Exogenous modulation of embryonic tissue and stem cells to form nephronal structures

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DEDICATION

This thesis is dedicated to my parents, Helga and Erich Sebinger, who have supported me throughout my life and accompanied me on my path of obtaining my PhD.
“Creativity in science, as in art, cannot be organized. It arises spontaneously from individual talent. Well-run laboratories can foster it, but hierarchical organizations, inflexible bureaucratic rules, and mountains of futile paperwork can kill it. Discoveries cannot be planned, they pop up, like Puck, in unexpected corners”

Max Ferdinand Perutz (1914 - 2002)

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“We shall dive down through black abysses… and in that lair of the Deep Ones we shall dwell amidst wonder and glory forever”

from the
“The Shadow Over Innsmouth”

Howard Phillips Lovecraft (1890-1937)
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Abstract

Renal tissue engineering and regenerative medicine represent a significant clinical objective because of the very limited prospect of cure after classical kidney treatment. Thus, approaches to isolate, manipulate and reintegrate structures or stimulating the self-regenerative potential of renal tissue are of special interest. Such new strategies go back to knowledge and further outcome of developmental biological research. An understanding of extracellular matrix (ECM) structure and composition forms thereby a particularly significant aspect in comprehending the complex dynamics of tissue regeneration. Consequently the reconstruction of these structures offers beneficial options for advanced cell and tissue culture technology and tissue engineering. In an effort to investigate the influence of natural extracellular structures and components on embryonic stem cell and renal embryonic tissue, methodologies which allow the easy application of exogenous signals on tissue in vitro on the one hand and the straight forward evaluation of decellularization methods on the other hand, were developed. Both systems can be used to investigate and modulate behaviour of biological systems and represent novel interesting tools for tissue engineering. The novel technique for culturing tissue in vitro allows the growing of embryonic renal explants in very low volumes of medium and optimized observability, which makes it predestined for testing additives. In particular, this novel culture set up provides an ideal opportunity to investigate renal development and structure formation. Further studies indicated that the set is universally applicable on all kinds of (embryonic) tissue. Following hereon, more than 20 different ECM components were tested for their impact on kidney development under 116 different culture conditions, including different concentrations and being either bound to the substrate or dissolved in the culture medium. This allowed to study the role of ECM constituents on renal structure formation. In ongoing projects, kidney rudiments are exposed to aligned matrix fibrils and hydrogels with first promising results. The insights gained thereof gave rise to a basis for the rational application of exogenous signals in regenerative kidney therapies. Additionally new strategies for decellularization of whole murine adult kidneys were explored by applying different chemical agents. The obtained whole matrices were analysed for their degree of decellularization and their residual content and composition. In a new straight forward approach, a dependency of ECM decellularization efficiency to the different agents used for decellularization could be shown. Moreover the capability of the ECM isolated from whole adult kidneys to direct stem cell differentiation towards renal cell lineage phenotypes was proved. The data obtained within this thesis give an innovative impetus to the design of biomaterial scaffolds with defined and distinct properties, offering exciting options for tissue engineering and regenerative kidney therapies by exogenous cues.
Nomenclature

2D Two-dimensional
3D Three-dimensional
BMP-2 Bone Morphogenetic Protein 2
BMP-7 Bone Morphogenetic Protein 7
BrdU 5-bromo-2-deoxy-uridine
CD 44 Cluster of Differentiation 44
CNS Central Nervous System
CO2 Carbon dioxide
CXCL1 Chemokine (C-X-C motif) ligand 1
DOPC 1,2-dioleoyl-sn-glycero-3-phosphocholine
ECM Extracellular Matrix
ES Embryonic Stem
FBS Fetal Bovine Serum
FITC Fluorescein isothiocyanate
GAG(s) Glycosaminoglycan(s)
GDNF Glial cell-derived Neurotrophic Factor
HA Hyaluronic Acid
HSC Hematopoietic Stem Cell
HSPG Heparan Sulfate Proteoglycans
IFNγ Interferon gamma
IgG Immunoglobulin G
KCM Kidney Culture Medium
LIF Leukemia inhibitory factor
MCP-1 Monocyte Chemotactic Protein 1
MMP Matrix Metalloproteinase
MSC Mesenchymal Stem Cell
MW Molecular Weight
PBS Phosphate Buffered Saline
PEG Poly(ethylene glycol)
PEMA Poly(ethylene-alt-maleic anhydride)
POMA Poly(octadecene-alt-maleic anhydride)
qRT-PCR quantitative Real Time Polymerase Chain Reaction
SEM Scanning Electron Microscopy
TE Tissue Engineering
TE/RM Tissue Engineering/Regenerative Medicine
TGF-β Transforming Growth Factor beta
TIMP1 Tissue Inhibitor of Metalloproteinase 1
TRITC Tetramethyl Rhodamine Iso-Thiocyanate
WT1 Wilms Tumor Protein 1
1 Introduction

Kidney failure is a common condition where kidneys fail to maintain their normal function, namely adequate filtration of toxins and waste products from the blood. This malfunction leads to increased fluid levels and accumulation of toxic substances in the body which affects the body's internal equilibrium [1]. Renal failure can be categorised into acute kidney injury and chronic kidney disease. Causes for renal failure can include genetic problems, injuries, drugs or medicines. Chronic renal failure and end stage renal insufficiency are severe illnesses, which only can be treated either by kidney transplants or by dialysis [2]. Currently, patients on dialysis have a five-year survival rate of 33 percent and 10 percent through 10 years, which is worse than the survival rate for many forms of cancer [3]. Most kidney diseases attack and irrecoverably destroy the nephrons, the basic filtration unit of the kidney. One of the reasons renal failure is so common and end stage renal failure or severe kidney injury cannot be cured by treatments or the body itself is that humans are unable to generate any new nephrons after the 36th week of gestation [4].

Thus new ways to repair damaged human kidneys have to be developed. Tissue engineering *in vitro* and regenerative therapies *in vivo* are new promising approaches for treatments of injured renal tissue. Those novel strategies use commonly the integration of cell sources, *ex vivo* assembled tissue, scaffolds and bioactive materials to replace damaged tissue or to induce and recruit the self regenerative potential of the tissue. Regenerating kidneys represent a particular challenge due to its structural complex organisation of numerous specialized cell types, including podocytes, mesangial cells, endothelial cells, fibroblasts, epithelial cells, and numerous stem and progenitor cell populations across the renal parenchyma into discrete, specialized functional units (nephrons) [5,6].

To give a background for this work, relevant scientific research topics, which represent the fundamentals of renal regenerative and tissue engineering approaches, are introduced in the following chapters.
2 Fundamentals

2.1 Kidney development and regeneration

2.1.1 Function of the kidney

The kidneys as part of the excretory system are indispensable organs which serve several essential regulatory duties in most animals [7]. Their homeostatic functions range from the regulation of electrolytes, maintenance of acid–base balance, and adjustment of blood pressure (via maintaining salt and water balance). In producing urine they filter the blood by removing metabolites such as urea and ammonium which are diverted to the urinary bladder (Fig. 1). But they are also responsible for the reabsorption of water, glucose, and amino acids. Moreover the kidneys produce hormones including calcitriol, erythropoietin, and the enzyme renin. Its functional unit is the nephron, which contains over 10,000 cells and at least 12 different cell types [8].

Figure 1. Overview of kidney function.
The functional units of the kidney, the nephrons, are responsible to filter the blood. (Adapted from [9]).

