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An improved method of renal tissue engineering, by combining renal dissociation and re-aggregation with a low-volume culture technique, results in development of engineered kidneys complete with loops of Henle

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Abstract

Background: Tissue engineering of functional kidney tissue is an important goal for clinical restoration of renal function in patients damaged by infectious, toxicological, or genetic disease. One promising approach is the use of the self-organizing abilities of embryonic kidney cells to arrange themselves, from a simply reaggregated cell suspension, into engineered organs similar to foetal kidneys. The previous state-of-the-art method for this results in the formation of a branched collecting duct tree, immature nephrons (S-shaped bodies) beside and connected to it, and supportive stroma. It does not, though, result in the significant formation of morphologically detectable loops of Henle - anatomical features of the nephron that are critical to physiological function.

Methods: We have combined the best existing technique for renal tissue engineering from cell suspensions, with a low-volume culture technique that allows intact kidney rudiments to make loops of Henle, to test whether engineered kidneys can produce these loops.

Results: The result is the formation of loops of Henle in engineered cultured 'foetal kidneys', very similar in both morphology and in number to those formed by intact organ rudiments.

Conclusion: This brings the engineering technique one important step closer to production of a fully realistic organ.

Keywords: embryonic kidney, re-aggregates, tissue engineering, low-volume culture, loop of Henle

Introduction

The primary goal of renal tissue engineering is to construct an organ that resembles the natural kidney as closely as possible [1-4]. One strategy for achieving this is to exploit the capacity of renal cells for self-organization.

Two years ago, Unbekandt and Davies invented a method to recapitulate the early stages of organotypic renal structure from simple suspensions of isolated embryonic renal cells [5]. Because the cell suspension was obtained by dissociation of early (E11.5, 'T-bud stage') embryonic mouse kidneys, the method was called the 'dissociation-reaggregation technique'. In its basic form, it produced immature nephrons arranged around ureteric bud tissue, but the ureteric bud cells were arranged as a multitude of small ureteric buds/ collecting duct trees rather than as one single coherent collecting duct tree system, and therefore failed to reproduce a key feature of normal renal anatomy tree [5,6]. The 'nephrosphere' technique developed by Lusis and colleagues in the same year [7] did not include collecting ducts at all, so suffered from an even more severe version of this problem.

To resolve this limitation, Ganeva et al. developed a serial dissociation and re-aggregation system [6]. They first used the original dissociation and re-aggregation system to make re-aggregates with multiple, small ureteric buds. They then manually isolated one of these small re-aggregated ureteric buds and combined it with fresh disaggregated and reaggregated mesenchyme: the result was development of immature nephrons that were arranged around one, highly-branched ureteric bud / collecting duct system. This was a good reflection of the structure of mouse embryonic kidneys at about 13 days gestation.

As normal kidneys mature, from about 14 days of mouse development [8], they develop distinct cortical and medullary zones. Bowman's capsules, proximal tubules and distal tubules are restricted to the cortex, while the medulla consists of collecting ducts and loops of Henle, elements of the nephron that extend radially inwards from the cortex. This arrangement is vital for normal physiology, particularly the recovery of water (which depends on loops of Henle making the medullary interstitium very hypertonic compared to normal body fluids). Any useful system for renal tissue engineering must therefore be able to reproduce this feature. The standard, Trowell-screen culture methods used for development of existing dissociation-reaggregation methods do not support efficient development of loops of Henle even when they are used to culture normal, intact kidney rudiments. There is therefore neither positive nor reliable negative evidence about the potential for reaggregated kidneys to organize themselves to produce realistic cortico-medullary zonation or loops of Henle.

A recently published novel culture method, based on growing rudiments on silicone-bounded glass slides with extremely low volumes of medium (just tens of microlitres), allows an intact kidney isolated directly from an E11.5 embryo to develop organotypic cortico-medullary zonation with loops of Henle over the course of 7-10 days [9]. In this short report, we combine the idea of tissue engineering, from cell suspensions by the serial dissociation-reaggregation method, with the low-volume culture method for cortico-medullary zonation. The result is the production, from cell suspensions, of kidneys with distinct cortical and medullary zones and with loops of Henle extending radially inwards. This marks a further step towards engineering a realistic foetal kidney from simple suspensions of cells, and provides a potential path by which renal stem cells could be used to make kidney rudiments for clinical applications.

