Unique Modulation of Cadherin Expression Pattern during Posterior Frontal Cranial Suture Development and Closure

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Key Words

Cranial Suture · Closure · *E-cadherin* · *N-cadherin* · Development · FGF-2 · Mouse

Abstract

Cranial suture development involves coordinated expression of multiple genes and tissue contribution from neural crest cells and paraxial mesoderm for timely sutural morphogenesis. Transcription factors, growth factors, and neural crest determinant genes play critical roles in calvarial growth ensuring normal development of the underlying brain. In vitro studies have implicated cell-cell adhesion molecules as a driving force behind suture closure. We performed cDNA microarray to study differential expression of adhesion molecules during the timing of suture closure in a mouse model where only the posterior frontal (PF) suture closes. Our results indicate increased expression of *E-cadherin* during the period of PF suture closure. Quantitative RT-PCR analysis of *E-* and *N-cadherin* in PF closing suture revealed a biphasic expression of *N-cadherin*, the first phase coinciding with cellular condensation preceding chondrogenesis followed by a second phase coinciding with *E-cadherin* co-expression and suture closure. Furthermore, expression analysis of the *N-cadherin* and *E-cadherin* transcriptional repressors *Wnt7a* and *Snail* indicate a specific

temporal regulation of these genes, suggesting their potential role as regulators of both *E-* and *N-cadherin* during the PF suture development and closure. Finally, given the in vitro evidence of fibroblast growth factor (FGF)-2 as a potential regulator of *E-* and *N-cadherin* we investigated the expression of *E-cadherin* during PF suture closure in *Fgf-2* deficient mice. In contrast to in vitro data previously reported, *E-cadherin* expression is normal in these animals, and PF suture closure occurs properly, probably due to potential redundancy of FGF ligands ensuring normal temporal expression of *E-cadherin* and PF suture closure.

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Abbreviations used in this paper

FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
PF	posterior frontal
qRT-PCR	quantitative reverse transcriptase polymerase
SAG	sagittal
SAM	significance analysis of microarray
SMA	Stanford microarray database
SSC	saline sodium citrate

Introduction

Cranial suture development is a complex process involving controlled regulation of multiple genes. Several genes, including transcription factors, growth factors, and their cognate receptors are implicated in this process [Wilkie, 1997; Opperman, 2000; Rice et al., 2003]. Dysregulation at any control level would potentially lead to premature suture fusion or craniosynostosis [Wilkie, 1997]. Mutations in several genes have been implicated in pathologic development of cranial sutures, including fibroblast growth factor receptors (FGFRs), transcription factors *MSX2* and *TWIST*, and fibrillin-1 (*FBN1*) [Bellus et al., 1996; Howard et al., 1997; Meyers et al., 1996; Muenke et al., 1994; Sood et al., 1996]. All of the above genes have been linked to osteoblast differentiation biology. How exactly mutations in these genes cause premature fusion of calvarial sutures has been a subject of intense study in the past decade. The overall picture of suture biology at the molecular level has become clearer in recent years using candidate gene approaches and microarray technology on cell cultures, organ cultures and in vivo sutures. Although growth and transcription factors have been clearly implicated in normal suture development, their downstream structural protein targets have not received much attention in this context.

The posterior frontal (PF) suture is of neural crest origin [Jiang et al., 2002], and is the only cranial suture which in mice closes during the second week of life [Sahar et al., 2005], while all other cranial sutures remain patent throughout life. The PF suture is endowed with a sophisticated architecture comprised of 2 layers of bone: the ectocranial and endocranial layer [Sahar et al., 2005]. We have previously demonstrated that the endocranial layer of PF suture closes through an endochondral ossification process, and *Sox9* gene is a key regulator in this process [Sahar et al., 2005].

Given the sophisticated PF suture architecture and previous in vitro studies demonstrating the importance of cell-cell adhesion molecules in chondrogenesis and osteogenesis, we hypothesized that the PF closure would involve cell-cell adhesion molecules. In our initial search for candidate genes of structural proteins involved in suture patterning and closing, we performed a comparative cDNA microarray analysis between mouse PF and sagittal (SAG) calvarial sutures to investigate differential gene expression in closing versus patent sutures, respectively. Our results indicated that among the genes encoding for cell-cell adhesion molecules that were differentially up-regulated in closing sutures, *E-cadherin* is one of the most prominent.

