# Differentiation of a contractile, ureter-like tissue, from Embryonic Stem cell-derived ureteric bud and ex-fetu mesenchyme.

# TITLE PAGE

## *Title*: Differentiation **of a contractile, ureter-like tissue, from Embryonic Stem cell-derived ureteric bud and ex-fetu mesenchyme.**

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## *Running title*: **engineering ureter tissue.**

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## Abstract:

BACKGROUND: There is intense interest in engineering replacement kidneys from stem cells. Current techniques produce renal tissue but include no ureter, which will be needed before engineered kidneys can be clinically useful. This paper addresses the problem of making a ureter from stem cells.

METHODS: Mouse embryonic stem (ES) cells were differentiated into ureteric bud tissue using a published sequence of growth factors and drugs. Isolated engineered ureteric buds (eUBs), differentiated from ES cells, were characterized in 3D culture and grafted into ex-fetu mouse kidney rudiments.

RESULTS: eUBs branched in 3D culture and expressed Hoxb7. When grafted into the cortex of ex-fetu kidney rudiments, they branched and induced nephron formation. eUBs grafted into peri-Wolffian mesenchyme, still attached to a kidney rudiment or in isolation, did not branch but made multilayer, uroplakin-positive urothelium and organized the mesenchyme into smooth muscle that contracted spontaneously with a period a little slower than natural ureteric peristalsis.

CONCLUSIONS: Mouse ES cells can be differentiated into ureteric bud cells, which can be induced by the peri-Wolffian mesenchyme of kidney rudiments to produce urothelium, while organizing the mesenchyme to produce of rhythmically contracting smooth muscle layers. This raises new possibilities for renal regeneration.

## Introduction:

The last decade has seen significant advances toward the goal of generating kidneys from various types of stem cell. It is now possible to produce renal organoids, representing immature kidneys and showing some physiological functions, from embryonic and induced pluripotent cells of mouse and human.[1-6] So far, these organoids have not featured a ureter. This report describes a technique for differentiating mouse ES cells into urothelium that can organize fetal peri-Wolffian mesenchyme around it to produce contractile muscle layers.

Our experiments rest on two bodies of prior work. One is an effort to explore the self-organizing properties of cells, specifically renogenic stem cells from young fetal mouse kidney rudiments. It was shown in 2010 that these cells, disaggregated then reaggregated, could interact to produce what have since come to be called renal organoids, containing small collecting duct trees and immature nephrons.[7] A series of technical advances[8-10], most of which break the symmetry of the system to create large-scale order, have improved the realism of the tissues produced, resulting in organoids with nephrons arranged around a single collecting duct tree with a single urothelial end .[10]

The other body of work aimed to produce kidneys from human induced pluripotent stem (iPS) cells. [11,12] From early application of sequences of signalling molecules to differentiate mouse embryonic stem (ES) cells into renal epithelia that could integrate into developing kidneys[13], methods were established to produce complete renal organoids from human iPS and ES cells[4-6] that are able to connect with host blood systems.[14] Like the 2010 ex-fetu mouse organoids, they lack proper large-scale anatomical organization. This issue has been partly addressed by Taguchi and Nishinakamura in 2017,[4] who generated the first anatomically organized kidney organoid derived mainly from mouse embryonic stem cells, with some ex-fetu material included. The organoids had a single collecting duct tree, but no ureter.

Here, we report the results of combining the Taguchi and Nishinakamura differentiation techniques[4] with grafting into ex-fetu peri-Wolffian mesenchyme to produce urothelial structures surrounded by smooth muscle coats showing spontaneous contractions.

## Materials and Methods:

### Animals:

Mice mated overnight, the morning of vaginal plug discovery was considered E0.25. Pregnant mice were sacrificed at E11.5 by trained UK Home Office licence holders, by methods listed in Schedule One of the UK Animals (Scientific Procedures) Act. Embryos were decapitated and dissected to obtain 'nephrogenic areas' (metanephros + ureter + nearby Wolffian duct + mesenchyme), or isolated metanephroi, according to the experiment.

