A Novel Zebrafish Embryo Xenotransplantation Model to Study Primary Human Fibroblast Motility in Health and Disease

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Abstract

Fibroblasts have a central role in the maintenance of tissue homeostasis and repair after injury. Currently, there are no tractable, cost-effective model systems for studying the biology of human fibroblasts *in vivo*. Here we demonstrate that primary human fibroblasts survive transplantation into zebrafish embryos. Transplanted cells migrate and proliferate, but do not integrate into host tissues. We used this system to study the intrinsic motility of lung fibroblasts from a prototype fibrotic lung disease, idiopathic pulmonary fibrosis (IPF). IPF fibroblasts displayed a significantly higher level of motility than did fibroblasts from nonfibrotic lungs. This is the first *in vivo* examination of primary human lung fibroblast motility in health and disease using zebrafish models.

Introduction

DURING THE PAST 2 DECADES, zebrafish has served as a highly informative model system for studying vertebrate developmental genetics.¹ More recently, zebrafish has emerged as a powerful tool for modeling human disease (reviewed in Refs. 2–4). This trend is exemplified by several xenograft-based zebrafish cancer models that have been successfully used to study innate cancer cell properties (e.g., invasiveness, metastasis, tumorigenesis, and angiogenesis)⁵ and for anti-cancer drug development.⁶ Here we demonstrate for the first time that the zebrafish embryo can serve as a host to nonmalignant primary human cells, and can be used to study their intrinsic motility and proliferative capacity.

Idiopathic pulmonary fibrosis (IPF) is a common, progressive, and lethal lung disease in humans. The disease process begins at the lung bases and periphery and progresses by extension of serpentine fibrotic bands inwards and upwards to form a constricting reticular network that leads to death by asphyxiation. The only effective therapy for human IPF is lung transplantation. Currently, there are no experimental or animal models that recapitulate the naturally occurring diseases, greatly limiting our ability to study the pathobiology of the primary disease effector cell—the fibroblast—*in vivo*. We propose a completely novel strategy for this purpose, using the developing zebrafish embryo to host fibroblasts derived from the IPF lung enabling their intrinsic biological properties to be studied. In principle, this system is suitable for examining disease mechanisms and for testing new therapies targeting patho-

logical fibroblast function. Here we demonstrate that following xenotransplantation, human primary lung fibroblasts survive, proliferate, and migrate in the developing embryo, and that IPF fibroblasts display a high level of motility compared to fibroblasts from nonfibrotic lungs.

Materials and Methods

Fish and embryos

Adult zebrafish (*Danio rerio*) wild-type breeding colony maintenance, fish husbandry, and embryo collection were performed at the University of Minnesota Zebrafish Research Core Facility using standard procedures¹⁶ with approval from the Institutional Animal Care and Use Committee (IACUC Protocol 0909A72712, Assurance of Compliance A3456-01, effective September 10, 2008–April 30, 2013). Freshly fertilized eggs were obtained through natural spawning. Embryos were kept at 28.5°C and staged according to Ref. 9. Xenotransplantations were carried out with IACUC approval (Protocol 0906A67146, 06-25-2009).

Tissue procurement and characterization

Lung tissue was obtained at the time of biopsy, autopsy, lung resection, or lung transplantation (this study was deemed exempt by the University of Minnesota Institutional Review Board for Human Subjects Research). Primary human lung myofibroblasts from de-identified donors ($n=16$) were utilized. These consisted of 8 specimens, designated HLF (histologically normal

lung adjacent to a variety of surgically removed lesions), and 8 lung specimens from patients with IPF (meeting ATS/ERS/JRS/ALAT diagnostic criteria)¹⁷ with a confirmed final pathological diagnosis of usual interstitial pneumonitis.

Cell line derivation and cultivation

Lung tissue explants were cultivated in 35 mm tissue culture dishes in explant medium (DMEM+20% FBS + antibiotics and antimycotics) at 37°C in 95% air, 5% CO₂. Cell outgrowth was evident in 5 to 7 days, and cells filled the dish in 2 to 3 weeks. Cells from each 35 mm dish were released with trypsin-EDTA and placed in 100 mm tissue culture dishes after trypsin was neutralized with fresh explant medium. These cells, designated passage 1, were cultivated in growth medium (DMEM+10% FBS + antibiotics) at 37°C in 95% air, 5% CO₂. Medium was replaced twice weekly, and cells were subcultivated weekly at a 1:4 split ratio. Cells designated myofibroblasts (both IPF and HLF) had typical spindle morphology, were vimentin- and alpha smooth muscle actin-positive; and factor VIII- and surfactant C-negative. Cells were used between passages 5 and 7.