Cadherins are calcium dependent cell-cell adhesion molecules that give cells the ability to create form in aggregate [Yagi and Takeichi, 2000]. These adhesion molecules are involved in tissue differentiation and morphogenesis [Gumbiner, 1996; Vleminckx and Kemler, 1999]. Cell differentiation and tissue organization require a specific repertoire of cadherins that provide cues for cell specification and commitment to a certain lineage. The failure of *E-cadherin* null mice to form epithelia or a blastocyst cavity demonstrates its pivotal role in basic morphogenetic development [Larue et al., 1994]. Moreover, differential expression of cadherin family members is responsible for cell sorting, tissue differentiation and organogenesis during development [Yagi and Takeichi, 2000; Jamora et al., 2003]. In addition, it has been demonstrated that N-cadherin and OB-cadherin expression is important in osteoblast biology in vitro [Okazaki et al., 1994] and in vivo [Kawaguchi et al., 2001]. Classic cadherins are a potential effector downstream of the *Fgf-2/Msx2* pathway. Both *N-* and *E-cadherin* are expressed in normal human calvarial pre-osteoblasts and osteoblasts [Lemonnier et al., 1998]. Studies in cells harboring FGFR-2 S252W gain-of-function mutation, a cause of syndromic form of craniosynostosis in humans, have shown increased cell aggregation and up-regulation of *E-cadherin* [Lemonnier et al., 2001].

Here we investigate the temporal-spatial expression of *E-cadherin* and *N-cadherin* during the first month of life in CD-1 mice. Our results demonstrated that *E-cadherin* and *N-cadherin* gene expression is tightly regulated during PF suture patterning and closure. Specifically, *E-cadherin* expression is up-regulated during the timing of PF suture closure, while the *N-cadherin* gene expression profile is biphasic, with a first up-regulation phase preceding chondrogenesis, followed by a second up-regulation phase coinciding with the period of PF suture closure, as well as *E-cadherin* co-expression. Furthermore, gene expression analysis of the *N-cadherin* and *E-cadherin* transcriptional repressors *Wnt7a* and *Snail* [Tufan and Tuan, 2001; Battle et al., 2000; Cano et al., 2000; Hajra et al., 2002] indicate a specific temporal regulation of these genes, suggesting their potential role as regulators of both *E-* and *N-cadherin* in the context of PF suture development and closure.

Finally, given the in vitro evidence of FGF-2 as a potential regulator of *E-cadherin*, we analyzed *E-cadherin* expression in PF suture of *Fgf-2* null mice. Our results demonstrate normal *E-cadherin* expression and lack of abnormal phenotype in PF suture of *Fgf-2* deficient mice.

Materials and Methods

Animals

The animals were cared for in accordance with the guidelines of the institutional animal care and the use committee at Stanford University. CD-1 mice obtained from Charles River Laboratories (Wilmington, Mass., USA) were kept in a temperature- and moisture-controlled animal facility. Animals were sacrificed on a specific post-natal (p) day that was based on birth date (day 0). Time points included: p1, p3, p5, p6, p7, p8, p9, p10, p11, p12, p13, p14, p15, p16, p17 and p25. Mice heterozygous for the *Fgf2*^{tm1Doe} targeted-mutant allele were obtained from Jackson Laboratories (www.jax.org). The mouse strain of *Fgf-2* null is C57BL/6J. Homozygous mice were selected by Mendelian inheritance and genotyping was performed according to Jackson Laboratories' protocol (stock number 003256).

Tissue Harvest

Mice were sacrificed by CO₂ asphyxiation and the calvariae were dissected. PF and SAG sutures were meticulously harvested from CD-1 mice using Surgical Acuity™ loupes. After clearly identifying the lambdoidal, SAG, coronal, PF and anterior frontal sutures, the PF and SAG suture complexes inclusive of the suture mesenchyme, adjacent osteogenic fronts of the opposing calvarial growth plates, and the underlying dura mater were carefully dissected, separated and harvested using fine, slightly curved sharp tip scissors (www.finescience.com). Suture width after harvest measured 0.6–0.8 mm. The length of sutures depended on animal age. The bregma was excluded. The sutures were snap frozen for RNA extraction and stored at –80°C.

Tissue RNA Extraction

One litter (on average 8 animals) of CD-1 mice or *Fgf-2* null mice was used for each time point. Pooled sutures for each condition and time point were homogenized in 0.6 ml of Trizol (Invitrogen, Carlsbad, Calif., USA) using a Pellet Pestle Motor (Kontes). RNA was extracted and precipitated using chloroform and 2-propanol. RNA was further washed with DEPC treated 70% ethanol according to the manufacturer's protocol.

