDlx5+, and mice without Wnt1-Cre or R26R-CAG-flu-Dlo5+/+ allele were studied as the control. R26R-CAG-flu-Dlo5+/+ mice were described previously11. β-galactosidase staining of Wnt1-Cre;R26R-CAG-flu-Dlo5+/+ (NCCDlx5+) visualizes the NCC. Wnt1-Cre;R26R-CAG-flu-Dlo5+/+ mice expressing enhanced yellow fluorescent protein (EYFP) were crossed with R26R-CAG-flu-Dlo5+/+ mice to generate Wnt1-Cre;R26R-CAG-flu-Dlo5+/+EYFP (NCCDlx5+EYFP), in which NCC expresses EYFP with NCCDlx5+ phenotype. The Wnt1-Cre;R26R-CAG-flu-Dlo5+/+EYFP control littersmates express EYFP in the NCC. The morning on which a vaginal plug was found was designated as embryonic day 0 (E0). Animal procedures were approved by the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University (170238A, A2018-48C, A2019-060A) and the University of Tokyo (P19-043). All experiments were carried out in accordance with the relevant guidelines and regulations of the Tokyo Medical and Dental University and the University of Tokyo. Animal studies were conducted following the ARRIVE guidelines.

In situ hybridization (ISH). Embryos of E9.0–12.5 were collected in ice-cold phosphate-buffered saline (PBS). Samples were fixed with 4% paraformaldehyde/PBS overnight at 4 °C. For frozen sections, samples were incubated in 25% sucrose in PBS, finally embedding in O.C.T. compound (Sakura Finetek, Japan) and stored at −80 °C. Heads of E15.5 were frozen freshly in O.C.T. compound. Frozen sections were cut at 12 μm thickness (Leica CM1850, Germany). For whole-mount in situ hybridization (WISH) purposes, the fixed samples were dehydrated in a graded series of methanol and stored in 100% methanol at −20 °C. DNA fragments of mouse Bmp2, Dct, Dlx5, Dermol1, Foxc1, Lmx1b, Msx1, Msx2, Runx2, Snai1, Sox9, and Sp7 shown in Supplementary Table S1 were subcloned into the pGEM-easy vector (Promega, USA), or pCRII vector (Invitrogen, USA). Mouse probes were labeled with digoxigenin (DIG) and used for in situ hybridization. DIG-labeled RNA probes were synthesized by Sp6 or T7 polymerase (Roche Diagnostics, Germany) and used for in situ hybridization. Gene expression was visualized by nitro blue tetrazolium chloride and 5-Bromo-4-chloro-3-indolyl phosphate (Roche Diagnostics, Germany). In situ hybridization experiments were performed at least three times on different samples.

Immunohistochemical (IHC) and immunofluorescent staining. Bromodeoxyuridine (BrdU) (Roche Diagnostics) was used for BrdU cell proliferating assay. BrdU in PBS was injected intraperitoneally to the pregnant mice at a dose of 100 mg BrdU/kg body weight one hour before dissection. E11.5 fetal heads were collected, fixed with 4% paraformaldehyde/PBS overnight at 4 °C, and prepared to be embedded in the O.C.T. compound. Other immunohistochemical analyses were performed on untreated fixed samples. Samples were cut at 12 μm. Sections were incubated with mouse anti-BrdU (1:200, 1170376001, Roche Diagnostics), rabbit anti-cleaved caspase3 (1:200, #9661, Cell Signaling Technology), or anti-Acetylated Tubulin (1:200, T7451, Sigma–Aldrich), double immunohistochemical staining with mixtures of rat anti-PDGFRα (1:500, 14-1401, eBioscience) and rabbit anti-SOX9 (1:1500, cat.#AB5535, Millipore), mouse anti-β-catenin (1:500, 610153, BD BioSciences) and rabbit anti-RUNX2 (1:200, #9661, Cell Signaling Technology). SOX9 and RUNX2 fluorescence signals were detected by anti-Rabbit Alexa Fluor 555 (1:300, A31572, Molecular Probes). Other stainings were processed with biotinylated anti-mouse IgG (1:200, ZA0409, Vector Laboratories), biotinylated anti-rabbit IgG (1:200, ZB1007, Vector Laboratories), biotinylated anti-rat IgG (1:200, BA-4001, Vector Laboratories) followed by Avidin–biotin complex (Vectastain) and 3,3′-diaminobenzidine (Sigma–Aldrich), or Alexa Fluor 488 streptavidin conjugate (1:300, S11223, Molecular Probes). IHC experiments were conducted at least three times on different samples.

