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Patch grafting of organoids of stem/progenitors into solid organs can correct genetic-based disease states

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ABSTRACT

Patch grafting, a novel strategy for transplantation of stem/progenitor organoids into porcine livers, has been found successful also for organoid transplantation into other normal or diseased solid organs in pigs and mice. Each organoid contained ~100 cells comprised of biliary tree stem cells (BTSCs), co-hepato/pancreatic stem/

Abbreviations: AHEP, adult hepatocytes; AFP, alpha-fetoprotein; ALB, albumin; BTSC, biliary tree stem cells; CFTR, cystic fibrosis transmembrane conductance regulator; EGF, epidermal growth factor; EpCAM, epithelial cell adhesion molecule; FAH, fumaryl-acetoacetate hydrolase; FGF, fibroblast growth factor; HBs, hepatoblasts; HGF, hepatocyte growth factor; HpSCs, hepatic stem cells; LGR5, leucine rich repeat-containing G protein coupled receptor 5 that binds to R-spondin; MMPs, matrix metalloproteinases; NANOG, a transcription factor involved with self-renewal; NCAM, neural cell adhesion molecule; NGN, neurogenin; NRG, NOD Rag**-/-IL2RgammaC-null**; PDX1, pancreatic and duodenal homeobox 1, a transcription factor important in pancreatic development; PBGs, peribiliary glands; SALL4, Sall-like protein 4 that is important for self-replication of stem cells; SOX9, a transcription factor associated with endodermal tissues (liver, pancreas, intestine); SOX17, a transcription factor associated with liver development; VEGF, Vascular endothelial cell growth factor.

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progenitors, and partnered with early lineage stage mesenchymal cells (ELSMCs), angioblasts and precursors to endothelia and stellate cells. Patch grafting enabled transplantation into livers or pancreases of $\geq 10^{8th}$ (pigs) or $\geq 10^{6th-7th}$ (mice) organoids/patch. Graft conditions fostered expression of multiple matrix-metalloproteinases (MMPs), especially secretory isoforms, resulting in transient loss of the organ's matrix-dictated histological features, including organ capsules, and correlated with rapid integration within a week of organoids throughout the organs and without emboli or ectopic cell distribution. Secondarily, within another week, there was clearance of graft biomaterials, followed by muted expression of MMPs, restoration of matrix-dictated histology, and maturation of donor cells to functional adult fates.

The ability of patch grafts of organoids to rescue hosts from genetic-based disease states was demonstrated with grafts of BTSC/ELSMC organoids on livers, able to rescue NRG/FAH-KO mice from type I tyrosinemia, a disease caused by absence of fumaryl acetoacetate hydrolase. With the same grafts, if on pancreas, they were able to rescue NRG/Akita mice from type I diabetes, caused by a mutation in the insulin 2 gene. The potential of patch grafting for cell therapies for solid organs now requires translational studies to enable its adaptation and uses for clinical programs.

1. Introduction

We have established a novel method, "patch grafting", enabling transplantation of large numbers of organoids of stem/progenitor cells or of suspensions of adult cells into solid organs and without emboli or ectopic cell distribution [1]. Engraftment and full integration throughout the organ occurred within a week. By ~14 days, the graft's biomaterials, hyaluronans, are cleared by the organ's native metabolic processes, enabling donor cells to mature into adult fates. This process is dictated by the organ's tissue-specific signaling environment of soluble endocrine and paracrine signals and extracellular matrix components.

The success of patch grafting strategies overcomes the long-standing impasse for transplantation of epithelial cells, especially epithelial stem/progenitor cells, and even more so of organoids, into solid organs [1–3]. Historically, transplantation of hemopoietic cells or mesenchymal stem cells has been done by delivery of cells via a vascular route or by direct injection. Donor cells float in blood or lymphatic or interstitial fluid and then attach to a target site when their adhesion molecules are activated due to environmental signals such as occur in inflammatory processes in a process referred to as "homing" [3–6].

Solid organs are different and require grafting methods to enable transplantation of cells to occur, as has long been demonstrated for skin [4], and now also for liver [1]. Other efforts at grafting strategies, such as "cell sheet engineering", have succeeded at transplanting a cell sheet of mature cells linked by their lateral adhesion molecules and by gap junctions to the surface of livers [7]. Cell sheets provide adult functions at the discrete site at which the sheet is attached, but their ability to overcome deficits is limited to the numbers of cells that are transplantable by this method and by the interactions with host cells that are confined to the graft site, since the cells fail to migrate into the tissue. By contrast, patch grafting involves delivery of cells into the host's organ where the donor cells become fully integrated and can be regulated by the full repertoire of organ-specific systemic and paracrine signals.

Patch grafting is also an improvement on previous attempts that involved the delivery of soluble signals to the target sites within grafting biomaterials [8,9]. By contrast, patch grafting delivers viable cells that became fully integrated within the host organ and demonstrate dynamic and synergistic interactions with the host cells. The graft's biomaterials are intended only for delivery of cells into the organ and thereafter are cleared or absorbed within 2–3 weeks.

In the current studies, we demonstrate the efficacy of patch grafting in transplantation of cells into normal pigs (*Sus scrofa domestic)* or mice *(mus musculus)*, including in wild type hosts without any condition demanding engraftment dependent on a regenerative response. The findings presented expand those already demonstrated for porcine livers [1] by showing that they are successful also for pancreas, for which experimental and clinical methods of cell therapies involving direct cell transplantation into pancreas have not been demonstrated previously. In addition, patch grafting was demonstrated to be successful also for murine livers and pancreas. Both pigs and mice are major animal species used in translational research and used increasingly as alternatives to non-human primates in preclinical studies [10,11].

Pig donor cells were recognizable by a transgene coupled to a fluoroprobe, enhanced green fluorescent protein (eGFP), fused to histone 2B (H2B), providing a biomarker located in nuclei [10]. Murine biliary tree stem cells were prepared from DsRed mice, Tg(CAG-DsRed*MST) 1Nagy/J, with a red transgene in all cells [12]. Human donor biliary tree stem cells were prepared from fetal or neonatal human tissues and identifiable in grafts by fluoroprobe-labeled antibodies to human-specific biomarkers such as human-specific albumin.

Importantly, we demonstrate that patch grafting, enabling transplantation of large numbers of donor cells, including organoids, to solid organs, can overcome disease states in immunocompromised mice with genetically based diseases. Specifically, we show that patch grafting with xenografts of porcine or human cells can reverse tyrosinemia in the *NOD*-Rag1$^{-/-}$IL2Rgamma C-null *(*NRG*)* fumaryl aceto-acetate hydrolase knockout mice [13,14] and allografts of murine donor organoids can reverse diabetes in the NRG Ins2Akita mouse [15], demonstrating efficacy in two distinct disease states and in two different solid organs, the liver and pancreas.

2. Results

2.1. Graft's cellular components—donor organoids

Donor biliary tissue was derived from transgenic (GFP+) pigs, transgenic mice (DsRed), or humans. Donor cell populations were easily recognized as distinct from murine host cells either as ones with a transgene (GFP + or DsRed) in unstained cells or were cells that were stained with antibodies to the transgene or to a human antigen or biomarkers.

Donor cells, whether derived from murine, porcine or human sources, were presented as organoids of biliary tree stem cells (BTSCs), determined endodermal stem/progenitors, precursors to liver and pancreas, and that were partnered with early lineage stage mesenchymal cells (ELSMCs), precursors to endothelia and/or to stellate cells. The BTSCs have been found in all mammals [16–18] and are located in niches, peribiliary glands (PBGs), throughout the intrahepatic and extrahepatic biliary tree and the accessory duct to the dorsal pancreas (see Table 1). The methods for isolation and establishment of BTSC/ELSMC organoids and characterization of them are provided in prior reports as summarized in a recent review [18] and in a report on the methods and strategies of patch grafting [1]. Those from pigs are newly discovered , have been characterized in more details, and have been compared with their human counterparts in a separate article [19].

Organoids were prepared from freshly isolated cells as described in the methods, given in the online supplement, and those published previously [1]. They were suspended in serum-free Kubota's Medium [20] and placed on low attachment dishes. Within 6–8 h, organoids had assembled and were found to be solid aggregates of BTSCs and

associated ELSMCs. As shown in Fig S1 cells on the inside of the aggregates were primitive, expressing pluripotency genes and no mature hepatic (or pancreatic) genes; slightly more differentiated cells formed a coat on the outside of the aggregates. Organoids self-assembled, and each one contained ~100 cells. They were characterized using immunofluorescence (IF), qRT-PCR, and RNA-seq. More extensive data on the porcine versus human organoids, including their genetic signatures, are provided in a separate report [19].

1 **Donor Epithelial Stem/progenitors in the Organoids**

Organoids expressed low levels of pluripotency genes (OCT4, SOX2); endodermal stem cell genes [EpCAM, SOX 9, SOX17, PDX1, LGR5, CXCR4, MAFA, NGN3 and sodium iodide transporter (NIS)]; but did not express mature hepatic genes (e.g. P450s or transferrin/Fe) nor pancreatic genes (e.g. islet hormones or acinar cell-derived digestive enzymes). An exception was that the slightly more differentiated cells forming the outer perimeters of the organoids contained cells with constitutive, low-level expression of albumin and EpCAM. Immunohistochemical characterizations are shown in Fig S1A-F; representative qRT-PCR assays are shown in Fig S1G.

2 **Donor Mesenchymal Cells in the Organoids**

The summary of the porcine and human antigenic profiles of the mesenchymal cell partners, similar to that published previously [1], had phenotypic traits consistent for those of angioblasts (CD117+, CD133+, VEGFr+, CD31-negative) and their immediate descendants, precursors to endothelia (CD133+, VEGFr+, $CD31^{+}$, Von Willebrand Factor+) and precursors to stellate cells [CD146+, ICAM-1+, alpha-smooth muscle actin+ (ASMA), vitamin A-negative]. We refer to these collectively as early-lineage-stage-mesenchymal-cells (ELSMCs) and the organoids as BTSC/ELSMCs.

3 **Donor BTSC/ELSMC organoids were prepared from the following sources (Table 1):**

a **Murine organoids** were prepared from DsRed transgenic mice [12,21] and were grafted onto the pancreases of NRG/Akita mice.

b **GFP + porcine biliary tree stem/progenitor organoids** were grafted onto the livers of wild type pigs**.** The transgenic pigs have a transgene, green fluorescent protein (eGFP), fused to histone 2B (H2-B), that provides a donor-specific biomarker located in nuclei [10,19]. Cells were grafted onto the livers of wild type piglets (or onto the livers of murine hosts). In the liver, cellular components such as lipofuscins have autofluorescence at wavelengths overlapping with those of GFP. Analysis in the liver, therefore, relied on the immunohistochemistry using an antibody to the transgene, GFP, and that was then coupled to a chromogen, Vector NovaRED (Vector Labs, Burlingame, CA). This yielded a distinct red brown stain in the nuclei of donor cells. Methyl green was used for the counterstain.

c **. Male porcine organoids were grafted onto the livers of female piglets**. There were concerns that the GFP transgene might prove immunogenic, and concerns for experimental needs given the limited availability of H2B-GFP+ pigs. Therefore, we tested a separate model using the Y chromosome as the biomarker to recognize donor cells. Donor BTSCs were prepared from the biliary tree of male pigs and grafted onto host livers of females. The paraffin sections of the fixed tissue were stained with an antibody to RBMY-1, a male germ cell-specific RNA-binding protein (the Y chromosome RNA chromosome recognition motif), expressed only in males [22]. The antibody was coupled to Vector NovaRED enabling recognition of donor cells.