2.1.2 Development of the metanephric kidney

The development of the kidney (Fig. 2) proceeds through a series of successive phases of a more advanced kidney: the pronephros, mesonephros, and metanephros [10]. The most immature and early form of kidney is the pronephros, while the metanephros is
The metanephros persists as the definitive adult kidney of the adult amniotes that are, the reptiles, birds, and mammals. As the nephric duct extends from these earlier kidneys to fuse with the cloaca at the posterior of the embryo, it emits a branch, the ureteric bud, which invades a specialised region of intermediate mesoderm called the metanephrogenic mesenchyme (MM) [12]. When the ureteric bud reaches the MM, a series of inductive signals are exchanged. The MM induces the bud to grow and to branch, thereby forming a tree-like collecting duct system. On the other hand the ureteric bud induces the MM to gain a stem-cell phenotype and to multiply. Hereupon it induces groups of stem cells to differentiate into nephrons [13]. As the ureteric bud continues to grow and branch, its tips get in touch with fresh stem cells and induce those into the nephrogenic pathway. This event results in a gradient of developmental age in a foetal kidney: the outermost cortex consists of stem cells which are not yet committed to differentiation, the next inner region contains cells undergoing the earliest phases of nephrogenic differentiation, and the innermost region comprises maturing nephrons and supporting stromal cells [14].

Figure 2. General scheme of development in the vertebrate kidney.

(A) The original tubules, which generate the pronephros, are induced from the nephrogenic mesenchyme by the pronephric duct during caudal migration. (B) The mesonephric tubules shapes when the pronephros degenerates. (C) The metanephros is initiated by the ureteric bud, which branches from the nephric duct, in the final mammalian kidney. (D) The image of an intermediate mesoderm of a 13-day old mouse embryo demonstrating the induction of the metanephric kidney (bottom) while the mesonephros is still visible. The duct tissue is immunostained to cytokeratin found in the pronephric duct and its derivatives (A-C after [15]; D courtesy of S. Vainio; adapted from [12,16]).
Differentiation of nephrons (Fig. 3): This is a complex process by which disorganised mesenchymal cells become highly organised epithelial tubules. For convenience, it can roughly be divided into four stages [5]:

1. **Condensation**, in which groups of about hundred cells condense tightly together to form a distinct cell mass [17].

2. **Epithelialisation**, in which condensed cells lose their mesenchymal characteristics and gain contrariwise epithelial ones; at the end of this stage the cells have formed a small epithelial cyst completed with a basement membrane, cell-cell junctions and a defined cellular apico-basal polarity [17].

3. **Early morphogenesis**, in which the cyst invaginates twice to form at first a comma and then a S-shaped body, one of these invagination sites will later become the glomerular cleft. At about this time, blood vessel progenitors invade the cleft to begin with the construction of the vascular component of the glomerulus [14].

4. **Tubule maturation**, in which the specialised transporting segments of the nephron differentiate. Also the complex morphogenesis of convoluted tubules is created.

Additional events are the vascularisation and the innervation of the developing kidneys together with the formation of a protective capsule [17].

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**Figure 3. Reciprocal induction in the development of the mammalian nephron.**

(A) While the ureteric bud enters the metanephrogenic mesenchyme, the bud is induced by the mesenchyme to branch. (B-F) At the tips of the branches, the epithelium initiates the mesenchyme to aggregate and cavitate to generate the renal tubules and glomeruli (where the blood filtration takes
2. Fundamentals

The mesenchyme digests the basement membrane of the ureteric bud cells that induced it and connects to the ureteric bud epithelium, when it has condensed into an epithelium. In general, the mesenchyme becomes the nephron (renal tubules and Bowman’s capsule), while the ureteric bud turns into the collecting duct system (for the urine). (Adapted from [15,16]).

2.1.3 Selfregenerative potential of the kidney

The loss of kidney function underlies many renal diseases [18]. Regenerative capacity of the kidney is still not fully elucidated. Mammals can partly repair their nephrons, but cannot form new ones [19,20]. The main four components of the nephrons are the glomerulus, tubules, the collecting duct and peritubular capillaries. The regeneration of the tubular component following an acute injury is well investigated [21]. Recently also the regeneration of the glomerulus has also been documented [22]. Following an acute injury, the proximal tubule is more damaged, and the injured epithelial cells detach from the basement membrane of the nephron. After an injury the surviving epithelial cells, however, migrate, dedifferentiate, proliferate, and redifferentiate to replenish the epithelial lining of the proximal tubule (Fig. 4), [23]. Recently, the presence and participation of kidney stem cells in the tubular regeneration have been shown [24]. In addition to the surviving of tubular epithelial cells and kidney stem cells, the bone marrow stem cells have also been shown to participate in regeneration of the proximal tubule [25,26]. However, the mechanisms remains controversial [27]. Recently, studies which investigate the capacity of bone marrow stem cells to differentiate into renal cells are emerging [28].

By contrast, lower vertebrates like fish add nephrons throughout their lifespan and regenerate nephrons de novo after injury [29,30]. Quite recently a previously unknown type of transplantable kidney cell responsible for this regeneration has been discovered in adult zebrafish. This cell type is found in small aggregations throughout the kidney [31]. These findings suggest that it might be possible to identify an equivalent regenerative cell in humans for therapeutic purposes.
Following acute ischemic or chemical injury, residual tubular epithelial cells and supposed renal stem cells proliferate and differentiate to replace lost tubular epithelial cells for structural and functional repair. It is believed that bone marrow stem cells, including hematopoietic stem cells (HSC) and mesenchymal stem cells (MSC) can also participate in the repair process by differentiation into renal lineages. MSC have been shown to decrease inflammation and enhance renal regeneration. (Adapted from [32]).

2.2 The extracellular matrix as biological scaffold

When we commonly speak of the extracellular matrix (ECM) we refer to the secreted extracellular part of animal tissue that usually provides structural support to the cells together with various other important functions such as segregating tissues from one another and regulating intercellular communication [33]. It is the defining characteristic of connective tissue in animals and regulates the dynamic behaviour of cells like differentiation, survival, migration, the ability to form tissue and overall tissue homeostasis by offering attachment sites for cell surface receptors and feedback-controlled reservoirs for signalling factors (Fig. 5), [34].

The formation and reorganization of the ECM, where proteases and metalloproteinases play a key role, is essential for processes like tissue growth and repair as fibrosis and wound healing. Additionally the ECM is involved in cancer biology [35]. An understanding of ECM structure and composition also helps in comprehending the complex dynamics of tissue regeneration, which can help to develop novel scaffolds for regenerative medicine [36].
Figure 5. Overview of the most important ECM functions.

ECMs are highly dynamic and can be modulated by cells that come into contact with them. A bidirectional mode of cell–matrix communication is generated. Interactions with the ECM may vary based on the chemical and mechanical composition of extracellular microenvironments. (Adapted from [37]).

2.2.1 Molecular composition of the ECM

Components of the ECM are produced intracellularly by resident cells, and secreted into the ECM via exocytosis [38]. After secretion these molecules aggregate with the existing matrix. The ECM is composed of a complex three dimensional network of structural and functional molecules, namely proteins, glycosaminoglycans (GAGs), glycoproteins and small molecules (Fig. 6). The composition is unique to each tissue [34,39].

