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Regulation of cell polarity in the cartilage growth plate and perichondrium of metacarpal elements by HOXD13 and WNT5A

Pia Kuss [a,b,1], Katerina Kraft [a,b], Jürgen Stumm [a], Daniel Ibrahim [a,b,c], Pedro Vallecillo-Garcia [a], Stefan Mundlos [a,b,c,*], Sigmar Stricker [a,b,**]

[a] Max Planck Institute for Molecular Genetics, Ihnestrasse 63-73, 14195 Berlin, Germany
[b] Institute for Medical and Human Genetics, Charité University Medicine, Augustenburger Platz 1, 13353 Berlin, Germany
[c] Berlin-Brandenburg Center for Regenerative Therapies (BCRT), Charité University Medicine, Augustenburger Platz 1, 13353 Berlin, Germany

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ABSTRACT

The morphology of bones is genetically determined, but the molecular mechanisms that control shape, size and the overall gestalt of bones remain unclear. We previously showed that metacarpals in the synpolydactyly homolog (spdh) mouse, which carries a mutation in *Hoxd13* similar to the human condition synpolydactyly (SPD), were transformed to carpal-like bones with cuboid shape that lack cortical bone and a perichondrium and are surrounded by a joint surface. Here we provide evidence that spdh metacarpal growth plates have a defect in cell polarization with a random instead of linear orientation. In parallel prospective perichondral cells failed to adopt the characteristic flattened cell shape. We observed a similar cell polarity defect in metacarpals of Wnt5a$^{-/-}$ mice. *Wnt5a* and the closely related *Wnt5b* were downregulated in spdh handplates, and HOXD13 induced expression of both genes in vitro. Concomitant we observed mislocalization of core planar cell polarity (PCP) components DVL2 and PRICKLE1 in spdh metacarpals indicating a defect in the WNT/PCP pathway. Conversely the WNT/β-CATENIN pathway, a hallmark of joint cells lining carpal bones, was upregulated in the perichondral region. Finally, providing spdh limb explant cultures with cells expressing either HOXD13 or WNT5A led to a non-cell autonomous partial rescue of cell polarity the perichondral region and restored the expression of perichondral markers. This study provides a so far unrecognized link between HOX proteins and cell polarity in the perichondrium and the growth plate, a failure of which leads to transformation of metacarpals to carpal-like structures.

Introduction

During endochondral ossification mesenchymal progenitor cells condense to form a cartilaginous template (anlage) of the future bones. Concomitantly, cells surrounding the condensation form the perichondrium consisting of flattened cells. At either end of the anlage a growth plate is established driving longitudinal outgrowth of the skeletal element. Here small, round chondrocytes (resting or reserve chondrocytes) differentiate to proliferating chondrocytes, which form stacks of discoid cells. Finally calls undergo prehypertrophic differentiation characterized by the expression of Indian hedgehog (*Ihh*) and subsequently become hypertrophic. At the same time cells from the perichondrium form the first bone collar (cortical bone) around the proliferating/hypertrophic chondrocytes. This stack-like arrangement of proliferative cells and its restriction by the perichondrium/bone is considered the main mechanical force enabling longitudinal growth of long bones as compared to spherical growth of, for example, wrist bones. The transition of spherical to discoid cell shape is achieved by cell polarization concomitant with lateral intercalation into a column in a process reminiscent of convergent extension movements seen in vertebrate gastrulation (Li and Dudley, 2009).

In the past years signaling by components of the WNT family of secreted factors have emerged as key players in this event (Romereim and Dudley, 2011). Classically, WNT pathways are divided into the "canonical" branch, acting via stabilization of β-CATENIN, and several alternative pathways (Angers and Moon, 2009) such as the WNT/planar cell polarity (PCP) pathway. WNT/PCP was demonstrated to regulate oriented cell divisions and cell

* Corresponding author at: Institute for Medical Genetics, Charité University Medicine Berlin, Charité University Medicine Berlin, Augustenburger Platz 1, 13353 Berlin, Germany. Fax: +49 30 450569915.
** Corresponding author at: Max Planck Institute for Molecular Genetics, Development and Disease Group, Ihnestrasse 63-73, 14195 Berlin, Germany. Fax: +49 30 84131385.
E-mail addresses: stefan.mundlos@charite.de (S. Mundlos), strick_s@molgen.mpg.de (S. Stricker).
[1] Present address: Sanford-Burnham Medical Research Institute, La Jolla, CA, USA.

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polarity in the chicken growth plate (Li and Dudley, 2009). WNT5A is a major ligand activating the PCP pathway in the mouse (Kikuchi et al., 2012; Komiya and Habas, 2008). Targeted disruption of *Wnt5a* leads to severely impaired skeletogenesis (Yamaguchi et al., 1999) with altered growth plate morphology (Yang et al., 2003). Moreover, it was recently demonstrated that WNT5TA controls cell shape during condensation of cartilage in mouse digits via a PCP pathway (Gao et al., 2011).

Hox genes control patterning, differentiation and morphogenesis during development. HOXD13 is a master regulator of autopod skeletal morphogenesis. In previous studies we examined the role of *Hox* genes in cartilage and bone formation in the limbs mainly using a *Hoxd13* mutant called synpolydactyly homolog ($Hoxd13^{spdh/spdh}$, hereafter termed spdh) which carries a seven-alanine expansion in HOXD13. In humans identical mutations in *HOXD13* lead to synpolydactyly (SPD) (Muragaki et al., 1996). The mutation results in the degradation of HOXD13 protein and thus a loss of function. In addition, mutant HOXD13 interacts with other co-expressed HOX proteins that contain poly-alanine stretches such as HOXA13 and inactivates them resulting in a combined inactivation of HOX proteins in the autopod (Bruneau et al., 2001; Villavicencio-Lorini et al., 2010). Spdh mice exhibit a transformation of metacarpals (long bones) to carpals (cuboid bones). Spdh metacarpals have a variable shape that ranges from a longitudinal appearance to almost fully round. Mutant metacarpals invariantly have no perichondrium and fail to form any cortical bone. Instead they are surrounded by joint-like structures and undergo secondary ossification similar to the ossification scheme observed in carpal bones (Villavicencio-Lorini et al., 2010).

