

Gene transfer *in utero* biologically engineers a patent ductus arteriosus in lambs by arresting fibronectin-dependent neointimal formation

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Closure of the ductus arteriosus requires prenatal formation of intimal cushions, which occlude the vessel lumen at birth. Survival of newborns with severe congenital heart defects, however, depends on ductal patency. We used a gene transfer approach to create a patent ductus arteriosus by targeting the fibronectin-dependent smooth muscle cell migration required for intimal cushion formation. Fetal lamb ductus arteriosus was transfected *in utero* with hemagglutinating virus of Japan liposomes containing plasmid encoding 'decoy' RNA to sequester the fibronectin mRNA binding protein. Fibronectin translation was inhibited and intimal cushion formation was prevented. We thus established the essential role of fibronectin-dependent smooth muscle cell migration in intimal cushion formation in the intact animal and the feasibility of incorporating biological engineering in the management of congenital heart disease.

Neointimal formation is common to vascular diseases but occurs as a normal developmental process in the ductus arteriosus (DA) in late gestation as a prerequisite for complete anatomical closure of this vessel after its constriction at birth^{1,2}. In a strain of dogs with hereditary persistent patent DA, neointimal formation does not occur². Furthermore, in premature infants³ and neonates exposed to rubella infection *in utero*⁴, lack of intimal cushion formation is associated with postnatal patency of the DA.

In neonates with congenital heart defects involving severe right- or left-sided obstruction, maintenance of ductal patency is essential as a 'bridge' to life-saving corrective surgery, and has been achieved with E-type prostaglandins^{5,6}. However, the use of prostaglandins is associated with many serious side effects, including hypotension, cardiac rhythm and conduction disturbances, hypoventilation and apnea, and pyrexia⁷. Therefore, strategies aimed at preventing the formation of intimal cushions in the DA might be advantageous in maintaining postnatal patency of this vessel.

Intimal cushion formation in the DA involves fragmentation of elastic laminae, and upregulation of smooth muscle cell fibronectin synthesis, which mediates the migration of these cells into a glycosaminoglycan-enriched subendothelium^{8,9,10}. Fibronectin synthesis is increased in fetal lamb DA smooth muscle cells at the onset of intimal cushion formation at 100 days of gestation, and decreases to baseline levels at 138 days of gestation, when the process is complete⁸.

Moreover, the increase in fibronectin synthesis is regulated by enhanced mRNA translation¹⁰. The mechanism involves the in-

teraction of an RNA binding protein, identified as light chain 3 (LC-3) of microtubule-associated proteins 1A and 1B (ref. 11), with an AU-rich element (ARE) in the 3'-untranslated region (3'-UTR) of fibronectin mRNA. Here, cultured DA smooth muscle cells were transfected with chloramphenicol acetyl transferase (CAT) expression plasmid encoding an mRNA in which the 3'-UTR of CAT was replaced with the 3'-UTR of fibronectin (pECE-CAT-FN). CAT activity was enhanced in these cells compared with its activity in cells transfected with plasmid encoding a mutated ARE in the fibronectin 3'-UTR (pECE-CAT-FNΔ), which would no longer bind LC-3. At the same time, transfection with the wild-type expression plasmid (pECE-CAT-FN) suppressed endogenous fibronectin synthesis and resulted in a switch from an elongated, motile cell phenotype to a stellate phenotype. This indicated that the CAT-fibronectin 3'-UTR mRNA was acting as a 'decoy' RNA, sequestering LC-3 away from endogenous fibronectin mRNA and thus inhibiting its translation¹¹. Although previous studies have used direct transfection of double-stranded DNA oligonucleotides as 'decoys' of transcription factors¹², this is the first, to our knowledge, demonstrating the use of plasmid expressed mRNA as a 'decoy' of a translational regulatory protein. An advantage of this expression construct is that it includes a coding sequence for the CAT reporter gene.

These observations indicated a strategy whereby transfection of the fetal lamb DA *in utero* with the pECE-CAT-FN plasmid would similarly result in sequestration of LC-3, reduction in fibronectin synthesis and inhibition of smooth muscle cell migration and intimal cushion formation. To test this, we transiently

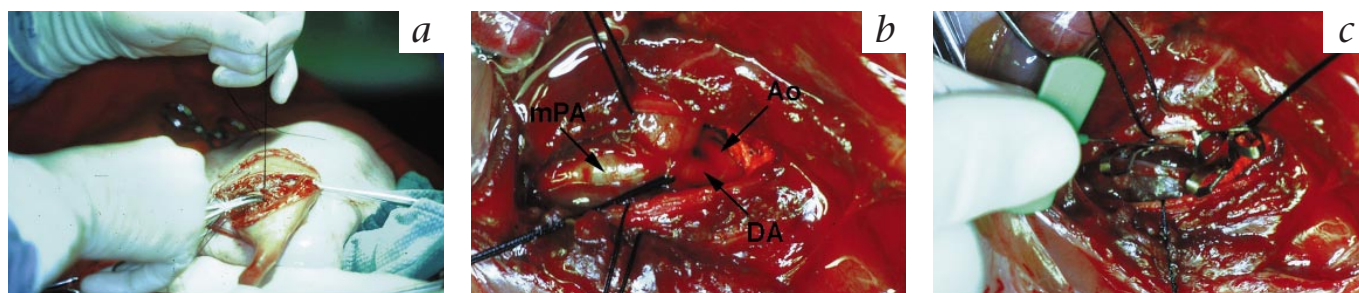


Fig. 1 Transfection of the fetal lamb DA *in utero* at 90 days of gestation. **a**, Accessing the thorax of the fetal lamb with the forelimb exteriorized. **b**, Isolation of the great vessels. DA, ductus arteriosus; Ao, aorta; mPA,

main pulmonary artery. **c**, Microseffarine occlusion of the main PA, left and right branches of the PA and distal DA at the aorta, followed by injection of the HVJ liposomes using a butterfly needle.