The components of the ECM can be classified into two categories of molecules: On the one hand in fibrous and adhesion proteins, which mediate mechanical stability and facilitate spatially defined and chemically specific adhesion sites. And on the other hand in proteoglycans and glycoproteins, which are in general composed of a core protein that is posttranslationally modified with mucopolysaccharide chains (gly-
coaminoglycans apart from hyaluronans), offering hydration and pressure resistance to the ECM [40]. Moreover, proteoglycans also support cell adhesion and act as depot for growth factors within the ECM [34]. Because of their overlapping functions, a strict division into structural and functional groups is impossible. The ECM underlies dynamic reciprocity and reacts on environmental cues. Thus the cell behaviour is a coordinated response to molecular interactions with the ECM molecules. Therefore ECM degradation, synthesis and reorganization involves a highly bidirectional flow of information between cells and the ambient ECM [41].

Figure 6. Extracellular matrix.
A natural scaffold consisting of structural proteins, polysaccharides and proteoglycans. (Adapted from [42]).

2.2.1.1 An overview of the main ECM components

Fibrous and adhesion proteins (Tab. 1)

- **Collagen:** In animals the most frequent protein family in the ECM are collagens. In the human body for examples it accounts for 90% of bone matrix protein content [43]. In the ECM, collagens are present in their fibrilar forms and give structural support to the cell environment. Exocytosed as procollagen (precursor form), collagen is then cleaved by procollagen proteases to enable extra-
cellular assembly. Due to their structures, collagens can be categorised into the following families [44]:

- **Fibrillar**: such as Type I, II, III, V, XI
- **Facit**: like Type IX, XII, XIV
- **Short chain**: as Type VIII, X
- **As part of the basement membrane** (Type IV)
- **Other**: like Type VI, VII, XIII

**Elastin**: Elastins, as their name implies, are responsible for the elasticity of a tissue. High amounts can be found in skin, the lungs, in blood vessels, and the ligamentum nuchae where they are expressed by fibroblasts and smooth muscle cells [45]. They are insoluble and the precursor molecule (tropoelastins) is secreted within a chaperone. By contact with a mature elastin fiber tropoelastin is released and deaminated to become incorporated into the elastin strand [46].

**Fibronectin**: By connect cells via their integrins with collagen fibers in the ECM, Fibronectins allowing cell migration through the ECM. Thereby the cytoskeleton of the cell is reorganized and enables cell movement. Fibronectins which are originally secreted in an unfolded, inactive form bind to their integrins so that the resulting dimers get activated. Fibronectins are also involved in wound healing by enabling cell migration to the affected area [38].

**Laminin**: As major part of the basal lamina (one of the layers of the basement membrane) of virtually all animals, laminins influence cell differentiation, migration, adhesion as well as phenotype and survival [47]. In contrary to the fibres formed by collagen, laminins generate networks of web-like structures that resist tensile forces in the basal lamina. Laminins can bind other ECM components like collagens, nidogens, and entactins [38].
Proteoglycans and glycosaminoglycans (Fig. 7)

By connecting with ECM proteins, glycosaminoglycans, as carbohydrate polymers, form proteoglycans. Because of their negative charge they attract positively charged sodium ions (Na+) which absorbs water molecules. Consequently the hydration of the ECM and the resident cells is ensured. Moreover proteoglycans are involved in trapping and storage of growth factors [51].

- **Heparan sulfate:** Heparan sulfate regulates a variety of biological activities, including developmental processes, angiogenesis, blood coagulation and tumour metastasis. Heparan sulfate occurs as a proteoglycan in which two or three linear polysaccharide chains are attached in closeness to cell surface or other extracellular matrix proteins, especially to components of the basement membrane, the multi-domain proteins perlecan, agrin and collagen XVIII [52,53].
2. Fundamentals

- **Chondroitin sulfate**: Chondroitin sulfates are conducive to the elasticity of cartilage, tendons, ligaments and walls of the aorta and are involved in neuroplasticity [54].

- **Keratan sulfate**: Keratan sulfates are large highly hydrated molecules which are able to act as a cushion to absorb mechanical shock [55]. In addition to the central nervous system they are mainly present in the cornea [56], cartilage and bones.

- **Hyaluronic acid (non-proteoglycan polysaccharide)**: Hyaluronan (or hyaluronic acid) is an anionic, nonsulfated glycosaminoglycan allocated throughout connective, epithelial, and neural tissues. It is unique among glycosaminoglycans in that it is not found as a proteoglycan, is nonsulfated, forms in the plasma membrane instead of the Golgi, and can reach large molecular weights by subunit assembling [57]. As one of the main components of the ECM, hyaluronan contributes significantly to cell behaviour such as proliferation and migration by interacting with a specific transmembrane receptor (CD44) [58]. Furthermore it is involved in processes like development, healing processes, inflammation and tumour development. Hyaluronic acid in the extracellular space gives tissues the ability to resist compression because of providing a counter-acting swelling force by absorbing huge amounts of water. Hyaluronans is also a chief component of the interstitial gel and can be found plentifully in the ECM of load-bearing joints [59].
2.2.2 Cell/tissue-matrix interactions

Cell behaviour in vivo such as cell adhesion, proliferation, migration, differentiation and cell death are crucially controlled by the environment manifested by the surrounding ECM [60]. Consequently the characteristics of the ECM, such as its mechanics, chemical composition and structure, provide key signals for cells and the formation of tissues. Biochemical cues of the ECM in general imply biomolecules, which are involved in binding and activation of cell membrane receptors, which activates downstream signalling events. Moreover the ECM mediates a storage ability for soluble signalling molecules [61]. Mechanical, structural and physical properties of the ECM on
the other side regulate tensile strength, cushioning and filter functions (as in kidney) and formation of boundaries as well as geometric and topographical guidance for cellular growth and tissue formation [62]. In fact, it is difficile to make a clear distinction between biomolecular and physical cues, since complex interwoven ECM assemblies in many cases unify both characteristics. Nevertheless the next sections will discuss separate and combinatorial instructive effects of the ECM which form the basis for beneficial engineering of cell environments.

2.2.2.1 Biochemical signals

Biochemical signals of the ECM are transmitted into the cell via cell surface receptors which recognise specific ECM adhesion domains of ECM molecules and display differential binding affinities towards their ligand binding sites [63]. Integrins form hereby the most important group of cell surface receptors (Fig. 8). They organize the crosstalk between biochemical and mechanical stimuli of the ECM to the interior cytoskeleton of the cell. Integrins are heterodimeric trans-membrane receptor glycoproteins with alpha and beta subunits, which allow 24 different combinations [64,65]. The composition of these subunits determines which ECM signals (or binding sites) are recognized. But cells also have other non-integrin cell surface receptors, which are transmembrane proteoglycans like syndecan, CD44 (for hyaluronans) [58] and laminin receptors. Depending on specific ECM signals, cells can change their phenotype, change cell surface receptor expression or actively rearrange the ECM environment by secreting ECM proteases [66]. Additionally the molecular composition of the ECM can be influenced by its interaction with growth factors, which effects cell behaviour on various levels. In mediating the binding and mobilisation of growth factors, cytokines and morphogens, proteoglycans and their GAG side chains play a key role in this process. The direct binding to the ECM controls the biological activity, spacial distribution and local concentrations of growth factors [67,68]. On the other hand, transcription, translation and post-translational modification of ECM macromolecules are regulated by various growth factors [69]. Interactions between growth factors and ECM molecules are important for cellular environments and regulate the genotype and phenotype of cells.
2. Fundamentals

**Integin-mediated signalling**

![Integin-mediated signalling](image)

*Figure 8. Simplified schematic diagram of cell behaviour control from integrins and growth factor receptors.*

Both growth factors and the ECM provide external signals that influence cell behaviour. (Adapted from [70]).