Here we propose that the underlying cause for this phenomenon is a defect in polarization and orientation of spdh metacarpal growth plate and perichondral cells. $Wnt5a^{-/-}$ mice have a similar phenotype and we provide evidence that *Hoxd13* is upstream of *Wnt5a*. The defects seen in spdh mice are accompanied by dysregulation of both the canonical WNT/β-CATENIN pathway as well as the WNT/PCP pathway. These data indicate that a local *Hox* code might influence shaping of skeletal elements by influencing cell shape via local induction of factors such as WNT5A.

Materials and methods

Mice

Spdh mice (Johnson et al., 1998) were obtained from The Jackson Laboratory (Bar Harbor, ME, USA), $Wnt5a^{-/-}$ mice (Yamaguchi et al., 1999) from E. Arenas (Karolinska Institute, Stockholm, Sweden) and $Axin2^{LacZ}$ reporter mice (Lustig et al., 2002) from W. Birchmeier (Max Delbrück Centre, Berlin, Germany). Genotyping was performed as described previously (Albrecht et al., 2002; Lustig et al., 2002; Yamaguchi et al., 1999).

Skeletal preparation, in-situ hybridization, immunolabeling

Skeletal preparation and in-situ hybridization was performed as described previously (Witte et al., 2010). Probes for *Hoxd13*, *Wnt5a* and *Wnt5b* were described in (Albrecht et al., 2002; Witte et al., 2009). For immunolabeling, antigen retrieval was performed using citrate buffer or high-pH buffer (Dako). After permeabilization (0.2% Triton X-100 for 15 min) and blocking (5% NGS for 1–5 h) primary antibodies (see Supplementary material) were applied at 4 °C overnight and detected with fluorescence-conjugated secondary antibodies (Molecular Probes, Eugene, OR, USA; 1:500) at room temperature (RT) for 1 h. Phalloidin-AlexaFluor-488 staining (Molecular Probes) was done 1:200 for 45 min at RT. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, St. Louis, MO, USA). For anti-RCAS staining, additional signal amplification using the Tyramide Signal Amplification system (Perkin-Elmer) was performed according to the manufacturer's protocol.

Polarity index, golgi apparatus orientation

After phalloidin labeling or differential interference contrast imaging, the maximum and minimum diameter of cells was determined using the AutMess tool integrated in the Zeiss AxioVision software (Zeiss, Oberkochen, Germany). The ratio of these parameters was used as an index for cell polarization. For Golgi orientation, the angle between the Nucleus–Golgi axis (Golgi apparatus stained via GM130 antibody) and the longitudinal axis of the cartilage element was determined using the AutMess tool (Zeiss). In each case at least three specimens were analyzed.

Real-time PCR

DF1 cells were infected with concentrated viral supernatants containing RCAS(A)–Hoxd13WT, RCAS(A)−Hoxd13+7Ala and RCAS(A)–GFP and grown for 3 or 7 days in DMEM supplemented with 10% FCS and 2% chicken serum. RNA was extracted with peqGold Trifast (PEQLAB, Erlangen, Germany). Real-time PCR for *Wnt5a* was performed as described before (Hecht et al., 2007) using three independent repeats for each condition.

Explant cultures

DF1 cells were transfected with RCAS(A)–Hoxd13 or RCAS(A)–Wnt5a together with RCAS(B)–GFP for visualization, or with RCAS (A)–GFP alone as control. Cells were grown for 7 days in DMEM supplemented with 10% FCS and 2% chicken serum. For implantation, DF1 cells were trypsinized and pelleted. Cells from the pellet were transferred to a mouth pipet by gentle aspiration. Embryos were harvested at E13.5 or E14.5. The limb culture protocol was modified from (Minina et al., 2001). Forelimbs were dissected and the explants were placed on cell strainer nets in limb culture medium (BGJb, 1% BSA, 1% L-Glutamine, 0.2 mg/ml ascorbic acid, 50 U/ml PenStrep, 0.05 mg/ml Gentamycin, 0.3 μg/ml Amphotericin B) at the liquid–air interface. The epidermis was slit with a tungsten needle and DF1 were implanted next to metacarpal condensations using a mouth pipet. Explants were incubated for 4 or 3 days, respectively. After incubation, placement of the implants was controlled via GFP visualization. Only specimens showing a strong GFP signal adjacent to metacarpal condensations were used for further analysis. To localize the implant on sections 3C2 antibody labeling and detection with the ABC staining kit (Vector Labs, Burlingame, CA, USA) was used. In-situ hybridization or quantification of cell polarization was performed in regions next to explants either on the same or on adjacent sections. In each case at least three specimens were analyzed.

Results

$Hoxd13^{spdh/spdh}$ and $Wnt5a^{-/-}$ metacarpals show lateral enlargement of condensations, lack of a defined border and cartilaginous fusion

We have shown before that postnatal spdh metacarpals show spherical growth and lack perichondrium and cortical bone (Villavicencio-Lorini et al., 2010). We thus re-analyzed the histological appearance of spdh metacarpal condensations during development. This confirmed the lack of perichondrium formation