### Induction of ureteric bud differentiation from mesenchymal ES cells:

A Hoxb7-GFP mouse ES cell line was a gift from Professor Ryuichi Nishinakamura's laboratory, Kumamato University, Japan. Cells were maintained in GMEM (Sigma G5154) supplemented with 10% FBS, GlutaMAX (1x, Gibco), MEM-NEAA (1x, Gibco), sodium pyruvate (1 mM, Gibco), β-mercaptoethanol (0.1 mM), and leukaemia inhibitory factor (LIF, 1 U/µl, Santa Cruz sc-4989). The mesenchymal ES (mES) cell line was differentiated into ureteric bud (eUB) using a slight modification of a method previously described by Taguchi et al., 2017.[4] Briefly, at 0h cells were dissociated with Accutase (Gibco), re-aggregated at 2,000 cells/aggregate in 96-well, U-bottomed, low cell-binding plates (Greiner 650970) and cultured to form embryonic bodies (EBs). At 48h, the medium was replaced by 'base medium', comprising 75% Iscove's modified Dulbecco's medium (Gibco 12440-046) and 25% Ham's F12 (Gibco 11765-054), with 0.5 × N2 (Gibco 17502-048), 0.5 × B27 (Gibco 12587-010), 0.5 × penicillin/streptomycin, 0.05% BSA (Sigma), 2 mM L-glutamine (Life Technologies), 0.5 mM ascorbic acid (Sigma), 450µM 1-thioglycerol (Sigma) with addition of 10 ng/ml human Activin A (R&D 338-AC) as step1. At 72h (all times are from 0h), the medium was changed for base medium containing 0.3 ng/ml human BMP4 (R&D 314-BP) and 10 µM CHIR99021 (TOCRIS 4423) step2. At 108h, the medium was changed for base medium containing 0.1 µM retinoic acid (RA; Sigma R-2625), 100 ng/ml human FGF9 (R&D 273-F9), and 10 µM SB431542 (TOCRIS 1614) step3. At 132h, the medium was changed for base medium containing 0.1 µM RA, 100 ng/ml human FGF9, and 5 µM CHIR99021 step4. At 156h, medium was changed for base medium containing 10µM Y27632 (Stem Cell Technologies 72302), 0.1 µM RA, 1 µM CHIR99021, 5 ng/ml human

FGF9, and 10% growth factor-reduced Matrigel (Corning 354230) step 5. At 180h, 3µm CHIR99021 and 1ng/ml GDNF (R&D 212-GD) were added to a fresh change of the medium used from 156h step 6. At 204h, this was changed to the same medium with 2ng/ml GDNF and without FGF9 as step 7. After 24hrs of step 7, the embryoid bodies (EBs) developed numerous ES cell-derived UB-like radiating tubules, which we refer to as 'eUBs' in this report.

For culture in Matrigel[4], projecting eUB tubules were isolated by manual dissection from EBs produced using the method described above, and were suspended in 20% Matrigel in DMEM/F12 medium containing 10% FBS, 0.1 µM RA, 100 ng/ml human Rspondin1 (R&D 4645-RS), 2ng/ml human GDNF (R&D 212-GD), and 100 ng/ml mouse FGF1 (Pepro Tech 450-33A) in U-bottomed, low-binding plates .

**Grafting of eUBs into cultured kidney rudiments:**

E11.5 kidneys were isolated from CD1 mouse embryos, and the rudiments were cultured on 24 mm, 0.4 µm-pore membranes (Transwells, Corning 3450) in kidney culture medium (KCM) comprising Minimum Eagle's Medium with Earle's salts (MEM; Sigma M5650) with 10% fetal bovine serum (FBS). Hoxb7-GFP eUBs were isolated manually from day 10 (~230h) EBs using sharpened tungsten needles, and grafted into either the metanephric mesenchyme (Fig1A) or the peri-Wolffian mesenchyme (Fig3A) of E11.5 embryonic kidneys in culture as above. The kidneys and grafts were cultured for 5 or 9 days in KCM, medium being changed every two days.

**Combination of eUB with peri-Wolffian mesenchyme or metanephric mesenchyme:**

Peri-Wolffian mesenchyme (PWM) was isolated by manual dissection from E11.5 nephrogenic areas using sharp Tungsten needles, and dispersed by incubation in 1x trypsin/ EDTA (Sigma T4174), at 37°C for 2 min. Around 150,000 cells were suspended in 150 µl KCM, and centrifugation (3 min at 3000 g) was used to obtain a cell pellet. Pellets were transferred to wells in 96-well, low cell-binding, U-bottom plates. The eUBs were dissected from day 10 organoids and added to the mesenchymal cell pellet, 1 eUB/well, and incubated

at 37°C and 5% $CO_2$. After 24 hrs, the combination had formed a compact spheroid, which was transferred to a 24 mm, 0.4 μm-pore Transwell membrane (Corning 3450) in a well containing 1.5 ml KCM (Fig4A). The same method was used to combine eUB with isolated metanephric mesenchyme (MM) (Fig2A), with the spheroids being cultured in KCM for 5 days, with addition of 50 μl KCM containing 10% Matrigel on top.

**Immunofluorescence:**

Samples were fixed by immersion in cold methanol, and the immersed samples were allowed to warm to room temperature (19°C ± 5°C) over 30 mins. They were washed with phosphate buffered saline (PBS) and blocked in staining buffer, consisting of 5 % bovine serum albumin (BSA) in PBS, overnight at 4 °C. For Krt15 and NP63 staining, samples were fixed in 4% PFA in PBS and blocked using 5% BSA and 0.2% Triton X-100 (Sigma) in PBS. After blocking, primary antibodies (Table 1S) diluted in staining buffer were applied to samples at 4 °C for 24hrs. Unbound primary antibody was washed off in PBS (3 x 5 min), and secondary antibodies (Table S1) in staining buffer were applied overnight at 4 °C. Samples were washed (3 x 15 min PBS), and mounted on a slide using Vectashield (H-1000; Vector Laboratories).