Materials and Methods

Organ culture

The main method is depicted in diagrammatically in Fig 1. Kidney rudiments were obtained from E11.5 CD1 mouse embryos (morning of plug check considered to be E0.5) by manual dissection in Eagle's Minimum Essential Medium with Earle's Salts (Sigma cat # M5650). For whole kidney conventional (Trowell screen) culture, they were placed on a 5 μm Isopore membrane filter (Millipore cat # TMTP02500) supported by a stainless steel grid in a 3.5 cm culture dish at the gas-medium interface. The medium was Kidney Culture Medium (KCM): Eagle's Mimimum Essential Medium (Sigma cat # M5650) with 10% foetal bovine serum (FBS: Invitrogen cat # 10108165) and 1% penicillin/ streptomycin (Sigma cat # P4333), as described by Unbekandt et al. [5]. For the low-volume culture system, we followed the method described in Sebinger et al. [9]. The Cone shape 'A' silicon ring (SARSTEDT cat # 94.6077.434) was attached to a 22×22 mm glass cover slip (VWR International cat # 631-0125) and the re-aggregates were placed in the centre of it in 85 μl of KCM.

Tissue engineering by re-aggregation

E11.5 embryonic kidneys from CD1 mice were dissected in MEM (Sigma cat # M5650). The kidney rudiments were then dissociated enzymatically and reaggregated exactly as described in Unbekandt and Davies, 2010 [5]. They were cultured in KCM on Isopore filters supported by metal grids as described above. For the first 24 h, the ROCK inhibitor, 1.25 μM glycyl-H1152-dihydrochloride (TOCRIS batch # 1A/93503), was added to KCM. This medium was then replaced with drug-free KCM for the remaining 3-4 days of culture, as described [5]. After this first incubation, single ureteric bud cysts were isolated from the whole-kidney re-

aggregates by manual dissection. Dissociated fresh metanephric mesenchyme was isolated as described by Ganeva *et al.* [6]; briefly, 10 to 15 kidneys were incubated in 2× trypsin/EDTA (Sigma cat # T4174) in MEM for 2 min at 37°C and quenched in KCM. Following this, the mesenchymes were peeled away from the ureteric bud. The mesenchymes were collected in a 500µl tube, dissociated by gentle pipetting and re-aggregated by centrifugation at 3000 rpm for 2 min in a micro-centrifuge. The reaggregated ureteric buds from the dissociation-reaggregation experiment were combined, singly, with reaggregated fresh mesenchymes on a membrane filter in the conventional culture system. They were then cultured for 1-2 days in KCM; during this time, they became solid enough to manipulate. They were removed from their filters and transferred to the low-volume culture system. They were incubated for a further 5-7 days (so to a total of 6-9 days from application of mesenchyme to bud cyst), medium being changed every 2 days.

Immunohistochemistry

Tissues were fixed using cold methanol initially at -20°C and allowed to warm up towards room temperature during the 15 min fixation. They were rinsed in PBS for 30 min at room temperature. Primary antibodies applied to the tissues were diluted 1:100 in PBS and applied overnight at 4°C; primary antibodies were mouse anti-Calbindin (Abcam cat # ab9481), mouse anti-pan cytokeratin (Sigma cat # C2562), chicken anti-laminin (Abcam cat # ab14055), rabbit anti-laminin (Sigma cat # L9393), and rabbit anti-Human Tamm-Horsfall glycoprotein (Bioquote cat # bt-590). The next day, tissues were washed for a few hours in PBS and secondary antibodies were applied overnight at 4°C. Secondary antibodies were goat FITC anti-chicken (Abcam cat # ab97134), goat FITC anti-mouse (Sigma cat # F2012) and goat TRITC anti-rabbit (Sigma cat # T6778), and were applied at 1/100 in PBS. Finally,

tissues were washed in PBS for few hours. Those grown on filters in Trowell culture were mounted, still on their pieces of filter, between two 22x64mm coverslips that had 22x22mm coverslips sandwiched between them at their ends as spacers, to keep the longer converslips apart and prevent the samples being crushed: the whole assembly was sealed with nail varnish (Portobello Pink, Rimmel) and mounted loosely on a microscope slide so that the coverslip assembly could be inverted if the filter-and-kidney combination happened to be upside-down. These samples were viewed on a Zeiss Axioscope epifluorescence microscope. Organs and reaggregates grown in the low volume system were viewed using a Zeiss Axiovert epifluorescence microscope.