By using these two pig models, we were able to assess the potential of patch grafting strategies with confidence that the findings pertinent to grafting could be discerned independently of issues, such as immunological ones, regarding the models used.

d **Human extrahepatic biliary tree tissue** was used to prepare organoids that were grafted onto immunocompromised murine hosts, NRG/FAH-KO mice. Thus, we were able to compare the effects of both porcine and human donor cells to rescue these mice from type I tyrosinemia. The grafted human donor cells were identifiable with antibodies to human albumin or other human biomarkers.

2.2. Hosts for assessment of patch Grafting's ability of correct disease states

For effects of patch grafts on diseases of the liver, we used a model of type I tyrosinemia [23]. We debated using the newly established pig model of type I tyrosinemia [24], but the costs of the pigs were prohibitive and, even more worrisome, there is an only partial ability to manage them with the drug, Nitisinone (NTBC), used to protect animals from adverse effects of tyrosinemia. The inability to control completely the effects in pigs of tyrosinemia on the kidneys as well as the liver, effects that can be especially severe, impacting interpretation of the data.

Therefore, for the mutant liver studies, we used *NOD*-Rag1$^{-/-}$IL2RgammaC-null *(*NRG*)* mice that were subjected by Dr. Lishan Su and associates to Crispr/Cas9 methods to delete fumaryl acetoacetate hydrolase (FAH), the key enzyme in tyrosine catabolism and that when aberrant or missing results in type I tyrosinemia [25]. The mice are referred to as NRG/FAH-KO. Given that they are immunocompromised, we were able to assess if porcine or human stem/progenitor organoids could rescue them from the FAH deficiency.

For the mutant pancreas studies, we did grafts onto the pancreases of diabetic, immunodeficient *NODRag1*^{null}*IL2rg*^{null}*Ins2*^{Akita} mice, referred to as NRG/Akita mice. These mice are immunocompromised. And so permissive for xenografts. Controls had to be given insulin for survival and humane treatment of the animal. 6–8 weeks (see Table 1).

2.3. Cell graft biomaterials

1 **Hyaluronan Hydrogels with Specific Viscoelasticity**

The details of the graft composition have been reported previously in the article on patch grafting strategies [1]. In brief, organoids were embedded in a soft (~100 Pa) hyaluronan (HA) hydrogel layer that was then plated onto a platform of silk impregnated with a more rigid (~700 Pa) HA hydrogel. The grafts were secured to the organ surface by sutures or surgical glue. The graft composition involved the use of thiol-modified hyaluronans (HA) hydrogels prepared with precise concentrations of HA and PEGDA to achieve defined levels of stiffness, as determined by rheological traits and expressed as the dynamic shear modulus (G*). The viscoelasticity level of 100 Pa or less was chosen for the layer containing the organoids based on prior studies showing that

Table 1
Donors and Recipients used for assessing Patch Grafting.

Donor BTSC/ELSMC Organoids		Hosts (Recipients)	Marker for Donor cells
Mouse (Table S3)	mBTSC/ELSMC from DsRed mice	NRG/Akita Mice	DS-Red transgene [12, 21]
Pig (Tables S1 and S2)	pBTSC/ELSMCs from GFP + transgenic pigs [1,10]	Wild type pig NRG/FAH-KO mice [25]	GFP transgene [10]
	pBTSC/ELSMCs from male, wild type pigs	Female, wild type pigs	RBMY-1 [22]
Human (Table S2)	hBTSC/ELSMCs from extrahepatic biliary tree tissue	NRG/FAH-KO mice [13]	Human albumin

under these conditions the donor cells remained as stem/progenitors able to express stem cell traits [26] and that includes both plasma membrane-associated and secreted isoforms of matrix metalloproteinases (MMPs) [1]. By contrast, increasing the rigidity of the hydrogel to 300 Pa or higher caused the cells to differentiate, to mute MMP expression and, in parallel, to cease migration [1]. The graft was sutured or glued to the organ surface at the corners of the silk patch such that the soft hydrogel layer containing the organoids was positioned against the target site. A hyaluronan hydrogel with a rigidity of ~200–300 Pa was sufficiently fluid to enable painting or coating the outside (serosal) surface of grafts. It was applied at the end of surgery and was found to minimize adhesions with surrounding tissues.

2 **Backing for the Grafts**

In prior studies [1], we tested a number of backings with a focus on ones already used clinically in abdominal surgeries. We chose contour SERI Silk Surgical Scaffolds (Sofregen, Medford, MA) that provided the best combination of mechanical support, neutrality with respect to effects on the donor cells, minimal adhesions and an ability to be attached to sites with significant curvature [27,28]. Adhesions were minimized further by application of 2X HA (~200-300 Pa) to the outside (the serosal side or free side) of the silk backing after attachment to the target site during surgery.

2.4. Grafts on livers versus pancreases of normal, wild type pigs

Preliminary studies were done to define how long it would take to see engraftment; the particulars of the engraftment process; whether only to the target organ or whether donor cells distributed also to ectopic sites; and how long the effects of grafts would last. The findings dictated the experimental designs and time points for more extensive characterizations at a week, three weeks, a month, and finally three months. The details of the graft composition, the surgeries, the sites chosen for the grafts and the general strategies for patch grafting were presented in a prior report [1].

1 **Patch grafts of organoids from transgenic GFP + pigs attached onto the liver or pancreas of wild type hosts (Fig. 1)**

Engraftment of organoids from GFP + transgenic pigs and into normal, wild type pig livers occurred within one week, a process associated with extensive remodeling with loss of most of matrix-dictated histological features, especially those near the graft site (Fig. 1). The Glisson's capsule had disappeared, and acinar and lobular histological features of the liver were missing or aberrant. At greater distances from the site at which the graft was attached to the liver, there were indications of early stages of maturation of the engrafted donor cells such that they expressed HNF4a or alpha-fetoprotein at boundary zones for lobules. There were donor GFP + cells throughout the tissues, especially concentrated in the bile ducts (Fig. 1B–E). Remarkably, the donor organoids had migrated within a week through the entirety of the piglet liver that had an average liver diameter of 10–14 cm. This was confirmed by qRT-PCR assays for the transgene in different regions of the liver (Fig S3).

2 **Patch Grafting of GFP + BTSC organoids onto the Pancreas of wild type host**

Patch grafts of BTSCs partnered with ELSMCs and derived from transgenic (GFP+) pigs were tethered to the pancreases of wild type piglets. To avoid triggering autolysis of the pancreas, the grafts were sutured to the duodenum on one side and attached with surgical glue to the connective tissue above the pancreas on the other. The donor cells fully migrated and integrated throughout the host pancreas within a week and had matured to functional pancreatic acinar cells expressing amylase (Fig S5) and islets expressing insulin and glucagon (Fig. 2). The transgene, GFP, was observed primarily in the nucleus of the islets even at one week. However, for the first 2 weeks, the transgene, GFP, was also observed in the cytoplasmic region in acinar cells. This proved a transient phenomenon with localization in the nucleus of the acinar cells of the transgene occurring by 2–3 weeks (Fig. 2C–E).

3 **Patch Grafts of Male Cells onto the Livers of Females**

To control for aberrant reactions due to use of cells from a transgenic pig, similar studies were done with patch grafts of biliary tree stem/progenitor organoids of male pigs tethered onto the livers of female pigs (Figs. 3 and 4). The sections were stained with an antibody to RBMY-1, a male germ cell-specific RNA-binding protein (the Y chromosome RNA chromosome recognition motif), expressed only in males [22]. The antibody was coupled to a chromogen, NovaRED, enabling donor cells to be readily identified and distinguished from host cells as rust colored, whereas host cells with the background stain of hematoxylin were a fuchsia or purple color.

The engraftment process at one week indicated donor cells clustered at the perimeters of the liver acini and lobules and as a loss of Glisson's capsule especially at the interface of the graft and the liver. The nuclear biomarker, RBMY-1, evident both in nuclei and during the remodeling phase, was observed also cytoplasmically. The loss of hepatic histological features was transient; those features were restored by three weeks at which time donor cells were found uniformly dispersed throughout the liver and the RBMY-1 biomarker was entirely and solely within the nuclei (Fig. 4).

4 **Engraftment Correlates with Expression of Matrix-Metalloproteinases (MMPs) Especially the Secreted Isoforms by Donor Cells (Fig. 5)**

The remodeling associated with migration and integration throughout the liver (or the pancreas) correlated with production of matrix metalloproteinases (MMPs) by the organoids. MMP production included both plasma membrane-associated and secreted forms, known for degradation of extracellular matrix components and correlated with cell invasion and migrations [29–31].

The findings from RNA-seq data were confirmed by IHC assays for the enzymes encoded by MMP genes (Fig. 5A). The organoids of extrahepatic biliary tree stem/progenitors expressed strongly a number of MMPs including MMP2, MMP7, and MMP13. IHC assays (Fig. 5B) showed the presence of the secreted forms of MMPs, such as MMP1, MMP2, and MMP9 and some plasma membrane-associated matrix metalloproteinases, (e.g. MMP15), especially in the regions of loss of typical hepatic histology. Protein expression of MMP1 was found in BTSCs/ELSMCs organoids and also in remodeling regions of grafts. However, existing data banks of RNA-seq findings do not include MMP1 due to a lack of an annotated species of porcine MMP1 for use in the analyses. Therefore, recognition of its presence in the remodeling zones was based on the IHC assays. The expression of multiple forms of MMPs lasted for one to 2 weeks after which expression of them became muted. This was followed by a restoration of Glisson's capsule and of the lobular/acinar histological features of the liver and of maturation of donor cells to adult fates.