**Paraffin wax-embedded tissue sectioning:**

Samples were fixed in methanol as above, and were then placed in an automatic wax processing machine (Sakura VIP E300; Sakura). Wax-infused samples were embedded in paraffin wax blocks, and 6 μm sections were cut using a Leica RM2245 microtome. Sections were floated out before mounting on slides (Superfrost plus; ThermoFisher) and dried in a 37 ºC oven. Samples were dewaxed in xylene (30 mins), rehydrated in an ethanol series (100%, 90%, 70%, 5 mins each), then placed under running water. Antigen retrieval was carried out by microwaving the dewaxed slides in sodium citrate buffer (10 mM Sodium citrate, 0.05% Tween 20, pH 6.0), for 3 x 15 mins. Immunohistochemical staining was carried out as above.

**Statistics:** For categorical (feature present/ absent) data, 95% confidence intervals were calculated using the binomial normal approximation interval, corrected for small sample sizes, as $\pm 1.96\ [\sqrt{(p(1-p)/n)}] + 1/2n$.[15]

## Results and Discussion:

### Engineering of GFP-labelled ureteric buds from mES cells.

We differentiated HoxB7-GFP mESC[4,16] to ureteric bud (UB) cells using the method of Taguchi and colleagues[4]. In agreement with their findings, by day 2, mES cells formed Hoxb7-GFP$^-$ embryoid bodies (Fig.1S A, B). By day 10, they developed numerous epithelial projections (Fig.1S C) expressing the ureteric bud marker, HoxB7-GFP (Fig.1S D: 6 runs, at least 6 EBs in each, all showing these features). When isolated and cultured in 3D Matrigel supplemented with GDNF, R-Spondin1, FGF1 and retinoic acid[4], the epithelial projections branched in a manner similar to natural ureteric buds in gel culture (Fig.1S F,G; 3 runs, 4 samples in each, all branching).[17] Expression of HoxB7-GFP was maintained (Fig.1S H), suggesting retention of ureteric bud character, and the UB markers Calbindin $D_{28k}$, pan-cytokeratin, Krt8, E-cadherin (Cdh1), Gata3 and Pax2 were present in 3/3 samples tested (Fig.1S I-L). Furthermore, the epithelia expressed the GNDF receptor c-Ret (Fig. 2S A-D; 5/5 samples) and the 'tip' marker Sox9 (Fig. 2S E, F; 6/6 samples). They did not, however, bind the 'stalk' maker Dolichos biflorus agglutinin[18] (DBA; Fig.2S G-H; 0/3 samples). The whole structure therefore had the character of ureteric tip, with no evidence of differentiation to stalk. We refer to tubular structures as ‘engineered ureteric buds’ (eUBs).

### eUBs differentiate into collecting duct-like epithelial trees in a metanephric mesenchymal environment.

Building on previous work, [19-21] Mills and colleagues grafted isolated tips or stalks of natural ureteric buds into either the metanephric mesenchyme or the peri-Wolffian mesenchyme of cultured kidney rudiments. They found that differentiation of the ureteric bud fragments was controlled by the identity of surrounding mesenchyme. Importantly for the current study, they found that ureteric tips grafted into peri-Wolffian mesenchyme expressed the urothelial marker, Uroplakin (UPK).[10]

We tested whether eUBs showed the same plasticity, beginning with grafting to the metanephric mesenchyme (Fig.1A). Grafted eUBs (Fig.1B) grew and branched to produce a

tree (Fig.1C: 10/10 branched; 100%, $CI_{95\%}$ ± 5%). As well as expressing Hoxb7-GFP (Fig.1C) and KRT8 (Fig.1D), they organized a nephrogenic response in the host MM. Their tips became surrounded by SIX2+ cap mesenchyme cells (Fig.1D; 3/3 samples tested, 100%, $CI_{95\%}$ ± 17%). Early-stage nephrons, with WT1+ glomerular poles and JAG1+ proximal tubules, formed near the grafted eUBs and, eventually, connected to them (Fig.1E, E'; 8/8 samples tested 100%, $CI_{95\%}$ ± 6%). All but one graft into metanephric mesenchyme were $UPK^-$, the $UPK^+$ host ureter acting as a positive staining control (Fig 1D; 5/6 tested). The exception was in a damaged host kidney that had lost its own ureter and had a torn mesenchyme. Across these experiments, UPK expression rate was therefore 17% ($CI_{95\%}$ ± 39%).

The host UB is not necessary for this response. When eUBs were grafted into isolated metanephric mesenchyme (Fig 2A), they still branched (Fig.2B) and induced the differentiation of nephrons with WT1+ glomerular poles and Jagged-1+ proximal tubules (Fig 2C; 3/3 samples tested, 100%, $CI_{95\%}$ ± 17%). Again, this observation confirms the prior work of Taguchi and colleagues.

**eUBs differentiate into ureter-like epithelia in a peri-Wolffian mesenchyme environment.**

When grafted instead into the peri-Wolffian mesenchyme of ex-fetu kidney rudiments (Fig.3A), eUBs did not branch and did not induce nephrons, though they retained Hoxb7-GFP expression (Fig.3B, C). They now showed robust expression of uroplakin (Fig.3D; 12/12 samples examined; 100%; $CI_{95\%}$ ± 4%, a range that does not overlap the 95% confidence interval of grafts into metanephric mesenchyme described in the last paragraph). In addition to expressing UPK, they acquired a smooth muscle layer expressing alpha smooth muscle actin (Fig.3D, E).