Tissue Processing and Staining

Following sacrifice of animals described above, the calvariae were harvested and immediately fixed in fresh, chilled 4% paraformaldehyde overnight at 4°C followed by 24–48 h of decalcification in Formical-2000 (Decal Chemical Company, Congers, N.Y., USA). The calvaria was divided coronally through the bregma to separate the PF from the SAG sutures. The specimen then underwent dehydration in a graded ethanol series and were paraffin embedded. The entire blocks were serially sectioned (5–7 µm) totaling to approximately 400 sections for each block. Every other slide was stained with routine hematoxylin and eosin for evaluation and the remaining slides were used for immunohistochemistry. The stained sutures were examined with Carl Zeiss Axioplan 2 (Zeiss, Thornwood, N.Y., USA) microscope. Images were captured by AxioVision (Zeiss) and combined by Adobe Photoshop (Adobe Systems, San Jose, Calif., USA). Fusion was defined as mature bony bridge between the osteogenic fronts.

cDNA Arrays

We utilized *Mus musculus* cDNA large-scale microarrays manufactured by the Stanford Functional Genomics Facility (www.microarray.org). Prior to use, slides were rehydrated in a hydration chamber for 20 min at room temperature followed by snap drying at 7°C. The poly-L-lysine surface was blocked by treatment with 0.05% succinic anhydride prepared in a buffer solution consisting of 350 ml of 1-methyl-2-pyrrolidinone and 15 ml of boric acid for 20 min on a shaker. The cDNAs on the slides were then denatured in distilled water for 2 min at 95°C followed by fixation with 95% ethanol. Forty micrograms of RNA per time point from PF and SAG sutures were reverse transcribed to fluorescently-labeled cDNA probes for hybridization using Cy3-dUTP and Cy5-dUTP (Amersham, Piscataway, N.J., USA). Our common reference point was RNA extracted from calvariae at several time points. After probe hybridization, the arrays were incubated overnight at 65°C followed by serial washing in saline sodium citrate solution. Arrays were immediately scanned using a GenePix Scanner (Axon Instruments, Foster City, Calif., USA). The array images obtained were analyzed by GenePix Pro software (Axon Instruments) and imported to the Stanford Microarray Database for further comparison and interpretation. In addition, we utilized Significance Analysis of Microarrays for statistical analysis of arrays.

Quantitative Real-Time PCR

Purified and quantified RNA was treated with DNase I (Ambion, Austin, Tex., USA) to clear genomic DNA. Five µg of total RNA from each time point was reverse transcribed to cDNA using random primer hexamers (Invitrogen). Primers were designed with Primer Express™ software (Applied Biosystems). Each primer was subjected to RT-PCR to ensure single primary amplicon as evidenced by 2% agarose gel electrophoresis to be between 100–150 bp. Primer sequences used: *E-cadherin* F: GC-CAATCCTGATGAAATTGGAA; R: CAGAACCACTGCCCTC-GTAATC, *N-cadherin* F: CCGTGAATGGGCAGATCACT; R: TAGGCGGGATTCCATTGTCA, *Snail* F: ACCCACTCGGATGTGAAGAGA; R: GGAAGATGCCAGCGAGGAT, *Wnt7a* F: CCGTTGGAAGCTGCTCAGCG; R: CCGCAGCGATAATCG-CATAG.

Amplified fragments were ligated into pGEM Easy Vector (Promega, Madison, Wisc., USA) and sequence was confirmed using DNA sequence analysis (Stanford Genome Technology Center, Stanford, Calif., USA). Samples along with primers and Syber Green Master Mix (Applied Biosystems) were loaded in a 384-well sealed plate and the reaction was run in ABI Prism 7900 HT (Applied Biosystems) according to the manufacturer's protocol. *Gapdh* (Applied Biosystems) was used for internal control. The standard curve method of quantitation was used to calculate expression of target genes relative to the housekeeping gene *Gapdh*. Four serial dilutions of cDNA (1:4) were made for the calibration curve and trend lines were drawn using Ct values versus log of dilutions for each target gene and *Gapdh* run in triplicate with correlation coefficient ($R^2 > 0.99$). Relative expressions were calculated using line equations derived from calibration curves and obtaining ratios of target gene to *Gapdh* for each time point. For each gene, experiments were run at least 3 times using 3 independent litters of mice. The error bars reflect variability between litters.

Statistical Analysis

The results are presented as the mean $\pm$ SE of 3 independent experiments. Statistical differences between the means are examined by Student's test or 1-way ANOVA between groups with Tukey's multiple comparison test on Prism software (Prism version 3.0 for Windows; GraphPad, San Diego, Calif., USA). A p value <0.05 was considered statistically significant.