isolated and transfected the DAs of fetal lambs at 90 days of gestation with pECE-CAT-FN or with the mutant, non-LC-3-sequestering construct pECE-CAT-FN Δ , and confirmed that fibronectin synthesis was inhibited with pECE-CAT-FN. The lambs survived to term (145 days) and delivered spontaneously. We confirmed the abrogation of intimal cushion formation and increased DA luminal patency at birth.

Results

We did gene transfer on fetal lambs at 90 days of gestation, so that it would precede the upregulation of fibronectin synthesis observed at the onset of intimal cushion formation (at 100 days of gestation in fetal lambs⁸). The DA was transiently isolated *in utero* by lateral thoracotomy, and hemagglutinating virus of Japan (HVJ) liposomes containing pECE-CAT-FN or the mutant plasmid pECE-CAT-FN Δ were injected (Fig. 1). To control for effects related to the surgical procedure alone, the DA was isolated and injected with saline instead of liposomes in a third group of lambs. A fourth group consisted of untreated twins of the transfected and saline-injected lambs.

To confirm that transfection with pECE-CAT-FN inhibits fibronectin synthesis at the time point associated with its upregulation, we collected transfected and twin untreated DAs at 100

days of gestation, and assessed fibronectin synthesis by gelatin sepharose purification after 4 hours of metabolic labeling (Fig. 2). Transfection with pECE-CAT-FN resulted in a greater reduction in fibronectin synthesis than did transfection with pECE-CAT-FN Δ ($P < 0.05$; Fig. 2), each being assessed relative to the respective twin, untreated control lamb DA.

Immunohistochemical analysis correlated with the biochemical assessments of fibronectin synthesis (Fig. 3a). Expression of fibronectin was abundant in the subendothelium and throughout the media of control, untreated DAs (Fig. 3a, untx) and was present in lamb DAs transfected with pECE-CAT-FN Δ (Fig. 3a, Δ), but was not detected in the media of DAs transfected with pECE-CAT-FN (Fig. 3a, wt). Western immunoblotting confirmed that total fibronectin was reduced in DAs transfected with pECE-CAT-FN compared with that in DAs transfected with pECE-CAT-FN Δ and in untreated controls (Fig. 3b). However, a reduction in fibronectin in DAs transfected with pECE-CAT-FN Δ compared with that in untreated controls was not detected (Fig. 3b). The CAT-fibronectin 3'-UTR mRNA might also serve as a 'decoy' RNA sequestering regulatory factors binding to other ARE-containing mRNAs. Therefore, we also used western immunoblotting to assess expression of proteins derived from ARE-containing mRNAs that might be relevant to intimal cushion formation, such as those involved in cell division and differentiation (Fig. 3b). Expression of c-myc and c-fos was unaffected by transfection with pECE-CAT-FN or the mutated, control plasmid pECE-CAT-FN Δ , compared with no treatment (twin controls) (Fig. 3b).

Immunohistochemical analysis of the bacterial reporter gene CAT at term reflected the sustained effect of gene transfer (Fig. 4). In the DAs transfected with pECE-CAT-FN that were collected from term lambs, CAT protein expression was present throughout the endothelium and the media where the smooth muscle cells were circumferentially oriented (Fig. 4, wt). In DAs transfected with pECE-CAT-FN Δ , CAT was mostly absent in the fully formed neointima, which may be due to loss of expression of the plasmid in actively proliferating smooth muscle cells (Fig. 4, Δ). The distribution in the media was 'patchy' but was evident in elongated smooth muscle cells oriented perpendicular to the intima and lumen of the vessel (Fig. 4, Δ).

The morphologic features of the term DA associated with normal anatomical closure postnatally have been described^{1,3,13,14}, including extensive intimal cushions as well as the formation of medial indentations¹⁴ attributed to postnatal DA constriction³. In addition, the closing DA has a smaller lumen because of medial thickening as a result of increased number of smooth muscle cell layers during the second half of gestation¹. Movat pen-