It is known that the ureteric stalk epithelium and the mesenchyme that surrounds it collaborate to produce a ureter via reciprocal inductive signalling. There is strong evidence that epithelium-derived SHH signalling to the mesenchyme is necessary for the mesenchyme to express BMP4 as a result of an internal FOXF1-dependent pathway and to become competent to differentiate into muscle.[22] BMP4 from the mesenchyme signals to the epithelium to drive urothelial differentiation[10], while the epithelium signals to the

mesenchyme to drive smooth muscle differentiation. This urothelium-to-mesenchyme communication involves beta catenin-mediated WNT signalling, probably by WNT7B and/or WNT9B, both present in the epithelium.[23] In addition, retinoic acid signalling is required for a correct balance of differentiation in both compartments.[24] Are local paracrine signals such as these sufficient to drive urothelial eUB differentiation, or are influences from the kidney or natural ureter needed? Testing this by combination of eUBs with isolated ex-fetu peri-Wolffian mesenchyme (Fig 4A) again resulted in the eUBs remained unbranched (Fig.4B), activating UPK expression and gaining a smooth muscle layer (Fig.4C; 3/3 cases examined, 100%, $CI_{95\%}$ ± 17%). This argues that local interactions between peri-Wolffian mesenchyme and the eUB are sufficient to induce differentiation.

When in the peri-Wolffian mesenchyme, of a kidney or isolated, the form of these grafts was fully or oblately spherical, with no evidence of elongation into a tube. Within the structures, the eUB-derived urothelium differentiated to form the layered structure similar to a natural ureter. At the core were cells showing strong expression of UPK (Fig.4C,D), a classic superficial ('S') cell marker (UPKIII).[25] In some samples, there was evidence of a lumen, albeit somewhat collapsed rather than inflated (Fig.4D). Between the superficial cells and the basement membrane were cells showing strong expression of KRT5 (Fig.4D; 3/3 samples), a classic basal ('B') cell marker.[25-27] Within the B cells' zone were occasional cells expressing KRT15 (Fig.4E; 5/5 samples), as in natural ureter.[28] In some places along the least-basal parts of the zone dominated by B cells were cells expressing no KRT5 and only very weak UPK (Fig.4D), and expressing strong NP63 (Fig, 4F; 4/4 samples), a pattern characteristic of intermediate ('I') cells.[25]

**Ureter-like tissues made by combination of eUBs with peri-Wolffian mesenchyme show spontaneous contractions.**

By 7 days after combination, the ureter-like tissues formed by grafting eUBs into peri-Wolffian mesenchyme of host kidneys started to show rhythmic contractions. These became stronger and more frequent by day 9 and were detectable in 3/3 of the samples filmed under time-lapse (100%, CI95% ± 17%; video 1S). To assess whether contractions of the graft were synchronised with those of the host ureter, this video was analysed frame-by-frame and the

times of peak contraction (minimum diameter) of the graft and host were recorded separately. Times of individual contractions are shown in Fig 3S. The period of contraction of the natural ureter, averaged over 8 intervals between 9 contractions in the recording, was 12s (sem. 0.8s), comparable to that in vivo[29]; the period of the graft, averaged over 7 intervals between its 8 contractions, was slightly slower at 15s (sem 0.6s). There was no obvious relationship between the timings, the graft sometimes leading and sometimes lagging the host (Fig 3S). No contractility was detected in any eUB grafted in the MM.

The asynchronous contraction of graft and host implies that the contractions of the muscles formed around the graft are spontaneous and independent of activity in the nearby natural ureter. To verify this, we filmed combinations of eUBs and pure peri-Wolffian mesenchyme with no host ureter present. These still showed large spontaneous rhythmic contractions (video 2S; Fig 4S), of period 11s (sem 0.8s) in one video and 20s (sem 0.7s) in another. This indicates not only that the muscles are functional, but that at least some have the 'pace-maker' activity usually ascribed to atypical muscle cells normally found at the proximal end of the ureter or renal pelvis.[30] Careful observation showed that, between these large contractions, there were very small contractions that, with the large contractions, formed a steady sequence with periods 6.4s (sem 0.4s) and 7.5s (sem 0.5s) in the same two videos. It is already known from electrical measurements that pacemaker activity in ureter smooth muscle cells runs at 2-4x the frequency of gross peristaltic contraction, due to the mechanism of muscle contraction having a refractory period[29]; the small contractions we observed between large ones may reflect this underlying clock. We did not observe small contractions in either host or grafted UBs in the whole-kidney samples described in the previous paragraph, perhaps because the more closely-packed stroma in these prevented visible small movements.