CFSE staining

When myofibroblasts in log phase growth reached ~ 80% confluence, growth medium was removed and replaced with Phosphate Buffered Saline (PBS) containing 5 μ M carboxy-fluorescein succinimidyl ester (CFSE) (Sigma) for 10 min at 37°C (shaking every 2 min). The PBS-CFSE was removed and replaced with growth medium for 2 h. Cells were released from the dish with trypsin-EDTA, pelleted by centrifugation (1000 g for 5 min) and resuspended in serum-free DMEM.

Engrafting

Xenotransplantation was carried out by microinjection. Microinjections and embryo observations were performed on the stage of a Stemi-2000 stereomicroscope (Carl Zeiss MicroImaging, Inc.) equipped with a FemtoJet injector (Eppendorf AG) at RT. Embryos (in chorions) at the oblong stage were lined up and restrained in agar grooves¹⁶ with their blastoderms upward. Injections were performed as previously described.¹⁸ Myofibroblasts were transplanted into the central portion of the blastoderm at oblong-sphere stages (as in Ref. 15) through a micropipette with a splinted sharp tip 50 μ m in diameter. Embryos were kept at 28.5°C.

Graft imaging in live embryos

At 48 hours post fertilization (hpf), embryos were dechorionated, immobilized with Tricaine (Sigma), and screened using a Zeiss Axiovert upright microscope equipped for fluorescence detection. Images (fluorescence, phase contrast and bright field) were taken using a Nikon 260 camera and AxioVision software (Release 4.7.2).

Fixation and sectioning

Following image acquisition, embryos were fixed overnight with paraformaldehyde (4%) at RT.

Frozen sections. Fixed embryos were rinsed in 6% sucrose/0.1 M Dulbeccos PBS (DPBS) at RT, infiltrated with

increasing concentrations of sucrose/PBS with rotation at 4°C over a 72 h period, and cryoprotected with 20% sucrose/DPBS overnight. In preparation for embedding in OCT (Sakura Tissue-Tek), the embryos were placed in a 1:1 solution of 20% sucrose in DPBS/OCT, and rotated overnight at 4°C. After cryoembedding in OCT, 5 μ m sections were cut and placed on silane-coated slides using a Leica 1950 Cryostat.

Paraffin sections. Fixed embryos were embedded in paraffin, 4 μ m sections were cut using a Leica RM2255 microtome and placed onto silane-coated slides exactly as described.¹⁹

Immunostaining, microscopy, and image analysis

Cryosections were fixed in -20°C cold 100% methanol for 10 min, air-dried, rinsed in DPBS, blocked with normal donkey serum for 30 min, and incubated with the primary antibody, mouse monoclonal CD 59 [MEM-43] (1:500) (Abcam, Inc.), overnight at 4°C. For detection of the primary antibody, donkey anti-mouse Cy-3 (1:500) (Jackson ImmunoResearch) was applied for 45 min at RT in a dark chamber. Images were collected on a Zeiss Axiovert 200 fluorescent microscope and analyzed using AxioVision (Release 4.7.2) imaging capabilities. Ki67 immunostaining was performed exactly as described.¹⁹ Briefly, sections were dewaxed, subjected to antigen-heat retrieval (Biocare Medical) for 20 min at 95–98°C, blocked with Background Sniper (Biocare Medical) for 1 h, and probed with rabbit anti-human Ki67 (1:400) (Thermoscientific, Clone SP6) overnight at 4°C in 10% Background Sniper solution. For antibody detection, a USA-HRP rabbit linking reagent (Covance) was used for 30 min, followed by USA-HRP Labeling Reagent (Covance) for 30 min. Sections were treated with diaminobenzidine (DAB) to detect signal, and counterstained with Harris hematoxylin (Surgipath Medical).

Statistical analysis

Graft morphometry data were analyzed using nonparametric statistics. For data ranking, we used clustering and the Mann-Whitney U test. To minimize bias, images taken at random were analyzed by two independent observers. The rank orders from each analyst were compared using ordinal ranking and the Mann-Whitney U test.

