Age-Dependent Differential Expression of Fibronectin Variants in Skin and Airway Mucosal Wounds

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Objective: To delineate age-dependent and tissue-specific molecular activities of the variant-inclusion fibronectin transcripts in fetal and postnatal skin and airway mucosal wounds during early events of the wound healing process. Fibronectin is involved in multiple steps of the wound healing process. The functional complexity of fibronectin is carried through its protein diversity, which is controlled in part by alternative RNA splicing, a coordinated transcription and RNA processing. From a rabbit model of airway mucosal wound healing, we isolated and cloned an RNA splicing factor, SRp20, that was coexpressed with Fn1 complementary DNA and suppressed in fetal wounds but induced in postnatal wounds. Previous studies revealed a link between the inclusion and/or exclusion of the alternatively spliced Fn1 variants (extra domain A [EDA], extra domain B [EDB], and a variable region [V]) and outcomes of wound repair.

Design: Skin and airway mucosal incisional wounds were made in fetal (gestational day 21–23), weanling (4–6 weeks), and adult (>6 months) rabbits. Tissues (nonwounded and wounded) were collected at 12 hours (all age groups), 24 hours, and 48 hours (weanling only) after wounding. The expression levels of the 3 Fn1 spliced domain (EDA, EDB, and V)-containing messenger RNA (mRNA) species were assessed by real-time polymerase chain reaction.

Results: Fn1 spliced variants were either suppressed or showed no change in fetal skin and airway mucosal wounds 12 hours after injury, whereas the spliced mRNAs were induced in postnatal wounds. The augmented molecular activities of Fn1 spliced variants were more prominent in airway mucosal wounds than in skin wounds. Furthermore, the EDA variant was dominantly selected in adult airway mucosal wounds (6-fold increase), which was strikingly different from the adult skin wounds (1-fold).

Conclusion: Our study suggests that the age-dependent activation of Fn1-EDA mRNA may play a fundamental role in differentiating fetal wound regeneration from postnatal wound scar formation during the early events of airway mucosal wound healing.

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UBGLOTTIC STENOSIS (SGS) IS A MAJOR CLINICAL PROBLEM, PARTICULARLY IN PREMATURE INFANTS FOLLOWING PROLONGED INTUBATIONS AND IN ADULTS FOLLOWING PERCUTANEOUS DILATIONAL TRACHEOTOMY. We previously demonstrated that fetal rabbit airway mucosal healing was regenerative and scarless. From this model, we isolated and cloned a pre-messenger RNA (pre-mRNA) splicing factor, SRp20, that was coexpressed with a Fn1 complementary DNA (cDNA). Both gene transcripts were simultaneously suppressed in fetal wounds and induced in postnatal wounds, and their expression levels in wounds were tissue specific during the early events of the wound healing process.

Fibronectin functional complexity is carried through its protein diversity. The Fn1 protein family consists of multiple isoforms, including plasma fibronectin, a soluble dimeric form in blood and cellular fibronectin filaments, a dimeric or multimeric form at the cell surface and in the extracellular matrix. On wounding, fibronectin, as a major component of the primary extracellular matrix, is expressed at high levels at the wound site. The plasma fibronectin is released from the α-granules of platelets that are activated by damaged blood vessels, and the cellular fibronectin is synthesized locally at the wound site. Fibronectin protein diversity is controlled by alternative pre-mRNA splicing, a coordinated transcription and RNA processing. All fibronectin isoforms are encoded by a single large gene, Fn1 (50 kilobases and 50 exons) and have 3 regions subject to alternative splicing, including extra domain A (EDA), extra domain B (EDB), and a variable region (V), depend...
Protein subunits are produced. In humans, approximately 20 different mRNA-encoding protein subunits are produced. The full-length nature of some variants has not been determined. Cellular fibronectin is produced by a wide variety of cell types and contains considerably higher proportions of alternatively spliced sequences compared with plasma fibronectin, suggesting that the alternative splicing of the Fn1 mRNA transcripts is tissue- and function-dependent. This process may assist tissue cells to produce a type of fibronectin that is the most suitable for the needs of a specific tissue or cellular function. Nevertheless, expression profiles and biological functions of the Fn1 spliced variants in fetal and postnatal airway mucosal epithelia and wound repair remain undefined.