This is not the first report of differentiation of ES and iPS cells into urothelial cells, but previous examples[31] lacked three-dimensional structure and both these and those of Santos and colleagues[32] lacked smooth muscle. A recent publication by Mullenders and colleagues described ureter organoids made from bladder cancers and from adult human tissue.[33] They adopted a cyst-like shape with a lumen, but with no organization of mesenchymal components or muscle and no evidence of contraction. Our approach is distinct in combining ES-derived ureters with ex-fetu mesenchymal cells to generate multiple epithelial layers and smooth muscle coat that contract spontaneously. Important future goals are to develop

techniques for differentiating peri-Wolffian mesenchyme from ES cells, and inducing the engineered tissue to elongate into a proper tube.

## Author Contributions

MS conducted experiments and led manuscript writing. AP, helped in data analysis and paper editing; CM in grafting experiments; JT in time-lapse video recording and paper editing; ME with thoughtful discussions; LM co-supervised MS' PhD work and made suggestions about the strategy for experiment; JAD is primary supervisor of MS' work, participated in overall project design and detailed experimental design, advised about methods of analysis, analysed timing of contractions and participated in writing the manuscript. All authors approved the final version of the manuscript.

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## Disclosures:

The authors declare that they have no competing interests.

**Supplementary Information**

| | |
|---|---|
| Table 1S | Antibodies used for immunofluorescence analysis. |
| Video 1S | A GFP-expressing eUB grafted into the peri-Wolffian mesenchyme of an intact nephrogenic zone, showing regular smooth muscle contractions. |
| Video 2S | An eUB combined with peri-Wolffian mesenchyme in the absence of an associated kidney, also showing regular smooth muscle contractions. |
| Figure 1S | Production of eUBs from Hoxb7-GFP mESC. |
| Figure 2S | ES-cell derived eUBs show tip markers. |
| Figure 3S | Contraction in grafted eUB-derived ureter-like tissue and in the natural ureter. |