2.5. Patch grafts deliver donor cells that can correct disease states

1 **Ability to Alleviate Tyrosinemia in NRG/FAH-KO Hosts (Figs. 6 and 7)**

NRG/FAH-KO mice were obtained from Dr. Lishan Su and associates, who had established the mice using CRISPR/Cas9 technology with NRG mice [25] to achieve a murine model of type I tyrosinemia due to deficiency in fumarylacetoacetate hydrolase (FAH) and, in parallel, one

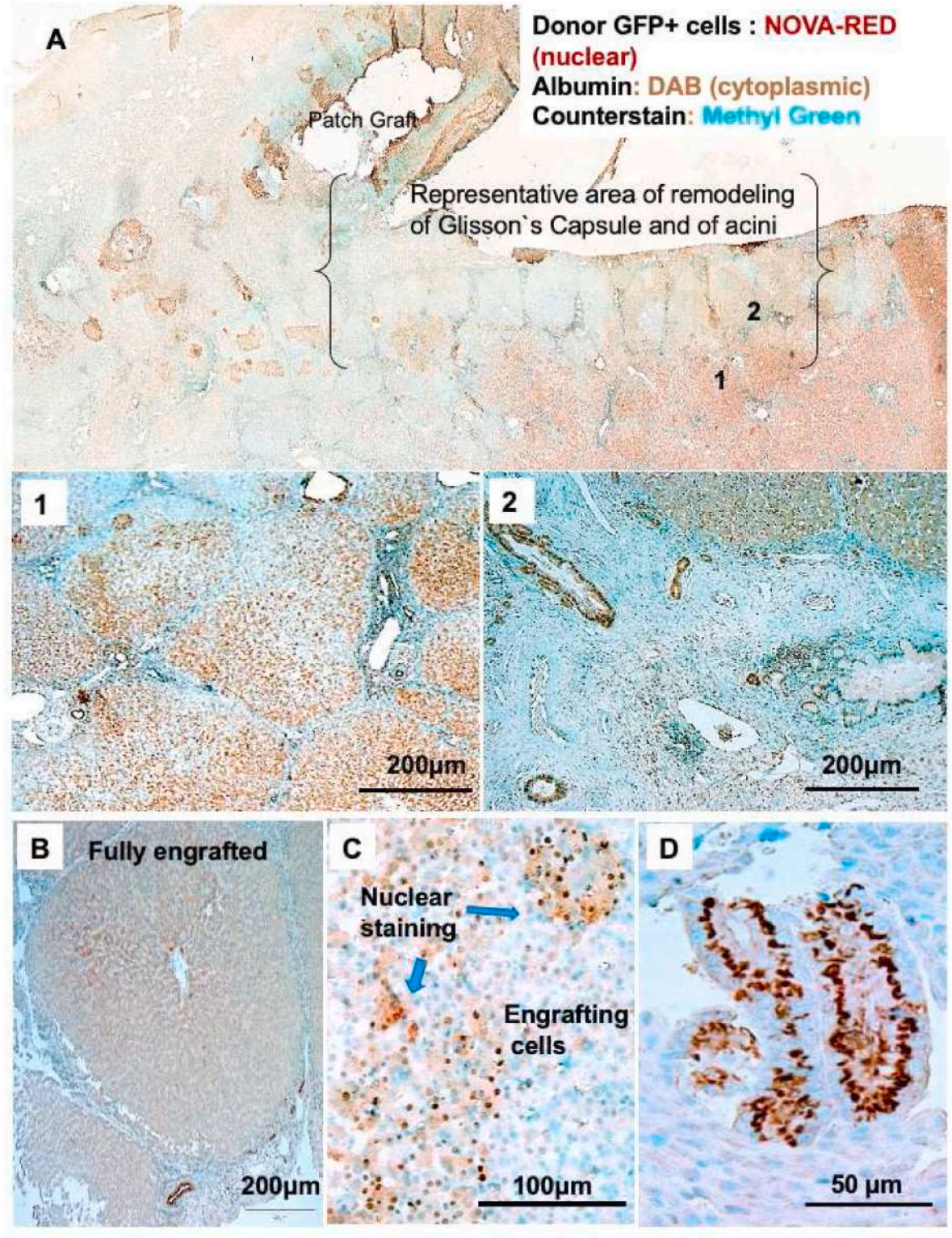

Fig. 1. Patch graft of GFP + biliary tree stem/progenitor organoids on the liver of a wild type piglet. A. IHC stain with GFP antibody on the FFPE liver sections of GFP + BTSCs grafted wild type host pig after 1-week post grafting. Donor cells have migrated extensively throughout the host liver and are reasonably uniformly dispersed in the acini and in the bile ducts. Shown are enlarged images of GFP + donor cells located in the liver plate zone (1) and ductal area (2). GFP + donor cells are indicated with an antibody to GFP and linked to a red chromogenic dye, NovaRED. The DAB (brown) cytoplasmic stain was used to indicate albumin expression. The section is counter stained with Methyl green. **B–C.** The region in the wild-type pig liver showing stages of partial versus complete engraftment of the donor GFP + cells. Donor cells were identified with an antibody to GFP and that was linked to Vector NovaRED; the counter stain was methyl green. Donor GFP + cells with co-expression of albumin were stained very dark red/brown; host cells expressing albumin but without co-expression of GFP were stained a pale brown. Nuclei that are positively stained are indicated in **C.** In a week in all of the grafted piglets, donor cells had migrated into and were found in every lobe of the liver (see Fig S3 with qRT-PCR proof of this). The piglet organs had diameters of 10–15 cm. **D.** Strong engraftment of GFP + donor cells in the bile ducts of the grafted liver. Timewise during the engraftment and migration process, evidence for donor cells was observed first in the bile ducts and secondarily in the parenchyma of the acini. See also Fig. 3 versus 4 showing the stages of engraftment at a week versus 3 weeks. The counter stain was methyl green.

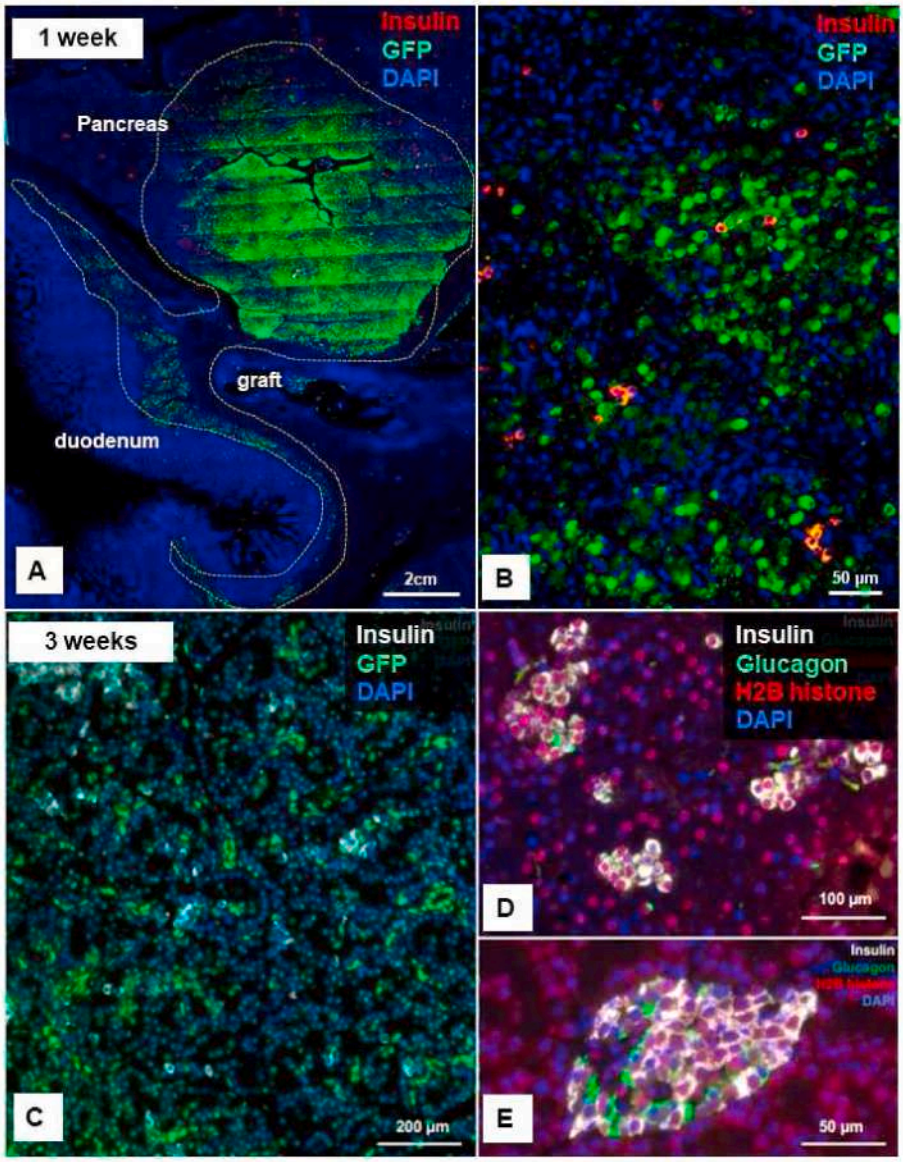

Fig. 2. Patch graft of porcine GFP + donor cells attached onto the pancreas of a wild type porcine host. In all of these figures, the background stain was the nuclear dye, DAPI (4′, 6-diamidino-1-phenylindole), yielding a blue color to the nuclei.**A.** Dramatic findings were observed using grafts of organoids from the GFP transgenic pigs and attached to the pancreases of wild type pigs and evaluated after one week. GFP + donor cells had engrafted and migrated throughout much of the pancreas and also into the submucosa of the duodenum, the location of Brunner's Glands, the start-point for the network of BTSCs [42]. See also Fig. S5 demonstrating maturation of donor cells to acinar cell fates indicated by expression of amylase. Bar = 2 cm. See also Fig. S6 for additional images of donor cells that matured to yield functional islets.**B.** Higher magnification images are of sections stained with a primary antibody for insulin and a secondary donkey anti-guinea pig antibody coupled to AlexaFluor 647. The positive signal was pseudocolored in red. The numbered arrows indicate (1) host islets (red); 2) donor islets (yellow/orange color from merger of GFP-fluoroprobe and the pseudocolored red signal from staining with antibody to insulin; 3) donor acinar cells (GFP+). Host acinar cells are blue from DAPI staining. The images reveal that the GFP transgene was expressed cytoplasmically in most of the donor cells, especially the donor acinar cells. Bar = 50 μm **C.** Evaluation of the grafts after 3 weeks showed that the remodeling phase has diminished with donor cells scattered throughout the pancreas and now demonstrating nuclear localization of the transgene. **D and E.** Enlargement of donor islets expressing insulin and glucagon.

permissive for xenografts. Normal liver and kidney functions were sustained in control mice by treatment with a drug, 2-(2-nitro-4--trifluoromethylbenzoyl)-1,3-cyclohexanedione, NTBC, also called Nitisinone, in the drinking water (20 μg/ml). NTBC has been shown to block the tyrosine pathway prior to the FAH enzymatic deficiency which prevents the buildup of toxic intermediates affecting both the liver and the kidney [14]. Interruption of NTBC administration leads to rapid death of the animal, typically within ~2 weeks in young adult animals (8–12 weeks old) and within ~4 weeks in older animals (4–9 months old). It is unknown what the age-dependent variable(s) is for the length of time for tolerance to tyrosinemia in these mice.

NRG/FAH-KO mice were treated with patch grafts of organoids of porcine BTSC/ELSMCs or in parallel experiments, with organoids of human BTSC/ELSMCs. Controls received grafts with the hyaluronan biomaterials but without cells. Preliminary studies were done to assess how fast to wean animals from NTBC and revealed that weaning could go from 20 μg/ml to 0 by 24 h after surgery, and this was the course of action taken with all subsequent experiments.