### 2.2.2 Mechanical signals

Physical cues of the ECM relates to the mechanical and structural signals acting upon the residing cells within a tissue [71]. Tissue elasticity arises from concrete ECM compositions and the structural processing of the ECM. A specific stiffness is featured by each tissue and thereby is representative for the mechanical environment of the resident cells within (Fig. 9). On the cellular level, elasticity is a result of the flexibility and extensibility of ECM molecules, such as collagens, elastins and hyaluronic acid. Mainly the mechanical interactions between the cell and the ECM are integrin-mediated cell adhesion and mechanotransduction processes[72]. Thus the mechanical properties of the ECM are associated with the cellular cytoskeleton, where the stress on the cytoskeleton is translated into cellular motility which can induce additional mechanical signals [73]. Through tensile forces cells can respond by rebuilding the ECM and take on differential morphologies [72]. Loops of a mechanosensitive local force geometry, mechanotransduction via integrin receptors and mechanoresponse by intracellular transduction of tensional integrity into biochemical signals result in regulations of cell behaviour and finally of tissue homeostasis [74].
2.2.2.3 Structural signals

In addition to biochemical and mechanical cues, cell surface receptors respond to specific structural features of the ECM [61]. So it is of crucial importance how and in which pattern mechanical and biomolecular signals, which are rarely isolated molecules, are presented. The ECM functionality is based on the presence of suprastructural elements which originate from ECM polymerisation into insoluble fibrils, micro-fibrils and networks that assemble to superior structures (i.e. fibres or basement membranes) [76]. The pattern of ligand topography is constituted by this suprastructural conformation. Within the environment the ECM structure acts as contact guidance for development, orientation, alignment and migration for the embedded cells. In this way more complexity and regulation opportunities are added to the anyway versatile ability of the ECM to direct cellular behaviour and tissue formation [77]. But of course further research is required to entirely reveal the influence of ECM suprastructural organisation on cell and tissue fate.

2.3 Tissue engineering for therapeutic purposes

2.3.1 An overview of tissue engineering and regenerative medicine

Langer [78] and Vacanti [79] have described tissue engineering as "an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ" [80]. This ambitious objective is executed by scientists, engineers, and physicians for the past decades by applying tools from a variety of fields to construct these substitutes on the one hand for diagnostic and research purposes and on the other hand to replace or regenerate diseased and injured tissues [81]. The difference from
conventional drug therapy lies in the integration of the *in vitro* engineered tissue within the patient, aiming a potentially permanent and specific cure of the medical condition [82].

The reason for tissue engineering and regenerative medicine is the disability of most human tissues to regenerate after an injury or disease. Even if a spontaneous regenerative potential of the tissue exists (for example in bone), it is often overstrained in large defects. In such case also so far applied conventional implants have limits in terms of temporal and mechanical durability [83].

Often tissue engineering is equate with regenerative medical approaches. However the difference between these related research fields are the site where regeneration is induced: In case of tissue engineering, novel tissue is produced *in vitro* by seeding cells into an artificial scaffold. The resulting tissue is subsequently implanted into the body. This has the advantage that the generated tissue can be evaluated prior implantation. In contrast, biomaterials, with or without integrated cells, are implanted into the body to promote regeneration of the tissue *in vivo* when we refer to regenerative medicine. The benefit hereby is the natural environment, under which the tissue is integrated and formed (Fig. 10).

![Figure 10. Strategies in tissue engineering (in vitro) and regenerative medicine (in vivo).](image)

In vivo tissue regeneration (regenerative medicine) as well as in vitro tissue engineering can be achieved from cells, signal molecules and scaffolds. For the tissue regeneration, three different combinations (signal molecules, signal molecules+cells, and signal molecules+cells+scaffold) can lead to tissue regeneration so far. The combination signal molecules+cells leads to cell based therapies. In this scheme recent attempts to create bioreactive scaffolds for direct application in tissue engineering and regenerative medicine are not considered. (Adapted from [84]).
To induce regeneration, the following constituents are utilized (Fig. 11): First, porous, absorbable synthetic and natural (bio)materials provide the scaffold for rebuilding tissue [85]. Second, differentiated and undifferentiated (stem) cells of autologous or allogeneic origin are used to form tissue [86]. Additionally soluble regulators such as growth factors can be added to regenerative approaches [87]. All of these components can be used separately or in combinations to induce regeneration in vivo or to generate tissue in vitro for implantation. Finally the environmental factors like the mechanical loading or the fluidic dynamics within bioreactors for tissue growth are worth to be mentioned because of their influences on the engineered tissue [88].

Figure 11. Exemplary scheme of a tissue engineering approach that involves seeding cells within porous biomaterial scaffolds.

(a) Cells are isolated from the patient or of allogeneic origin and may be efficiently expanded (b) in vitro on two-dimensional surfaces. (c) Afterwards, cells are seeded in porous scaffolds together with supporting (e.g. growth factors. The scaffolds serve as a mechanical and shape-determining support. Their porous character provides high mass transfer and waste removal. (d) The cell-material constructs are further cultivated in bioreactors supplying optimal conditions for development into a functioning tissue. (e) Once an efficient tissue has been successfully engineered, the construct is transplanted on the side of injury to restore function. (Adapted from [89]).

Current tissue engineering faces challenges in biocompatibility of matrices [90]. Furthermore optimized geometry, architecture and composition as well as the ideal lifespan of scaffolds and surfaces have to be found for the best possible contribution to regenerate tissue. The development and application of permanent and biodegradable materials is also an important aim [91]. Of course, to provide greater insights into and
understanding for tissue engineering, further basic research on the selfregenerative potential of tissues and the behaviour of cells, as well as their controllability and manipulability, have to be accomplished. Special attention is drawn on vascularization of engineered or regenerated tissue in vivo and in vitro [92]. How scaffolds and tissue can be transferred and incorporated most efficiently into the body is another important task of tissue engineering and regenerative medicine [91].

2.3.2 Biomaterials for tissue engineering and regenerative medicine

The term biomaterial is generally understood to describe any matter, surface, or construct that interacts with biological systems. Biomaterials serve as supporting structures or mechanical scaffolds for growing cells and tissues in and outside the body. Additionally biomaterials can be engineered to modulate the cellular response through specific adhesion receptors (e.g. integrins), through the release of bioreactive factors or through self-degradation, changing both their chemical and mechanical properties over time [85]. Biocompatibility, biofunctionality, accessibility and mimicry of biologic functions are key tasks for designing and applying of biomaterials [91,93]. Due to their origin, biomaterials can be classified into either biologic, synthetic or hybrid biomaterials. They are composed of biologic (e.g. ECM molecules) and/or synthetic (e.g. modified polymers) building blocks [94]. As mentioned before, the ECM is a highly sophisticated natural biomaterial that features mechanical integrity and affects cell/tissue fate and function. The whole ECM can be extracted from natural tissue by decellularization [95]. Purified natural single polymers can be employed as biomaterials as well, like collagen and polysaccharides, which can be assembled to porous sponges or hydrated gels [96]. These biofunctional hydrogels (also in combination with synthetic components or binding site sequences) are water swollen cross-linked polymer structures. They can absorb diverse factors, which can be controlled released and encapsulate cells, which then are affected in their function and shape [97]. Biomaterials can also be formed by molecular self assembly of peptides or peptide-amphiphiles [98]. Also synthetic simplified protein fragments can be designed to interact with cells by emulating key molecular features of the ECM. Thereby the essential chemical character of ECM influences is distilled into simple chemical functionalities, which reduces the complexity of biomaterials massively [41].
An increasingly important tendency in tissue engineering research is that rather than attempting to recreate the complexity of living tissues in vitro, attempts are being made to develop synthetic biomimicking materials that establish key interactions with cells in vivo in ways that unlock the body's innate potential of organization and self-repair [99].