Immunohistochemistry

Selected paraffin embedded sections were chosen and heat antigen retrieval was performed. Vectastain ABC system (Vector Laboratories, Burlingame, Calif., USA) was used according to the manufacturer's protocol. Primary antibody against E-cadherin (H-108) and N-cadherin (H-63; Santa Cruz Biotechnology, Santa Cruz, Calif., USA), were used in 1:50 to 1:500 dilutions. Biotinylated secondary antibody was used in 1:200 dilutions. Controls for each primary antibody consisted of probing with normal (irrelevant) rabbit IgG (sc-2027; Santa Cruz Biotechnology), followed by incubation with biotinylated secondary antibody. Immunostaining with the irrelevant IgG did not detect any staining (data not shown).

Results

cDNA Microarray Analysis of PF and SAG Suture on p5 and p15

To search for candidate cell-cell adhesion molecules differentially expressed in PF suture during its closure, we performed cDNA microarray on PF and SAG sutures on days p5 and p15, before and after the period of suture closure. Our results revealed up-regulation (approximately 9-fold) of *E-cadherin* gene expression in the PF suture at day p15 compared to p5 (fig. 1a). In contrast, the expression of *E-cadherin* remained steady in SAG suture during the same time points. In order to validate the data obtained from microarray analysis, we performed quantitative real-time PCR (qRT-PCR). As shown in figure 1b, qRT-PCR validates the microarray results. Thus, these results indicated that *E-cadherin* is differentially expressed in patent versus closed PF suture.

E-cadherin Expression Pattern during PF Suture Development and Closure

Next, to investigate the potential relationship between *E-cadherin* expression and PF suture biology, we analyzed the expression of this gene during the first month of life in the PF suture, using qRT-PCR. As shown in figure 2a, an increase in *E-cadherin* expression is noted as early as day p9. Maximal expression is observed on day p15 with down-regulation by day p17. This period coincides with the period of PF suture closure, which starts around p10 and mostly completes by p15 [Sahar et al.,

Fig. 1. Differential *E-cadherin* expression in cranial sutures. **a** Microarray profile of *E-cadherin* gene on SAG and PF suture shows that the gene is up-regulated exclusively in the PF suture at p15. Green = Lower than mean expression; red = higher than mean expression. **b** Quantitative real-time RT-PCR confirmed the up-regulation of *E-cadherin* gene expression in PF suture at p15. Quantified mRNA values were normalized by the amounts of *Gapdh* mRNA, and results are given as fold induction. The results are presented as the mean $\pm$ SD of 3 independent experiments.

2005]. To define the precise spatial localization of E-cadherin protein we performed immunohistochemistry on PF suture sections obtained from different time points from the beginning to the end of suture closure time. As shown in figure 2b, immunohistochemical analysis performed using an E-cadherin-specific antibody detected at days p5 and p7 E-cadherin positive cells mainly at the osteogenic front, in the pericranium and dura mater, while very few positive cells were present in the suture mesenchyme. At day p9 some chondrocytes and pre-hypertrophic chondrocytes present in the suture mesenchyme stain positively. More positive E-cadherins cells were observed in the differentiating suture mesenchyme

at days p9 and p11. Finally, at day p15 a strong E-cadherin staining was observed in the ossified endocranial layer of PF suture, overlying pericranium and underlying dura mater. In addition, immunostaining was also observed in the area of suture populated by red blood cells, vessels and lymphocytes. Thus, the immunohistological data confirm the gene expression profile. Moreover, the expression profile of the *E-cadherin* gene on SAG suture during the first month of life did not reveal any specific up-regulation of this gene compared to that observed in PF suture (fig. 2c).

The tightly regulated expression of the *E-cadherin* gene suggests a potential role of this cell-cell adhesion molecule in PF suture closure.

Biphasic Expression Pattern of N-cadherin during PF Suture Development and Closure

PF suture closure occurs by endochondral ossification of endocranial layer [Sahar et al., 2005]. Given the role of N-cadherin in mesenchymal condensation and osteoblast differentiation [Oberlender and Tuan, 1994; Ferrari et al., 2000; DeLise and Tuan, 2002; Tuan, 2003; Tuli et al., 2003; Kii et al., 2004; Stains et al., 2005], we performed qRT-PCR to examine the expression pattern of *N-cadherin* during PF suture closure (fig. 3a, top panel). Interestingly, the profile of *N-cadherin* appeared biphasic. The first peak expression was observed on day p7, a period when mesenchymal cell condensation is noted in the endocranial plate of the PF suture [Sahar et al., 2005]. While, the second peak was detected at days p13–p15, a period marked by osteogenesis and closure of PF suture [Sahar et al., 2005], as well as by up-regulation of *E-cadherin* expression (fig. 2a, b). Of interest, the expression profile of the *N-cadherin* gene on SAG suture during the first month of life did not reveal any specific up- and/or down-regulation of this gene, its expression profile was steady (fig. 3a, bottom panel). The expression of *N-cadherin* gene in PF suture was further confirmed by immunostaining performed with specific N-cadherin antibody (fig. 3b). At days p5 and p7, N-cadherin immunostaining was detected in cells present in the suture mesenchyme of the endocranial layer of PF suture, as well as at osteogenic fronts and in the pericranium. By day p9 very faint staining was observed in the PF suture, only the few chondroblasts present in the suture mesenchyme stained positively, while chondrocytes were negative. At day p11 N-cadherin positive cells were completely absent in the differentiating suture mesenchyme. N-cadherin protein was detected later in the endocranial layer of PF suture, at p13 and p15 when the PF suture closes. Immunohisto-