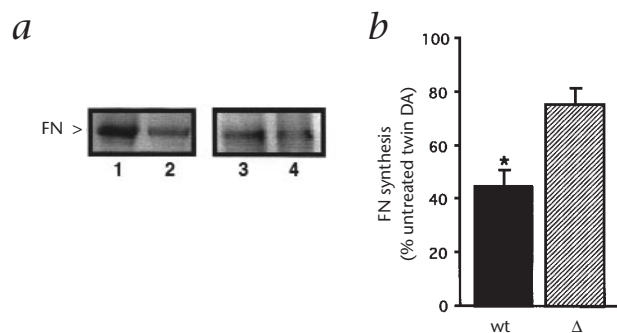
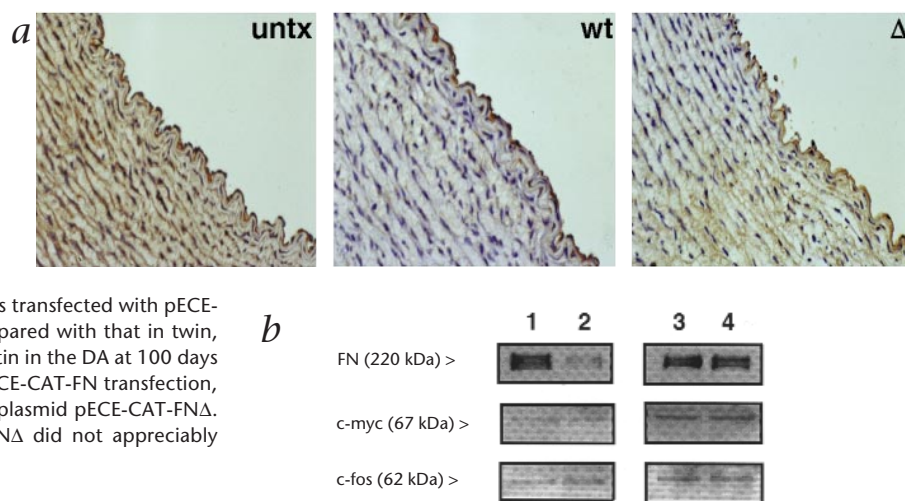


Fig. 2 Effect of pECE-CAT-FN transfection on fibronectin synthesis and distribution in the DA at 100 days of gestation. **a**, Affinity-purified, ³⁵S-methionine-labeled fibronectin (FN), separated by SDS-PAGE, from DAs transfected with pECE-CAT-FN (lane 2) or pECE-CAT-FN Δ (lane 4) compared with their respective twins' untreated, control DAs (lanes 1 and 3), metabolically labeled for 4 h in organ culture at 100 days of gestation. **b**, Densitometric analysis of fibronectin synthesis in transfected DAs expressed as a percentage of synthesis in twin, untreated controls. Relative to twin, untreated controls, fibronectin (FN) synthesis in DAs transfected with pECE-CAT-FN (wt) was less than that in DAs transfected with pECE-CAT-FN Δ (Δ); * $P < 0.05$. Bars represent mean \pm s.e.m. ($n = 3$ sets of twins).

Fig. 3 Effect of pECE-CAT-FN transfection on fibronectin distribution and expression of fibronectin, c-myc and c-fos. **a**, Immunohistochemical analysis of fibronectin expression. The abundant expression of fibronectin in the media of the untreated (untx) DA is lost in the DA transfected with pECE-CAT-FN (wt), but persists although somewhat less, in the DA transfected with pECE-CAT-FN Δ (Δ). **b**, Immunoblot analysis (representative of two experiments with similar results) of fibronectin (FN), c-myc and c-fos expression in DAs transfected with pECE-CAT-FN (lane 2) or pECE-CAT-FN Δ (lane 4) compared with that in twin, untreated controls (lanes 1 and 3). Total fibronectin in the DA at 100 days of gestation was considerably decreased after pECE-CAT-FN transfection, but not after transfection with the mutated ARE plasmid pECE-CAT-FN Δ . Transfection with pECE-CAT-FN or pECE-CAT-FN Δ did not appreciably alter c-fos or c-myc expression.



tachrome staining of the DA transfected with pECE-CAT-FN at term showed inhibition of intimal cushion formation (Fig. 5) and lack of medial indentations, features observed in DAs transfected with mutant pECE-CAT-FN Δ as well as in saline-injected or untreated controls (Fig. 5a). In addition, the lumen was visibly larger in the DAs transfected with pECE-CAT-FN compared with those transfected with pECE-CAT-FN Δ or injected with saline or with twin, untreated control DAs (Fig. 5a) and the distances between elastic lamellae were reduced (Fig. 5b).

Morphometric analysis demonstrated that intimal thickness was reduced by about 60–70% in DAs transfected with pECE-CAT-FN compared with those transfected with pECE-CAT-FN Δ or injected with saline, or with untreated controls ($P < 0.05$ for all comparisons; Fig. 6a). Medial thickness relative to vessel perimeter was decreased by about 20–25% in DAs transfected with pECE-

CAT-FN compared with those transfected with pECE-CAT-FN Δ or injected with saline, or with twin, untreated control vessels (Fig. 6b). The difference between vessels transfected with pECE-CAT-FN and those injected with saline or untreated control vessels was significant ($P < 0.05$; Fig. 6b). DA luminal area relative to medial area was significantly increased in vessels transfected with pECE-CAT-FN compared with that in both vessels injected with saline and in untreated controls ($P < 0.05$; Fig. 6c).