We report herein age-dependent differential expression profiles of the 3 alternatively spliced domain (EDA, EDB, or V)-containing Fn1 mRNAs in fetal, weanling, and adult rabbit skin and airway mucosal wounds during the early phase of wound repair. Results indicate that the age-dependent selection of the Fn1-EDA mRNA during the early phase of airway mucosal wound healing may play a fundamental role in differentiating fetal wound regeneration from postnatal wound repair and scar formation.

**METHODS**

**ANIMAL MODEL**

Adult Pasteurella-free New Zealand white rabbits (pregnant and nonpregnant) were obtained from a US Department of Agriculture–approved supplier (Hazelton Research Products, Denver, Pennsylvania). At least 3 animals or fetuses were used in each group (nonwounded and wounded), including fetuses at gestational days 21 to 23 (term=31 days), weanling (4-6 weeks), and adult (>6 months) rabbits. Animal maintenance and experiments were conducted following a protocol approved by the Animal Research and Care Committee of the Children’s Hospital of Pittsburgh, Pittsburgh, Pennsylvania.

Rabbit skin and airway mucosal wounds were produced following well-established techniques as previously applied in our laboratory. Briefly, anesthesia was induced with a mix of ketamine hydrochloride (35 mg/kg) and xylazine hydrochloride (5 mg/kg) via intramuscular injection. For fetal surgery in pregnant rabbits, anesthesia was maintained with a mix of 1% to 3% halothane, 2% oxygen, and 1% nitrous oxide delivered by spontaneous mask ventilation at a rate of 1 L/min. The abdominal hair was shaved, and the skin was prepared with povidone iodine. The site for the incision was treated locally with subcutaneous infiltration of the skin with lidocaine, 2%. A lower midline laparotomy incision was made extending from the umbilicus to the most caudal set of nipples. The size, number, and position of the fetuses were determined by palpation. Surgery was performed on every second fetus to reduce the risk of spontaneous abortion and every other fetus was left unwounded for controls. A purse-string suture was placed through all layers of the uterus and a hysterotomy incision was made within the borders of the uterus. The fetal animal was partially delivered through the opening to expose the areas intended for wounding and then carefully replaced. All skin incisional wounds were made on the dorsal skin in an identical pattern (approximately 1 cm long). For airway mucosal wounds, a midline thyrotomy was made, followed by cricoideotomy and circumferential mechanical injury of the subglottic mucosa. Sterile isotonic sodium chloride solution was added to reconstitute the volume of the lost anesthetic fluid and the purse-string suture was closed in layers with running sutures. Similar procedures for generating skin and airway mucosal wounds were applied to weanling and adult rabbits, as previously described.

After 12 hours, a second surgery was carried out to deliver all the fetuses, and the mother was given a lethal intracardiac injection of a mix containing sodium pentobarbital (30 mg/kg) and pentobarbital sodium–phenytoin sodium (0.2 mL/kg) while under a deep anesthesia. Nonwounded and wounded skin and airway mucosal tissues from individual age groups were immediately collected, rinsed in cold physiological saline, rapidly frozen in liquid nitrogen, and stored at −80°C. For weanling rabbits, tissues samples were also collected at 24 hours and 48 hours after wounding.

**REAL-TIME POLYMERASE CHAIN REACTION**

**RNA Extraction**

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, California) and then treated with DNase I (Ambion Inc, Austin, Texas) following the instructions of the manufacturers. Total RNA was pooled from each age group because of a sparse amount of RNA from each animal and used for reverse transcription and real-time polymerase chain reaction (PCR) quantification.

**Gene-Specific Primers**

To assess the gene expression levels of the Fn1 variants, gene-specific primers coding for the 3 Fn1 spliced fragments were designed based on the published sequences from human Fn1-EDA (GenBank No. X07718), rat Fn1-EDB (GenBank No. L20801), and human Fn1-V (GenBank No. X04530). Primers for rabbit 18S ribosomal RNA (rRNA) (GenBank No. X06778) were designed and used as an endogenous control for data normalization. Primer sequences are listed in the Table.