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## FIGURES

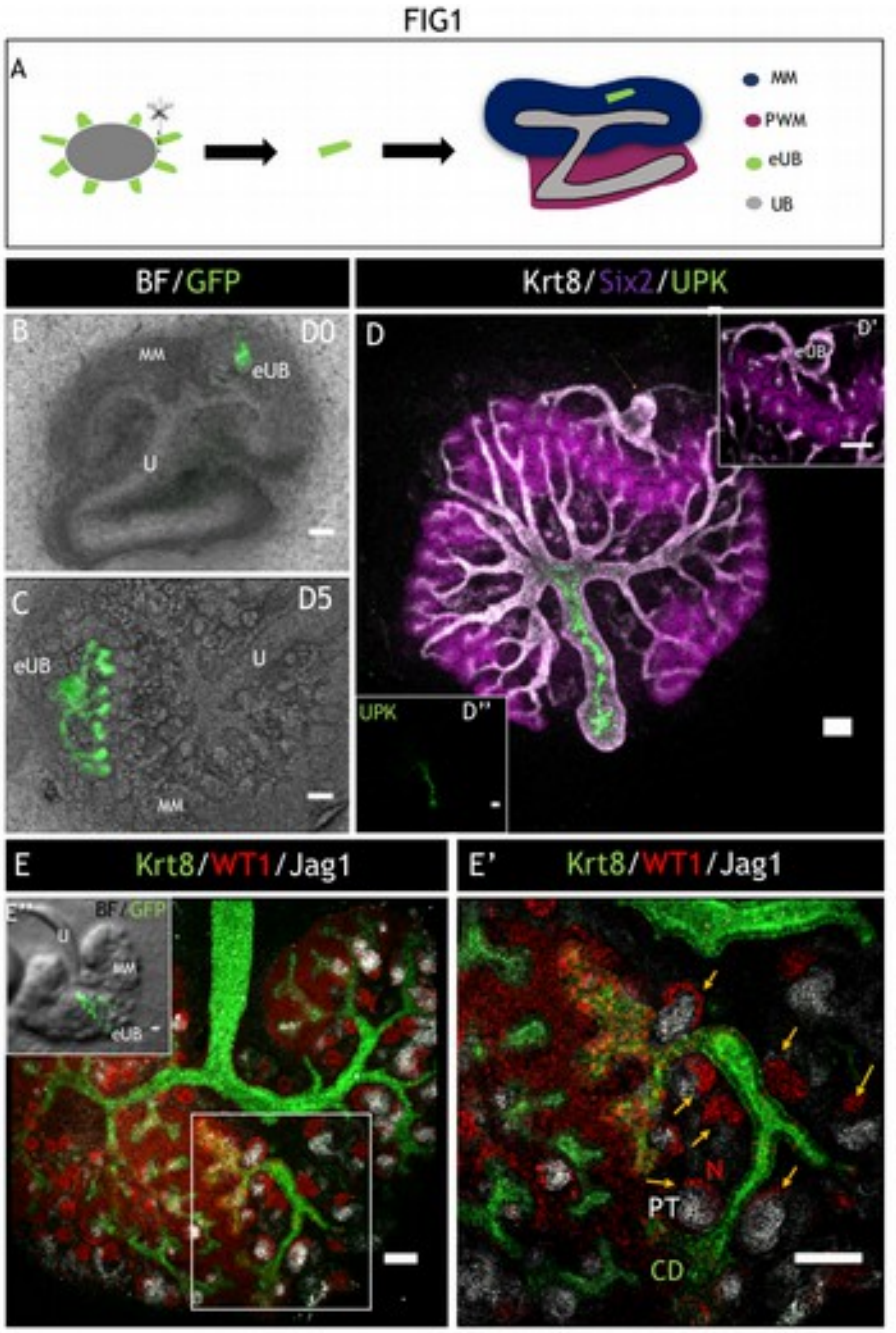


**Figure 1. eUB branches and induced nephrogenesis in renal metanephric mesenchyme.**
**(A)** Steps of grafting eUB into MM. **(B-C)** Combined bright field and GFP fluorescent image of a HoxB7-GFP mESC-derived eUB grafted into the MM of an E11.5 kidney at (**B**) 0, and (**C**) 5 days of culture. **(D)** Immunofluorescence of image C, the grafted eUB arrowed (the sample has been rotated by 90 $^{o}$ because of the differences between microscopes); **(D')** region of D showing the eUB tips surrounded by SIX2+ nephron progenitor cells, like those of the natural UB. **(D")** Image D with only the UPK channel, showing UPK expression in the host ureter but not the eUB graft. **(E)** Immunofluorescence image of an eUB graft in MM showing eUB branching and induction of JAG1+ early nephrons. **(E')** Magnified image of the boxed area in E showing the early nephrons (arrows) associated with the eUB. **(E")** Combined bright-field and GFP image of E showing the location of the GFP-eUB graft (MM; metanephric mesenchyme, eUB; engineered UB, U; natural ureter, PT; proximal tubule, CD; collecting duct). Scale bar =100µm

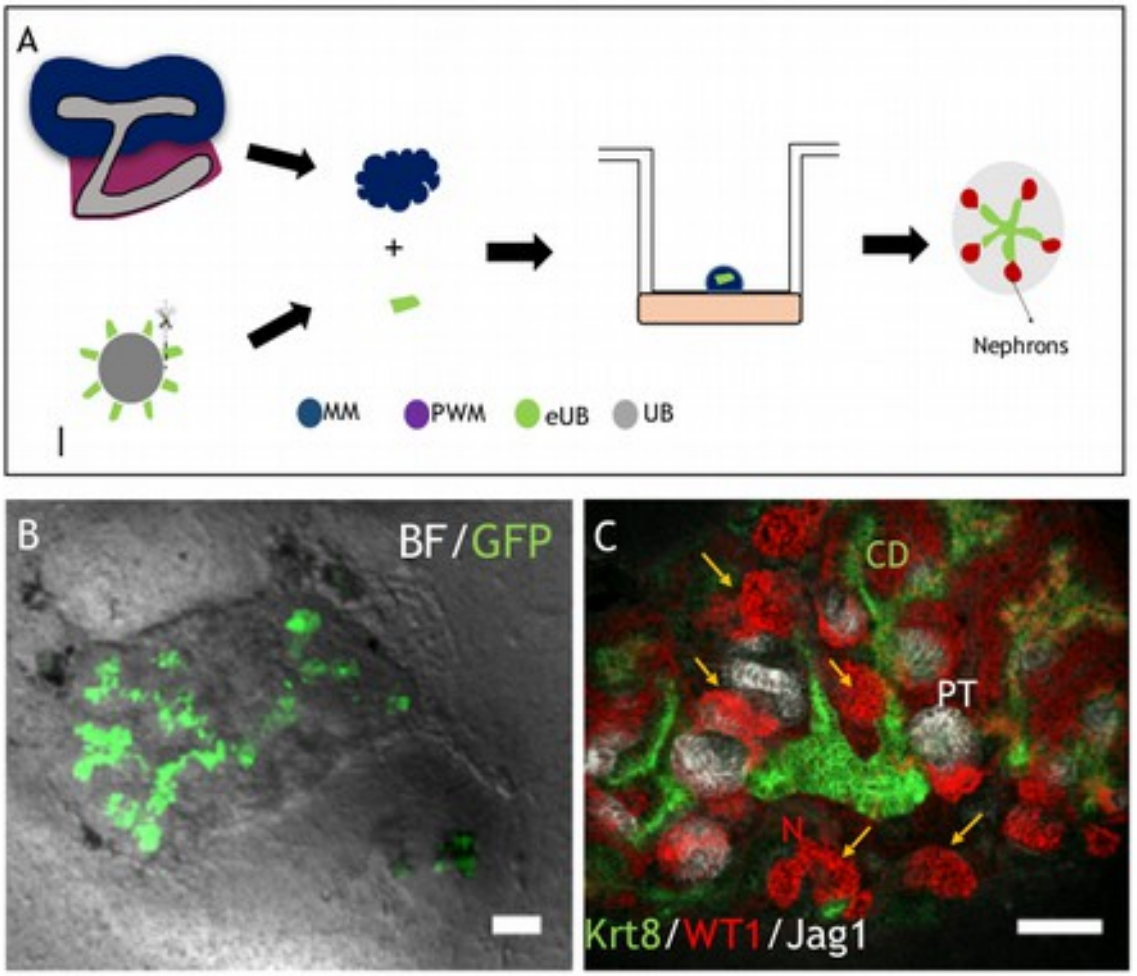


**Figure 2. eUBs branch and induce nephrons when placed into isolated metanephric mesenchyme.**
**(A)** Steps of recombination of eUB with isolated MM cells. **(B)** Combined bright-field and GFP image of Hoxb7-GFP eUB recombined with MM, showing branching of the eUB, survival of the MM and induction of nephron differentiation. **(C)** Immunofluorescence showing the eUB epithelial branching (KRT8 expression), nephrogenic condensates (indicated by arrows and WT1 expression), and early nephrons expressing strong WT1 at their glomerular poles and the proximal tubule marker JAG1, connecting to the eUB.

