Wnt11 Is Required for Oriented Migration of Dermogenic Progenitor Cells from the Dorsomedial Lip of the Avian Dermomyotome

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Abstract

The embryonic origin of the dermis in vertebrates can be traced back to the dermomyotome of the somites, the lateral plate mesoderm and the neural crest. The dermal precursors directly overlying the neural tube display a unique dense arrangement and are the first to induce skin appendage formation in vertebrate embryos. These dermal precursor cells have been shown to derive from the dorsomedial lip of the dermomyotome (DML). Based on its expression pattern in the DML, Wnt11 is a candidate regulator of dorsal dermis formation. Using EGFP-based cell labelling and time-lapse imaging, we show that the Wnt11 expressing DML is the source of the dense dorsal dermis. Loss-of-function studies in chicken embryos show that Wnt11 is indeed essential for the formation of dense dermis competent to support cutaneous appendage formation. Our findings show that dermogenic progenitors cannot leave the DML to form dense dorsal dermis following Wnt11 silencing. No alterations were noticeable in the patterning or in the epithelial state of the dermomyotome including the DML. Furthermore, we show that Wnt11 expression is regulated in a manner similar to the previously described early dermal marker cDermo-1. The analysis of Wnt11 mutant mice exhibits an underdeveloped dorsal dermis and strongly supports our gene silencing data in chicken embryos. We conclude that Wnt11 is required for dense dermis and subsequent cutaneous appendage formation, by influencing the cell fate decision of the cells in the DML.

Introduction

The presence of a connective tissue layer of the skin, called dermis, is the prerequisite for the development of cutaneous appendages. The somitic origin of the back dermis has been shown by Mauger in 1972 [1], [2] and later on, using quail-chick grafting technique, the medial origin of the dorsal dermis was demonstrated [3]. During embryonic development, dermis in vertebrates takes its origin from three different sources. The dense dorsal dermis, which will be addressed mainly in this work, originates from the lateral plate mesoderm [1], [2]. In addition to being a source for the dorsal dermis, the dermomyotomes also give rise to striated muscle, blood and lymphatic vessels, and in the neck region to the scapular blade [6], [7], [8], [9], [10], [11].

The development of the dermal layer of the skin and its appendages is a complex and highly regulated process. Although late stages of dermis development and notably the formation of cutaneous appendages have been studied to some extent, molecular data on the early fate of dermal progenitor cells has only begun to surface [12], [13], [14], [15], [16]. The progenitor cells for the dermis of the back are established during somitic compartmentalization, while their differentiation into dermis as connective tissue layer of the skin occurs only later during development. In our study, we focus on the early specification of dermal progenitors and their oriented migration towards the dorsal midline which is shown here to be dependent on the presence of Wnt11. Wnt11 has previously been reported to maintain the DML in an epithelial state [17], however, our data suggest that EMT of dermogenic progenitors relies on Wnt11.
Based on the spatially restricted expression pattern of a number of regulatory genes in chicken embryos, the dermomyotome has been divided into a medial, a central and a lateral region. All three regions can be distinguished by markers, such as Wnt11 in the DML, En-l in the central region and Sim1 in the lateral region [14]. As well as being a source for the dermal precursor cells that collect under the ectoderm to form the dorsal dermis, the dermomyotome is furthermore a source of myoblasts that enter the myotome [18], [19], [20]. These regions of the dermomyotome are, thus, consisting of multipotent progenitor cells that yield myogenic and dermogenic precursors determined by the surrounding signals. While the DML and the central dermomyotome yield the dermal progenitors destined to form the dense dermis above the neural tube, the lateral part corresponding to the Sim1 positive region is responsible for the loose dermis located in the normally apertic region of the chicken back [15].

In chicken embryos, the first visible signs of dermis development appear from day E5 (HH26) onwards, and in mouse, at an equivalent developmental stage day 9.5 (E9.5) [16], [21]. In the chicken embryo, the somite-derived dermal progenitor cells undergo EMT and organize to form a mesenchyme of noticeable thickness underneath the ectoderm between E3 and E5, adjacent to the neural tube and the differentiating myotome. It is only by E6 that the mesenchyme condenses locally and on E7 the epithelio-mesenchymal dialogue between dermis and epidermis is initiated which will eventually lead to the morphogenesis of cutaneous appendages.

The earliest dermis marker described to date is Twist2 (mDermo-1) in the mouse [22] and its orthologue cDermo-1 in the chicken [23]. The expression of Twist2 appears first at E10.5 in cells of mesodermal and mesectodermal origin. Its expression in the dermal progenitor cells increases progressively and is prominently detectable under the overlying ectoderm from E12.5 onwards. The expression of Twist2 (mDermo-1) in this domain of the prospective dermis is maintained until birth. In the chicken embryo, cDermo-1 has been shown to influence the density of the dorsal dermis and to act upstream of Fgf10 during dermis development [24]. Overexpression of cDermo-1 leads to an increase in dense dermis with formation of ectopic and larger feather buds [24]. cDermo-1 expression is controlled by BMP signaling and can be inhibited by the BMP antagonist Noggin [24], [25].

Although the origin of the dorsal dermis has been an object of interest for several research groups, so far no functional experimental data is available showing the direct relationship between gene expression in the dermomyotome and dermis formation. Based on its temporospatial expression pattern in the dorsomedial lip (DML) of the avian dermomyotome and subectodermal mesenchyme [26], Wnt11 has been proposed to play a role during dermis development in addition to its described role in myogenesis [27]. A comparable expression pattern of Wnt11 has been described in mice in the DML starting from E9.5 [28].

During somite development, two essential processes involving the cellular organization of the embryonic tissue occur: mesenchymal-epithelial transition (MET) occurs during somitogenesis and epithelio-mesenchymal transition (EMT) occurs during compartment formation and differentiation of the somites. Snail1 is a member of the Snail family of zinc-finger transcription factors with an important role in triggering EMT during the development of the neural crest and somites [29]. So this marker is playing a key role in normal chicken embryo development and the cell fate decision of the cells located in the somites.

The present work aims to investigate the role of Wnt11 in dense dorsal dermis formation which is derived from the medial somite. Its function is analysed by means of loss-of-function experiments in the chicken and mouse embryo. We are able to show that knockdown of Wnt11 in chicken embryos leads to compromised dense dorsal dermis formation due to a defective recruitment of dermal progenitor cells from the DML, causing a delay in feather bud development. This is corroborated by the analysis of knock-out mice for Wnt11 which demonstrates a decrease in dorsal dermis thickness with a reduced number of hair follicles in comparison with their WT littermates. qPCR analysis for several skin markers in wild-type mice and homozygous Wnt11 mutants points towards a defect in the formation of dense dorsal dermis in the absence of Wnt11 as seen by a significant decrease in the number of Twist2 (mDermo-1)-positive cells. In addition, we show that Wnt11 is regulated in a fashion similar to cDermo-1 in response to dermogenic and dermis inhibitory signals suggesting the involvement of Wnt11 and Dermo-1 in a common pathway controlling dermis formation.