2.3.2.1 Decellularization approach as tool to extract natural matrices

Decellularization in general is the process by which tissue or organs are treated to remove all resident cells to obtain the ECM. The resulting decellularized tissues and organs serve as effective tools for tissue engineering/regenerative medicine applications. Meanwhile manifold decellularization methods are utilized for a huge variety of tissues and organs [100]. The applied methods as well as the kind of tissue or organ, especially their density, organization, geometric and biologic properties, determine the efficiency of cell removal, the preservation of the architecture as well as a potential loss of surface structures and composition [101]. Different treatments influence thereby the biochemical composition, tissue architecture and mechanical behaviour of the remaining ECM scaffold, which affect the host response to the material [100]. The agents used for decellularization can roughly be divided into chemical (like detergents), biologic (like enzymes) and physical (e.g. temperature) ones. These agents are also combined to increase decellularization efficiency [95].

To summarize, common techniques to apply the various decellularization agents are whole organ perfusion, induction of a pressure gradient across tissue, usage of super-critical fluid (carbon dioxide) and immersion and agitation [100]. The increasing employment of biological scaffolds for tissue engineering and regenerative medicine approaches justify further work and intensive research on decellularization protocols [102].

2.3.3 Tissue engineering and regenerative medicine in kidney treatment

Renal TE/RM represents a significant clinical objective because of the very limited prospect of cure after classical clinical treatment of severe injured kidneys, kidney failure or chronic kidney disease.
Cell based induction of regenerations in models of (chronic) kidney disease can improve renal physiology [103–105]. Also, host-specific renal progenitor cells are activated in response to the insertion of bioactive cell populations [106], like mesenchymal stem cells (MSCs) [107]. It is believed that a paracrine signalling is responsible for provoking diverse reactions of the host tissue, like angiogenesis, inflammation and fibrosis, while simultaneous mobilization of resident stem and progenitor cells occurs. Direct injection of stem cells into the renal vasculature or renal parenchyma even causes apoptosis or efflux of most applied cells from the kidney within short time [108]. There exits anyway little if any evidence that the exogenously applied stem or progenitor cells undergo site-specific engraftment and directed differentiation and if they do, uncontrolled differentiation of these cells appears [109]. So renal cell therapy methodologies show mixed results and the mechanisms behind cell based approaches still remain unclear. Therefore primary renal cell/biomaterial constructs are being investigated for therapeutic bioactivity [110] with promising outcome including de novo nephron formation in healthy kidneys (Fig. 12).

Figure 12. Example for regenerated renal tissue.

The upper image (A) show cortico-medullary junction of healthy adult rodent kidney, 8 weeks after injection with regenerative construct composed of selected regenerative renal cells and gelatin hydrogel [110]. Trichrome X10 low power image of kidney cross-section showing biomaterial aggregate. Inset indicates panel magnified below. Higher magnification (B) of boxed area demonstrates regenerative response induction consistent with neo-kidney tissue formation (as an example, trichrome stained, ×400, circled in yellow). (Adapted from [111,112]).
Exosomes or microvesicles, which are involved in cell to cell communication, could possibly be utilized for regenerative assays [113]. Decellularized kidney scaffolds provide interesting matrices for attempts to induce neo-organ maturation *ex vivo* because of their ability to induce differentiation of exogenously applied ES cells along defined renal lineages [114]. Synthetic biomaterials are also promising candidates for tissue engineering and regenerative medical applications of kidneys, as they are able to mimick bioactive factors like growth factors and ECM based peptides [115], which have the potential to modulate cell and tissue behaviour. *In vitro* applied nephrogenic cocktails, composed of activin A, retinoic acid [116], and BMP7 [117], have the ability to induce the formation of early kidney structures [116] and expression of markers of the early metanephric mesenchyme [117]. Such cocktails may also be interesting for a widened application with other cell types or being directly injected into tissue. Also the embryonic metanephroi after implantation into animals have the ability to form mature tubules and glomeruli by simultaneous enlargement and vascularization within tissue [118]. Moreover the embryo itself is deployed as an “organ factory” for generating neo-organs. Inserted transgenic MSCs can trigger formation of neo-kidney-like structures in the presence of the native developmental regenerative milieu by expressing GDNF [119,120]. Interestingly, cells involved in early kidney development have the potential for self-organisation and formation of early kidney structures [121,122]. It still needs to be tested though, whether this potential exists in renal stem cell populations derived from the adult kidney, which would allow reconstruction of nephrons in the mature organ. The application of hyaluronic acid itself, or biomaterials based on this glycosaminoglycan, could serve as basis for the development of novel tissue engineering or regenerative therapeutic strategies. The reason is that exogenously added hyaluronic acid, which is expressed in the early kidney [123], modulates branching morphogenesis in *ex vivo* cultured kidneys [124]. Injections of hyaluronic acid particles in healthy adult kidneys were unable to induce *de novo* structure formation so far [111], but nevertheless HA could help to design tissue engineering scaffolds like gel-based biomaterials, especially in combination with other types of regenerative constructs. In general, biofunctional hydrogels can serve as matrices for tissue reconstructing approaches [125]. Additionally hydrogels with or without incorporated bioactive components can act as ideal shuttle systems for cells or for *in situ* controlled factor re-
lease when injected into injured tissue within the body. Promisingly implantation of re-combined, *ex vivo* assembled kidney-like tissue within animal models, leads to formation of obviously vascularized glomeruli structures within 14 days [126]. Implantable kidney structure formed from cells instead of embryonic explants would greatly facilitate clinical translation of this kind of developmental engineering methods. Precursory progress toward this direction is shown by experiments investigating the potential of renal cell cultures to generate kidney progenitor tissue [127]. The self-organizing potential of renal cell populations [121] could play a key role in these attempts.

In general, tissue engineering, cell-based therapies and other regenerative medical technologies can contribute to potential prospective treatments for loss of renal tissue function. Thereby the abilities to isolate, manipulate and reintegrate embryonic structures or stimulating the selfregenerative potential of the tissue are of special interest [128]. Such new strategies go back to knowledge and further outcome of developmental biological research. In the next years it will be necessary to evaluate especially these methodologies within preclinical models of renal disease.

### 2.4 Organ and tissue culture as tool for tissue engineering

*In vitro* growth techniques of organs/tissues, in which their various components (e.g. mesenchyme and epithelium in early kidney organ anlagen) are preserved in terms of structure and function, so that the culture organs resemble closely the corresponding organs *in vivo* by using specialized media, substrates, and atmospheres, are called organ culture.

The reasons for *in vitro* organ culture are manifold: *In vivo* studies and observations of developing organs or their functionality as mature tissue are usually difficult. Additionally the different cell types or functional units (alveoli, glomeruli, islets, etc.) involved in these processes are often hard to identify. The visceral organs vary in their spatial and temporal development pattern within the embryo, which complicate manipulations like gene knock-out, knock-down or drug testing assays *in vivo*. Furthermore the temporal variation causes inconsistencies of results by examination of a series of organ or tissue samples.