Identification and counting of loops of Henle

Loops of Henle were identified and counted primarily by morphological criteria because the existence of an actual *loop* (rather than mere expression of marker genes, some of which appear in the S-shaped body before real loops form) has greater physiological relevance: see 'Results and Discussion'. Anti-laminin staining (see above) was used to trace the shapes of all tubules. The criterion used to define the presence of an LoH, and to count them, was the existence of a tube that was bent sharply back on itself like a hair grip (US: 'bobby pin'), extending from the mid-portion of a nephron. The straight part of the tube had to be more than a tubule diameter in length before it was considered to be a *bona fide* loop of Henle (in practice, they were much longer). As an additional test, immunostaining for Tamm-Horsfall glycoprotein was used in some experiments to confirm that the morphologically identified loops do indeed express this loop of Henle marker in the expected manner (they do: see Results and Discussion). Counting was done using a low-power image of the whole kidney and using high-power views to confirm loop morphology as shown in Figs 2-4.

We also recorded whether loops of Henle extended towards the middle of the kidney. To do this, two lines were drawn on an image: one ran along the long axis of the loop itself and the other, radius line, ran from the centre of the kidney to and beyond the tip of the loop ('centre of the kidney' defined as the first branching point of the collecting duct system). The angle between the axis of the loop and the radius line beyond the tip was then measured, to assess how accurately the loop of Henle was orientated radially towards the centre of the kidney: perfect radial alignment would yield an angle of zero. Where the angle was less than 45 degrees, the loop was counted as extending towards the centre ('centripetal').

Results and discussion

The aim of the work described here is to produce morphologically detectable and correctly positioned loops of Henle. We emphasize development of a proper loop morphology, rather than just presence of early Loop of Henle gene expression markers in the central section of an S-shaped body, because the function of the loop of Henle depends critically on its anatomy: just having marker expression with no extending loop would not be physiologically useful. The principal criterion used to define the presence of a LoH was therefore the existence of a tube, bent sharply back on itself like a hair grip (US: 'bobby pin'), extending from the mid-portion of a nephron. Immunostaining for Tamm-Horsfall glycoprotein was used as an additional confirmation that the criterion for identifying loops of Henle described above, does correlate with expression of this marker (see below).

As has been described before [10], intact kidneys in conventional organ culture produced well-branched collecting duct trees that had nephrons developing next to them and connecting with them (Fig 2A,B). Even after 10 days of culture, there was little evidence of morphologically-detectable loops of Henle: on average, only a mean of 1.4 (σ=0.74) formed per culture, and those that could be found tend to be very short. An example of one of these rare loops is arrowed in Fig 2B. Kidneys engineered by serial reaggregation from cell suspensions, by the method in Fig 1, and cultured conventionally, also produced nephrons arranged around a well-branched collecting duct system (Fig 2C,D) but, again, loops of Henle were rare, with a mean number per culture of 1.0 (σ=1.4). There was no significant difference between the number of these loop rudiments produced in intact and engineered kidneys cultured conventionally on Trowell screens ($p = 0.65$ by 2-tailed t-test).

To overcome this restriction, we used the low-volume culture method of Sebinger *et al*. [9]. For intact kidneys, this technique was used exactly as published. For serial re-aggregates, the combination of a ureteric bud cyst from the first reaggregation (Fig1, step 1) with fresh mesenchyme (from Fig1, step 2) was cultured conventionally for 1-2 days before being transferred to the low-volume culture system (Fig1, step 3). This period of conventional culture before low volume culture had to be used because the low-volume culture method requires an organ rudiment to be placed in a specific place (the centre, where the medium is at its shallowest so that surface tension presses down on the tissue [9]). This accurate placement was not possible until reaggregation had proceeded far enough to make a solid 'tissue' that could be manipulated by pipette.