Results and Discussion

Primary cell labeling and engrafting

IPF and control (HLF) myofibroblasts were prepared and characterized as previously described.⁷ To distinguish human cells from zebrafish embryonic cells, we labeled primary human cells with a vital dye, CFSE, which is stable and dilutes stoichiometrically as cells divide.⁷ CFSE-labeled fibroblasts were slightly opaque, which allowed for visual control of microinjections and facilitated xenotransplantation into transparent embryos at the late blastula stage. Early embryos tolerated engraftment of approximately 100 myofibroblasts, which were easily detected among the transparent host cells. With the onset of gastrulation, transplanted cells aggregated within the ectoderm, generally adhering to each other to form a distinct solitary graft. Typically, grafts acquired a position close to the animal pole, at the geometric center of the blastoderm (Fig. 1). In the embryo, the deep cells of the blastodisc are motile and constantly mix prior to epiboly.^{8,9} Thus, it is

FIG. 1. Representative images of grafts at mid-gastrula (3 h post transplantation). A solitary graft positioned close to the geometric center of the blastoderm. (A) Phase contrast; (B) fluorescence; (C) merged image. Shown is a lateral view with the animal pole upwards. *Arrows* indicate graft location. Scale bar: 100 μm .

FIG. 2. Formation of the fibrotic reticulum. Representative images of the IPF and HLF grafts localized to the head and trunk regions of the 1 and 2 dpf host embryos. *Left panel:* Fluorescence: High cell motility is characteristic of the IPF grafts in 2 dpf embryos. *Arrows* pointing at the processes, wedge indicating the graft margin, * marking the foci. Yolk autofluorescence (*background*) and no signal in head and trunk regions in embryos without grafts (CON). *Right panel:* Brightfield images showing graft-related malformations in the head (unilateral anophthalmia and microphthalmia) and trunk (lump, axial deformities) regions in the graft-bearing embryo, and no deformities in embryos without grafts (CON); e, eye; ov, otic vesicle; p, pericardium; Y, yolk sack. Lateral view. Scale bar: 250 μm .

likely that grafts were pushed towards the blastula apex mechanically by host cells. According to the zebrafish fate map,⁹ the apical position predetermines cell restriction to the head region of the developing embryo. Indeed, out of 115 grafts we inspected, the majority of grafts (80%) were observed in the head and only 20% in the trunk region of the host embryos (Fig. 2, left panel). All grafts remained visible in live embryos for 3 days.

Early zebrafish embryos as hosts for primary human cells

We chose late blastula (oblong stage) embryos for xenotransplantation for several reasons. First, there is no

FIG. 3. Proliferation of the engrafted cells in the 2 dpf host embryos. (A) Representative image of the embryo cross-section immunostained with anti-human cd59/Cy-3 antibodies. (1) FITC: CFSE-labeled graft cells; (2) TRITC: CD59/Cy-3-positive cells; (3) DAPI; (4) Merged image. *Arrows* point to the areas with CFSE-/CD59+ cells. Scale bars: 50 μm . (B) Representative image of the embryo sagittal section immunostained with human anti-Ki67 antibody and HRP. *Arrow* points to the graft area in the head region of the embryo. *Inset:* Ki67+ cell nuclei in the graft body. *Arrows* point to the labeled nuclei of the engrafted cells. Scale bars: 100 μm in B and 30 μm in B inset.

FIG. 4. Graft morphology and measurements.

detectable innate immune response for up to 22 h of development and no adaptive immunity until the larval stages.¹⁰ As expected, host cells did not exert any detectable adverse effect on engrafted cells, and no morphological evidence of graft rejection was found during the 2-day observation period. Second, fibroblast growth factors (Fgfs) are expressed in early embryos, providing stimuli for fibroblast survival, growth, and proliferation. With the exception of Fgf9, zebrafish fgf subfamilies are similar to their human orthologs.¹¹ In the embryo, extracellular Fgf morphogen gradients are present where they are utilized for proper gastrulation,¹² for axis patterning,¹³ and for organ specification.¹⁴ Third, primary human fibroblasts and zebrafish embryonic cells at the oblong stage are of comparable size.