Organ culture has the following characteristics: The cultured organs/tissues maintain their physiological features and new growth occurs in form of differentiated struc-
tures (for example nephrogenesis during embryonic kidney culture). Furthermore the morphogenesis in cultured foetal tissues is analogous to that *in vivo*. Cellular outgrowth from periphery of explants can be controlled and manipulated by modulating culture conditions.

Models of organ explant culture provide several crucial advantages for studies of patho-physiologic mechanisms like differentiation, structure development, secretion or cell injury, as compared to cell culture [129]. By applying organ culture the histiotypic relationships among cells of an organ without any disturbance of the cellular or tissue architecture is preserved, as it is usually the case *in vivo*. In contrast to that, cells are enzymatically, chemically or mechanically separated during classical cell culture techniques. Because results are easier to obtain, in some cases, organ culture can replace animal experiments [130]. But the limit of organ culture is reached when the metabolism of the whole body is involved in experiments e.g. the influence of drugs on the whole organism. Also the limited culture period restricts the application of conventional organ culture systems. Other disadvantages of organ culture systems are the diminished visibility of the cultured tissue and the infeasibility for “automation”. Some of these methods are also difficult to handle and need high volumes of culture medium. Additionally the convertibility and the modifiability of the culture substrates are limited (chapter 2.4.1).

Organ culture is basically employed on organs/tissues to be exposed to experimentally varied environments where the maintenance of structural tissue organization is important (for example testing growth factors, drugs or radiation) [129]. Studying the anatomical aspects of mammalian organ development [131], morphogenesis and function in both normal and mutant animals, is important for basic biology as well as for regenerative medicine and tissue engineering. Another aspect of tasks, which are investigated with the help of organ culture systems, is the comparison of the development of isolated organs with the development of their equivalents *in situ* [132,133].
2. Fundamentals

2.4.1 Common organ culture systems

Several different attempts to culture explanted tissues and organs in vitro have been developed so far:

- **Plasma clot method:** Here the explant is placed on the top of clotted (originally owl) plasma containing an embryo extract inside a watch glass. The set up can be closed by a glass lid. This has been the original technique for studying the morphogenesis in embryonic organ rudiments [134,135].

- **Raft method:** A raft of lens paper or rayon acetate, which is floated with culture medium in a watch glass, serves here as a culture surface for isolated explants. Treating the rafts with silicon enhances hereby the floatability. This method is also combinable with the set up stated above by placing the raft on the plasma clot [136].

- **Grid method:** This method is an advancement of the raft method as it utilizes small tables formed of wiremesh or perforated stainless steel. The explants are first placed on rafts, which are then kept on the grid table in a culture chamber filled with culture medium. This prevents the sinking of explants into culture medium (Fig. 13), [137].

- **Agar gel method:** The explants were cultured on in agar gelled culture medium, containing all required nutritions. Thereby the sinking of the organs/tissues is avoided. Additionally the gel surface provides soft, more tissue like surface which allows studying many developmental aspects [138].

- **Hanging drop assays:** In this method the isolated explants were cultured in hanging drops of culture medium. The explants were initially placed in small drops on a Petri dish lid, which is then turned around and situated on a liquid filled Petri dish. The liquid serves to keep the explant containing drops from dehydration (Fig. 13), [139].
2. Fundamentals

Figure 13. Schematic representations of Trowell-type organ culture and Hanging-drop techniques.

Sketches of the Trowell and hanging drop set ups are presented under (A and B). An example of a Trowell-type organ culture dish, where the metal grid supports six pieces of filters placed on the holes punched in the grid is given under (C). Close-up image shows (D) one cultured explant lying on a filter in the Trowell-type culture dish (Picture C courtesy of Otso Häärä). (Adapted from [140]).

3 Objectives and Motivation

The reconstruction and isolation of natural ECM biopolymer structures offer beneficial options for advanced cell and tissue culture technology and tissue engineering approaches. Thereby assays, which provide the application of multiple extracellular components to study cell- or tissue-matrix interactions, are of special interest. To test this interplay in vitro, the development of potent cell and tissue culture systems is necessary. Thus, in an effort to investigate the influence of natural extracellular structures and components on embryonic stem cell and renal embryonic tissue to support nephrogenesis, the following main goals had to be addressed within this thesis.

A) The establishment of a novel organ and tissue culture system which allows the facile application of exogenous signals in various ways and simultaneously improves observability of cultured tissue (Chapter 4.1, Appendix A).
3. Objectives and Motivation

B) The direct exposure of embryonic kidney rudiments to a large set of either surface-immobilized or soluble ECM molecules and growth factors using the new organ culture set-up to explore the role of ECM constituents on renal structure formation (Chapter 4.2, Appendix B).

C) The isolation of whole kidney ECMs and the investigation of their differing compositions caused by treatment with different chemical agents (Chapter 4.3, Appendix C).

D) The determination of the various resulting whole ECM constructs’ abilities to influence mouse embryonic stem cell differentiation and renal tissue behaviour (Chapter 4.4, Appendix C).

Finally, these studies could help to create a knowledge base for ECM-supported approaches in kidney regenerative therapies.

Figure 14. The strategy of the thesis: Generating biomaterial scaffolds which act as exogenous signals for renal tissue engineering purposes.
(Adapted from [41]).
4 Results and Discussion

4.1 A novel, low-volume method for organ culture of embryonic kidneys that allows development of cortico-medullary anatomical organization

This section refers to the following publication (see Appendix A):


In the frame of this work a novel organ culture method has been developed to allow the easy and varying application of exogenous signals to embryonic (kidney) rudiments *in vitro*.

As discussed in the introduction for organ culture systems (Chapter 2.4.1), several approaches have aimed to culture (embryonic) organs and tissue *in vitro*. In the fifties and sixties of the last century, whole organ metanephric culture systems have started to be designed to study developmental aspects of mammalian embryonic kidney development. In the meanwhile, several improvements have been archived: clotted owl plasma [134,135] was replaced by a Millipore filter [141,142], acting as support for the growth; these Millipore filters were again replaced by polycarbonate filters, which have been recently fixed on inserts [143] for better handling. But retrospectively, the principle of culturing kidney rudiments has not really changed since the first trials by Clifford Grobstein [144]. Kidneys are grown on the top of a supporter (filter), provided with medium from underneath.

As part of this thesis, a new path in culturing embryonic kidney rudiments and possibly embryonic organs in general, has been struck. Kidney rudiments are grown in the medium on glass, fixed by the surface tension (Fig. 15). This new strategy of organ culture has several advantages compared to currently used ones: The most obvious benefit is the visibility, which enables the observation of the growing organ rudiments all the time. Another virtue is the reduction of the culture medium, especially interesting if
Results and Discussion

expensive or rare additives are used during the culture period. Surprisingly the novel culture method has an exceedingly positive influence on the development of the cultured embryonic kidneys, resembling more naturally the in vivo organ embryogenesis.

Long-term (10-day) culture in our low volume system resulted in a substantial increase in area of the organ rudiment and the formation of distinct cortical and medullary zones. The forming glomeruli were restricted to the cortical zone and the medullary zone contained ureteric bud / collecting duct tubules and also straight tubules of nephrons’ loops of Henlé. The new culture system therefore has the substantial advantage, beyond economy with reagents, that it shows improved renal development.