Materials and Methods

Ethics Statement

According to German legislation, the use of embryonic vertebrates in an animal experiment needs approval only if the animal is in the last third of its embryonic development. In the case of chicken, this means that experiments done on animals before embryonic day 14 (E14) are not regarded as an animal experiment by the Tierschutzgesetz, and therefore, do not need approval or governmental permission.

The chicken embryos sacrificed for this work were between developmental stages HH21 (E3.5) and HH38 (E12). All embryos were sacrificed at the end of the study by opening the shell and tearing the allantois and amnion with forceps. Thereafter, the embryos were immersed in 4% PFA/PBS solution for fixation. No permits were required for the described study, which complied with all relevant regulations.

The mice embryos used in this study were provided by Medizinische Hochschule Hannover and were previously described [30]. All animal work conducted for this study was approved by H. Hedrich, head of the animal facility at Medizinische Hochschule Hannover and performed according to German legislation. All mice were maintained on an outbred (NMRI) background. Mice were kept with regulated temperature embryos, heterozygous mice were intercrossed. Vaginal plugs were checked in the morning after mating, for timed pregnancies noon was taken as E0.5. Female mice were sacrificed by cervical dislocation. Embryos were harvested in PBS, decapitated, fixed in 4% paraformaldehyde overnight and stored in 100% methanol at −20°C before further use.

Genomic DNA prepared from yolk sacs or tail biopsies was used for genotyping by PCR.

Chicken Embryos

Fertilized chicken eggs (White Leghorn, Gallus domesticus) were obtained from a local poultry farmer in Freiburg and a local breeder in Möhnesee. The eggs were incubated at 37°C under 80% humidity in a poultry egg incubator (Ehret). The progress of development was determined according to the staging system of [31].

In ovo Electroproporation (IOE)

Electroporation of the chicken embryos was performed as described before [32], [33]. Care was taken in the placement of the electrodes for effective transfection of the DML. Electroproporation was carried out using the Intracel TSS20 OVODYNE Electro-
porator. Following electroporation, the eggs were sealed with tape and reincubated. The first EGFP expression could be detected 4 hours after electroporation.

Gene Silencing Studies

The target sequences for Wnt11 were selected using RNAi target selection tools from Ambion and Promega. Four target sites in the chicken Wnt11 mRNA were chosen for the purpose of gene silencing (see Table S1). The selected nucleotide sequences chosen for targeting were subjected to nucleotide-nucleotide BLAST (blastn) analysis provided on the NCBI server (www.ncbi.nlm.nih.gov/BLAST) prior to the synthesis of corresponding shRNA-expressing constructs. This was done to precheck possible off-target silencing. For further preparation of the constructs two b-cis-tronic vector systems were used. These vectors that had been modified from the pCMV-EGFP vector, differ in the type of the RNA polymerase III promoter, which is either H1 or U6. They share the EGFP sequence and the insertion place for the shRNA cistron [33]. The pEGFP-H1-shRNA and pEGFP-U6-shRNA vectors were pre-restricted with BamHI and HindIII enzymes. Each of the shRNA encoding cistrons was introduced into both mentioned vectors. The associated EGFP reporter sequence incorporated into the shRNA-expressing constructs permitted to detect the success and position of DML transfection. The efficiencies of all four target sequences was individually tested by in ovo electroporation. Eventually for the silencing experiments, two cocktails were used one containing the H1 and the other one containing the U6 promoter.

Additional approaches employed for interfering with Wnt11 expression or signaling, repectively, included a miRNA-RFP construct targeted against Wnt11 (obtained from the lab of Christoph Marcelle, Monash University, Australia) [27] and a dominant-negative (DN) construct for Wnt11 (from the lab of Philippa Francis-West, London) [34]. The DN-Wnt11 RCAS was co-electroporated along with an EGFP reporter vector. The silencing efficiency of Wnt11 RNAi vectors was assessed by ISH analysis of embryos, transfected with an mRNA probe specific for avian Wnt11.

Controls were performed by electroporation of scrambled DNA with an EGFP reporter.

In Situ Hybridization

Embryos, electroporated with Wnt11 RNAi constructs, were tested using the following probes: cDermo-1 probe of 1,029 bp [23]; En-1 probe of 800 bp [35]; Paxax probe of 1,2 kb [36]; Pax3 probe of 660 bp [37]; Shh probe of 1,5 kb [38]; chicken Wnt11 probe of 1,000 bp [39]; Smad 600 bp in pBlueSript and Smadl 550 bp pGEMT. Easy probes were kind gifts from Olivier Pourquie (IGBMC, Illkirch, France). miTiss12 probe (400 bp insert in p17T3) was kindly provided by Ernst-Martin Fuchthauer (Aarhus University, Denmark). The riboprobes were labeled using a digoxigenin RNA labeling kit from Roche Diagnostics, Germany. Whole-mount in situ hybridization was performed as described previously [40].

TUNEL Assay

The TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) assay for detection of apoptotic cells was performed on paraffin sections. Tissue sections were treated with 0.5% pepsin in Aqua, HCl, at 37°C. For positive controls bovine pancreas DNase I treatment was performed for 30 minutes at 37°C. All sections were quenched of endogenous peroxidase in 3% hydrogen peroxide in methanol for 5 min.

For TdT labeling the sections were incubated for 10 min in TdT buffer and then incubated in labeling mixture for one hour at 37°C. The sections were incubated with Streptavidin-HRP for 30 minutes at room temperature. For AEC (3-amino-9-ethylcarbazole) reaction one AEC substrate reagent was prepared (4 ml deionized water, 2 drops Acetate Buffer, 1 drop AEC Chromogen, Sigma, 1 drop 3% hydrogen peroxide). Two drops of this substrate reagent were applied on each slide, for 10 minutes.

Bead Implantation

Heparin-coated acrylic beads were used, approximately 40–80 μm in diameter (Sigma, Germany). Before being transferred into the BMP2 protein solution, the beads were rinsed 2–3 times for a few hours in PBS solution.

After the washing steps in PBS, the beads were further soaked in BMP2 protein solution (100 μg/ml). For obtaining different concentrations, the proteins were diluted in PBS+0.1% bovine serum albumin (BSA). BMP2 protein was obtained from the Genetics Institute, Cambridge, MA, USA.

HE (Hematoxylin-Eosin) Staining

Paraffin sections of Wnt11 mutant mice and wild-type mice were first deparaffinized. The sections were washed twice for 5 minutes in Rotihistol and then twice for 1 minute in 100% ethanol, followed by short washing steps of 30–45” in 95%, 90%, 80%, 70%, 50%, and 30% ethanol. Finally, a short wash in deH2O was followed by a 3–4 minutes wash in Hemalaun and 10 minutes wash in running water. 1.5 minutes in 0.1% eosin in 70% ethanol were followed by short washes in 70%, 80%, 90%, 90x100% and 2x rothilid. The slides were embedded in Entellan and then covered with cover slips.