Graft size and embryo viability

Primary human cells did not integrate into the germ layers, but rather interfered with embryo development, giving rise to a variety of abnormalities. The severity of malformations and embryo survival rate depended on the size and localization of the graft. Grafts containing >100–150 cells impeded gastrulation and caused 100% embryo lethality. Smaller grafts did

not prevent major axis patterning, but rather hampered proper organ formation and led to a variety of deformities. As evidenced by fluorescence microscopy, grafts localized to the posterior head and trunk regions caused trunk and tail underdevelopment and malformations, while grafts positioned in the anterior and mid-head regions predominantly hindered brain development, causing microphthalmia, bilateral or unilateral anophthalmia (Fig. 2, right panel). Of note, phenotype screening for malformations greatly facilitated selection of graft-bearing embryos at 2 days post fertilization (dpf). Despite these abnormalities, the majority of host embryos survived for 5–6 days.

Graft morphology

Fluorescence from xenografts containing CFSE-labeled cells was detectable in live host embryos for 3 days. During this period, there were major changes in graft shape and fluorescence intensity. At day 1, grafts were compact, showing strong adhesion of the CFSE-positive cells to each other. Grafts were spheroid in shape, with indistinct outer edges and a few (2 to 5) short protrusions of approximately 0.1 to 0.5 graft diameters in length (Fig. 2, upper panel). By day 2, graft

TABLE 1. GRAFT CHARACTERISTICS

Cell type	Grafts, N	Graft size area (μm^2)	Margin size width (μm)	Foci, N	Processes, N	Processes: length MAX (μm)	Processes: length SUM (μm)
IPF 82	5	6343 $\pm$ 487	29 $\pm$ 11	5 $\pm$ 3	8 $\pm$ 3	218 $\pm$ 19	975 $\pm$ 119
IPF 74	5	4034 $\pm$ 319	27 $\pm$ 15	2 $\pm$ 1	6 $\pm$ 3	240 $\pm$ 15	748 $\pm$ 225
IPF 37	10	10581 $\pm$ 588	45 $\pm$ 12	1 $\pm$ 0	8 $\pm$ 2	135 $\pm$ 17	811 $\pm$ 67
IPF 49	3	6332 $\pm$ 599	33 $\pm$ 9	2 $\pm$ 1	7 $\pm$ 4	219 $\pm$ 23	868 $\pm$ 87
IPF 66	3	7685 $\pm$ 418	62 $\pm$ 11	3 $\pm$ 2	8 $\pm$ 4	90 $\pm$ 21	569 $\pm$ 175
IPF 129	8	1562 $\pm$ 388	24 $\pm$ 12	2 $\pm$ 1	10 $\pm$ 3	186 $\pm$ 12	933 $\pm$ 82
IPF 188	4	6120 $\pm$ 218	74 $\pm$ 22	1 $\pm$ 1	8 $\pm$ 3	156 $\pm$ 14	763 $\pm$ 200
IPF 75	13	12104 $\pm$ 715	60 $\pm$ 17	7 $\pm$ 3	12 $\pm$ 4	227 $\pm$ 22	1268 $\pm$ 159
HLF 130	7	3099 $\pm$ 233	50 $\pm$ 15	0	4 $\pm$ 3	104 $\pm$ 12	319 $\pm$ 77
HLF 78	10	11383 $\pm$ 446	60 $\pm$ 10	2 $\pm$ 1	6 $\pm$ 2	110 $\pm$ 13	507 $\pm$ 112
HLF 142	11	1226 $\pm$ 517	10 $\pm$ 4	3 $\pm$ 1	7 $\pm$ 3	67 $\pm$ 12	495 $\pm$ 84
HLF 63	8	4947 $\pm$ 734	98 $\pm$ 16	2 $\pm$ 1	5 $\pm$ 2	86 $\pm$ 9	464 $\pm$ 76
HLF 99	7	8872 $\pm$ 799	76 $\pm$ 15	0	4 $\pm$ 2	94 $\pm$ 14	324 $\pm$ 89
HLF 136	9	2866 $\pm$ 331	33 $\pm$ 9	3 $\pm$ 1	6 $\pm$ 1	147 $\pm$ 17	618 $\pm$ 114
HLF 89	2	3952 $\pm$ 628	22 $\pm$ 12	0	7 $\pm$ 1	116 $\pm$ 12	543 $\pm$ 247
HLF 57	10	1692 $\pm$ 174	88 $\pm$ 14	0	5 $\pm$ 2	100 $\pm$ 9	314 $\pm$ 62