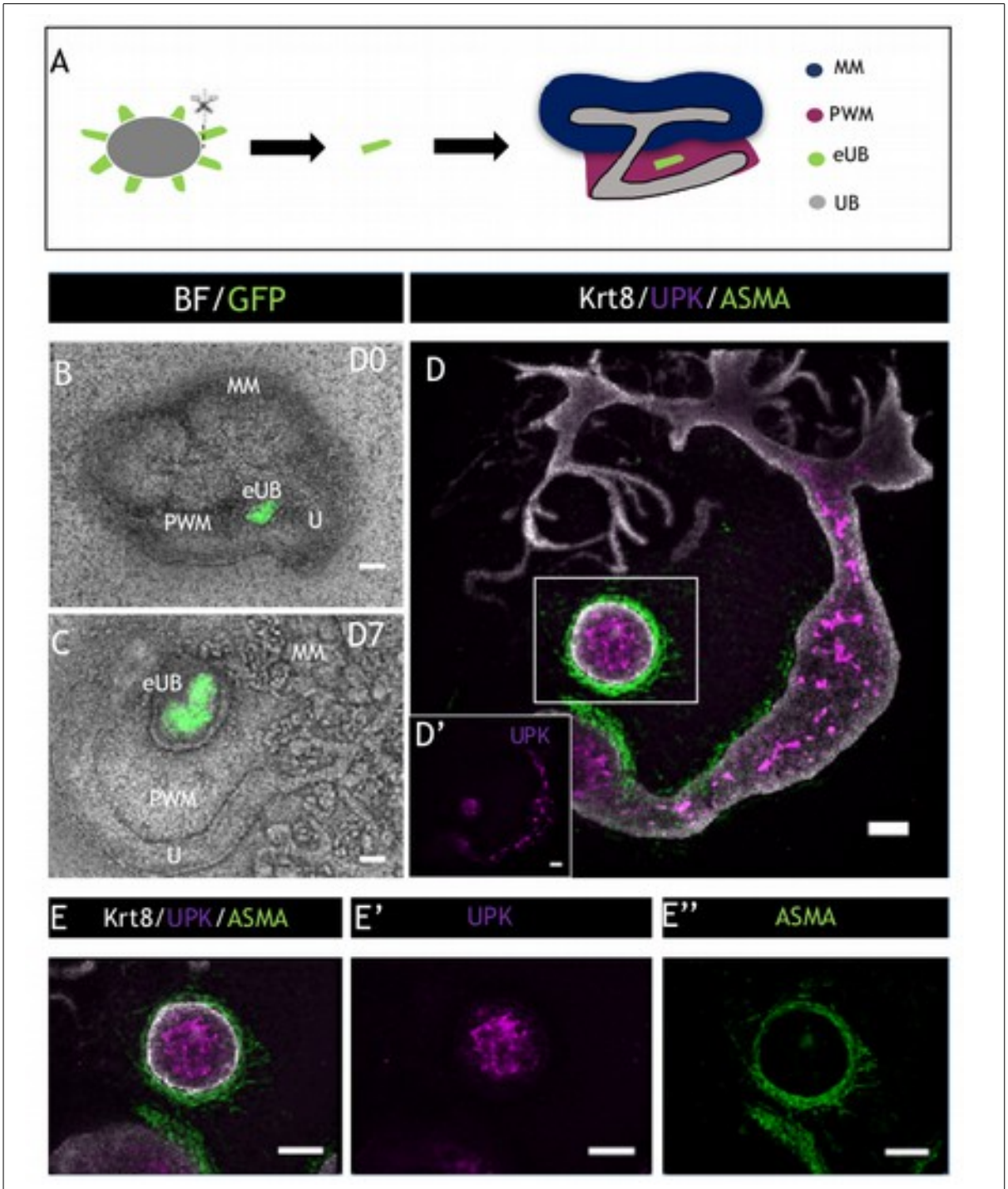


**Figure 3. mESC-derived eUB differentiate into ureter tissue when grafted into peri-Wolffian mesenchyme.**
**(A)** Steps of grafting eUB into peri-Wollfian mesenchyme (PWM). **(B)** A combined bright-field and GFP image of a Hoxb7-GFP eUB grafted into PWM cells at the time of grafting, and **(C)** 7 days later**. (D)** Immunofluorescence stain of an eUB grafted into PWM showing expression of uroplakin (UPK) in the epithelium, KRT8 in the epithelial layers, and smooth-muscle actin (ASMA) around the epithelium. **(E)** Shows detailed views of the graft with separated channels.