**Reverse Transcription and Real-Time PCR**

Expression levels of the gene transcripts were quantified using real-time PCR as previously described. Briefly, the reverse transcription reaction included 230 ng of DNA-free total RNA pooled from each group, random primers, and SuperScript II (Invitrogen) and was incubated at 25°C for 10 minutes, 48°C for 30 minutes, and 95°C for 5 minutes in a 9600 thermocycler (Applied

**Abbreviations:** EDA, extra domain A; EDB, extra domain B; V, variable region; rRNA, ribosomal RNA.
postnatal induction of alternative splicing is development-age dependent. Second, some degree, suggesting that trauma-induced Fn1 mRNA alternative splicing is development-age dependent. Second, postnatal induction of Fn1 variants was more prominent in airway mucosal wounds than in skin wounds, indicating that wound-induced Fn1 mRNA alternative splicing is tissue specific. Quantitative data of the 3 Fn1 variants also indicate that the tissue-specific selection of Fn1 variants may contribute to the total levels of Fn1 transcripts by comparison between skin and airway mucosal wounds, which were correlated with the coexpressed pre-mRNA splicing factor, Sfsr3 (Figure 3). Third, the 3 Fn1 domains were dramatically induced in adult airway mucosal wounds compared with that in adult skin wounds. Finally, the EDA variant was dominantly selected in both mucosal and skin wounds by comparison with the other 2 domain (EDB and V)-containing variants.

EXPRESSION PATTERNS

Age-dependent and tissue-specific expression patterns of the 3 Fn1 spliced variants were depicted at 12 hours after wounding (Figure 4). In skin wounds, the 3 spliced domains showed a similar expression pattern, suppressed in prenatal wounds and induced in postnatal wounds. In airway mucosal wounds, however, EDA was dramatically induced in the postnatal wounds in an age-dependent fashion compared with the EDB and V variants.
Our results suggest that Fn1 alternative splicing is indeed age dependent and tissue specific in wound healing and that EDA may be a key factor in promoting postnatal airway mucosal wound scar formation.

Time-dependent patterns of the 3 Fn1-spliced variants in postnatal weanling wounds are further schematically expressed in Figure 5. The 3 variants in skin wounds were slightly induced at 12 hours and then were gradually and persistently augmented with time (to 48 hours). The EDA variant was preferentially selected, followed by EDB and V in skin wounds. In airway mucosal wounds, however, EDA was dominantly selectively induced, followed by EDB and then V. The molecular activity of the Fn1-EDA transcript was quickly and dramatically induced at 12 hours, remained persistently high at 24 hours, and then gradually decreased to 48 hours in airway mucosal wounds. Our results indicate that, in spite of a similar abundance of the transcripts in both normal skin and airway mucosal tissues (Figure 1), differential selection of Fn1 mRNA-spliced variants (EDA > EDB > V) is more pronounced in postnatal airway mucosal wounds than in skin wounds.

Enhanced alternative splicing of Fn1 variants were observed in cutaneous wounds,24-26 corneal wounds,27-30 gastric ulcer healing,31 liver wound repair32 and hepatic fibrogenesis,33 myocardial infarction,34 acute major trauma,35 proliferative glomerulonephritis,36,37 and peripheral nerve injury.38 Assays of the plasma fibronectin and fetal fibronectin variants have been applied to the clinical diagnosis and therapeutic administration of fibronectin to human diseases.39-42 However, to our knowledge, development-age–dependent and tissue-cell–specific expression and regulation of Fn1 spliced variants in airway mucosal wound repair in vivo have not been previously described.

Fetal isoforms of Fn1 mRNAs, which include EDA, EDB, and V, are differentially included or excluded during embryonic development43-45 and wound healing10,13 in a cell-type specific manner.46-48 The pattern of Fn1 RNA splicing in the early embryo is, however, different from that seen later in development, but if adult skin is in-

![Figure 2](http://archotol.jamanetwork.com/pdfaccess.ashx?url=/data/journals/otol/11932/) Postwounding messenger RNA (mRNA) expression levels for the 3 Fn1 spliced variants in fetal and postnatal skin and airway mucosal wounds. Data were normalized to rabbit 18S ribosomal RNA and adjusted to a 0-fold level for the nonwounded group.

![Figure 3](http://archotol.jamanetwork.com/pdfaccess.ashx?url=/data/journals/otol/11932/) Postwounding expression levels (12 hours) for Sfrs3 and Fn1 total messenger RNA (mRNA) in fetal, weanling, and adult skin and airway mucosal wounds. Data were normalized to rabbit 18S ribosomal RNA and adjusted to a 0-fold level for the nonwounded group.