Wnt11 Knock-out Mice and Dermis Volume

Wnt11 knock-out mice were created by using a targeting construct containing 4.8 kb of 5’ and 4.0 kb of 3’ homology regions, which was transfected into 5x10 7 R1 ES cells derived from 129 Sv strain [41] using a BioRad pulser.

Wnt11 homozygous mutant mice (Wnt11−/−) were identified by Southern blot and genomic PCR analyses. The absence of the exons IV and V in the targeted allele was confirmed by Southern blot analysis [30].

The volume of the dermis of E18.5 in wild-type and Wnt11 knock-out mouse embryos was analysed in paraffine sections after HE staining. We have used rectangular shaped dermis cross-section samples with 9 μm length and 20 μm withth (thickness of the cross-sections). The height of the dermis was taken at 3 different locations (measuring the thickest, thinnest and medium points) on each cross-section and the average of these measurements was used to calculate the volume of the dermis in each slide (volume of dermis = 9×20×average height of dermis). For wild-type and Wnt11 knock-out mice we have used for each 10 slides representing 3 mice embryos.

The average of dermis volume for wild-type and Wnt11 knock-out mice embryos were plotted as a graph (Figure 1). The standard error was used for the error bar. The photos were taken at 40× magnification.

qPCR

Mice dorsal skin samples derived from embryos of E12.5 and E16.5 were dissected and collected in screw cap tubes. Each tube contained a tissue quantity from which we could extract around 5
microgram of mRNA (approx. 500 mg of tissue). The dissected tissue was frozen immediately in liquid nitrogen.

mRNA from frozen tissue was extracted with Trizol (Invitrogen) and reversely transcribed with Quanti reverse transcription kit (Qiagen). For the qPCR, specific primer pairs (Table S2) for each analyzed gene were ordered from Invitrogen. 18s rRNA was used as reference gene. SYBR green qPCR master mix (Applied Biosystems) was used for the PCR reaction. Subsequent qPCR was carried out with DNA engine Opticon 2 Real-Time PCR Detection system (Biorad). The experiments were conducted with three independent sets of samples. Each PCR reaction was performed in triplicate. The threshold cycle (Ct) was acquired at the point where the fluorescence signal reached a value of 0.01. The result was presented as relative expression ratio (R) formulated as follows,

\[
R = \frac{2^{(Ct_{\text{reference}} - Ct_{\text{target}})}}{2^{(Ct_{\text{reference}} - Ct_{\text{target}})}}_{\text{sample}} \frac{2^{(Ct_{\text{reference}} - Ct_{\text{target}})}}{2^{(Ct_{\text{reference}} - Ct_{\text{target}})}}_{\text{control}}
\]

The data was introduced in REST software [42] for the calculation with the Pair Wise Fixed Reallocation Randomisation Test [43].

Live-cell Imaging

Slices of chicken embryos (HH20) at the DML region were prepared under visual control with a binocular microscope. Specimens were aseptically cut into 175 μm thick transverse slices on a McIlwain tissue chopper, and then attached to collagen-faced (Sigma-Aldrich, C7661) glass coverslips (32 mm, Kindler, Freiburg, Germany) fixed by a plasma clot (Sigma, P3266) coagulated with thrombin (Calbiochem, 605157).

Time-lapse imaging was performed by the aid of confocal laser scanning microscopy (CLSM, Zeiss LSM 510) and
10x-Apochromate lens (Plan-Neofluar, NA 0.3) equipped with a laser module containing an Ar laser (488 nm) and a HeNe laser (543 nm). To maintain the incubation settings at 37°C and 5% CO₂ on the microscope stage, a CTI controller 3700 digital, O₂ controller, Temp control 37-2 digital, and the Incubator SO₂ oxygen together with the heating insert P (Zeiss) are used. Typically, live-cell imaging of DML cell dynamics starts 24 hours after electroporation. Therefore coverslips are placed in a Rose chamber and covered with 2 ml phenol red-free nutrient medium to get best results in terms of brightness, contrast, acuity and resolution. To reduce phototoxicity of the cells and photobleaching of the FP-signal the excitation intensity was reduced to a minimum (488 nm, 2%, 543 nm, 11%) and images were taken every 10 minutes for a period of up to 10 hours [44].

Results

Wnt11 Expressing DML is a Source of Dorsal Dermis Precursors

In the chicken embryo, local electroporation can be employed to perform gain and loss of function studies by either overexpressing or silencing genes of interest, respectively. This method is very convenient for spatially restricted manipulations of somatic cells, making the study of different developmental processes possible. In our work, we transfected the DML of embryos at stage HH14-17 with three different Wnt11 RNAi producing plasmids and with scrambled DNA for control experiments and analysed changes in the development of the DML, the dermomyotome and its derivatives.

To confirm the efficacy of our in ovo electroporation protocol, we electroporated the DML of chicken embryos using an EGFP-containing control plasmid, reincubated and followed up the development of electroporated embryos. 24 hours later, EGFP was detectable in the DML (Figure 2 A) and after 3.5 days of reincubation following electroporation (HH29), EGFP positive cells were detected in the myotome, as already described [32]. Additionally, EGFP expressing cells were located above the neural tube where the future dorsal dermis will form (3 out of 5 embryos) (Figure 2 B–C). This was very clearly visible in our time-lapse experiments, when the EGFP-positive cells could be followed in their migration towards subectodermal space above the neural tube (seen in 5 out of 5 movies) (Figure 3/Movies S1 and S2). In another set of experiments, we silenced Wnt11 gene expression in the DML by electroporation of shRNA-EGFP constructs directed against Wnt11 mRNA (Figure 2 D). The expression of shRNA from these constructs was driven by either an H1 or a U6 promoter. Four Wnt11 gene targeting constructs were individually tested for their efficiency and then used in a cocktail (see Material and Methods). This was followed by tracing the fate of EGFP-transfected cells while the silencing effect of the Wnt11 RNAi vector was assessed by ISH analysis of the transfected embryos with mRNA probe specific for avian Wnt11. An effective silencing of the Wnt11 gene expression was seen in the transfected region indicated by the EGFP signal (15 out of 20 embryos) (Figure 2 G–H). In the transfected embryos with Wnt11 RNAi, after 3.5 days of reincubation time following electroporation (HH29), no EGFP-transfected cells were detected in the subectodermal region, however, many EGFP transfected myofibbers were detected in the myotome. This was validated by our time-lapse experiments, when no EGFP-positive cells were observed to move towards subectodermal space overlying the spinal cord as usual. The migration into the myotome was unaffected (seen in 3 out of 3 movies) (Figure 4/Movies S3 and S4). The time-lapse experiment has been performed using two different constructs to interfere with Wnt11, Wnt11 RNAi (Movie S3) and a DN-Wnt11 RCAS construct (Movie S4). As already shown by Gros and colleagues in 2009, we also noticed the disorganized arrangement of the myofibers after interfering with Wnt11 signaling (11 out of 11) (Figure 2 E). This confirms the efficiency of our RNAi constructs when compared to a previously described phenotype [27].