FIG. 5. Motility of engrafted fibroblasts measured by aggregate length of graft's processes. (A) Box-plot showing all data for each of the 8 IPF and 8 HLF cell lines; (B) Boxplot showing aggregated data for IPF versus HLF.

outgrowth increased significantly and graft shape became irregular. The central highly fluorescent area was surrounded by an uneven fine mesh-like margin with diminished fluorescence due to partitioning of the CFSE dye between daughter cells as engrafted cells proliferated. In other regions there were highly fluorescent processes resulting from cell migration. The processes varied in number, length, and trajectory, with occasional focal swellings at the outer surface of the host embryos (Fig. 2, mid- and lower panels). Of note, these reticular features were independent of graft location (head vs. trunk) or type/severity of host embryo malformation (Fig. 2, mid-panels). By day 3, graft fluorescence was diminished with multiple areas of reduced fluorescence observed in the embryos (data not shown).

To determine whether the observed loss of fluorescence indeed reflected cell proliferation and was not simply due to the death of engrafted cells, we took two approaches. First, we used anti-human CD59/Cy-3 antibodies to identify cells of human origin. We found CFSE+ /CD59+ cells in the central part of the graft. At the graft margin, however, we observed a large number of CFSE-CD59+ cells. These were cells of human origin that had diluted the CFSE dye, apparently due to cell division (Fig. 3A). To determine if the engrafted cells were mitotically active or had simply lost the CFSE dye for some other reason, we used anti-human Ki67 antibody to identify cycling human cells. We found cells with Ki67+ nuclei in the central part of the graft (Fig. 3B), while the host cells stained negative. Typically, 2–5 Ki67+ nuclei were present per graft section. Of note, the distribution of cycling cells per 500 μm^2 in

grafts (3.7 ± 0.5 , $n=8$) and original human lung tissue samples (4.7 ± 0.9 , $n=8$) was comparable ($p=0.25$), suggesting a similar proliferation rate. Second, in a separate study, we injected GFP-labeled fibroblasts into the zebrafish embryos. The grafts remained fluorescent for up to 6 days, indicating persistence of the engrafted cells (data not shown). These data indicate that the engrafted cells remained viable and retained proliferating potential.

Cell motility

To compare the intrinsic motility of IPF and HLF myofibroblasts, we examined graft dimensions 48 h post engraftment. Grafts were examined in live embryos, and fluorescent 2D images were collected. At present, there is no satisfactory mathematical model to capture the geometrically complex graft morphology that developed as a result of cell migration from the initial graft site. In building such a model, we focused on quantifiable characteristics and parameters (Fig. 4). We characterized 115 xenografts from 16 human primary myofibroblast cell lines: 8 IPF and 8 HLF. Using Axiovision imaging capabilities, we measured the size of the central graft area, maximum width of the graft marginal zone demarcated by decreased fluorescence, number of processes and foci, and process length (Table 1). There were no differences in graft size between IPF and HLF. All IPF and 75% of the HLF grafts developed multiple processes that differed in length and branching frequency. In the IPF grafts, processes extended up to 1.5 to 3 graft diameters in length; whereas the processes in

HLF were considerably shorter. While the graft margin width tended to be larger in HLF grafts, there were more processes and more discrete graft foci among the IPF cohort. To quantify these differences as an index of intrinsic migratory capacity, we calculated the aggregate length of all processes (Table 1). Based on this parameter, we ranked grafts using the Mann-Whitney U test, presenting the data graphically as a box-plot (Fig. 5). All IPF cell lines showed a high level of motility. In contrast, their nonfibrotic HLF counterparts were generally far less motile, a result in accord with a prior report describing human dermal fibroblasts.¹⁵

Thus we have developed a highly tractable *in vivo* model system amenable to examining key phenotypic differences between human fibroblasts participating in both physiological and pathological processes. Here we provide the first report of a stable phenotypic difference between lung fibroblasts from patients with IPF and their nonfibrotic counterparts in a zebrafish *in vivo* system. Our work provides strong corroboration of the growing number of *in vitro* reports documenting durable differences between fibrotic and control fibroblasts across several dimensions including: integrin repertoire, growth factor signaling networks, and genome-wide pattern of gene expression. By providing a system that is amenable to genetic and pharmacological manipulation of fibroblasts prior to grafting, this model can be used to study the mechanistic basis of these differences in an *in vivo* context.

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Disclosure Statement

No competing financial interests exist.

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