Discussion

Here we used a gene transfer approach *in utero* to inhibit intimal cushion formation and achieve increased luminal patency of the DA at term. Fetal lambs were able to survive thoracotomy and extensive manipulation of the great vessels at 90 days of gestation, developed normally and delivered spontaneously at term. Using a strategy that produced 'decoy' RNA by transfection with expression plasmid encoding recombinant CAT-fibronectin 3'-UTR mRNA, we successfully targeted fibronectin-dependent smooth muscle cell migration at the onset of intimal cushion formation. In contrast, controls transfected with pECE-CAT-FN Δ , in which the ARE site of the fibronectin 3'-UTR was mutated such that it would not bind LC-3, showed no substantial difference in intimal cushion thickness or medial wall thickness compared with lambs that had undergone the same surgery with saline injection or with no treatment of the DA.

The HVJ liposome transfection method was chosen because it is a highly efficient gene transfer technique in blood vessels^{12,15,16}. Here we confirmed its efficacy by showing CAT immunostaining at term, 55 days after initial transfection. There was no evidence

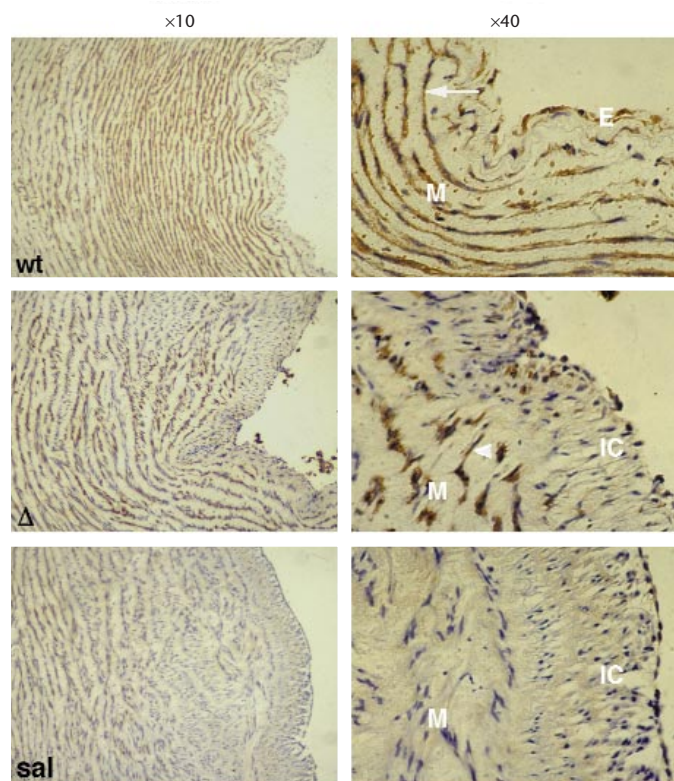


Fig. 4 CAT reporter gene expression at term in DAs transfected with pECE-CAT-FN or pECE-CAT-FN Δ DAs. Medium-power (×10) and high-power (×40) magnification of CAT immunohistochemistry in term DAs transfected at 90 days of gestation with pECE-CAT-FN (wt) or pECE-CAT-FN Δ (Δ) or injected with saline (sal). CAT reporter gene expression was detected in both the endothelium (E) and media (M) of DAs transfected with pECE-CAT-FN, whereas in DAs transfected with pECE-CAT-FN Δ , CAT is mostly absent in the intimal cushion (IC) and is 'patchy' in the media (M). CAT-expressing smooth muscle cells (arrow) from the inner media of the DAs transfected with pECE-CAT-FN have a circumferential orientation, in contrast to the smooth muscle cells of DAs transfected with pECE-CAT-FN Δ or saline, which have their long axes oriented towards or perpendicular (arrowhead) to the lumen of the vessel. CAT immunoreactivity is absent from the saline-injected control vessels.