No increase in cell death was detected at the site of Wnt11 RNAi transfection in comparison with the control slides, evidenced by the TUNEL assay (Figure 2 I–K).

We can thus conclude that the DML does not only give rise to the myotome, but also to the subectodermal mesenchyme overlying the neural tube and that silencing Wnt11 leads to an altered EMT of a subpopulation of DML-derived cells, resulting in an absence of subectodermal cells originating from the DML.

Wnt11 is Required for Dense Dorsal Dermis

Once the silencing capability of our Wnt11 shRNA constructs was established, we moved on to study the effects of Wnt11 silencing on known dermal markers. The expression pattern of cDermo-1 is noticeable from stage HH24 at low levels throughout the subectodermal mesenchyme of the trunk, being much stronger along the dorsal midline in the mesenchyme between dorsal ectoderm and neural tube. The expression intensifies at stage HH26 and is maintained until stage HH29. In older stages, the expression is strong in the mesenchyme of the nascent feather buds, underneath the ectodermal placodes. At stage HH36 the expression gets restricted to the mesodermal core of the feather buds [23].

In order to study the effects of Wnt11 silencing on dermis and skin appendage development, the electroporated embryos were allowed to develop at least until the stage when the expression of the dermal marker cDermo-1 starts or later to embryonic day 6 (HH29) when feather buds start to form. After 3.5 days of reincubation time following RNAi treatment, in situ hybridisation shows a significant downregulation of cDermo-1 in the dorsal midline of the back (4 out of 5 embryos) (Figure 5 A–C). After a longer reincubation period following electroporation (when the embryos reached the stage HH31), we could observe the delay in formation of the first rows of feather buds in the manipulated area (3 out of 3 embryos) (Figure 5 D–F).

The retarded growth of feather buds was also demonstrated by analyzing older embryos (12 days), which were hybridized with an additional feather bud marker gene, Shh. This marker revealed the presence of much smaller and more flattened feather buds, expressing less Shh on the manipulated side with Wnt11 RNAi (3 out of 3 embryos) (Figure 5 G–H).

These results support our hypothesis that Wnt11 plays a role in dermis formation. Wnt11 silencing leads to a reduction of dense dermis, resulting in a reduced cDermo-1 expression and also in a retarded and compromised formation of feather buds.

Central and Lateral Dermomyotomal Identities are Maintained Following Wnt11 Silencing in the DML

The dorso-medial lip and the central part of the dermomyotome are able to give rise to dense dermis competent for dermal condensations which will later bear feathers, while the lateral part is responsible for sparse dermis formation, characteristic of aperic regions. It is also known that while the dorsomedial lip of the dermomyotome expresses Wnt11, the central and the lateral compartments can be identified by their expression of two transcription factors, En-1 and Sim1, respectively.

To check if these compartments are affected following silencing of Wnt11 in the DML, we performed ISH for En-1 and Sim1 on Wnt11 RNAi-treated embryos. None of the electroporated
The results revealed that the silencing of the Wnt11 gene in the DML did not affect other parts of the dermomyotome. This is probably due to the fact that silencing of Wnt11 in the DML affects only the dorsal dermis, whereas the central and the lateral regions of the dermomyotome and the tissues that arise from it remain unaffected by this manipulation and develop independently of the DML.

**Figure 2. Wnt11 expression in the DML is important for recruitment of dorsal dermal progenitors.** A. EGFP-transfected DML of chicken embryo at stage HH18-19, 20 hours after electroporation. B. The embryo in A after 3 days of reincubation following electroporation. Note the presence of EGFP positive cells in the myotome and region of the future dorsal dermis (dotted circles). The white dotted lines squares delineate the somites. C. Cross-section of the embryo in B. EGFP-positive cells can be seen to be migrating into the subectodermal space overlying the spinal cord (white arrows). D. Wnt11 RNAi on chicken embryo. DML of EGFP-Wnt11 RNAi construct transfected chicken embryos at HH18-19, 20 hours after electroporation. E. After 3 days of reincubation following electroporation, the EGFP-Wnt11 RNAi expressing cells are only to be found in the myotome in a disorganized manner, whereas EGFP-Wnt11 RNAi positive cells are missing in the dorsal dermis anlage. F. In cross-section of the embryo in E, only very few cells (nearly undetectable) migrating from DML are present into the subectodermal space (white line), White lines were traced along the neural tube for a better orientation. G. Targeting of the DML at stage HH14-17 by EGFP-Wnt11 RNAi constructs after 24 hours reincubation and the corresponding Wnt11 silencing as seen by ISH (area between black arrows in H). I. There is no evidence of increased cell death at the sites of EGFP-Wnt11 construct transfection as seen by TUNEL staining. J. TUNEL staining of an electroporated embryo with scrambled DNA was used as control for our TUNEL assay. K. Represents the untreated control (Control 2) for TUNEL assay. Photos in I, J, K show the DML of the corresponding sections after TUNEL assay. Black arrows in I, J, K point towards Tunel-positive cells. NT: neural tube. DML: dorso-medial lip. Scale bar: 100 μm. doi:10.1371/journal.pone.0092679.g002

Effect of Wnt11 Silencing on Genes Expressed in Dermomyotome

Paraxis [45] and Pax3 [46], [47] are further transcription factors expressed in the dermomyotome [48]. Pax3 is a progenitor cell marker for the entire premyogenic and myogenic lineage and also plays a role in maintaining the dermomyotomal cells in an uncommitted state. To assess if Wnt11 silencing has an effect on the epithelial state and commitment of the dermomyotome, we looked for possible alterations in expression of Pax3 and Paraxis after electroporation. ISH for Paraxis did not reveal any alterations in its transcript distribution (40 embryos out of 40) (Figure 6 E, F). In contrast, we observed a strong upregulation of Pax3 (Figure 6 G, H), strictly corresponding to the EGFP signal in the electroporated embryo. Section through one of the embryo hybridized with Pax3
probe shows a strong upregulation of this gene in the DML, but no changes in the structure of the dermomyotome (Figure 6 K). Electroporation of scrambled shRNA constructs affected neither Pax3 nor Paraxis expression. These results show that the cells which would normally adopt a dermogenic fate remain uncommitted, as shown by the strong upregulation of Pax3 (25 embryos out of 25). This is an indication that DML cells require Wnt11 for unfolding their future fate as dermogenic cells.