As summarized in Figs. 6 and 7 and S4, all the animals grafted with porcine BTSC/ELSMCs organoids and weaned from NTBC remained healthy for at least 4 weeks and gained weight (~1 gm/every 2–3 days). In replicate experiments, animals grafted with either porcine or human organoids (Fig. 7H) were maintained for 3 months and remained healthy throughout the experiment. The controls lost weight (~1 gm/day) and comprised those with no grafts and those with grafts with the hyaluronan biomaterials but no organoids. Controls of young adults (8–12 weeks old) had to be euthanized by day 16 post-surgery. If grafted as older animals (4–9 months), their ability to tolerate tyrosinemia was greater, and the animals survived for ~4 weeks. However, regardless of age at the time of transplantation, animals that were not transplanted with donor cells and not given NTBC did not survive or had to be euthanized within, at most, ~4 weeks. Therefore, to demonstrate a comparison in the health of the experimental animals at a time point when the controls had all died, the majority of the experimental animals were terminated by 4 weeks, and their livers and kidneys were evaluated. Mice transplanted with organoids of BTSC/ELSMCs (porcine or human) had livers and kidneys with histological features similar to those of animals maintained on NTBC. The liver was healthy and composed of evenly distributed central veins and portal tracts in low magnification images, and with hepatocytes arranged as cords indicated in the high magnification images (Fig. 6C). The kidney presented normal histology with intact outer cortex and inner medulla of kidney parenchymal cell

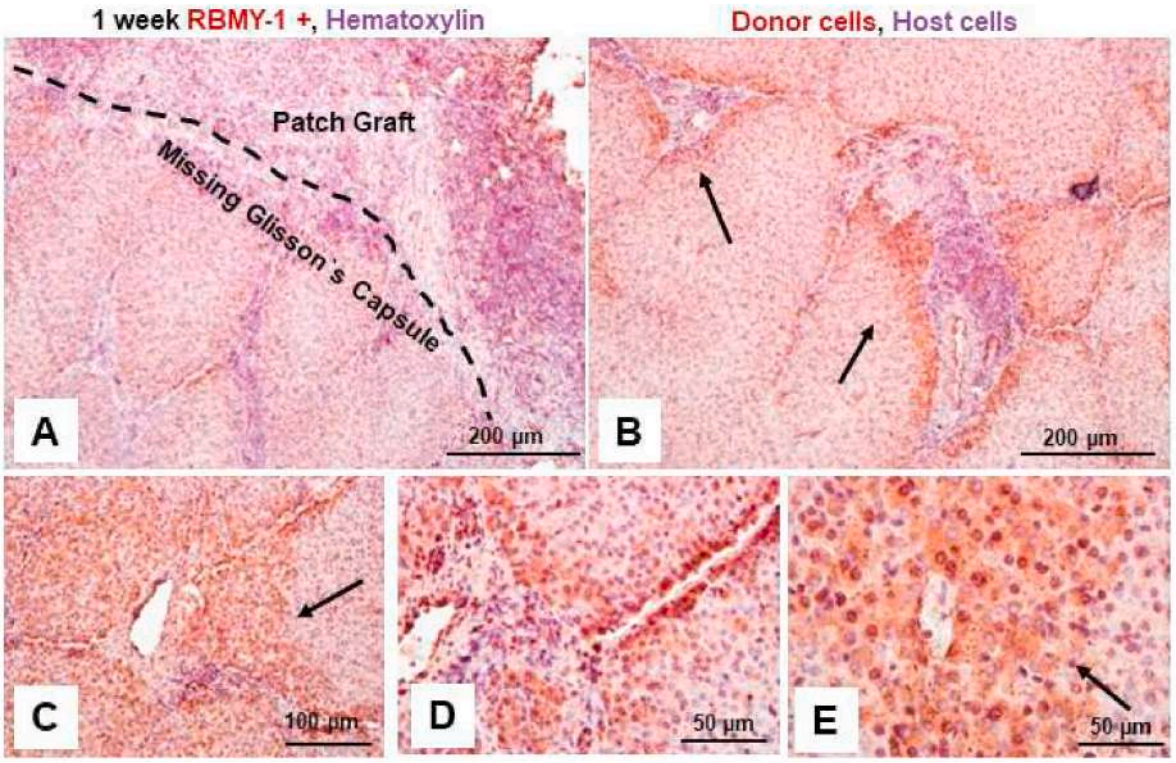

Fig. 3. Engraftment of biliary tree stem/progenitor organoids prepared from males onto the livers of female pigs. One-week post-transplantation. Images of grafts and subjacent hepatic tissue in the first week after transplantation, the phase in which there was extensive remodeling due to organoid-derived matrix metalloproteinases (MMPs). Transplants containing organoids of porcine biliary tree stem/progenitors prepared from male piglets, were attached to the livers of female pigs. The paraffin sections of the fixed tissue were stained with an antibody to RBMY-1, a male germ cell-specific RNA-binding protein (the Y chromosome RNA chromosome recognition motif), expressed only in males. The background stain is hematoxylin. Donor cells are recognized in the images by a rust color and host cells by a fuchsia color. **A.** In the remodeling phase (first week), the Glisson's capsule disappears (black line indicates its former location) along with blurring or loss of the matrix boundaries defining liver acini and lobules. Bar = 200 μm. **B–C.** Images of regions of the liver showing donor cells, identifiable by the rust color, are present at the boundaries of lobules and acini. Black arrows note some of the regions with engrafting cells. B. Bar = 200 μm. C. Bar = 100 μm. **D-E.** Higher magnification images indicating that the nuclear biomarker (RBMY-1) is evident both in the nucleus and in the cytoplasm of cells. Bar = 50 μm.

structures in the low magnification image and healthy glomerulus and glomerular capsule of the renal corpuscle in the high magnification image (Fig F2).

By contrast, there were massive injuries in the livers and kidneys of control animals that were not provided with cells and not provided with NTBC (Fig. 6B, F1). Pathological damage in the liver, primarily reflected in large numbers of necrotic liver parenchyma, and in apoptosis of hepatocytes with cytoplasmic vacuolization and necrosis in the high magnification image (Fig. 6B). In the kidney, necrosis was found in the renal medulla region, and there were abnormal structural features in the renal cortex due to the loss of renal tubular epithelial cells (Fig F1). The effects on livers and kidneys are known due to toxins from tyrosine metabolism in hosts with FAH deficiency [25]. GFP + donor cells were found throughout the host liver (Fig. 6) and the donor cells expressed porcine FAH (or human depending on the donors).

The experiments were repeated, and the hosts maintained for up to 3 months (Figs. 6D, 7C–E and S4). Those given the grafts survived for the length of the experiments and were found to have livers and kidneys demonstrating normal histology. Hosts with grafts were evaluated for porcine biomarkers for GFP or FAH (Fig S4) or for porcine albumin (Fig. 7). Those with grafts of human cells were assessed also for donor cells expressing human albumin (data not shown). A summary of the findings of these xenografts is provided in the table (Fig. 7H).

2 Patch Grafting Used for Rescue of Hosts from Type 1 Diabetes (Fig. 8)

Organoids of murine BTSCs/ELSMCs, derived from DsRed mice, were grafted onto the pancreases of diabetic, immunodeficient *NODRag1*^{null}*IL2rg*^{null}*Ins2*^{Akita} (NRG/Akita) mice. Whereas controls without grafts or with grafts with no organoids and without treatment with insulin did not survive more than a week or two, the grafted mice survived for at least 4 weeks and demonstrated a rapid reversal of hyperglycemia (Fig. 8B–D). A progressive reduction of blood glucose levels started at 1 week after patch grafting and euglycemia was established at 3–4 weeks. Moreover, the intraperitoneal glucose tolerance tests showed that, in NRG/Akita mice grafted with BTSCs/ELSMCs organoids, serum glucose levels progressively returned to control levels after glucose bolus injections. New functional insulin was produced in the engrafted diabetic mice by around 7–10 days and reached a peak at around 21–28 days correlating with increasing serum levels of C-peptide. In addition, we observed that murine serum C-peptide and insulin levels were minimal in untreated diabetic mice, in diabetic mice with patches without cells, and on day 0 for diabetic mice with patches containing cells. Moreover, to trace the engrafted BTSCs in the host pancreas, insulin protein expression was examined in the recipient pancreas using immunohistochemistry. In the pancreas of NRG-Akita mice that had patch grafts with organoids, insulin-positive cells were observed inside the areas of the patch that was grafted onto the pancreas and were absent in diabetic littermates without donor cells in the patch. Taken together, these data indicate that donor BTSCs differentiated into functional β cells, leading to considerable attenuation of hyperglycemia and improved glucose tolerance in diabetic mice. Allografts of organoids from DsRed mice patch grafted onto the pancreas of NRG/Akita mice were able to rescue them from type I diabetes with restoration of insulin production and stable normo-glycemia.