Semi-quantification of ISH, IHC and immunofluorescent staining. The number of Dct-positive cells was the total number counted in the EMM of three sections: middle of the eye, back of the eye, and behind the eye, at 10× magnification (n = 3). The percentage of BrdU positive cells was calculated by the number of BrdU positive cells divided by the total number of cells in the designated area, in four representative views at 40× magnification of each NCCDlx5+ mouse (n = 3) and four corresponding views of each control (n = 3). Semi-quantifying experiments were conducted with negative control sections without the primary antibody. Fluorescent images were treated equally with reference to the negative control sections by Photoshop (Adobe, USA). Semi-quantification of fluorescent signals was processed by ImageJ, relative signal was compared by a ratio of mean brightness values (brightness per area) of the NCCDlx5+ to the control (n = 3).

3-Dimension reconstruction of the trigeminal ganglion. Heads of E17.5 fetuses of the Wnt1-Cre;R26R-CAG-flu-Dlo5+/+ and the control were embedded in O.C.T. compound, sectioned at 12 μm thickness, and stained by Mayer’s Hematoxylin and 1% Eosin Y solution (HE) (Muto Pure Chemical, Japan). 3-Dimension structures were constructed from serial histological sections by Avizo 6.3 (Visualization Sciences Group, USA). Volume of the trigeminal ganglion of the control (n = 3) and the NCCDlx5+ (n = 3) was measured after reconstruction.

Alizarin red and alizarin blue skeletal staining. Post-natal day 0 (P0) mice were skinned, followed by fixation in 96% ethanol for one week. Skeletal staining was performed in a mixture of 0.02% alizarin blue (Sigma, 05500-10G), 0.005% alizarin red (Wako, 013-25452), 5% acetic acid in 70% ethanol for three days with rocking.
at room temperature. Samples were then washed by distilled water and optically cleared by glycerol in 0.5% KOH until the bone and cartilage were visible.

**Computed X-ray microtomography (μCT).** Heads of P0 mice were fixed in 70% ethanol overnight. μCT was taken by inspeXio SMX100CT (Shimadzu, Japan). The data were analyzed by Avizo 6.3. μCT scans were uploaded to Avizo 6.3 as DICOM files and visualized using Isosurface in Avizo 6.3. The NCC-derived bone length was measured in three-dimension at the midline, tracing the top of the calvaria (Fig. 3g–j, n = 3). The bone volume was calculated by Avizo 6.3, including all the bone components within the frontal bone forming area.

**Histological analysis.** Heads of fetuses at E15.5 were fixed in Bouin’s fixative solution for 48 h. The samples were washed by 70% ethanol, then dehydrated in a gradient of ethanol until 100%, followed by xylene treatment and embedded in paraffin. Sections were cut at 5 μm thickness (Leica RM2235, Germany), then stained by 1% alcian blue (Sigma, 05500-10G) in 3% acetic acid, followed by Mayer’s Hematoxylin and 1% Eosin Y solution. Comparisons were made among at least three independent littersmates.

**Transmission electron microscopy (TEM).** Heads of E15.5 fetuses were trimmed to collect the targeted tissues, then fixed in 2.5% glutaraldehyde/0.1 M phosphate buffer (PB) for 72 h. After washing in PB overnight at 4 °C, samples were postfixed with Osmium tetroxide (OsO4) for 2 h. Samples were then dehydrated in ethanol, followed by infiltration of epon resin and propylene oxide catalyst, then embedded in epon resin. Semi-thin sections at 1 μm and toluidine blue staining were utilized to examine the samples. Ultrathin sections at 80 nm were collected and double-stained with uranyl acetate and lead citrate on carbon-coated copper grids. Sections were observed by transmission electron microscopy (Hitachi H-7100, Japan) (n = 3).

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**Author contributions**

T.H.V, M.T designed, conducted experiments, analyzed data and wrote manuscript. M.S, T.K provided preliminary data. H.H, A.I and H.K advised on the experiments, managed mice. S.I designed, conducted experiments, analyzed data, revised and approved manuscript.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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