Snail1 has been described in context of deepithelialisation. Loss-of-function studies for Snail1 in chicken embryos resulted in delayed EMT with the maintenance of the epithelial structure of the dermomyotome [29]. Following initiation of EMT in the dermomyotome, the Snail1 positive cells can be detected in the myotome, dermomyotome, sclerotome as well as in the subectodermal space above the neural tube (dermogenic progenitor cells). We analysed the effect of Wnt11 RNAi on the expression of Snail1 in the DML following electroporation. A remarkable decrease in the Snail1 expression was observed after Wnt11 silencing in the DML of the dermomyotome (8 out of 9 embryos)
Wnt11 in Dermis Development

Previous findings in our group have shown that BMPs promote dermogenesis [25]. Implantation of beads soaked in different concentrations of purified BMP2 protein in chicken embryos of stage HH13-17 upregulates dDerma-1 expression. On the other hand, BMP-antagonist Noggin is able to downregulate dDerma-1 when applied at appropriate stages of dermal development. To test our hypothesis concerning the role of Wnt11 during dermis development, we were interested to see if Wnt11 expression would also respond to dermogenic and anti-dermogenic signals in a similar pattern (Figure 7). Using BMP2-coated beads, we could show that the Wnt11 expression domain could be broadened (5 out of 6 embryos), while Noggin, a BMP inhibitor, was able to downregulate Wnt11 expression in the somites (6 out of 6 embryos) (Figure 7 A–D). These experiments reveal that Wnt11 is regulated similarly to cDermo-1, as both respond to BMPs and Noggin in a similar fashion thus supporting our hypothesis that Wnt11 plays a role in dermis development.

Compromised Hair Follicles and Dermis in Knock-out Mice for Wnt11

To validate our loss-of-function and gain-of-function findings for Wnt11 from chicken embryos, we went on to look for alterations also in the dermis in Wnt11 knock-out mice [30]. In situ hybridizations performed for Twist2 (mDermo-1) on Wnt11 mutants of E14.5 revealed a large area in the dorsal midline region of homozygous mutants devoid of hair placodes in comparison to the wild-type embryos of the same developmental stage (Figure 8 A–B). The hair placodes on the back of the hybridized embryos were counted in identical squares drawn on photos obtained using standardized magnification parameters. We could document a decrease of approximately 35% in hair follicles number in homozygous mutant embryos as compared to the wild-type littermates (6 embryos analyzed each for homozygous and wild-type) (Figure 8 A–B). As a graph of hair follicles number comparison between Wnt11 mutants and wild-type mice is presented in Figure 1. By inspection of vibratome sections of these embryos that had previously undergone whole mount ISH, a decrease in the depth of the Twist2 (mDermo-1) expression domain of the mutants in comparison to the wild-type embryos became evident (Figure 8 C–D).

Additionally, the skin from wild-type mice and homozygous mutants of Wnt11 of day E 18.5 was compared in paraffin sections. Hematoxylin-Eosin staining of these sections revealed a noticeable decrease in size of the dermis layer in Wnt11 homozygous mice compared with their wild-type littermates. Upon quantification, we observed a significant decrease in dermal volume of about 35% (Figure 8 F, Figure 1) associated with a reduced number of hair follicles (35% decrease) in the trunk region of homozygous Wnt11 mutants in comparison to the wild-type embryos (10 slides representing 3 mice embryos were analysed for each genotype) (Figure 8 E, Figure 1).

Expression Analysis of Knock-out Mice for Wnt11 by qPCR

Further quantitative analyses of the expression of regulators of dermis development were performed using qPCR (Real-Time PCR).

An early marker of dorsal dermogenesis has been considered to be the homeobox gene Msx1 which at the same time also stains cartilage precursor cells of the vertebral spinoar process in chicken embryos, although at earlier stages [49]. Msx1 has been shown to control the process of differentiation in the dorsal-most dermis lying above the neural tube. During their migration from the DML towards the subectodermal space, precursor cells of the dorsal dermis express Msx1 [12]. At stage E12.5 when Msx1 is expressed in the subectodermal space (the place where in the chicken dDerma-1 is expressed in a similar developmental stage) above the neural tube, we found that Msx1 expression was considerably less (P<0.001) in the Wnt11 mutant mice than in the wild-type animals (Figure 9). At this stage, mDermo-1 expression has not yet started medially in the space above the neural tube. Therefore the downregulation of Msx1 at E12.5 in the mouse is comparable with the downregulation of dDerma-1 at day 6.5 of expression.
Figure 5. Decrease of dermal markers after Wnt11 RNAi. A. Embryo showing the region of transfection after electroporation with EGFP-Wnt11 RNAi. The embryo has been electroporated at stage HH14-17 and reincubated for 20 hours. B. The embryo in A after 2.5 days reincubation time following manipulation (HH28). Starting with stage 24, cDermo-1 expression pattern can be found in the subectodermal mesenchyme of the trunk, and at stage 26 it is very strong along the dorsal midline. Following additional 2.5 days of reincubation after EGFP signal documentation for embryo in A, cDermo-1 is remarkably reduced along the dorsal midline in the transfected region (area between white arrows in B). C. Higher magnification of the embryo in B. D. Electroporated embryo with Wnt11 silencing construct at stage HH14-17 and photographed 20 hours later, at stage HH20. E. After longer reincubation periods following electroporation (4.5 days of the embryo in D), the embryo shows a retarded feather bud development as seen after cDermo-1 ISH, which is expressed in this stage (HH31) in the mesenchyme of the nascent feather buds (the white arrowheads in E and F mark the missing row of feather buds on the manipulated side in D). F. Higher magnification of the embryo in E. G. Embryo showing the region of transfection after electroporation with EGFP-Wnt11 RNAi at stage HH14-17 and photographed 24 hours later, at stage HH20. H. After 8 days reincubation for embryo in G after EGFP documentation (HH38) and hybridisation with a Shh probe we have noticed a retarded feather bud development as seen with cDermo-1. In situ hybridisation for Shh also reveals a changed morphology of the eventually formed feather buds, which are smaller and more flattened, with lower expression of Shh on the manipulated side (white arrow and arrowheads in H). The white square presents in a higher magnification the altered feather buds formation (yellow arrow). Scale bar: 100 μm. doi:10.1371/journal.pone.0092679.g005
Embryonic development in the chicken embryo. Unchanged expression levels of mDermo-1 were not surprising, considering that the source of the analysed mice skin pieces was the most dorsal (medial) back where expression of mDermo-1 had not yet started.