chemistry performed with irrelevant IgG did not detect any staining (data not shown). Thus, immunohistochemistry analysis mirrored the gene expression profile. The peculiar expression pattern may reflect a dual role for *N-cadherin* during PF suture closure: during cellular condensation preceding PF suture chondrogenesis, and later during osteogenesis when the PF suture closes.

Expression of N- and E-cadherin Transcriptional Repressors Wnt7a and Snail

Temporally and spatially localized expression of *N-* and *E-cadherin* in the PF suture during the period of suture closure suggests strict regulation of these 2 molecules. Previous in vitro studies have shown pre-chondrogenesis condensation events preceding overt chondrogenesis to be associated with *Wnt7a*-mediated *N-cadherin* regulation [Tufan and Tuan, 2001; Tufan et al., 2002; Tuli et al., 2003]. Particularly, it has been demonstrated that *Wnt7a* mis-expression negatively affects N-cadherin expression during limb mesenchymal chondrogenesis [Tufan and Tuan, 2001]. Given the unique and tight expression pattern of *N-* and *E-cadherin* during the first month of life, we investigated the expression pattern of their transcriptional regulators *Wnt7a* and *Snail*, during the same period. Interestingly, *Wnt7a* was up-regulated in the PF suture during pre-chondrogenesis, as well as during chondrogenesis (p7–p11) and its expression returned to baseline during the period of osteogenesis (p13–p15). More important, the maximal up-regulation of *Wnt7a* coincided with the maximal down-regulation of *N-cadherin*. (fig. 4a, b) This pattern of expression would suggest that *Wnt7a* is a potential mediator of *N-cadherin* expression in PF suture and thus, cellular condensation, is a prerequisite to differentiation along the chondrogenic pathway.

Similarly, an important transcriptional repressor of *E-cadherin* is the transcription factor *Snail* [Batlle et al., 2000; Cano et al., 2000]. Given the highly regulated expression of *E-cadherin* during the period of PF suture patterning and closure, we analyzed the expression of *Snail* during the first month of life. As shown in figure 4c, an up-regulated expression of *Snail* was noted only during the period of pre-chondrogenesis (p5–p8), marking the mesenchymal condensation period. *Snail* expression returned to a basal level during suture closure and osteogenesis, a period marked by up-regulation of *E-cadherin* (fig. 4d). The above expression patterns suggest that *Wnt7a* and *Snail* may be potential regulators of *E-* and *N-cadherin* expression during the PF suture patterning and closure.

E-cadherin Expression Pattern in Fgf-2 Knockout Mice

Several studies have demonstrated that in vitro FGF-2 induces expression of *E-cadherin* [El-Hariry et al., 2001; Debiais et al., 2001; Strutz et al., 2002; Marie et al., 2002]. Furthermore, the activating *FGFR-2* mutation also causes up-regulation of *E-cadherin* expression in vitro [Lemonnier et al., 1998]. We have previously showed that *Fgf-2* is up-regulated during PF suture development and closure. Specifically, the expression of *Fgf-2* increases at day p9 [Sahar et al., 2005]. Thus, up-regulation of *Fgf-2* expression followed by up-regulation of both, *E-* and *N-cadherin*, may suggest a causal role. Therefore, this observation prompted us to analyze the expression pattern of *E-cadherin* in PF suture of *Fgf-2* null mice. qRT-PCR analysis indicated that *E-cadherin* expression is not down-regulated in PF suture of *Fgf-2* null mice (fig. 5a), as would be predicted based on previous in vitro data. Indeed, *E-cadherin* expression is similar to that observed in wild-type animals (fig. 2a). A possible explanation for the apparent

contradiction between these in vivo data and in vitro data previously reported could be the redundancy of FGF ligands in the PF suture context. For instance, FGF-18, which is also expressed in the PF suture [Sahar et al., 2005] may compensate for the lack of FGF-2 ligand.