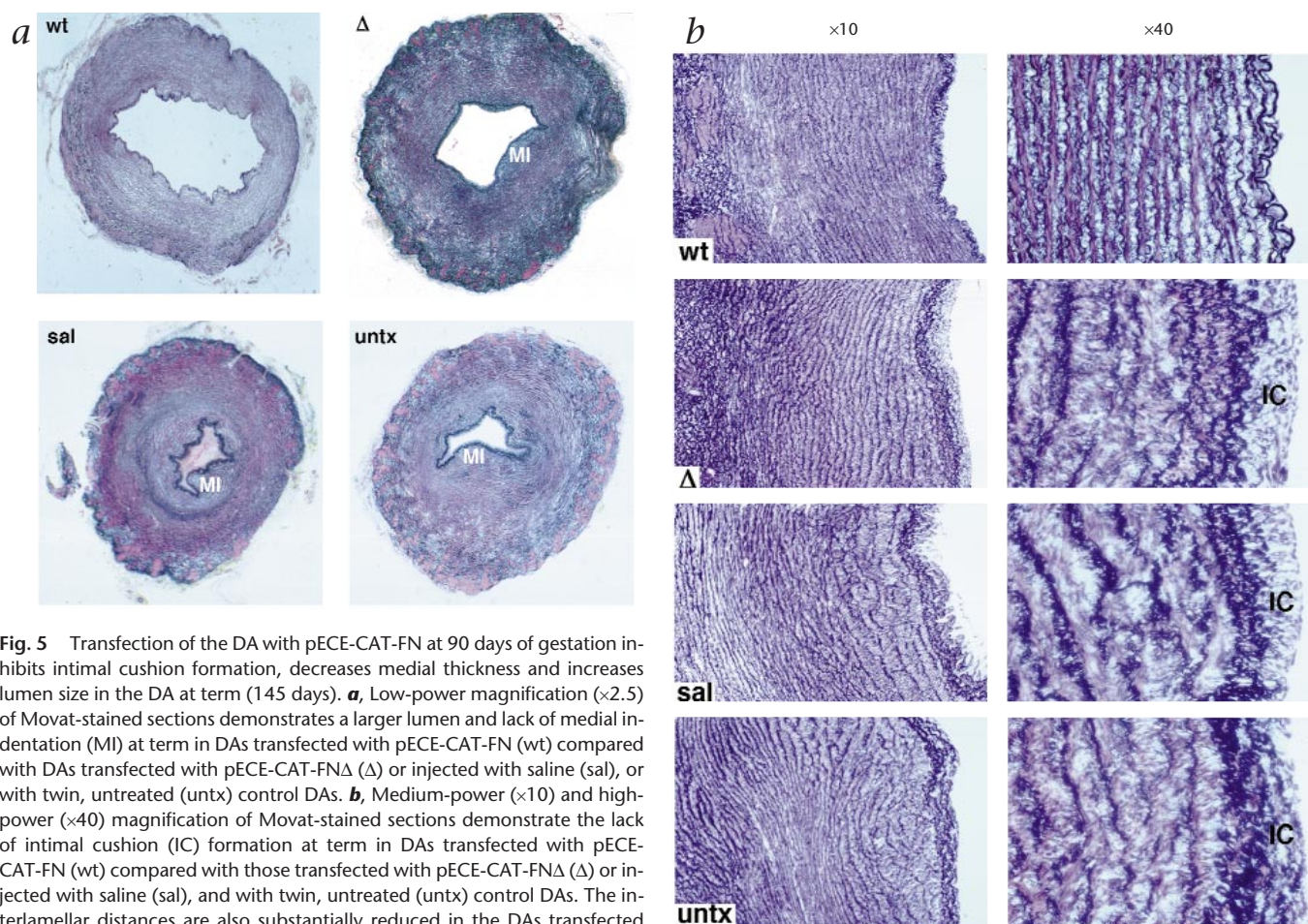


Fig. 5 Transfection of the DA with pECE-CAT-FN at 90 days of gestation inhibits intimal cushion formation, decreases medial thickness and increases lumen size in the DA at term (145 days). **a**, Low-power magnification ($\times 2.5$) of Movat-stained sections demonstrates a larger lumen and lack of medial indentation (MI) at term in DAs transfected with pECE-CAT-FN (wt) compared with DAs transfected with pECE-CAT-FN Δ (Δ) or injected with saline (sal), or with twin, untreated (untx) control DAs. **b**, Medium-power ($\times 10$) and high-power ($\times 40$) magnification of Movat-stained sections demonstrate the lack of intimal cushion (IC) formation at term in DAs transfected with pECE-CAT-FN (wt) compared with those transfected with pECE-CAT-FN Δ (Δ) or injected with saline (sal), and with twin, untreated (untx) control DAs. The interlamellar distances are also substantially reduced in the DAs transfected with pECE-CAT-FN (wt) compared with those of the control groups.

of inflammatory response or morphologic abnormalities in adjacent blood vessels such as the pulmonary artery or aorta, and there were no evident systemic effects that could be attributed to the HVJ liposomes. Although we chose to use a direct surgical approach to isolate the ductus arteriosus, the same gene transfer strategy could be applied *in utero* using a catheter-based technique¹⁷. This would require design of a double-balloon catheter appropriately tailored to occlude both ends of the ductus. Future studies should also address how long after birth patency is maintained and whether an inflammatory response is mounted in older animals.

In collagen gel assays, migration of cultured smooth muscle cells collected from lamb DA at 100 days of gestation is increased compared with that of cells from the aorta, and the enhanced migration could be inhibited by disrupting the cell–fibronectin interaction with either RGD peptides or fibronectin antibodies⁹. Here we establish the essential importance of fibronectin-dependent smooth muscle migration in the formation of the DA intimal cushions. We also show that the post-transcriptional mechanism responsible for upregulation of fibronectin synthesis in cultured DA smooth muscle cells¹¹ functions in the intact animal. Cell culture studies have demonstrated that transfection of smooth muscle cells with LC-3 results in increased fibronectin synthesis without altering fibronectin mRNA levels¹¹. Further examination of the mechanism involved has demonstrated that by binding the ARE in the fibronectin 3'-UTR, the microtubule-

associated protein LC-3 facilitates recruitment of fibronectin mRNA to the polyribosomal translational machinery, a process that requires an intact microtubule network¹⁸.

The ARE-specific effect of pECE-CAT-FN transfection on cell migration was particularly evident in the analysis of CAT expression by immunohistochemistry, in which smooth muscle cells transfected with pECE-CAT-FN expressed CAT but maintained a migratory phenotype, in contrast to the cells transfected with pECE-CAT-FN Δ , which became stellate. The ARE specificity was also demonstrated by the reduction in fibronectin synthesis caused by pECE-CAT-FN compared with that caused by pECE-CAT-FN Δ transfection. The small effect of pECE-CAT-FN Δ on fibronectin synthesis compared with the effect of no treatment (control DAs) may have been related to the transfection method. It was not evident when total fibronectin levels were compared by western immunoblotting, which may reflect a lack of sensitivity of this method in detecting small changes in protein expression. Because neointimal formation was not affected by transfection with the control pECE-CAT-FN Δ plasmid, there may be a threshold of fibronectin synthesis needed to allow for smooth muscle cell migration, which was still achieved in these vessels.