In stage E16.5, skin samples processed for qPCR showed a complete absence of Wnt11 expression in the Wnt11 knock-out mice. Compared to the wild-type animals, Fgf10 also showed a lower expression in homozygous mice. Fgf10 is expressed in the dermis, with a role in mesenchymal-epithelial signaling during skin development [50]. In the chicken, Fgf10 is expressed in the dermis of nascent feather primordia [51]. and Dermo-1 acts upstream of this early dermal marker [24]. The transcription factor Twist2 (mDermo-1) is expressed in dermis during mouse embryonic development [22] and acts redundantly with Twist1 [52]. There was no significant change of expression levels for Twist1 and Twist2 (mDermo-1) comparing the knock-out and wild-type mice. At E16.5, we also used the dorsal dermis from the midline of the trunk region. At this time, the expression domain of Twist2 (mDermo-1), has already spread dorsally. However, when performing the qPCR, we normalized the expression analysis for the amount of tissue. As shown in Figure 8C and D, there are fewer cells in the dorsal domain, so the expression levels of Twist2 were not reduced, but the number of cells expressing Twist2 (mDermo-1) were. However, for induction of the skin appendages the density of dermis is critical.

No change was observed also in the case of Krt5 between knock-out and wild-type mice. Krt5 is a type II cytokeratin produced in mitotically active keratinocytes, in the basal layer of the epidermis, that maintains the proliferation potential of this layer [53].

**Discussion**

Dorsal skin morphogenesis can be divided into three successive phases during embryogenesis. In the first phase, the dermogenic progenitors delaminate from the dermomyotome in an epithelial-to-mesenchymal transition (EMT) and migrate under the ectoderm to form dense dermis. This initial event is followed by a cross-talk between the dermal condensations and the overlying epidermis leading to the formation of skin appendage primordia (epidermal placodes) and the final and third phase consists of the organogenesis of the appendages. Although several publications have arisen over recent years aiming to dissect the mechanisms

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Figure 6. Effects of Wnt11 silencing on dermomyotome. A, C, E, G and I. Chicken embryos electroporated with Wnt11 RNAi at stage HH14-17 and after a reincubation period of 24 hours. B. The embryo in A (HH20) hybridized for En-1, shows that the central dermomyotomal compartment marked with En-1 was unaffected in the electroporated area (the space between white arrows in B). D. The photo represents the embryo in C after ISH for Sim-1 probe. The lateral dermomyotomal compartment marked with Sim-1 (white arrows in D indicate the electroporated area) did not show any change in its expression after Wnt11 silencing. F.ISH for Paraxis of the embryo presented in photo E. Paraxis transcripts seems not to be altered following Wnt11 silencing in the lateral dermomyotomal compartment (area between white arrows in F). H. The dermomyotomal marker Pax3, in contrast, is significantly upregulated at the site of Wnt11 RNAi transfection (area between white arrows in H). K. The cross-section through the embryo in H in the manipulated area shows a strong upregulation of Pax3 in the DML, while the DM remains normal when compared to the control side. L. Electroporated embryo at stage HH14-17 with Wnt11 RNAi and after 24 hours reincubation (HH19). J. Hybridized embryo from photo I for Snail1 probe. At stage HH20 the EMT has already started and the Snail1 expression can be seen in the myotome, dermomyotome, sclerotome and in the space above the neural tube (dermal progenitor cells). Whole-mount ISH of the embryo electroporated with Wnt11 RNAi shows a decreased Snail1 expression in the DML (area between white arrows in H). Scale bar: 100 μm.

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Figure 7. BMP2 signaling controls Wnt11 expression in DML. A, B. BMP2 beads implanted into somites of chicken embryos (black arrow in A) lead to an upregulation of Wnt11 as seen by ISH in vibratome sections (black arrow in B; section level indicated by red line in A). C, D. In contrast, grafting of Noggin cells (BMP antagonist) lead to a downregulation of Wnt11 transcripts on the operated side (C) also visible in vibratome sections of the same embryo (black arrow in D; section level indicated by red line in C). Scale bar: 100 μm.
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regulating the second and third phase of skin morphogenesis [16], [54], [55], little is known about the signaling and the molecular players involved in the initial phase of dermis development. In our study, we address the first phase of dermogenesis and only use the formation of skin appendages as a readout for the successful formation of dense dermis.

In avians, the dorsal dermis is derived from the medial part of the somitic dermomyotomes which is responsible for the formation of the dense dermis competent for feather bud induction, while the lateral compartment is the source of sparse dermis specific for aperic regions [16]. Based on its embryonic expression (Figure S1) in the dorsomedial lip of the dermomyotome and subectodermal mesenchyme, Wnt11 was proposed as a candidate gene involved in the formation of dermis [26]. At the same time, Wnt11 has been extensively linked to morphogenetic movements such as cell migration [56] and convergent-extension movements [57] during development. Although Wnt11 is not restricted to the dorsal dermis progenitor cells, but throughout the nascent dermis, the

Figure 8. Dorsal dermis is decreased in dermis thickness and number of hair follicles in the Wnt11 knock-out mice. A, C, E. Homozygous Wnt11 knock-out embryos. B, D, F. Wild-type embryos. Embryo in A represents a homozygous Wnt11 knock-out embryo of embryonic day E 14.5. In the dorsal mid-line region of this embryo a large area devoid of hair placodes is detectable. The white square shows a decrease number of hair placodes in comparison to the WT embryo of similar developmental stage (B). Section through the embryo in A shows that the cDermo-1 signal is diminished with corresponding decrease in dermal thickness in the mutant (black arrow in C) as compared with a similar section of the same magnification of a WT embryo (black arrow in D). Hematoxylin-Eosin staining in paraffin sections of knock-out mice of E 18.5 shows a remarkable decrease in dermis thickness (45%), as well as a reduced number of hair follicles (35%). (E) In comparison with a Hematoxylin-Eosin treated section of a WT mouse of the same developmental stage (F). Double-headed arrows in E and F show the remarkable difference in dermis thickness between the mutant mouse embryo as compared to the normal thickness of dermis in a wild-type embryo (photographs were taken at the same magnification). The sections were taken at the trunk regions of the mice. The analysis was restricted to the same anatomical area of the dorsal skin for all embryos investigated. Scale bar: 100 μm.

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avian dermomyotome represents a region of special interest which lends itself readily to the electroporation. In our study, we found that knocking-down Wnt11 affects the patterning behaviour of somite-derived dermal progenitor cells leading to a reduced expression of the early dermal marker cDermo-1 [23].

We could furthermore show that Wnt11 is also regulated in a manner similar to cDermo-1. Our results obtained in chicken embryos are corroborated by our analysis of murine Wnt11 mutants which show thinner dermis than their wild-type littermates, and impaired hair follicle development in the trunk region. In combination with previous studies, our results pinpoint the role of Wnt11 in dermis development, in particular that of dense dermis.