Interestingly, in *Fgf-2* null mice the up-regulation of *E-cadherin* expression occurs earlier than in wild-type mice (fig. 2a). The trend of this expression profile has been consistently observed at least in three independent experiments. However, we cannot rule out that the earlier up-regulation of *E-cadherin* expression observed in the PF suture of *Fgf-2* null mice is merely due to the different genetic background existing between wild-type and *Fgf-2* null mice. Finally, histological analysis of PF suture did not show abnormal phenotype, but closure occurred approximately 2 days earlier than in CD-1 mice (data not shown). Furthermore, analysis of *N-cadherin* did not reveal any differences in the expression pattern between *Fgf-2* null and wild-type CD-1 mice (fig. 5b).

Fig. 2. a *E-cadherin* expression in posterior frontal suture during the first month of life. Quantitative real-time PCR for *E-cadherin* on PF suture reveals up-regulation of *E-cadherin* gene starting at day p9 with maximal expression at days p13–p15, a period coinciding with active bone deposition in the PF suture and suture closure. Quantified mRNA values were normalized by the amounts of *Gapdh* mRNA, and results are given as fold induction. The results are presented as the mean $\pm$ SD of 3 independent experiments from 3 independent litters. * $p < 0.05$.

Fig. 2. c *E-cadherin* expression in posterior frontal suture during the first month of life. Quantitative real-time PCR for *E-cadherin* performed on SAG suture and its comparison with expression profile observed in PF suture. Expression of *E-cadherin* gene in SAG suture remains steady during the first month of life. The results are presented as the mean $\pm$ SD of 3 independent experiments from 3 independent litters.

Fig. 2. b *E-cadherin* expression in posterior frontal suture during the first month of life. Immunohistochemistry analysis of *E-cadherin* protein performed using a specific *E-cadherin* antibody, shows at day p5 absence of positive cells in the suture mesenchyme PF suture (boxed area), while positive cells are detected in the surrounding osteogenic fronts. At day p7, a faint *E-cadherin* staining of few cells is observed, *E-cadherin* staining is also detected in pericranium and dura mater cells. Starting from day p9, more *E-cadherin* positive cells are detected in differentiating mesenchyme of the endocranial layer of PF suture. At day p15 an intense *E-cadherin* staining is observed in the bony bridge of closed endocranial layer of PF suture (boxed area). Boxed areas corresponding to the suture mesenchyme undergoing to endochondral ossification are magnified in panels on the right column. Dashed lines mark the osteogenic front of approaching bone plates. Note, immunostaining is also observed in the area of suture populated by red blood cells, vessels and lymphocytes (asterisk). Pr = Pericranium; DM = dura mater; PF-endo = PF endocranial suture. Scale bar: 200 μ m (at $\times 20$) and 50 μ m (at $\times 40$).

Discussion

The anatomy of PF suture is characterized by a complex architecture, and the molecular control of PF suture closure is a highly coordinated process characterized by a unique endochondral ossification of the endocranial layer [Sahar et al., 2005]. *Sox9*, the master regulator of chondrogenesis, is one of the genes controlling this process. To get more insights into the molecular mechanism controlling the PF suture we have performed a cDNA microarray analysis on mouse PF and SAG calvarial sutures from time points before and after closure to investigate differential gene expression in closing and patent sutures. The expression study reported here provides a basis for future functional studies with the aim to identify other genes controlling PF suture closure.

Adhesive interactions are essential not only in embryonic development, but also in a variety of other biological processes, including the differentiation and maintenance of tissue architecture. Cell-cell adhesion plays a critical role in the organization of cells to tissues during embryogenesis as well as adult life. Cadherins are a family of cell surface glycoproteins that function in promoting Ca^{2+} -dependent cell-cell adhesion and serve as the transmembrane components of adherens junctions. The cadherins are a major class of membrane proteins with prominent roles in cell adhesion, and the regulation of tissue organization and morphogenesis.

Our interest focuses on cell-cell adhesion molecules N-Cadherin and E-cadherin since these molecules are regulators of chondrogenesis and osteogenesis, the 2 major events controlling the PF suture patterning and closure.