We cannot exclude the possibility that sequestration of LC-3 affected other mRNAs that might be important in the formation of the intimal cushions, or that the 'decoy' RNA sequestered other ARE-binding proteins, influencing the expression of other

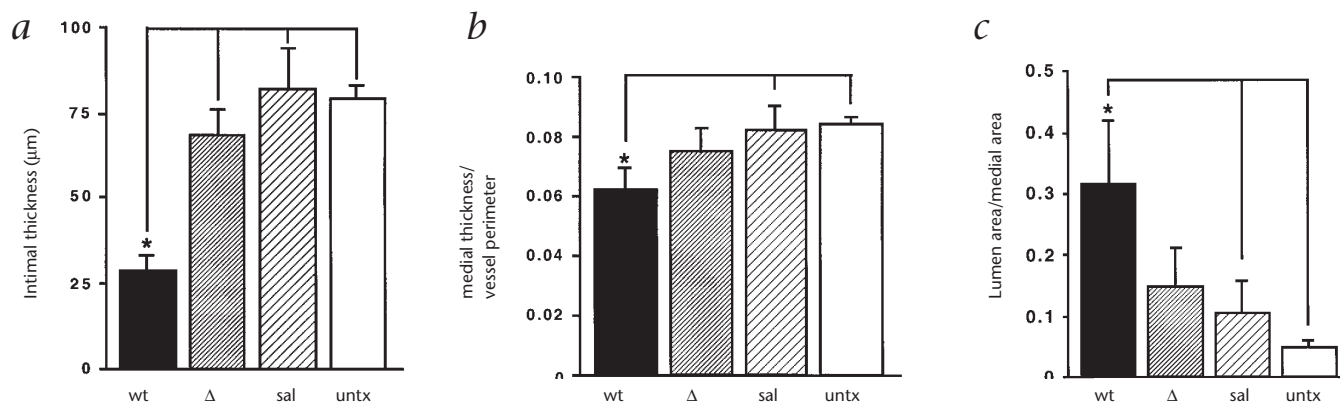


Fig. 6 Quantitative analysis of mean intimal thickness (**a**), mean DA medial thickness relative to vessel perimeter (**b**) and luminal area relative to medial area at term (**c**) in DAs transfected with pECE-CAT-FN (wt) compared with those transfected with pECE-CAT-FN Δ (Δ) or injected with saline (sal) or with twin, untreated (untx) control DAs. Transfection with pECE-CAT-FN causes a decrease in mean intimal thickness compared with pECE-CAT-FN Δ transfection, saline injection and no treatment (twin con-

trols) (*, $P < 0.05$ for all comparisons) and a decrease in mean medial thickness compared with saline injection and no treatment (twin controls) (*, $P < 0.05$ for each). Luminal area relative to medial area was increased in vessels transfected with pECE-CAT-FN compared with those injected with saline and with untreated controls (*, $P < 0.05$ for each). Bars represent mean \pm s.e.m.; $n = 4$ except for the twin, untreated control group, for which $n = 7$.

mRNAs relevant to this process. LC-3 upregulates expression of apolipoprotein D (A. Burry and M.R., unpublished data), but no other mRNAs have been identified. AREs are found in mRNAs that encode proteins known to regulate cell differentiation as well as proliferation, including c-myc, c-fos and others¹⁹⁻²³. We therefore compared expression levels of c-myc and c-fos in vessels transfected with pECE-CAT-FN or pECE-CAT-FN Δ , but found them to be similar.

Medial thickening in the DA may be responsible for the medial indentations observed after ductal constriction at term in untreated vessels, vessels injected with saline and vessels transfected with pECE-CAT-FN Δ , which were not apparent in DAs transfected with pECE-CAT-FN. Increasing medial thickness during late gestation has been documented in the developing human DA. This thickening of the DA wall contributes to lumen narrowing near term and would thus facilitate postnatal closure of the DA (ref. 1). In the DA transfected with pECE-CAT-FN, medial thickness is decreased compared with that of DAs transfected with pECE-CAT-FN Δ and untreated, control DAs, a feature that was associated with a reduction in the distances between elastic lamellae. This effect may be related to a difference in the orientation of the smooth muscle cells or to the reduced contribution of fibronectin to the extracellular matrix.

Our gene transfer strategy was developed to test the role of fibronectin in DA intimal cushion formation. Increased fibronectin expression has also been shown in human vascular diseases such as pulmonary hypertension^{24,25}, restenosis²⁶, atherosclerosis^{27,28} and post-cardiac transplant coronary arteriopathy²⁹, the last three involving fibronectin induction by cytokines. In an experimental model of post-cardiac transplant coronary arteriopathy, inhibition of fibronectin-T-cell interactions resulted in reduced neointimal formation³⁰. Because cytokine-mediated induction of fibronectin is also regulated by LC-3 (C.A.E.M and M.R., manuscript in preparation), the gene transfer strategy described here has potential mechanistic and therapeutic implications for a variety of occlusive vasculopathies.