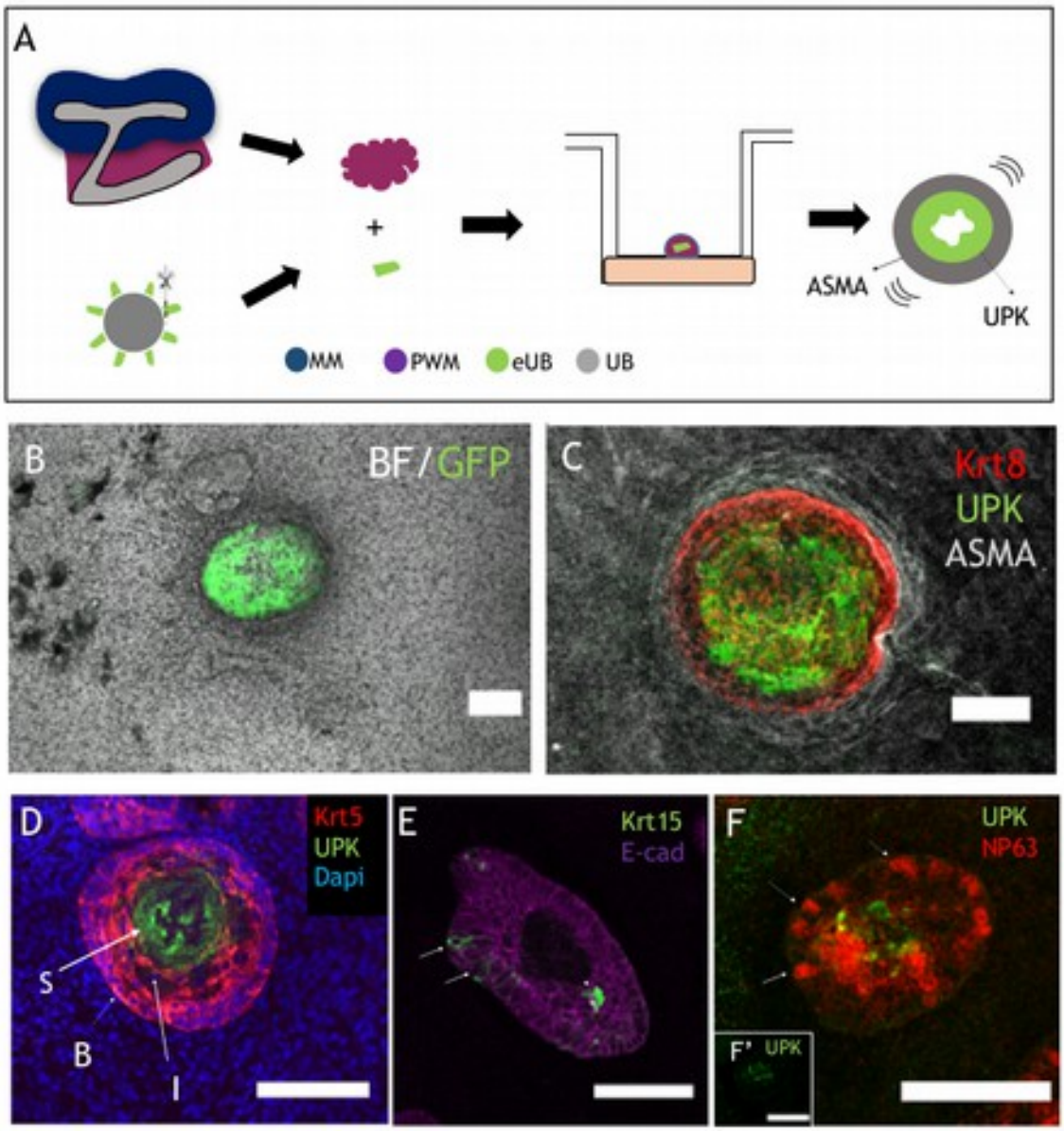


**Figure 4. Urothelial differentiation in pure peri-Wolffian mesenchyme.**
**(A)** Steps of recombination of Hoxb7-GFP-eUB with PWM cells. **(B)** A combined bright-field and GFP image of a Hoxb7-GFP eUB recombined with PWM cells. **(C)** Immunofluorescence stain of an eUB recombined with PWM cells showing expression of Uroplakin (UPK) in the adluminal epithelium, KRT8 in the urothelium as a whole, and smooth- muscle actin (ASMA) around the epithelium. **(D)** A 6µm section of an eUB combined with PWM shows UPKIII expression in superficial ('S') cells, KRT5 in basal ('B') cells and also the presence of KRT5- intermediate ('I') cells in the less basal zone of the area otherwise dominated by 'B' cells. (**E**) Krt15 is expressed by occasional cells in the B cell layer; the counter- stain E-cadherin (Cdh1) marks all epithelial cells of the eUB graft. (F) Shows cells expressing the intermediate cell marker NP63 (arrows) and others expressing UPK; the insert shows the UPK channel alone, for clarity.