In order to differentiate along the chondrogenic pathway mesenchymal cells tightly gather together, a process known as mesenchymal condensation. This process results from active cell movement that causes an increase in mesenchymal cell packing density. N-cadherin is one of the cell-adhesion molecules implicated in this process. The unique biphasic expression pattern of *N-cadherin* in PF suture supports a potential role of this cell-cell adhesion molecule during PF patterning and closure. Our data indicate that N-cadherin expression is temporally up-regulated during the time coinciding with the mesenchymal condensation, while a dramatic down-regulation is observed during the period of chondrogenesis followed by a second up-regulation at the end of chondrogenic differentiation when endochondral ossification and, therefore, closure of PF suture has occurred. These data strongly suggest that N-cadherin may be involved in early events of PF suture patterning and closure, such as mesenchy-

mal condensation preceding cartilage formation, and later during osteogenesis when the final step of PF suture occurs, while E-cadherin is mainly involved during the late steps of PF suture closure when the bony tissue replaces the cartilage (fig. 6). Our hypothesis is strongly supported by previous studies. Evidence indicating the involvement of N-cadherin mediated cell-cell adhesion in cellular condensation and chondrogenesis comes from studies designed to perturb N-cadherin function. A pre-

Fig. 3. a *N-cadherin* expression in the posterior frontal suture during the first month of life. Top panel: Quantitative real-time PCR for *N-cadherin* on PF suture reveals a biphasic expression profile of *N-cadherin* gene. *N-cadherin* is first up-regulated on p7, a period coinciding with a cellular condensation in the endocranial plate of the PF suture. A second phase of *N-cadherin* gene up-regulation is observed on p13–p15, a period marked by osteogenesis and closure of PF suture. Bottom panel: Quantitative real-time PCR for *E-cadherin* on SAG suture shows that the expression is steady overtime. Quantified mRNA values were normalized by the amounts of *Gapdh* mRNA, and results are given as fold induction. The results are presented as the mean $\pm$ SD of 3 independent experiments from 3 independent litters. * $p < 0.05$.

Fig. 3. b *N-cadherin* expression in the posterior frontal suture during the first month of life. Immunolocalization of *N-cadherin* protein on PF suture using a specific anti-*N-cadherin* antibody mirrors the biphasic gene expression profile and localizes *N-cadherin* protein in the suture mesenchyme of the endocranial layer of PF suture during cellular preconditionation at day p5 and p7. By day p9 only a few chondroblasts present in the differentiating suture mesenchyme stain positively. At day p11 none of the cells present in the suture stain positive. In contrast, by day p13 a strong *N-cadherin* staining is observed in the endocranial layer of PF suture. Day p15, which marks the closure of PF suture, is also characterized by strong *N-cadherin* staining in the area of endochondral ossification (boxed area). Pericranium, osteoblasts and dura mater, also stain positively for *N-cadherin*. Boxed areas are magnified in panels on the right column. Pr = pericranium; DM = dura mater; PF-endo = PF endocranial suture. Scale bar: 200 μm (at $\times 20$), 50 μm (at $\times 40$).

Fig. 4. Expression of *N-* and *E-cadherin* transcriptional repressors *Wnt7a* and *Snail*. **a** Quantitative real-time PCR for *Wnt7a* on PF suture reveals up-regulation of *Wnt7a* gene as early as p7, with maximal expression on p11 and return to baseline by p13. **b** The inverse gene expression correlation between *Wnt7a* and *N-cadherin*. Note that *Wnt7a* gene expression is specifically up-regulated during the down-regulation of *N-cadherin* gene. **c** Quantitative real-time PCR for *Snail* on PF suture reveals a moderate up-

regulation of *Snail* gene starting on p5, with a decrease on p9 and a return to baseline by p15. * $p < 0.01$. **d** Graph showing the inverse gene expression profile of *Snail* and *E-cadherin* during the PF suture patterning and closure. Quantified mRNA values were normalized by the amounts of *Gapdh* mRNA, and results are given as fold induction. The results are presented as the mean $\pm$ SD of 3 independent experiments. * $p < 0.05$.

vious study demonstrated a substantial inhibition of cellular condensation and chondrogenesis both in vivo and in vitro using a function-blocking monoclonal antibody directed against N-cadherin [Oberlender and Tuan, 1994]. Moreover, it has been demonstrated that functional N-cadherin is required in a temporally and quantitatively specific manner for normal cellular condensation and chondrogenesis to occur. Interestingly, chondrogenesis is also suppressed by over-expression of either wild-type or mutant forms of N-cadherins, suggesting that temporally inappropriate cell-adhesion activities are in

fact inhibitory to chondrogenic differentiation, possibly because of inappropriate timing of signaling events. In vitro work demonstrated that N-cadherin is required for mesenchymal cell specification in early phases of embryonic limb bud development, and perturbation of cadherin-mediated interactions disrupts chondrogenic cell condensation [Haas and Tuan, 1999; Tuan, 2003; Tuli et al., 2003]. However, persistence of N-cadherin expression prevents further progression from precartilaginous condensation to chondrocyte development, probably by stabilizing cell-cell adhesion [DeLise and Tuan, 2002].