Here we have biologically engineered a developmental process in the DA by manipulating a program that regulates smooth muscle cell motility. Although the direct therapeutic implica-

tions of our study address duct-dependent congenital heart defects, biological interventional cardiology could be useful in other settings in which congenital heart defects alter vascular morphology as a primary or secondary feature of the disease. Our study should therefore lead to other approaches to treatment through biological intervention.

Methods

Preparation of HVJ liposomes with pECE-CAT-FN or pECE-CAT-FN Δ .

Plasmid expression vectors were previously constructed in which the 3'-untranslated region of a reporter gene encoding the bacterial protein CAT (chloramphenicol acetyl transferase), expressed under the control of the SV40 viral promoter, was replaced with the 3'-UTR of fibronectin mRNA containing the AU-rich element to which LC-3 binds (UUAUUUAU)(pECE-CAT-FN; ref. 11). Expression vector constructs were also made in which the ARE of the fibronectin 3'-UTR was mutated to GGAGGGAG such that it would not bind LC-3 (pECE-CAT-FN Δ ; ref. 11). HVJ liposomes were prepared as described³¹. Lipid layers for liposome preparation were constructed by evaporating lipid mixtures under nitrogen gas using a rotary evaporator (Yamato Scientific, Orangeburg, New York). For each liposome preparation, phosphatidylserine (6 mg; Avanti Polar Lipids, Alabaster, Alabama), phosphatidylcholine (1.25 mg) and cholesterol (2.5 mg; Sigma), were dissolved together in chloroform and evaporated under nitrogen gas to form a thin film of lipid on a glass tube. Plasmid DNA (200 μ g in 80 μ l) was pre-incubated for 1 h at room temperature with 65 μ g HMG (high mobility group)-1,-2 protein (WAKO, Richmond, Virginia). HMG-1,-2 has been shown to facilitate plasmid DNA entry into the cell nucleus³¹. The DNA-HMG-1,-2 complex was added to the lipid layer, vortexed for 30 s and then incubated at 37 $^{\circ}$ C for 30 s; this cycle of vortexing and incubation was repeated eight times, followed by sonication in a bath type sonicator and a final vortex for 30 s.

Ultraviolet-inactivated HVJ on the surface of the liposomes facilitates the transfer of DNA into the cells of the vascular wall^{12,16}. HVJ was propagated in embryonated eggs in the laboratory of Y. Kaneda (Osaka University, Japan), and purified from chorioallantoic fluid as follows: Chorioallantoic fluid (100 ml) was centrifuged at 1,000 g for 10 min at 4 $^{\circ}$ C. Supernatants were collected and pelleted at 27,000 g in a Sorvall centrifuge using an SS34 rotor. Pellets were resuspended in 20 ml balanced salt solution (BSS: 137 mM NaCl, 5.4 mM KCl, 10 mM Tris-HCl pH 7.6) and pelleted again at 27,000 g . The resultant pellet was kept in 2.5 ml fresh BSS at 4 $^{\circ}$ C for 12 h and then gently resuspended in BSS and centrifuged at 1000 g for 10 min. The titer of virus in the supernatant was determined by spectrophotometry. An absorbance of 1.0 at 540 nm corresponds to 15,000 hemagglutinating units (HAU) of HVJ. HVJ was inactivated by ultraviolet irradiation with 198 mJ in a

Stratalinker 1800 UV cross-linker (Stratagene, La Jolla, California).

Liposomes containing 200 µg of DNA (pECE-CAT-FN or pECE-CAT-FNA) were mixed with 37,000 HAU of ultraviolet-inactivated HVJ and placed on ice for 10 minutes. BSS (2.5 ml per HVJ liposome mixture) was added and the mixture was incubated at 37 °C for 90 min with shaking (120 rpm). HVJ liposomes were then separated from free HVJ on a sucrose density gradient consisting of 30% and 60% sucrose. HVJ liposomes were collected from the top of the 30% sucrose layer after ultracentrifugation at 62,800 g for 3 h at 4 °C in a SW55 Ti rotor in an L5-65 ultracentrifuge (Beckman Instruments, Mississauga, Ontario, Canada). The HVJ liposomes were then washed in BSS and pelleted again at 27,000 g.

Surgical procedure. All experiments were done following a protocol approved by the Animal Care Committee of The Hospital for Sick Children and in accordance with the guidelines of the American Physiological Society. Each pregnant ewe at 90 days of gestation was anesthetized with intravenous sodium thiopental (1 g, intravenously), intubated, and ventilated with halothane (1.5%) and oxygen. After a midline laparotomy, the uterus was incised to expose the left forelimb and chest of the fetus. A left thoracotomy was then done on the fetus and the pericardium was incised to expose the great vessels and ductus arteriosus. The ductus was first clamped at the aorta using a vascular microserrafine (Fine Science Tools, Vancouver, British Columbia, Canada). The main pulmonary artery was clamped at its bifurcation, and the right and left pulmonary arteries were then clamped at their origin (Fig. 1). Pre-warmed saline (1 ml) was used to flush out the isolated area through a butterfly needle (22 gauge). The HVJ liposome mixture or saline control (1 ml) was injected through the same needle and left for 10 min. The needle was then removed and the clamps were released. The chest of the fetus was closed, and the fetus was placed back in the uterus. Saline was used to replace lost amniotic fluid before closure of the uterus. Penicillin (500 mg) and streptomycin (1 g) were administered to the ewe before and at 2 and 4 days after surgery. To assess fibronectin synthesis and possible changes in the expression of other ARE-containing genes, fetal lambs from each transfected group were killed at 100 days of gestation, and DA tissue was obtained. To assess endpoints of neointimal and medial thickening and lumen area, as well as CAT immunohistochemistry, lambs were killed after spontaneous delivery at term (145 days of gestation).