In summary the new strategy of culturing kidney rudiments allows a much more controllable, visible and economical scenario than the conventionally applied procedures and will provide new approaches for organ culture in general.

![Figure 15. 3D outline of the low-volume culture method.](image)

The central reaction chamber is formed by a silicon ring attached to a glass coverslip, which contains the culture medium and the organ or tissue units fixed by the surface tension in the middle. The whole set up is placed in a Petri dish filled up with fluid for stabilising humidity within the set up.

4.1.1 Additional evidences (to Appendix A) for stress reduction of kidney rudiments cultured in the novel system than those grown in conventional organ culture.

By comparing the different culture systems, the relative gene expression of Timp1 (tissue inhibitor of metalloproteinase 1) and its antagonist Mmp9 (matrix metalloproteinase-9) were measured by qRT-PCR during a twelve day cultivation period. This confirmed the results from the cytokine arrays (Appendix A), where the medium from
the low volume glass culture system showed lower concentrations amounts of Timp1, which is an cytokine secreted by stressed tissues [145]. Filter cultured kidneys showed a massive over-expression of Timp1 and Mmp9 in comparison to kidneys cultured on glass (Fig. 16).

It seemed that more stress is acting on kidneys cultured under conventional conditions, which is eventually responsible for the up-regulation of Timp1. Timp1 is a key player in the constant turn-over of the ECM. Together with its antagonist, Mmp9, these proteins are responsible for rebuilding and growing the ECM in the tissue [146].

Reasons for this over-expression of ECM reactive proteins of conventional cultured kidneys on the one hand and important marker genes for development of kidney cultured under novel conditions on the other hand, could be the surface material or different acting surface tension of the filter culture set-up.

It is already known that different polymer surfaces like polystyrene or polycarbonate can have strong, different impacts on cell behaviour compared to glass as a cell and tissue culture surface [147–149].

Figure 16. Relative, comparative Expression of Timp-1 and Mmp-9.

Total RNA was extracted from nine embryonic kidneys cultured for 0, 2, 4, 6, 8, 10, 12 days and a kidney from an adult NRMI strain mouse using RNeasy silica columns (Quiagen), according to the manufacturer’s instructions. 50ng of total RNA from each sample was reverse transcribed including a digestion step for genomic DNA (QuantiTect RT, Quiagen). The expression levels of the target genes were quantified by RT-PCR using the SYBR Green Master Mix (Quiagen) and validated primers for Timp-1 and Mmp-9 (QuantiTect Primer Assay, Quiagen). The relative expression of the target genes was calculated based on the individual CT values, which were normalized to that of Gapdh mouse housekeeping gene applying the 2-ΔΔCT method and assuming 100% PCR efficiency after [150].
4. Results and Discussion

4.1.2 Additional evidences (to Appendix A) for corticomedullary zonation and improved development of kidney rudiments cultured in the novel system for a period of 12 days

Additionally to results from microscopy, where embryonic kidneys showed corticomedullary zonation when cultured in our novel system, the relative expression of important marker genes [151] during *in vitro* kidney development for the first 12 days was examined by qRT-PCR. Mesenchymal (Pax-2, K-cadherin and NCAM-1) genes were expressed, but between glass and filter cultured rudiments no distinct differences in expression were observed. The expression of ureteric bud markers (ret and Hoxb7) shows a light increase of expression in favour to the glass cultured kidneys. To determine the potential of improved differentiation within embryonic kidneys cultured in our novel system, the relative expression of basic markers for terminally differentiated epithelia in glomeruli (podocytes; CD2-Ap, Podoplanin and Podocalyxin), proximal tubules (Aqp1, Clc5 and Sglt1), loop of Henle (Bmn1 and Nkcc2), loop of Henle or distal tubules (Clck2 and polycystin 2) and distal tubules (ENaC and polycystin 1) were investigated and compared with those cultured in the conventional way. Indicating an improved development, almost all relevant markers [151] were expressed more strongly in kidneys cultured with the novel system especially at later time points of cultivation (Fig. 17). Therefore the novel system with glass surface seemed to be more suitable for any long term organ culture experiments. Interestingly, after about four days a decline of expression of the majority of marker genes of kidneys, which were grown on filter, was observable. Maybe this is a result of the reduced culture abilities of the conventional system.

![Comparative Expression of Genetic Markers for Mesenchym](image1.png)

![Comparative Expression of Genetic Markers for Ureteric Bud](image2.png)
4. Results and Discussion

Figure 17. Relative, comparative expression of markers relevant for kidney development and function.

Total RNA was extracted from nine embryonic kidneys cultured for 0, 2, 4, 6, 8, 10, 12 days and a kidney from an adult NRMI strain mouse using RNeasy silica columns (Qiagen), according to the manufacturer’s instructions. 50ng of total RNA from each sample was reverse transcribed including a digestion step for genomic DNA (QuantiTect RT, Qiagen). The expression levels of the target genes were quantified by RT-PCR using the SYBR Green Master Mix (Qiagen) and validated primers (QuantiTect Primer Assay, Qiagen) for: Pax2; K-Cadherin (Cdh6); Ncam1; c-ret (Ret); Hoxb7; Cd2ap; Podoplanin (Gp38); Podocalyxin (Podxl); Gp38; Podxl; Podocalyxin (Podxl); Aqp1; Clc5 (Clcn5); Sglt1 (Slc5a1); Brn1 (Pou3f3); Nkcc2 (Slc12a1); Clck2 (Clcnkb); Polycystin2 (Kcnj2); ENaC (Scnn1g); Polycystin1 (Pkd1); Gapdh. The relative expression of the target genes was calculated based on the individual CT values, which were normalized to that of Gapdh mouse housekeeping gene applying the 2−ΔΔCT method and assuming 100% PCR efficiency after [150].
4.1.3 Additional evidences (to Appendix A) for the application of the glass based low volume culture system for other organs

Based on the promising results for kidney culture, the novel low volume glass culture system was utilized for culturing E 11.5 mouse embryonic lungs (Fig. 18). The experimental set up seems to be suitable for culturing other tissues/organs, because obviously well developed lungs were obtained on glass, although presumably without an improvement of development caused by the novel culture set-up but with an overall improved visibility compared to conventional culture approaches [152].

![Embryonic Mouse Lungs](image)

**Figure 18.** The low-volume glass culture technique can also be used with embryonic lungs.

Bright light pictures of embryonic murine lungs cultures on filter for 48 h (A), on glass for 48 h (B), on filter for 96 h (C) and on glass for 96 h (D).

These results showed that this novel approach is also feasible for embryonic lung cultivation. Thus, an interesting impact on further developments of other organ cultures was initiated.

Multiple opportunities are offered now by our novel glass cultivation assay for applications: Apart from all kinds of developmental biology studies, the new culture system is predestined for tissue engineering with whole organs. Because of the small and
4. Results and Discussion

defined culture chamber, it provides more controllable conditions as the conventional used techniques, which are big advantages in respect of the reproducibility of such a sensitive experiment as the organ culture. Therefore it is obvious that our glass-surface based culture technique offers new strategies in observing living, growing organs with spectroscopic methods as for example high-resolution bright light time lapse. Also long time culture of organs is uncomplicated and allows new spectacular insights in the development *in vitro*, additionally considering observations of zonal formation of cortex and medulla. Among other things, our procedure enables the simple usage of biomaterials, medications and/or other additives. Generally the variation of the environmental conditions, especially biophysical signals, can be easily varied and applied in our new culture chamber, to prove the influence on organ development. We even think that this new system enables the development of “organ array” approaches or similar systems.
4.2 ECM modulated early kidney development in organ culture

This section refers to the following publication (in preparation, see Appendix B):

David D. R. Sebinger, Andreas Ofenbauer, Petra Gruber, Susann Malik, Carsten Werner.
“ECM modulated early kidney development in embryonic organ culture.”
Manuscript in preparation for submission in *Biomaterials*. (Accepted in May 2013).