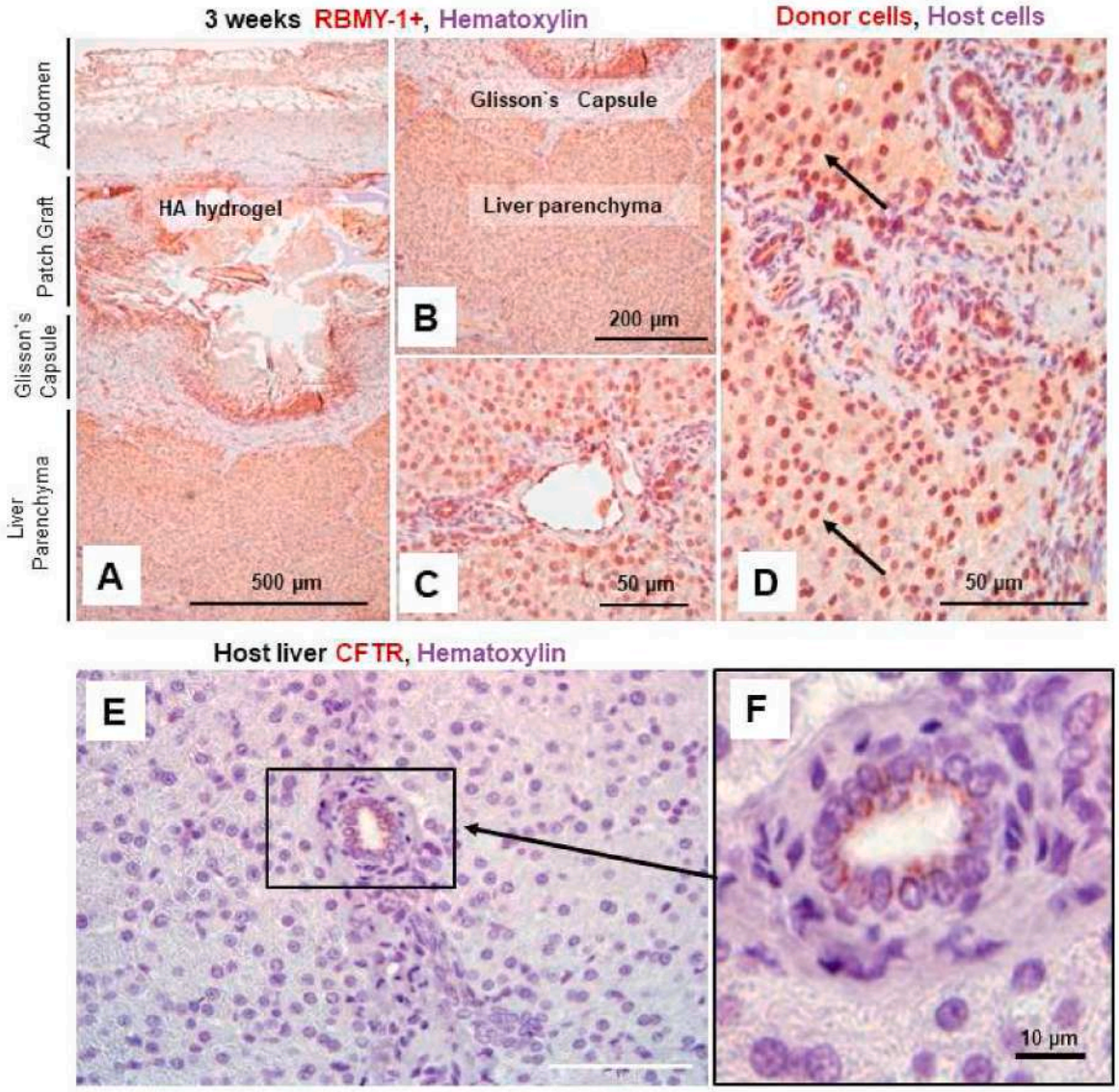

Fig. 4. Engraftment of biliary tree stem/progenitor organoids prepared from males onto the livers of female pigs. Three weeks post-transplantation. In the restoration of hepatic histological features by three weeks after attachment of patch graft containing organoids of male BTSCs/ELSMCs tethered to porcine female livers. The staining of the sections is as for Fig. 3. A. Low magnification image to show the entire depth of the serosal surface containing fat tissue, the patch graft with residual hyaluronan hydrogel, the restored Glisson Capsule, and the subjacent liver with restored histological features. Bar = 500 μm B. Higher magnification images to show that donor cells were now evident throughout the liver and are dispersed uniformly amidst host cells. Bar = 200 μm **C and D.** Higher magnification to show that the nuclear biomarker (RBMY-1) was now found entirely in the nucleus, and that donor cells are fully integrated throughout the parenchyma. Bar = 50 μm. **E and F.** IHC staining of cells expressing CFTR (cystic fibrosis transmembrane conductance regulator) in host pig intrahepatic bile duct and in low and high magnifications. E. Bar = 50 μm. F. Bar = 10 μm.

2.6. General findings

Conditions that maintained the donor cells in an immature state and able to express the full repertoire of MMPs were found to be critical for successful engraftment of donor cells. Factors causing differentiation of donor cells resulted in an absence or minimization of engraftment. These factors included soluble signals (serum, certain hormones), insoluble ones (matrix molecules such as type I collagen that promotes differentiation), and various backings (summarized in the methods paper on patch grafting [1]).

3. Discussion

We previously established patch grafting, a novel method to transplant organoids of stem/progenitors or adult cells, hepatocytes, into porcine livers, and with negligible evidence for emboli or for ectopic cell distribution [1]. Here we provide additional data to indicate that this grafting method is generic and can deliver large numbers of cells to liver or pancreas, in both mice and pigs, and whether the hosts are wild type (so no physiological condition influencing engraftment is required), or can be used with hosts having a disease condition that can both influence engraftment and also can make evident if donor cells can correct or alleviate the disease state. Within a week in all situations, there was engraftment of large numbers of organoids ($\geq 10^{8th}$/graft in pigs; $\geq 10^{6th}$-^{7th} /graft in mice). Few donor cells remained at the transplantation site. Differentiation of the donor cells to adult fates occurred in parallel with engraftment and was completed within approximately another week.

Simple surgical procedures were needed for tethering the patch grafts to the organs with the qualifier that the attachments can be done

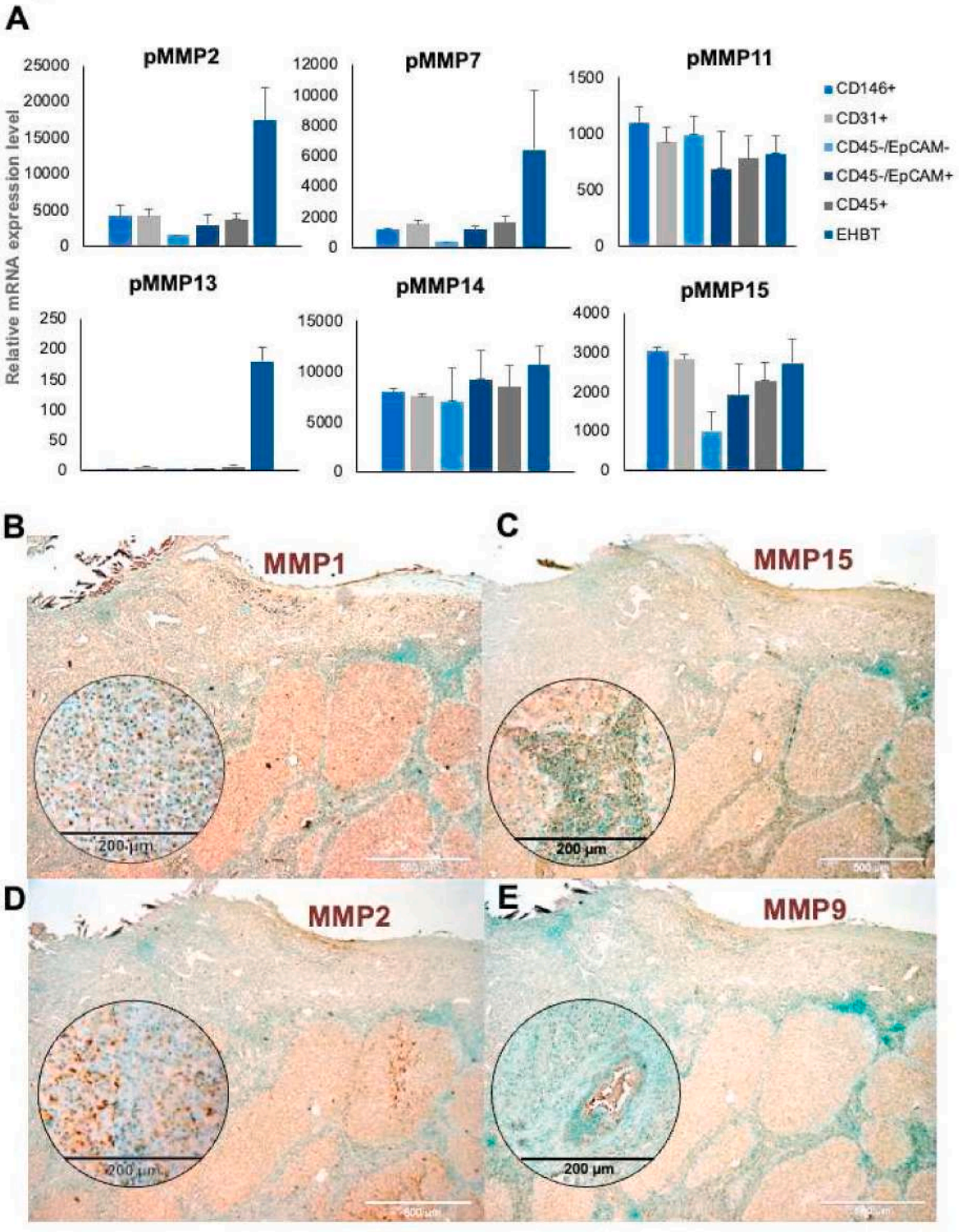

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Fig. 5. Expression of Matrix Metalloproteinases (MMPs). A. RNA-seq analyses for porcine matrix metalloproteinases (pMMPs) in subpopulations of donor cells. Comparisons were made of pMMPs expression in immunoselected porcine cells: stellate cells (CD146+), endothelia ($CD31^+$), hemopoietic cells ($CD45^+$), the hepatic progenitors ($CD45^-$, EpCAM+), the mature hepatocytes ($CD45^-$, EpCAM), and the organoids of extrahepatic biliary tree stem/progenitors (EHBT). Although plasma-membrane-associated MMPs were found strongly expressed in both stem/progenitors and mature cells, the secreted MMPs were expressed by the biliary tree stem/progenitors but not by or minimally by the other categories of cells and minimally by mature cells. B. There was widespread evidence of effects of MMP activity, such as the absence of the Glisson's capsule and the loss of matrix boundaries that define hepatic acini and lobular features. Immunohistochemistry (IHC) for expression of specific porcine isoforms of MMPs in the livers after patch grafting. The IHC data matched the RNA-seq findings. The caveat or exception is that existing RNA-seq data banks for porcine genes do not include MMP1 due to a lack of an annotated species of porcine MMP1 for use in the analyses. Therefore, proof that MMP1 was a contributor to the engraftment and migration was based on IHC data. Positive indications of MMP1 effects were the diffuse pattern, without matrix barriers, in the grafted host liver parenchyma. Nuclei staining was given in the encircled image with higher magnification. Methyl green was used as counterstain. Bar = 500 μm in the low magnification image; Bar = 200 μm in the high magnification image.C. IHC staining of MMP15 in the same grafted site in B. Primary antibodies were linked to a NovaRED chromogen, Methyl green was used as the counterstain. Bar = 500 μm in the low magnification image; Bar = 200 μm in the high magnification image. D. IHC staining of MMP2 in the grafted host liver. Positive signal was indicated with NovaRED, Methyl green was used as the counterstain. Bar = 500 μm in the low magnification image; Bar = 200 μm in the high magnification image.E. IHC staining of MMP9 in the grafted host liver. Primary antibodies were linked to a NovaRED chromogen. Methyl green was used as the counterstain. Bar = 500 μm in all of the low magnification images; Bar = 200 μm in high magnification images.

to connective tissue in close association with the organ if, as is the case for pancreas, there is a propensity for autolysis. Donor cells were found widely distributed within the target organs; necrotic cells were not observed in the target organs or elsewhere; and donor cells were not found in any ectopic sites as assessed by immunohistochemical assays for donor cells' biomarkers on serially sectioned ectopic tissues or by means of qRT-PCR analyses for the donor cells' transgene in ectopic tissues.

3.1. Matrix metalloproteinases (MMPs)

As demonstrated previously, success with patch grafting correlated with expression of matrix metalloproteinases (MMPs), both membrane-associated and especially secreted isoforms (e.g. MMP 1, MMP2, and MMP7), produced by donor cells when they were maintained in an immature state [1] (Fig. 5). The high expression of MMPs, especially secreted isoforms, are known contributors for engraftment, integration and migration processes [26,32–35]. This was followed by a merger and integration of donor and host cells and a distribution of cells throughout significant regions of the organ within a week.