Fig. 5. *E-cadherin* expression is not down-regulated in the PF suture of *Fgf-2* null mice. **a** *E-cadherin* expression in PF suture of *Fgf-2* null mice during the first month of life was assessed by quantitative real-time PCR. Up-regulation of *E-cadherin* gene is observed starting on p5 with maximal level on p9 and return to baseline by p11. Note that up-regulation of *E-cadherin* gene in *Fgf-*

2 null mice occurs earlier than in CD-1 wild-type mice. **b** *N-cadherin* expression in PF suture of *Fgf-2* null mice also shows a pattern similar to that of wild-type CD-1 mice. Quantified mRNA values were normalized by the amounts of *Gapdh* mRNA, and results are given as fold induction. The results are presented as the mean $\pm$ SD of 3 independent experiments. * $p < 0.05$.

Fig. 6. Temporal expression of *N-* and *E-cadherins* and their regulators, *Wnt7a* and *Snail*. The diagram summarizes the temporal gene expression profiles and the key differentiation events occurring during PF suture closure.

The PF suture closure occurs through endochondral ossification, and interestingly our study reveals a peculiar expression pattern of *N-cadherin* during this process characterized by a biphasic up-regulation of this gene. A first up-regulation of *N-cadherin* gene expression is observed at early time points when mesenchymal condensation occurs (pre-chondrogenesis) followed by a second up-regulation at late time points when suture closure is completed.

It is known that *N-cadherin* down-regulation in pre-cartilage condensation is a prerequisite step for mesen-

chymal cells to differentiate into chondrocytes. Therefore, the precise modulation of *N-cadherin* gene expression observed during the PF suture closure represents a crucial and permissive condition for the occurrence of suture closure. Indeed, our *N-cadherin* expression results are fully in agreement with previous observations.

Studies suggest that a cross-talk between *N-cadherin* and *Wnt7a* signaling occurs during the regulation of limb mesenchymal condensation. It has been demonstrated that misexpression of *Wnt7a* inhibits chondrogenesis in vitro, whereas *Wnt5* a ligand activating the non-canoni-

cal Wnt signaling does not [Tufan and Tuan, 2001]. Moreover, Wnt7a mis-expression influences the expression and/or turnover of N-cadherin, thereby preventing it from being down-regulated at transition from condensation to chondrogenic differentiation. Interestingly, during PF suture closure we found a modulated expression of Wnt7 gene characterized by an up-regulation inversely correlating with that of N-cadherin. These results suggest that Wnt7a is a potential negative regulator of N-cadherin function during the chondrogenic differentiation occurring in the closing PF suture.

The late up-regulation of *E-cadherin* gene during the PF closure timing would suggest a potential role for this molecule during the final step, when osteogenesis takes place. Of interest, a previous study performed on OB-cadherin null mutant mice showed a reduced calcified area at the PF suture that caused a round-shaped calvaria with increasing animal age to 3 months [Kawaguchi et al., 2001]. Indeed, this observation strongly supports the potential role of cadherins in the patterning and closure of PF suture.

Several studies indicated that *E-cadherin* gene expression is regulated by Snail, a transcriptional repressor which by binding to 3 E-boxes present in the *E-cadherin* promoter represses its transcription [Batlle et al., 2000]. Our analysis revealed in the closing PF suture at early time points an up-regulation of *Snail* gene overlapping with the down-regulation of *E-cadherin* expression. In contrast, at late time points *Snail* expression was down-regulated concomitantly with the up-regulation of *E-*

cadherin expression. These results would suggest that *E-cadherin* expression in PF closing suture might be under the control of the transcriptional repressor Snail.

It is noteworthy that cadherins, also can modulate the canonical Wnt signaling. N-cadherin and E-cadherin can interact directly with β -catenin and, thereby, may decrease Wnt signaling [Linask et al., 1997; Xu et al., 2002; Nelson and Nusse, 2004]. Moreover, a novel mechanism by which N-cadherin interacts with the Wnt co-receptor LRP5 and regulates canonical Wnt/ β -catenin signaling in osteoblasts has been described [Hay et al., 2009].

Taken together, our results demonstrate that during the patterning and closure of the PF suture there is a precise and unique modulation of the 2 major adhesion molecules N- and E-cadherin. We believe that this modulation may represent timing of signaling events governing the patterning and closure of PF suture. Our current results and ongoing experiments would suggest that E- and N-cadherins may represent a potential control of canonical Wnt signaling in PF suture. Indeed, the expression study presented herein sets the stage for future investigations aimed to further elucidate the molecular signaling underlying the patterning and closure of PF suture.

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