The origin of the dorsal dermis directly above the neural tube in mouse and chicken has been a matter of controversy as its origin was initially proposed to lie in the sclerotome as well as in the dermomyotome [15]. This idea arose from the fact that progenitor cells were shown, these processes being temporally separated. The dermomyotomal cells will deepiphteliazie and migrate towards the dorsal neural tube to form dermis and neural arches and the dermomyotomal origin of the dorsal region. In combination with previous studies, our results pinpoint the role of Wnt11 in dermis development, in particular that of dense dermis.

The earliest known marker of dermal precursors, cDermo-1 has been shown to be expressed from HH24 onwards in the subectodermal space of the dorsal midline region of chicken embryos [23]. It was later demonstrated that BMPs influence the expression of cDermo-1 in a positive manner promoting dense dermis formation and epidermal appendage formation in a distinct

Figure 9. Relative expression of dorsal skin development-related genes in Wnt11 knock-out mice and wild-type. A. At stage E12.5, Msx1 shows much less expression in mutant mice than wild-type mice. (***P<0.001). B. At stage E16.5, Wnt11 expression in Wnt11 knock-out mice is undetectable compared to wild-type mice. Fgf10 has less expression in the homozygous mice. Twist1, Twist2 and Krt5 expression does not show significant differences between wild-type and homozygous animals. doi:10.1371/journal.pone.0092679.g009

and we can confirm these findings by our experiments. Employing cell lineage tracing techniques with electroporation of EGFP reporter plasmid into the DML, we show here that the marked EGFP-expressing cells not only move ventrally to enter the myotome, but also delaminate and migrate dorsally above the neural tube under the ectoderm in the dorsal dermis anlage. Sclerotomal cells expressing EGFP were never found in our cell tracing experiments indicating that only the dermomyotomes were targeted. Thus using a different cell labelling technique and live imaging, we show that the DML is a source of dorsal dermal precursors. Cells from the DML delaminate and migrate to the subectodermal region above the dorsal neural tube to form the future dense dermis capable of skin appendage induction. This technique has revealed the presence of dorsal dermis precursors precisely from the DML using live-imaging for the first time to our knowledge.

The dorsomedial lip (DML) has been described to contain a complex population of multipotent stem/progenitor cells [58]. RNAi silencing of Wnt11 in the DML prevented the cells from migrating from the DML towards the subectodermal space. In contrast to the dermogenic lineage, the silencing effect of Wnt11 in the DML does not appear to quantitatively affect the migration of myogenic precursors into the myotome. We regularly find in our experiments EGFP expressing myoblasts and myofibers in the myotome following Wnt11 shRNA treatment in comparable numbers to the normal situation, as has previously been reported by others [27]. This is in accordance with data showing that although myotome cells do form after Wnt11 silencing, their arrangement is altered [27] which served in our study as a positive control for the efficacy of our silencing constructs.

The earliest known marker of dermal precursors, cDermo-1 has been shown to be expressed from HH24 onwards in the subectodermal space of the dorsal midline region of chicken embryos [23]. It was later demonstrated that BMPs influence the expression of cDermo-1 in a positive manner promoting dense dermis formation and epidermal appendage formation in a distinct
and time-dependent manner [23]. The application of exogenous BMP proteins leads to local upregulation of cDermo-1, while the BMP antagonist Noggin results in a downregulation of cDermo-1 transcripts and a lack of dermal tissue [24]. Based on the hypothesis that Wnt11 may play a role in dermis development, we tested the effect of BMPs and Noggin on Wnt11 expression. The parallel effects observed on Wnt11 and cDermo-1 expression following application of BMP and Noggin further strengthen the hypothesis that Wnt11 plays a role in dermogenesis.

We observed a loss of cDermo-1 following Wnt11 RNAi in the region where dorsal spinal pterygae are known to develop. Silencing of Wnt11 in the DML did not affect more laterally located regions of the dermomyotome also known to contribute to dermis formation. This was reflected by an unchanged expression pattern of E-cad and Sim1 following Wnt11 silencing in the DML, implying that Wnt11 does not influence the formation of these dermomyotomal compartments and showing that the derivatives of the dermomyotome maintain a topographical relationship with their epithelial ascendants [4].

In our experiments, silencing of Wnt11 in the DML did not alter the state of the already epithelial dermomyotome as reflected by the unaltered Paraxis expression and size of the dermomyotome. Paraxis is known to maintain the epithelialisation of the dermomyotome [45]. Another transcription factor, Pax3, known to keep the dermomyotomal cells in an undifferentiated state, is upregulated after RNAi of Wnt11.

The dorsomedial lip of the dermomyotome not only provides post-mitotic myoblasts for the myotome, but also contributes to the medio-lateral growth of the dermomyotome [59]. It has been reported earlier that Wnt11 acts as an epithelialization factor for the medial dermomyotomal lip [17]. Our results, obtained by a loss-of-function approach for Wnt11, do not support this idea. Dermomyotomes were normal in appearance and size in all of our experiments. Moreover, we even observed a decrease in the deep epithelialization marker Snail1 following Wnt11 RNAi. The release of dermal and myogenic progenitors from the dermomyotome is preceded by an epithelio-mesenchymal transition (EMT) event that ensures a timely and ordered allocation of these cells into their respective topographical destinations. Our data are more consistent with the observation that after Wnt11 RNAi, the EMT marker Snail1 was downregulated. This would indicate that the processes in which this gene is involved were disturbed, and as a consequence, the cells are not realizing their native fate decision, as shown by the strong upregulation of Pax3 in the DML. We could easily visualize the process by using live-cell imaging, where the distribution of the DML cells in the dermal and myogenic compartments are observed as expected using control constructs. FGFR signaling from the nascent myotome has been shown to act via the MAPK/ERK pathways leading to the activation of the downstream transcription factor Snail1 in the dermomyotome [29]. The Snail family of genes controls EMTs in many cellular contexts both during embryonic development as well as in cancer metastasis [60], [61]. Ectopic expression studies of Snail in the chick hindbrain show that the induction of EMT leading to neural crest formation is a conserved role associated with the Snail family of zinc-finger transcription factors [62]. In the early chicken embryo, Snail1, and not Snail2 is expressed in the somites [63]. These findings suggest that Wnt11 functions in the opposite way to the previously reported one in the dermomyotome, as we observed that the absence of Wnt11 rather serves for the maintenance of the epithelial dermomyotome by a decrease in the EMT as evidenced by the downregulation of Snail1 in our study. Live-cell imaging performed on sections of electroporated chicken embryos, treated with a construct for Wnt11 silencing or treated with a DN-Wnt11-construct, shows a restriction in the distribution of the cells from DML and their absence from the subectodermal space above the neural tube. The migration into the myotome where cells keep close contact to the dermomyotome was not affected. Thus, it appears that dermis progenitor cells cannot undergo EMT from the dermomyotome.