MMPs comprise a family of calcium-dependent, zinc-containing endopeptidases that degrade extracellular matrix components. Using RNA-seq studies, we found a pattern of stem/progenitor-associated MMPs, comprised of high levels of secreted forms (e.g. MMP2, MMP7) as well as membrane-associated forms (e.g. MMP14, MMP15). IHC assays indicated that protein levels of the secreted MMPs (MMP1, MMP2, MMP7) were richly expressed in areas of remodeling.

Conditions (soluble growth factors, cytokines, serum, matrix components, mechanical forces) that caused donor cells to differentiate resulted also in reduction or muting of secreted isoforms of MMPs and, in parallel, abrogation of engraftment. Therefore, transplantation of cells into solid organs requires a source of MMPs, especially secreted isoforms, and ideally a cellular source that can generate the full repertoire of multiple, secreted and plasma-membrane-associated isoforms of MMPs necessary for the engraftment, migration, and integration processes.

Evidence for that conclusion was indicated also by our prior findings in which we attempted to use patch grafting to transplant mature hepatocytes [1]. Grafts containing mature hepatocytes, partnered with mature mesenchymal cells (endothelia or stellate cells), expressed plasma-membrane-associated MMPs but negligible amounts of secreted isoforms of MMPs and were found not to engraft; they survived at the surface of the liver at the graft sites. Partial engraftment occurred when the adult cells were co-transplanted with fetal liver-derived mesenchymal stem cells that provided some, but not all, of the MMPs found expressed by the stem/progenitor organoids [1]. The partial engraftment was indicated by the fact that the donor cells remained near the graft site at the surface of the organ.

3.2. Cytoplasmic localization of the transgene, GFP, or other nuclear biomarkers of donor cells

During the 1∼2-week remodeling phase there was often cytoplasmic expression of the transgene (GFP) label (Fig. 2B) or the nuclear biomarker, RBMY-1 (Figs. 3–4), in addition to nuclear staining, in the donor cells. The GFP transgene was designed to express a fusion protein of eGFP with porcine H2B histone and requires nuclear localization. However, the cytoplasmic expression of both the GFP transgene and of the nuclear biomarker, RBMY-1, proved transient, and the nuclear localization was restored by 2–3 weeks (Fig. 2C–E; Fig. 4). The phenomena parallels others associated with inflammatory processes [36] or with transiently damaged nuclear membranes resulting from rapid migration [37].

In summary, patch grafting strategies proved to be generic methods for safe and rapid delivery of large numbers of organoids or of suspensions of cells to solid organs that are normal or diseased and in mice and pigs of any postnatal age. The remodeling phase in patch grafting can be associated with transient expression of nuclear biomarkers but this resolves to nuclear localization of these markers once the period of high expression of secreted MMPs is completed.

Patch grafting with adult cells will require additional investigations given that only partial success occurred with studies to date. We hypothesize that greater engraftment and integration should occur if adult epithelia are co-transplanted with organoids to provide the secreted MMPs from both epithelial and from mesenchymal cells and so optimizing the expression of the requisite MMP isoforms and the potential for synergistic effects among secreted and plasma membrane-associated MMP forms. Alternatively, if the key secreted MMPs are identified, then it is plausible that cloned forms of these secreted MMPs might be incorporated into the graft biomaterials to facilitate engraftment of adult (mature) epithelia or to overcome difficulties with certain disease states such as severe cirrhosis.

3.3. Correction of the disease states

The second, important realization was that the large numbers of cells delivered are able to generate functional adult cells, ones able to correct or alleviate disease states. Transplantation of porcine or human stem/progenitor organoids attached to the livers of NRG/FAH-KO mice, followed by withdrawal of NTBC, resulted in maturation of donor cells to become mature parenchymal cells that enabled mice to have normal liver and kidney histology and functions for up to 3 months. By contrast, control mice that were not transplanted with cells and that were weaned from NTBC, had to be euthanized by 2–3 weeks if transplanted as young adults (6–12 weeks) and by 4 weeks if transplanted as older adults (4–6 months of age). The transplanted cells enabled the mice to survive for up to 3 months or longer without NTBC and gave rise to mature hepatocytes expressing FAH and albumin (Figs. 6 and 7) and animals with normal hepatic and kidney histology.

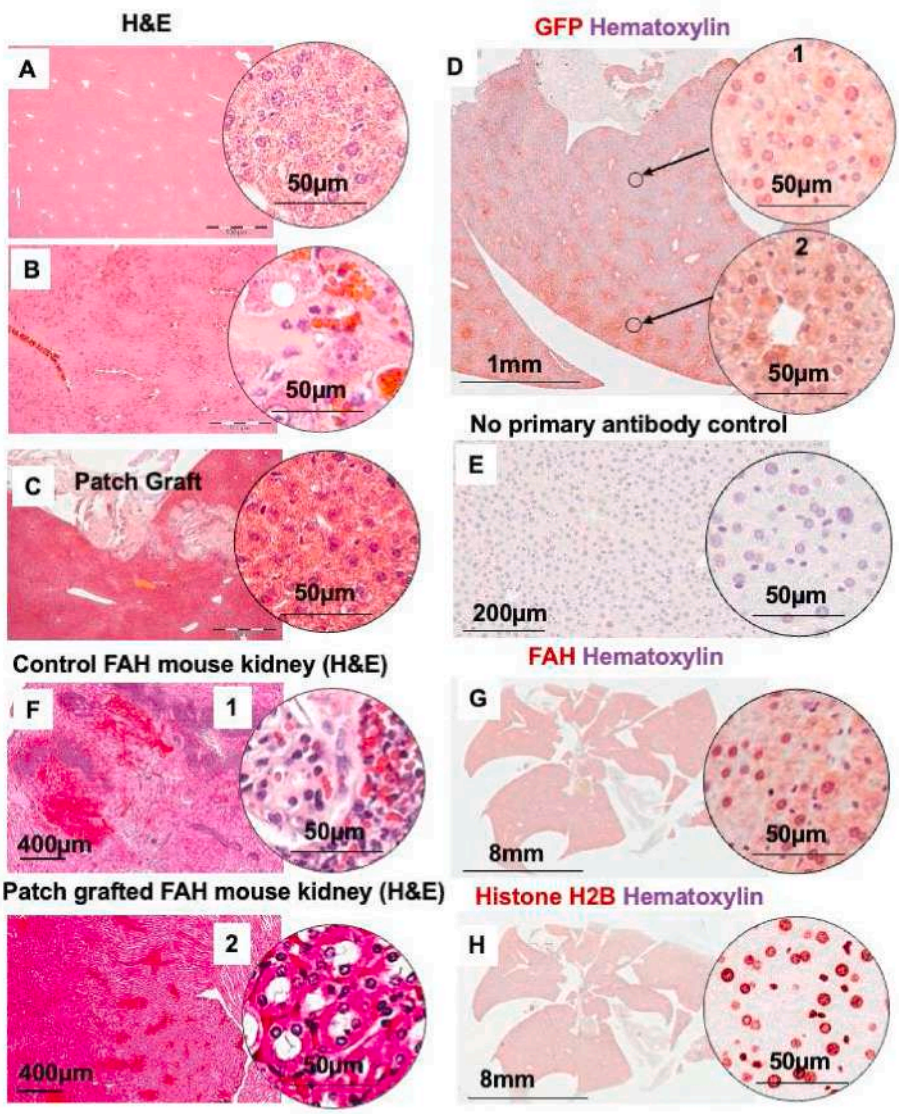
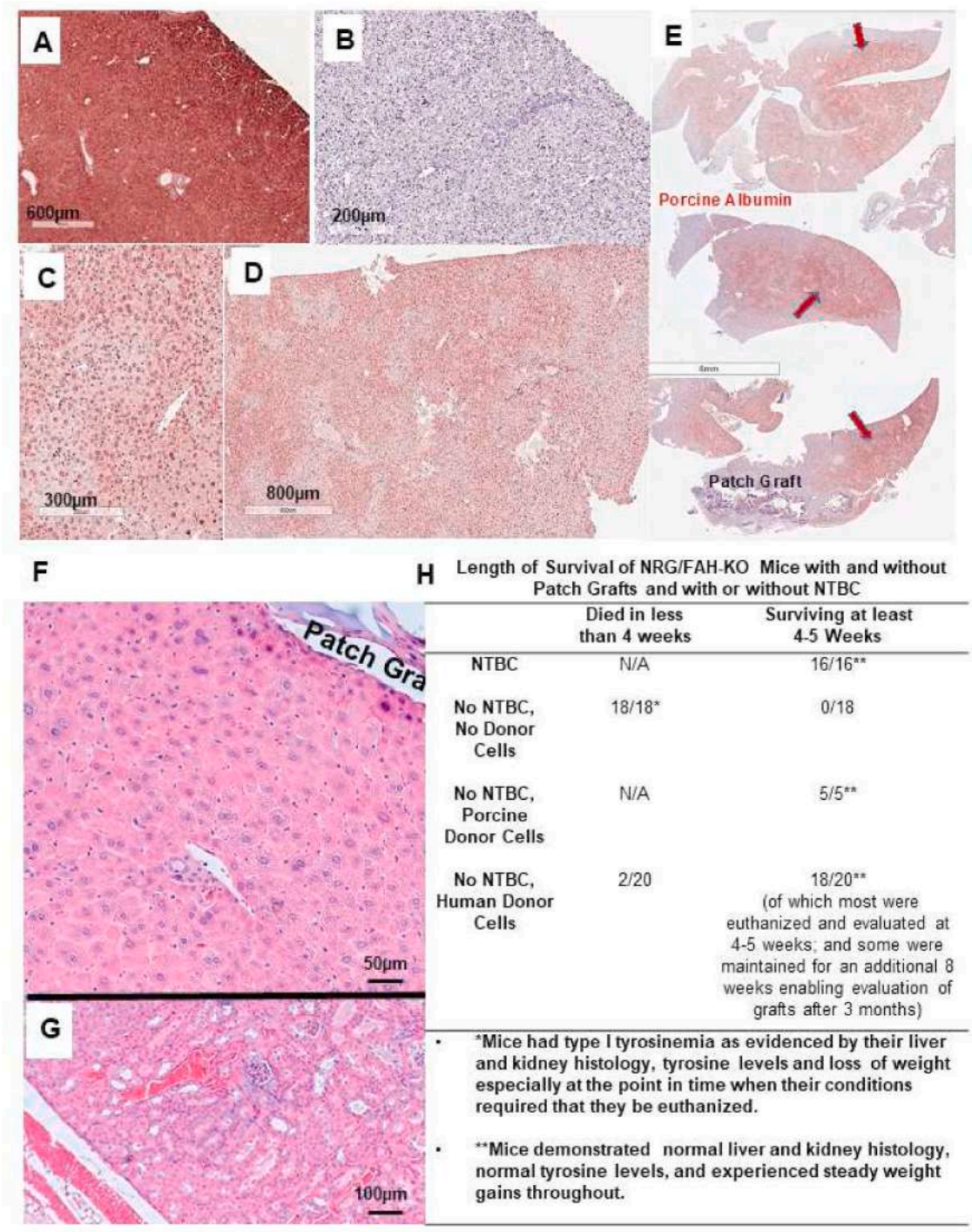