Intact kidneys behaved in the low volume culture system exactly as has been described before [9]. The organ rudiments spread over a large area and formed a well-branched collecting duct tree (Fig 3A,B). Under these culture conditions, loops of Henle could be seen; a mean of 16.3 (σ=3.0) per culture, some long and extended and some shorter but still identifiable: examples of both can be seen, arrowed, in the higher magnification view of the sample of Fig 3A that is presented in Fig 3B. This increase in loop production was highly significant ($p = 0.00004$ by a 2-tailed t-test). Most (95 of 98: 97%) loops extended correctly towards the middle of the kidney ('centripetally', defined for measurement purposes as heading towards the first ureteric branch with an error of less than ± 45 degrees).

Kidneys engineered by serial reaggregation, pre-incubated in conventional culture for 2 days and then transferred to low-volume culture, also spread over a large area and formed a well-branched collecting duct system (Fig 3C, D). Except for the fact that the intact kidneys had

an overall polarity arising from the ureteric bud entry point while the engineered ones had no unique entry point and therefore showed radial symmetry, these engineered organs were difficult to distinguish from their intact counterparts (compare Fig 3C with Fig 3A). Importantly, under these culture conditions the engineered kidneys produced morphologically identifiable loops of Henle, which can be seen in Fig 3C and more easily in the successively higher magnification views, Fig 3D and 3E. Quantitatively, the engineered kidneys produced a mean of 14.0 (σ=1.73) morphologically identifiable loops per culture. This was highly significantly different from loop production in conventional culture ($p= 0.0005$ by a 2-tailed t-test). Encouragingly, it was not significantly different from the performance of intact kidneys in low-volume culture (a 2-tailed t-test yields $p = 0.19$; no significant difference). Once again, most (40 of 42: 95%) loops were orientated towards the centre of the kidney. The quantitative behaviour of kidneys and engineered kidneys in these culture systems, with respect to Loop of Henle formation, is shown in Fig 3F.

To confirm morphological identification of loops of Henle, we examined the expression of Tamm-Horsfall protein (THP), which is expressed strongly in the ascending limb of the mature loop of Henle [11,12]. In intact kidneys and in serial reaggregates, THP expression could be seen in the growing loops of Henle (Fig 4A-C). It is striking to note that, in these early kidneys (both intact and engineered), THP expression is particularly strong near the bend of the growing loop. THP expression begins a little after loop emergence, which means that some shorter morphologically defined loops did not express THP. Nevertheless, counting only the THP-positive loops shows the same pattern (Fig 4D): very few in conventional culture and significantly more ($p=0.02$ by 2-tailed t-test) in low-volume cultures of intact and

engineered kidneys. Again, there was no significant difference ($p=0.90$ by 2-tailed t-test) between the numbers in intact and engineered.

The development of loops of Henle brings engineered kidneys an important step closer to being properly representative of kidneys that have developed normally in vivo. Essentially, it makes the anatomical development of the epithelial tubules very similar to that found in a normal late-gestation foetal murine kidney. Important remaining steps include the introduction of properly patterned and integrated vascular and nervous systems.