Analysis of fibronectin synthesis. DAs at 100 days of gestation were cut into 1-mm² pieces and placed in organ culture for 1 h with 2 ml serum-free and cysteine/methionine-free medium 199 containing 1% bovine serum albumin. The culture medium was replaced and 10 µCi/ml of ³⁵S-methionine (Amersham) was added for 4 h. Total secreted protein synthesis was then analyzed in triplicate from 50-µl aliquots of culture medium by trichloroacetic acid precipitation followed by liquid scintillation spectrometry. To extract newly synthesized fibronectin, 1-ml aliquots of culture medium were incubated with 100 µl gelatin 4B-sepharose beads overnight at 4 °C. The beads were washed three times in PBS. Bound fibronectin was then eluted from the beads into 100 µl SDS-PAGE sample buffer (5% β-mercaptoethanol, 2% SDS, 10% glycerol, 62.5 mM Tris-HCl, pH 6.8) by boiling for 5 min, and the samples were separated by 6% SDS-PAGE. Gels were fixed in 5% acetic acid/10% methanol and soaked in Amplify (Amersham) for 15 min before being dried under vacuum at 80 °C on a Model 443 slab dryer (Bio-Rad, Hercules, California). Dried gels were exposed to Kodak X-OMAT AR film for 2–4 days and the 220-kDa bands corresponding to fibronectin were compared using densitometric analysis with Molecular Analyst software for Macintosh computers. Identification of the 220-kDa band as fibronectin has been confirmed in our laboratory by western immunoblotting⁸.

Morphometry. Image Pro Plus software was used to obtain measurements of DA intimal and medial thickness, luminal area and vessel perimeter from Movat pentachrome-stained sections. Mean intimal and medial thickness were calculated using the program based on the entire intimal and medial area of the vessels, the latter corrected for vessel external perimeter. Because of the variability in vessel size, we calculated luminal area relative to medial area.

Immunohistochemistry. Immunoperoxidase staining of DAs and aortae from transfected and control sheep at birth was done on 3% paraformaldehyde-fixed tissue sections embedded in paraffin. Paraffin was extracted with two washes for 15 min in xylene. Sections were re-hydrated by successive 5-min washes in decreasing concentrations of ethanol. The sections were then washed twice in 0.1% BSA/PBS and immersed in 1% hydrogen peroxide/methanol to block endogenous peroxidase activity. After two washes in 0.1% BSA/PBS, the sections were blocked for non-specific cross-reactivity in normal horse serum (1:10 dilution, for fibronectin) or in normal goat serum (1:10 dilution, for CAT). The sections were then incubated overnight at 4 °C in one of the following primary antibodies: monoclonal antibody to human fibronectin (1:100 dilution; Chemicon, Temecula, California), or polyclonal antibodies to CAT (1:100 dilution; 5'→3', Boulder, Colorado). For all immunohistochemical analyses, negative controls were incubated with mouse or rabbit IgG instead of the primary antibody. The sections were then washed and incubated for 1 h at room temperature in secondary antibody: goat anti-mouse horseradish peroxidase or goat anti-rabbit horseradish peroxidase (1:10,000 dilution; Vectastain, Burlingame, California) before being developed in 3,3'-diaminobenzidine (25 mg in 50 ml 0.006% H₂O₂, 50 mM Tris, pH 7.6) and counterstained with hematoxylin.

Western immunoblot analysis. Protein extracts (15 µg per lane) from DAs collected at 100 days of gestation were separated by SDS-PAGE and transferred to a PVDF membrane in non-reducing conditions. Blots were blocked for 1 h in Tris-buffered saline (TBS) containing 0.1% Tween-20 and 5% nonfat dry milk, and probed with monoclonal antibodies to fibronectin (1:100 dilution; Sigma), c-fos (1:100 dilution) or c-myc (1:100 dilution; Oncogene Research Products, Cambridge, Massachusetts) for 1 h at room temperature. Blots were washed with TBS containing 0.1% Tween-20 followed by incubation with peroxidase-conjugated goat anti-mouse IgG (1:3,000 dilution; Vectastain, Burlingame, California). The blots were washed and developed using enhanced chemiluminescence western blotting detection reagents (Amersham).

Statistical analysis. A one way analysis of variance (ANOVA) was used to detect differences in DA fibronectin and morphometric assessments of intimal and medial thickness and luminal area described above, and post-hoc comparison of individual groups was done using a Fisher's test. In all analyses, differences were considered statistically significant with $P < 0.05$.

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