Fig. 6. Alleviation of type I Tyrosinemia in NRG/FAH-KO mice with grafts of organoids of pBTSC/ELSMCs. Transplantation of organoids of pBTSCs/ELSMCs into the livers of NRG/FAH-KO mice and then given regular water vs in the controls water with Nitisinone (NTBC). Animals transplanted with organoids were stable and gained weight, whereas controls, those with grafts without cells, deteriorated rapidly and had to be euthanized by day 16 if transplanted as young adults (8–12 weeks old) or by about 4 weeks if transplanted in animals at older ages (4–9 months of age). Bar = 50 μm in all of the high magnification images. **A.** Positive controls. Low and high magnification image of H&E-stained section of NRG/FAH-KO mouse liver in mouse maintained on NTBC. The liver showed as healthy and was composed of evenly distributed central veins and portal tracts in low magnification images, and with hepatocytes arranged as cords in high magnification images. Bar = 500 μm for the low magnification image; Bar = 50 μm for the high magnification image. **B.** Negative controls—transplanted using a graft with the biomaterials but without cells. Low and high magnification image of H&E-stained section of NRG/FAH-KO mouse livers weaned from NTBC treatment and evaluated at day 16. There was obvious pathological damage in the liver, which is mainly reflected in a large number of necrotic liver parenchymal cells in low magnification images, and in apoptosis of hepatocytes with cytoplasmic vacuolization and necrosis in the high magnification image. Bar = 500 μm for the low magnification image; Bar = 50 μm for the high magnification image. **C.** Patch grafted liver in a NRG/FAH-KO mouse. Transplanted using a graft with pBTSCs/ELSMCs organoids prepared from transgenic (GFP+) pigs. Low and high magnification image of H&E stained section of NRG/FAH-KO mouse livers weaned from NTBC treatment and evaluated at day 30. The patch graft was easily identified by the SERI silk fibers forming the backing. Histology images of low and high magnifications were identical to **A**. Bar = 500 μm for the low magnification image; Bar = 50 μm for the high magnification image.**D**. Low and high magnification images of the liver of an NRG/FAH-KO mouse grafted with organoids of pBTSCs/ELSMCs. The staining patterns in the first week or two after grafting included both nuclear and cytoplasmic staining of the donor nuclear biomarker, GFP+ (linked to histone H2B), patterns that transitioned to entirely or primarily nuclear staining after 3 weeks. The nuclei staining pattern (**1**) and residual cytosolic staining pattern (**2**) of GFP + donor cells in host liver are presented at a higher magnification (50 μm). The section was assessed with a GFP antibody linked to a NovaRED chromogen to identify donor cells engraftment in the liver. Bar = 1 mm **E.** Control. Low and high magnification images of livers grafted with organoids of pBTSCs/ELSMCs and under control conditions (no primary antibody controls for rabbit antibodies). Bar = 200 μm for the low magnification image; the higher magnification is at 50 μm. **F (1):** Kidney of mouse with graft with biomaterials but no organoids; Necrosis was found in the renal medulla region; an abnormal structure of the renal cortex was also observed due to the loss of renal tubular epithelial cells; **(2)** Kidney of mouse with graft with biomaterials containing BTSC/ELSMC organoids. Kidneys had normal histology with intact outer cortex and inner medulla of kidney parenchymal structure in the low magnification image, and healthy glomerulus and glomerular capsule of renal corpuscle in the high magnification image. Bar = 400 μm for the low magnification images; Bar = 50 μm for the high magnification images.**G.** Liver of NRG/FAH-KO mouse grafted with organoids of pBTSCs/ELSMCs. The section was assessed for fumaryl acetoacetate hydrolase (FAH), expressed by donor but not host cells. Bar = 8 mm for the low magnification images; Bar = 50 μm for the high magnification images. **H.** Liver of NRG/FAH-KO mouse with a patch graft containing organoids. Low and high magnification image of section stained for Histone H2B indicating expression primarily in the nucleus but with faint expression in the cytoplasm of cells; the cytoplasmic expression faded by 2 weeks leaving expression entirely within the nucleus. Bar = 8 mm for the low magnification images; Bar = 50 μm for the high magnification images.

Patch grafting of BTSC/ELSMC organoids onto pancreas yielded the most dramatic results observed with donor cells integrating amidst host cells and, in parallel, maturing into functional acinar cells and islets. The ability to transplant donor cells into pancreas has long been blocked with autolysis, obviating efforts to do cell therapies for pancreatic diseases. Historically, islet transplantation has been done with adult cells administered into the portal vein into the liver, leading to losses of up to 70% of the graft within the first 72 h after implantation, as the sensitive beta cells experience massive cell death due to hypoxia, mechanical stress, and nutrient deficiency [38]. As a result, more than 50% of islet transplant recipients require multiple procedures to achieve euglycemia [39,40].

Using the patch grafting techniques, we were able to deliver large numbers of cells (more than 10^{8th} cells in pigs; more than $10^{6th-7th}$ cells



H Length of Survival of NRG/FAH-KO Mice with and without Patch Grafts and with or without NTBC

	Died in less than 4 weeks	Surviving at least 4-5 Weeks
NTBC	N/A	16/16**
No NTBC, No Donor Cells	18/18*	0/18
No NTBC, Porcine Donor Cells	N/A	5/5**
No NTBC, Human Donor Cells	2/20	18/20** (of which most were euthanized and evaluated at 4-5 weeks; and some were maintained for an additional 8 weeks enabling evaluation of grafts after 3 months)

- *Mice had type I tyrosinemia as evidenced by their liver and kidney histology, tyrosine levels and loss of weight especially at the point in time when their conditions required that they be euthanized.
- **Mice demonstrated normal liver and kidney histology, normal tyrosine levels, and experienced steady weight gains throughout.

Fig. 7. Three-month grafts of organoids in NRG/FAH-KO Mice to treat Type I Tyrosinemia. Patch grafts containing GFP + organoids, prepared from transgenic pigs (or from human tissue), were attached to the livers of NRG/FAH-KO mice and monitored for up to 3 months. As indicated in the Table, the controls all died within a few weeks; only the grafted mice survived for longer. The control mice provided NTBC survived indefinitely. Those weaned from NTBC and given regular water died within ~2 weeks if the grafts were placed in young adult animals (8-12-week-old hosts) or within ~a month in older, adult animals (4-9-month-old hosts). Histology and immunohistochemistry of the liver and kidneys of the animals without NTBC and without cells indicated classic signs of type I tyrosinemia comprising toxic effects in the liver and kidney due to the absence of fumaryl-acetoacetate hydrolase (FAH). With only 2 exceptions, all of the mice transplanted with organoids of pBTSCs/ELSMCs survived indefinitely. **A. Positive control.** Expression of albumin in wild type pig liver. Bar = 600 μm **B. Negative control:** the same section as A but without the primary antibody. Bar = 200 μm.**C-E. Porcine albumin expression** in the grafted livers of NRG/FAH-KO mice transplanted with organoids of porcine biliary tree stem/progenitors. The donor cells had matured sufficiently to produce porcine albumin; arrows indicate regions of the tissue that are replete with donor cells. The porcine albumin was detectable by immunohistochemistry throughout the host organ. The bars = 300 μm (**C**); 800 μm (**D**); and 6 mm (**E**). The different magnifications enable one to realize how extensively and fully integrated the donor cells are within the livers by 3 months. **F.** Histology (H&E) of the liver of a mouse grafted with human BTSCs/ELSMCs and evaluated after 3 months. Bar = 50 μm. **G.** Histology (H&E) of kidney of mouse grafted on the liver with human BTSCs/ELSMCS and evaluated after 3 months. Bar = 100 μm. **H.** Table summarizing the findings of the patch grafts of porcine and human organoids on NRG/FAH-KO mice.