Acknowlegements

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Figures

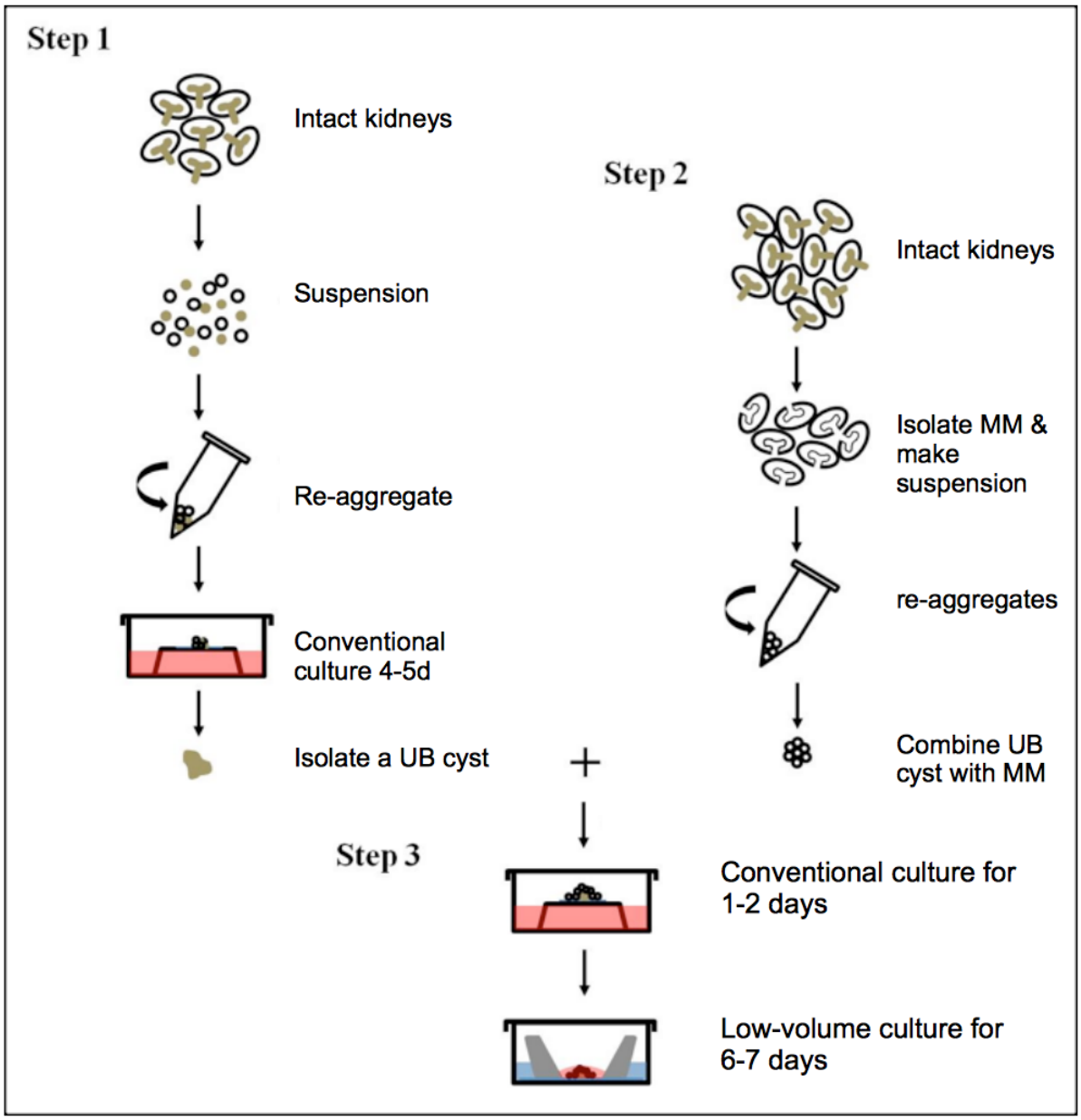

Figure 1. Schematic description of the method to incubate the re-aggregated kidney cells in the conventional and low-volume culture system.