Wnt11 has previously been reported to be involved in the cell shape changes associated with convergent extension movements during embryonic development [57], [64]. Furthermore, acting via the evolutionary conserved PCP pathway, Wnt11 ensures the proper orientation of the early myoblasts in the myotome [27]. Wnt11 is an orthologue of Wnt11-R in Xenopus. It is known from Wnt11-R mutants that Wnt11 is required for somitic precursor migration towards the dorsal fin [56]. This dorsally directed migration in anamniotes may have set the grounds for the formation of dorsal dermis in amniotes during evolution, thus employing the same set of molecules in new contexts. It appears that the observed effects on Wnt11 silencing can be attributed to an endogenous role of Wnt11 in an autocrine/paracrine mechanism in the silenced cell population in the dermomyotome as shown by our work here and other groups [27]. Taken together, these results confirm our hypothesis regarding the direct relationship between Wnt11 and dense dermis formation. Our analysis of Wnt11 knock-out mice supports our results in the chicken as we observed a significant decrease in the dorsal dermal volume and reduction in hairs in the mutants. Furthermore, our interpretation of the results is also supported by quantitative PCR showing that the dermally expressed genes Fgf10 and Msh1 are decreased in mutant embryos in comparison to the wild-type.

Although there appear to be differences between mouse and chicken regarding the control of mDermo-1 (Twist2), this may be partially attributable to the different experimental approaches in the two species. Experimental work in the chicken targets individual cells at a particular time point, while in the mouse, the system has a long time to adapt to the loss of Wnt11 and other genes or mechanisms may compensate for this. This is further supported by the fact that Msh1 is strongly downregulated in the mutant at E12.5 indicating that the migration from the DML is initially impeded also in the mouse. The cells that successfully make their way to the dermis do not downregulate mDermo-1, but there are fewer cells, which can be seen by ISH. However, it will be difficult to detect in qPCR, as this approach is normalized for the loaded amount of tissue. So, the number of cells expressing mDermo-1 were reduced, but the expression levels of each cells was not. However, for induction of the skin appendages the density of dermis is critical.

In summary, our results show that Wnt11 is required for the formation of dense dorsal dermis which is capable of forming cutaneous appendages. In the absence of Wnt11, dermal progenitor cells fail to acquire their fate and consequently fail to populate the subectodermal space overlying the neural tube. The downregulation of Snail1 in the dermomyotome after Wnt11 silencing argues for a compromised EMT at the DML. Successfully electroporated cells can however still be found in the myotome. Interfering with EMT leads to a decreased number of dermal precursors that can reach the subectodermal space resulting in a lower cell density of the dermis thereby diminishing their effectiveness to form feathers [13]. We suppose that these cells remain either in the DML in an undifferentiated state, as shown by strong upregulation of Pax3 in the absence of Wnt11 or enter by default into the myotome. Most EGFP-positive cells are found in these two locations. However, as none of these structures seem to have increased in size, an adjustment of proliferation could be simultaneously involved.
To conclude, our results show that Wnt11 is necessary for dermomyotomal cells in acquiring the dermogenic fate and migration towards the dermal anlage under the ectoderm. Thereby, we could experimentally substantiate the hypothesis based on its expression pattern that Wnt11 is essential for dermis development and effective cutaneous appendage formation.

Supporting Information

Figure S1 Expression of Wnt11. A. Whole-mount ISH for Wnt11 at stage HH18. E. Cross-section through the embryo in A. Expression of Wnt11 is limited to the dorso-medial lip of the dermomyotome (black arrowhead). C. Whole-mount ISH for Wnt11 at stage HH20. As the embryo develops, the expression domain extends more caudally, retaining its prominent expression cranially. F. In the cross-section of the embryo in B, we can observe a stronger expression in the dorso-medial lip (black arrow) and on top of the neural tube (red arrowhead). C. The whole-mount ISH for an HH22+ stage embryo shows a prominent expression of the Wnt11 transcripts, maintaining its cranio-caudal gradient. G. The cross-section through the embryo HH22+ reveals a broader area of the Wnt11 gene expression, including the area between the DML and neural tube, the anlage of the future dorsal dermis (black arrow) and above the neural tube (red arrowhead). Its expression pattern in the dorso-medial lip is also increased. D. Whole-mount expression of a stage HH24 embryo. The strong expression of the Wnt11 transcripts becomes obvious. H. The cross-section of the HH24 stage embryo hybridised for the Wnt11 gene shows clearly the large area of cells positive for Wnt11, including the subectodermal space (black arrow and black arrowhead) and also an increased expression on top of the neural tube (red arrowhead), which later on will form the dorsal dermis. All the sections showed in this figure were performed at the interlimb level. Scale bar: 100 μm. (TIF)

Table S1 Selected nucleotide sequences for silencing of Wnt11 mRNA. This table presents the four selected target sites. The first target site starts at +217 nt, the second target site starts at +326 nt, the third target site starts at +761 nt and the fourth target site starts at +1034 nt. The forward and reverse strands of the shRNA inserts were designed as presented in this table. (PDF)

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Table S2 Primers used for qPCR. Specific primer pairs used for qPCR of the genes analysed. 18sRNA was used as a reference gene. (PDF)

Movie S1 Time-lapse of control EGFP electroporated embryo 1. 175 μm thick slice of a HH20 embryo electroporated with a control EGFP plasmid (24 hours after transfection). The dynamic migration of the cells from the DML towards the subectodermal space and above the neural tube were observed. The migration was followed for 10 hours. (MP4)

Movie S2 Time-lapse of control EGFP electroporated embryo 2. Similar section as described in Movie S1 reconfirms the high number of the cells moving from the DML towards the subectodermal space and above the neural tube. (MPG)

Movie S3 Time-lapse of Wnt11 RNAi electroporated embryo. 175 μm thick slice of a HH20 embryo electroporated with Wnt11 RNAi plasmid (24 hours after transfection). Very few cells migrating towards the subectodermal space and above the neural tube were observed in comparison with the control time-lapse experiment. The migration of the EGFP –positive cells to the myotome is not affected by Wnt11 RNAi. (MP4)

Movie S4 Time-lapse of DN-Wnt11 RCAS electroporated embryo. 175 μm thick slice of a HH20 embryo electroporated with DN-Wnt11 RCAS construct (24 hours after transfection). Consistent with the Wnt11 RNAi result and contrary to the control EGFP electroporation, very few migratory cells towards the subectodermal space and above the neural tube were observed. The migration of the EGFP –positive cells to the myotome is not affected by DN Wnt11 plasmid. (MP4)

Author Contributions

Conceived and designed the experiments: GMP BBS FY. Performed the experiments: GMP ABR JC. Analyzed the data: GMP BBS FY GZ JC. Contributed reagents/materials/analysis tools: THWL AK. Wrote the paper: GMP BBS FY. Immunostaining and sample preparation: GMP MAK. Time-lapse imaging: CT. Supervision: BBS.
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