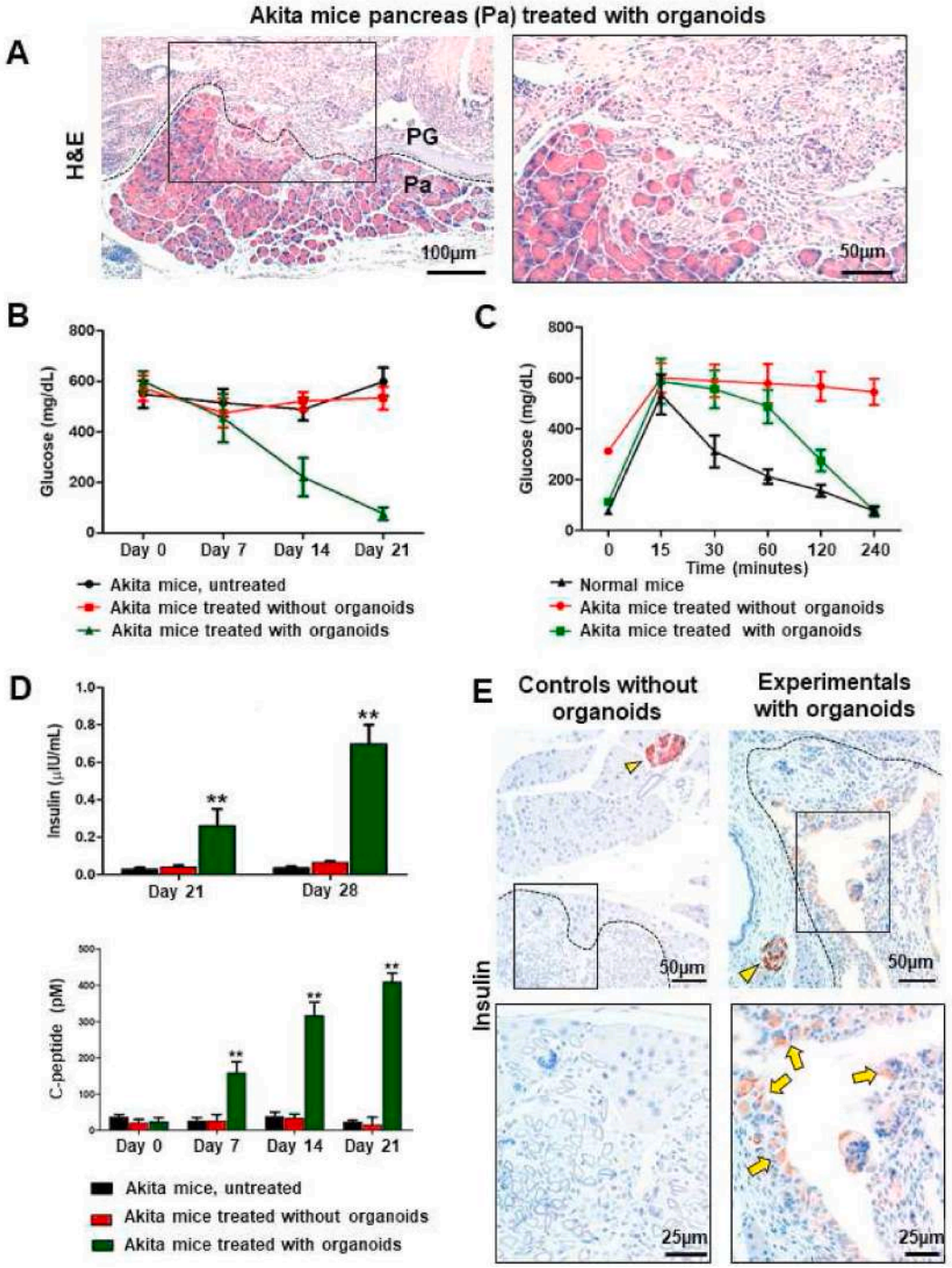


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Fig. 8. Rescue of NRG/Akita Mice from type I diabetes by patch grafting with murine BTSCs/ELSMCs organoids. Patch grafts containing murine organoids of BTSCs/ELSMCs were tethered to the pancreases of NRG/Akita mice and, for controls, to the pancreas of five non-diabetic NRG mice bred from NRG/Akita/+ mice. An additional control consisted of NRG/Akita mice with grafts that did not contain organoids. The plasma glucose and C-peptide levels in the two control groups did not show any significant difference suggesting that the grafted BTSCs/ELSMCs organoids did not affect recipient non-diabetic NRG mice. The only mice that showed a response were the NRG/Akita mice with patch grafts containing organoids. Some insulin-positive cells in organoids remained within the patch, but organoids also engrafted extensively within the pancreas in recipient NRG/Akita mice. The pattern of distribution of the donor organoids was different in the pancreases of NRG Akita/mice versus pigs. In the pigs, donor organoids exhibited rapid, wide-spread distribution throughout the pancreas (see Fig. 2), whereas in the NRG/Akita mice, they remained closer to the graft sites. This distribution pattern did not change even if additional organoids were added to the patch (from 1×10^6 to 1×10^7 organoids). The reason is unknown. There is no evidence for residual immunological reactions based on assays for serum pro-inflammatory cytokines and for histological evidence for local inflammation by 30 days by which time such reactions typically occur. Importantly, the restoration of insulin expression and glucose regulation indicates that engraftment was sufficient to yield functional results is indicated in the assays summarized below. **A.** Hematoxylin and eosin (H&E) stain on section of NRG/Akita mouse pancreas one month after transplantation. Dotted line identifies the interface between the transplant site (PG-patch graft) and the pancreatic tissues (Pa). Bar = 100 μm. The area in the box is magnified in the panel on the right. Bar = 50 μm. B. The graph shows the levels of glucose in untreated Akita (diabetic) mice (black/circles), in Akita mice controls without organoids (red/squares) and in Akita mice transplanted with BTSCs/ELSMCs organoids (green/triangles) at different time points. C. Intraperitoneal glucose tolerance test. The graph shows the levels of glucose in normal mice (black/triangles), in Akita mice controls without organoids (red/circles) and in Akita mice transplanted with organoids (green/squares) at different time points after intraperitoneal injection of glucose. N = 4 mice per experimental group. **D.** Histograms show means and standard deviation for serum insulin (on the top) and c-peptide (on the bottom) in untreated Akita mice (black, first columns), in Akita mice controls transplanted with biomaterials but without organoids (red, second columns) and in Akita mice transplanted with organoids (green, third columns) at different time points. ** = $p < 0.01$ vs. other groups. N = 5 mice per experimental group.**E.** Immunohistochemistry for insulin in tissues from Akita mice treated with biomaterials without organoids (panels on the left) and transplanted with organoids (panels on the right). An islet in the image of the upper control panel is representative of residual islets in Akita mice that were insufficient to mediate glucose regulation; more commonly, the pancreas tissue was devoid of islets as indicated in the higher magnification image below. Insulin-positive cells (arrows) are visible within the pancreas (identified by the dotted line) but only in Akita mice transplanted with organoids. Bar = 50 μm. Areas in the boxes are magnified in the panels below. Bar = 25 μm. Arrowheads indicate pancreatic islets (positive controls for insulin).

in mice) with no evidence of cell death, leading to consistent resolution of tyrosinemia or of diabetes after a single procedure. Engraftment occurred rapidly (within a week), with donor GFP + cells distributed throughout the pancreas at 7 days, and without evidence of autolysis (Fig. 2, Fig S5). To our knowledge, this has never before been observed even with attempts to transplant cell suspensions, much less organoids. Maturation of the donor cells to become functional islets (positive for insulin, glucagon) or acinar cells (amylase) was evident even in the first week. The functionality of the donor cells was proven further in the allografts of murine organoids grafted onto the pancreases of NRG/Akita mice and with rescue of those hosts from type I diabetes.

3.4. Patch grafting strategies compared with other transplantation strategies for epithelia

The shift to grafting strategies [3] for transplantation of epithelia is occurring with the efforts of a small but growing number of investigators [8,9]. Other grafting strategies reported recently are plating MSCs onto amnions and attaching the amnions to the target site [41] and cell sheet engineering [7]. In grafting using amnions as substrata, the MSCs confer paracrine signaling alleviating adverse conditions in the liver, but they are unable to differentiate into epithelial cells that might replace aberrant or damaged epithelia. Therefore, grafts of MSCs plated onto amnions can help the tissue by paracrine signaling but are of limited efficacy for certain types of injury or disease states.

In cell sheet engineering, sheets of cells are prepared *ex vivo* on thermo-sensitive dishes and then transferred to the surface of organs. The design provides support for transplantation of mature epithelial cells that then can provide relief for some dysfunctions in an organ. The limitations for cell sheet engineering, and for all grafting methods that maintain the cells at the surface of the target site, are in the numbers of cells that can be transplanted and in the minimization of interactions of the donor cells with host cells and so the extent of dysfunction that can be managed. By contrast, the graft site in patch grafting is temporary with intent for its dissolution within a few weeks. Once delivered, the cells integrate amidst the host cells and undergo the usual cell-cell interactions of the target organ or tissue along with its complex regulation by matrix, paracrine and endocrine signaling.

Candidate materials for backings include other materials as long as they can provide protection from adhesions and shear forces that occur amidst internal organs and, in parallel, are neutral with respect to effects on the donor cells. A backing of silk impregnated with hyaluronans and triggered to yield a hydrogel at ~700 Pa was a logical first choice, given that it can be industrially scaled. However, we expect that other backings likely to be successful include the amnion and the omentum, hypotheses being tested.

4. Conclusions

Importantly, we demonstrate that patch grafting, enables transplantation of large numbers of donor cells, including organoids, to solid organs, can overcome disease states in immunocompromised mice with genetically based diseases. Specifically, we show that patch grafting with xenografts of porcine or human cells can reverse tyrosinemia in the NOD-Rag1$^{-/-}$IL2RgammaC-null (NRG) fumaryl aceto-acetate hydrolase knockout mice [13,14] and allografts of murine donor organoids can reverse diabetes in the NRG Ins2Akita mouse [15], demonstrating efficacy in two distinct disease states and in two different solid organs, the liver and pancreas.

Additional research is required to learn of other pathologies amenable to correction or alleviation by patch grafting of stem/progenitor organoids. We predict that patch grafting should be successful with genetic-based defects and with conditions with minimal fibrosis. By contrast, severe cirrhosis is expected to be representative of a condition that might be alleviated but unlikely to be curable given the propensity of such conditions to cause differentiation of donor cells and so mute expression of the MMPs required for engraftment. We speculate that once the requisite secreted MMPs are identified, it should be possible to used cloned forms of MMPs in the graft biomaterials and so possibly overcome this limitation as well as offer enhanced conditions required for patch grafting of adult epithelia.

Patch grafting was found to be safe and effective for transplantation of organoids of stem/progenitors into the liver or pancreas. The strategies enabled functional replacement of diseased hepatic cells to correct type I tyrosinemia or diseased pancreatic cells to correct type I diabetes, examples of disease states amenable to patch grafting strategies. Patch grafting provides far greater potential than existing transplantation methods and even of other forms of grafting methods such as cell sheet engineering, because of the exponentially greater numbers of donor cells that can be transplanted; because the use of organoids ensures lineage-stage appropriate epithelial-mesenchymal interactions; and because the transplanted cells are integrated fully amidst host cells and so available for all known regulation by soluble and matrix systemic and paracrine signals.

Patch grafting methods and the graft conditions (other than the organoids) are generic and proved successful in preliminary studies (data not shown) with every other solid organ tested (e.g. also kidney and intestine). We hypothesize that the methods should be logical for ectodermal organs with grafts of ectodermal stem/progenitor organoids and for mesodermal organs with grafts of mesodermal stem/progenitor organoids. A key requirement will be to maintain the organoids in a stem cell state, conditions challenging for variables (e.g. muscle contractions) that could cause differentiation of the stem/progenitor organoids and so mute the requisite expression of MMPs. Collaborators have begun to assess these hypotheses in transplantation of organoids to various organs.

The next phases should be translational research investigations to adapt patch grafting for use in clinical programs initially for liver and pancreas and eventually for other solid organs.

Author contributions

W. Zhang performed most of the experimental studies; prepared analyses and interpretations of findings; and did the preparation and editing of the manuscript through all the drafts. Dr. Piedrahita and his associate, S. Simpson, provided the GFP transgenic and experimental pigs and managed them pre- and post-surgery. K. Mathews and C. Adin conducted the abdominal transplant surgeries on the liver and pancreas in the pigs. C. Suit and J. Ezzell provided technical support. G. Prestwich was a consultant guiding the use of the hyaluronan hydrogels used as biomaterials in the grafts. E. Wauthier handled the lab management, the overall supervision of bench work, and did many of the efforts establishing the methods of isolation of the biliary tree stem cells and of organoid formation.

The experiments and analyses on patch grafting on the pancreas in pigs were done by G. Lanzoni and with consulting to him by J. Dominguez-Bendala. Those on the NRG/Akita mice were done by D. Overi and Xianwen Yi. D. Gerber and C. Adin were essential contributors to the experimental designs of those experiments, Especially Dr. Adin provided text and interpretation of the findings and preparation of the text relevant to pancreas studies.

The experiments and analyses on patch grafting on the liver in pigs were done by W. Zhang. W. Zhang and H. Hani did patch grafting in NRG/FAH-KO mice. Investigators at Sapienza University (E. Gaudio, D. Alvaro, G. Carpino, V. Cardinale) provided expertise in analyzing the grafts with respect to known hepato/biliary and pancreatic biology and pathobiology.

L. Reid handled the overall management and supervision, was the primary contributor on the ideas of the determined stem cells and grafting strategies and prepared the grants that funded most of the research. Drs. Zhang and Reid did the preparation of the initial and all subsequent drafts of the manuscript; all authors helped to edit the drafts. Dr. Adin and D. Gerber contributed especially in the writing regarding the pancreatic patch grafts. A. Allen provided animations and artwork.

Declaration of competing interest

The authors declare that they have no conflict of interest.

Data availability

. All RNA-seq data in the form of bam and/or fastq files will be made available on Gene Expression Omnibus (GEO). All code used to analyze the data will be made available on GitHub and/or freely shared upon request by anyone in the research community.

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Appendix A. Supplementary data

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