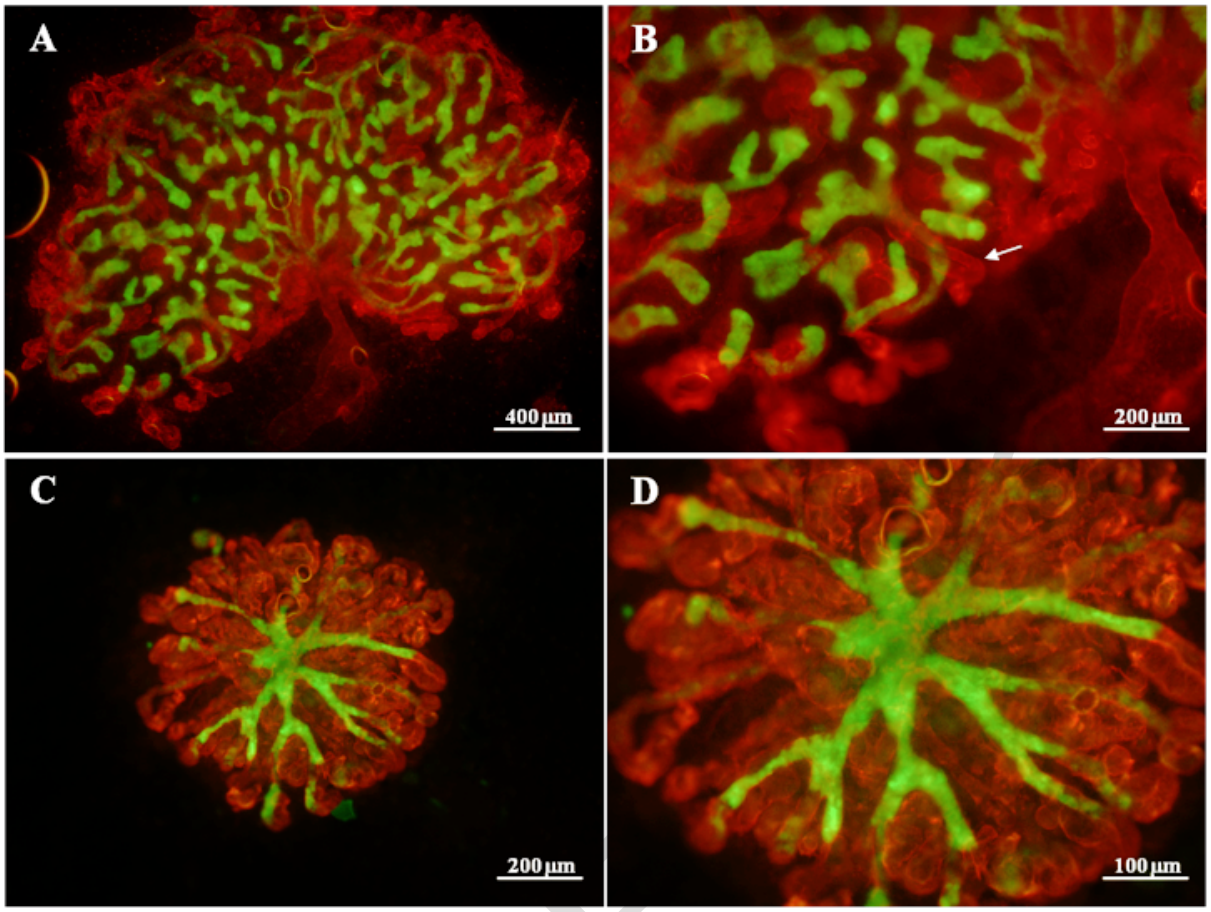

Figure 2. In 10-days of conventional Trowell-screen culture, both intact kidneys (A, B) and kidneys engineered (C, D) through serial reaggregation produce an organotypic arrangement of nephrons around a single collecting duct tree, but there is little sign of development of loops of Henle (one rare example is arrowed in B). Green shows the ureteric bud marker, Calbindin-D-28k, and red the basement membrane marker, laminin.