![Figure 4](http://archotol.jamanetwork.com/pdfaccess.ashx?url=/data/journals/otol/11932/) Age-dependent messenger RNA (mRNA) expression patterns for the Fn1 alternatively spliced variants extra domain A (EDA), extra domain B (EDB), and variable region (V) in skin and airway mucosal wounds 12 hours after injury. Data were normalized to rabbit 18S ribosomal RNA and adjusted to 0-fold level for the nonwounded group. For easy comparison and visualization of the age-dependent change, messenger RNA (mRNA) levels were hypothetically line connected between age groups for each variant.

**COMMENT**
jured, the pattern of Fn1 RNA splicing in the base of the wound switches back to the pattern seen in early development. These observations suggest that Fn1 isoforms produced in the early embryo and in wound healing are especially important for promoting the cell migration and proliferation required for tissue development and repair. Nevertheless, the expression entity of Fn1 spliced variants responsible for differentiating fetal scarless wound healing and postnatal wound repair/scar formation remains elusive.

We first evaluated the baselines of Fn1 mRNA variants in both normal skin and airway mucosal wounds and found that the expression levels in both normal tissues were similar, indicating that the differential selections of Fn1 variants after wounding are independent of the original transcript levels in tissues. Second, we observed that the age-dependent, preferential selection patterns of the Fn1 variants appear similar in the rabbit model of the skin and airway mucosal wound healing. The expression levels of the 3 Fn1 spliced domains were either suppressed or unchanged in fetal skin and airway mucosal wounds 12 hours after injury, whereas they were induced in the postnatal wounds, indicating that the transcripts of Fn1 variants were not actively modulated in fetal wounds at 12 hours after wounding. Fn1 deposition was observed at 1 to 4 hours in the fetal wounds, suggesting that the regulation of the Fn1 genes in fetal wounds in this study may occur much earlier. On the other hand, the normal preexistence of the active splicing process during embryonic development may prevent further alternative selections of the Fn1 spliced variants triggered by wound healing signals.

We further demonstrated that the induced postnatal gene expression of the 3 Fn1 spliced variants was more prominent in the airway mucosal wounds than that in skin wounds. Specifically, at 12 hours after wounding, the Fn1-EDA variant was dominantly included in postnatal airway mucosal wounds, which was strikingly different from that in skin wounds. Time-dependent expression patterns of the Fn1 spliced domains in weanling wounds confirmed that the Fn1-EDA variant is dominantly selected in a tissue-specific fashion in postnatal wounds.

Enhanced alternative splicing of Fn1 variants was observed in cutaneous wounds. Macrophages and fibroblasts express embryonic fibronectin during cutaneous wound healing. The expression of EDA was increased in the skin of patients with cutaneous graft-vs-host disease, suggesting that Fn1-EDA is a marker of skin fibrosis. Expression of EDA- and EDB-spliced variants in bone indicates that these 2 spliced variants are strong markers for active fibrogenetic and osteoid-forming processes in human bones. Fibronectin-spliced variants containing the EDA exon were prominently expressed in the vasculature of a variety of human tumors but not in their normal adult tissue counterpart. Previous studies and our results suggest that Fn1-EDA may be a crucial target for postnatal airway mucosal wound scar formation.

Our results further suggest that Fn1-EDA may be an important biomarker associated with airway mucosal wound repair. Interestingly, homozygous mouse strains with complete exclusion or inclusion of the EDA exon were viable and developed normally, indicating that the alternative splicing at the EDA exon is not vital during embryonic development. Conversely, mice without the EDA exon in the Fn1 protein displayed abnormal skin wound healing, whereas mice having constitutive inclusion of the EDA exon showed a major decrease in Fn1 levels in all tissues. Indeed, a deletion of the alternatively spliced EDA domain in mice with atherosclerotic lesions reduced atherosclerosis.

Figure 5. Time-dependent gene expression patterns of the extra domain A (EDA), extra domain B (EDB), and variable region (V) in weanling skin and airway mucosal wounds 12 hours, 24 hours, and 48 hours after injury. Data were normalized to rabbit 18S ribosomal RNA and adjusted to a 0-fold level for the nonwounded group. For straightforward comparison and visualization of the time-dependent change, messenger RNA (mRNA) levels were hypothetically line connected between time points for each variant.

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