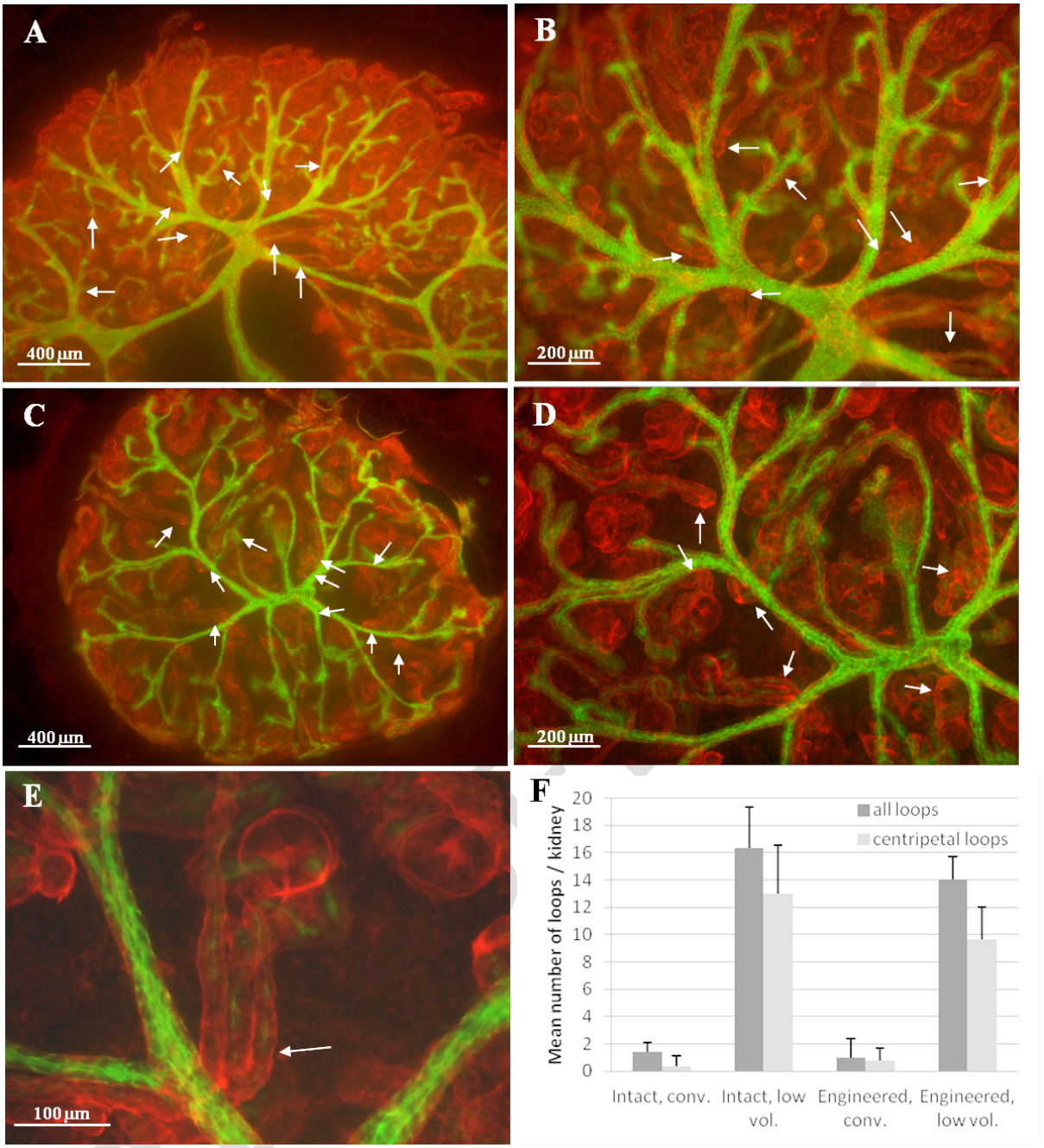

Figure 3. In low volume culture, kidneys engineered by series reaggregation form loops of Henle like those formed by intact kidneys in low volume culture. (A) shows a low-power view of an intact kidney in low-volume culture. The arrows point to positions of developing loops of Henle, identified by examination of higher magnification images; (B) shows an example of a higher magnification image, with loops of Henle marked with arrows (the arrows point at the loops and have nothing to do with the orientation of the loops). (C) shows a low-power view of a kidney engineered by serial reaggregation in low-volume culture. Again, the arrows point to developing loops of Henle, identified by examination of higher magnification images; (D), (E) show examples of successively higher magnification images,

with loops of Henle marked with arrows. (F) Shows the average total number of loops of Henle per kidney formed in each method, and the average number of loops that extend radially inwards (heading towards the first ureteric branch with less than a 45 degree error): these are called 'centripetal', ie centre-seeking, on the graph). Intact kidneys were cultured for 10 days and serial reaggregates for 2 days of conventional culture (from final aggregation of UB cyst with fresh MM) followed by 6 days of low-volume culture. Error bars show standard deviation; p values are given in the main text; the groups contained 8, 6, 4 and 3 cultures respectively. Green shows the ureteric bud marker, Calbindin-D-28k, and red the basement membrane marker, laminin.

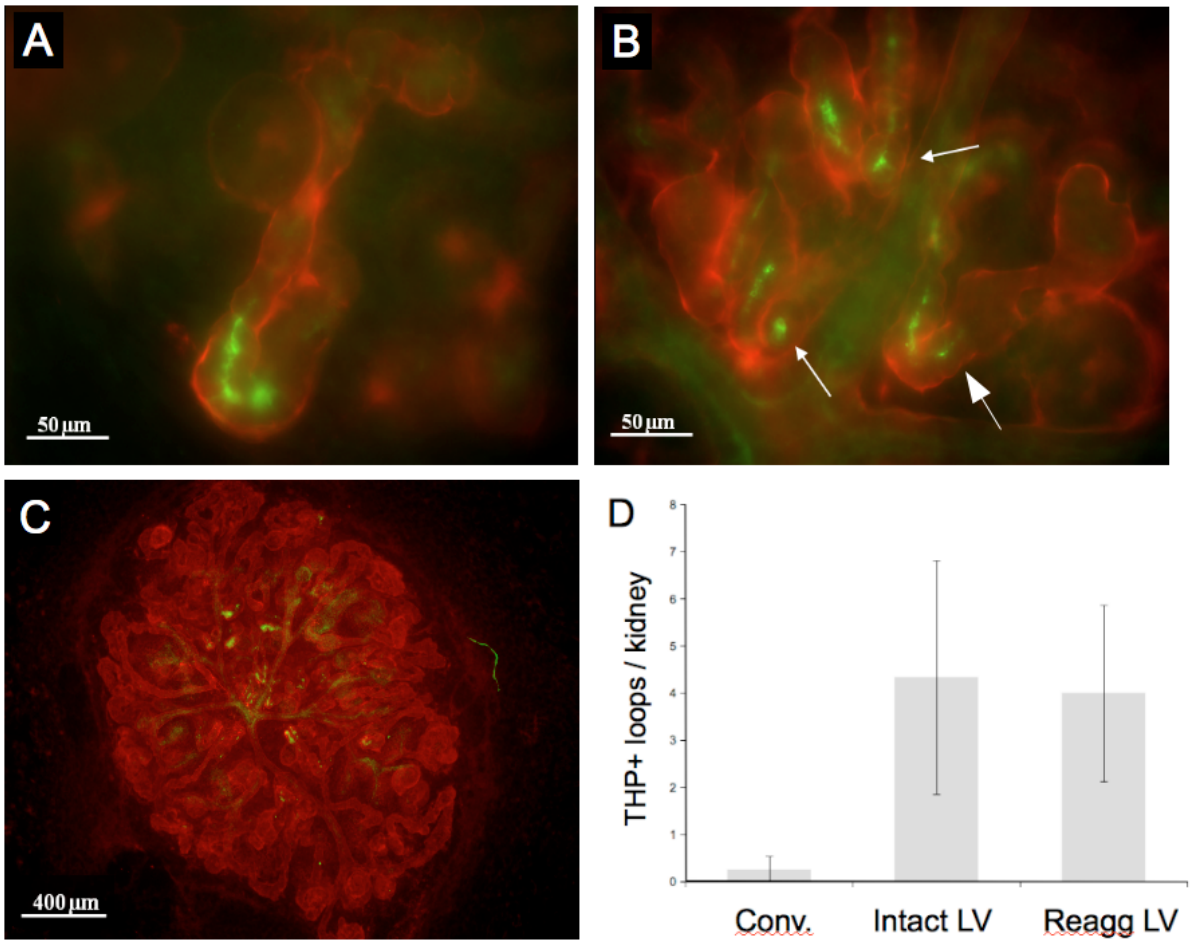

Figure 4. THP expressed loops were shown in the intact and engineered kidneys in low volume system. (A) shows an intact kidney cultured in the low-volume system, with a loop of Henle (arrow) marked with U-shaped adluminal expression of THP (green), the basement membrane again being stained for laminin (red). (B) shows a similarly stained image of a serial reaggregate kidney, with several loops visible (arrowed: the arrow with the larger head marks a loop, the bend of which can clearly be seen as such in this plane of focus). (C) shows a low-power view to demonstrate the specificity of anti-THP for loops of Henle (ie absence of stain in other parts of the kidney). Graph (D) shows the average number of THP-expressing loops of Henle per kidney formed in each method (error bars = standard error of the mean). Intact kidneys were cultured for 9 days and serial reaggregates for 2 days of conventional culture followed by 6 days of low-volume culture.

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