Referring to the introduction for tissue engineering and regenerative medicine (see Chapter 2.3), regenerative therapies require an in-depth understanding of development, maintenance, turn over and repair of the respective organ. Tissue specific cellular microenvironments massively influence cellular fate and function [41,153] by triggering different signalling pathways of cells [154–157] (see Chapter 2.2.2). These mechanisms ultimately determine assembly and function of tissues and organs [143,158,159]. In this context the ECM plays an indispensable role in the development and function of organs (see Chapter 2.2).

By utilizing conventional *in vitro* kidney organ culture, various regulators of renal development, including ECM components and growth factors, have been investigated by applying those to the culturing medium and observing the reaction and morphological changes of the organ. The ECM components hyaluronic acid [124], collagen I [143], endostatin [160], matrix metalloproteinases [161] as well as growth factors, including BMP-2, BMP-7 [162], pleiotrophin [163] and multiple fibroblast growth factors [164], were shown to exogenously influence branching morphogenesis and growth of explanted embryonic renal tissue. However, conventional organ culture setups in the cited studies varied due to the principle of culturing tissues and the way of applying additives of interest (Chapter 2.4.1). For example, different filter inserts and well plates are used, which results in varying amounts of media (400µl to ml range). This makes it difficult to compare the studies since nominal concentrations of applied additives referred to solutions additionally added to these conventional settings.

Thus, one objective behind this work was the analysis of these previously tested ECM components in a single study, by additionally deciphering the role of so far un-
tested ECM components in kidney development and function [165]. This resulted in a knowledge base, which should contribute to ECM-supported approaches in kidney regeneration therapies. To achieve this goal, the recently established embryonic organ culture system [166] (Chapter 4.1), suitable for embryonic kidney rudiments, was applied as a powerful technique for studying organ development that recapitulates many aspects of early in vivo development. In contrary to former studies, always the same culture parameters (e.g. media volume of 85µl, glass as surface, rudiments fixed by the surface tension …) were acting on the developing renal tissue, while offering excellent observability throughout all experiments. A broad range of ECM components and growth factors (Appendix B, Table 1), previously considered to be involved in kidney development and repair, including components that were reported to support nephron formation (BMP-7 [167], heparan sulphate [168,169]) and the development of glomeruli (collagen IV [170,171], laminin 8 [172] and laminin 10 [173]) and others that seemed to interfere with branching morphogenesis and tubule formation (endostatin [160], fibronectin [174,175], heparin [168], hyaluronic acid (HA) [124], laminin 1 [176], nephroduct [177] and pleiotrophin [163]), were tested using the novel culture approach. To reveal possible unknown interactions, some components apparently not involved in kidney development (although some of them are expressed in the kidney) were also included in the experiments (aggrecan, brevican, collagen I, meteorin, neurocan, osteopontin, tenascin-C and vitronectin).

ECM components were presented to the developing organ using two different forms of application: either covalently immobilized on pre-treated cover slips or added to the culture medium in different concentrations, drawing benefit from the low volume character of our organ culture system. Moreover immobilized adult kidney ECM extracts obtained by decellularization (Chapter 4.3, Appendix C) and reactive polymer films without any biomolecular components were included as additional reference systems.

Taken together, the impact of more than 20 different ECM components on kidney development was tested under different conditions in vitro. By using a quantification of characteristic structural parameters, such as the number of ureteric bud tips and developing nephrons as well as the size of the developing kidneys, effects of the differently presented ECM components were systematically analysed. The resulting 116 datasets were visually summarized in a colour coded array-like overview ( Appendix B,
Table 2). Generally this approach intended to broaden the spectrum of exogenous cues to include not only structure formation promoting factors (such as collagen I, laminin 1 or pleiotrophin), but also components that inhibit development (for example BMP-7, heparin or meteorin). Collectively, these substances can become utilized in directing the formation of complex tissue structures. Moreover, the usage of ambivalent constituents which affected kidney organogenesis in dependence on the mode of application, like Collagen IV, HA or Vitronectin are expected to become of particular interest in the engineering of complex kidney structures such as glomeruli or whole nephrons. The identified properties of the biomaterials applied in our approach should finally lay the fundament to develop new innovative treatments for injured kidney.
4.3 Establishing and evaluating decellularization techniques to isolate whole kidney ECMs from adult murine kidneys.

This section refers to the following publication (see Appendix C):

Andreas Ofenbauer, David D. R. Sebinger, Marina Prewitz, Petra Gruber, Carsten Werner.

“The dewaxed ECM: An easy method to analyze cell behavior on decellularized extracellular matrices.”

Accepted for publication in *Journal of Tissue Engineering and Regenerative Medicine* in October 2012.

As discussed in the Introduction, tissue decellularization is a way of generating acellular ECMs. As they are derived from a natural origin, they are able to resemble tissue specific microenvironments and composition. This fact was the primary motivation to investigate in the development of new decellularization protocols as a further source for exogenous modulation of developing tissue or cells in general.

The small size of murine kidneys allows decellularization based on the principles of diffusion and agitation in favour of utilizing a perfusion device, which would actively pump decellularizing agents through the organ. Without the need of designing and applying a perfusion device, more time could be invested in testing different chemicals and develop decellularization protocols which led to distinguishable ECMs with distinct characteristics. The developed protocols included:

1. A hypotonic solution, which should lyse cell via osmosis [95].
2. Sodium dodecyl sulfate (SDS), which is very effective in removing nuclear remnants and cytoplasmatic proteins, but causes damage to the ECM ultrastructure and may remove ECM components such as GAGs, growth factors and collagen [100,178].
3. A polyethylene glycol solution (PEG, Mw=1000), which was analyzed as a potential decellularization agent for the first time on kidneys [179,180].

Three developed protocols were analysed histologically (H&E, Alcian Blue) and immunohistologically (laminin, hyaluronic acid). Including a PEG 1000 step before SDS treatment resulted in a better matrix composition, thus the use of PEG 1000 may have the potential to stabilize ECM structures during decellularization processes.
4. Results and Discussion

4.4 The ability of whole decellularized ECM constructs to influence murine embryonic stem cell differentiation and renal tissue behaviour in a new straightforward approach.

This section refers to the following publications (see Appendix B and Appendix C):

David D. R. Sebinger, Andreas Ofenbauer, Petra Gruber, Susann Malik, Carsten Werner.
“ECM modulated early kidney development in embryonic organ culture.”
Manuscript in preparation for submission in Biomaterials. (Accepted in May 2013.)

Andreas Ofenbauer, David D. R. Sebinger, Marina Prewitz, Petra Gruber, Carsten Werner.
“The dewaxed ECM: An easy method to analyze cell behavior on decellularized extracellular matrices.”
Accepted for publication in Journal of Tissue Engineering and Regenerative Medicine in October 2012.

Acellular matrices derived from the decellularization protocols discussed in the last chapter were tested for their potential to modulate tissue and cell behavior. The newly developed embryonic kidney culture system allowed the application of binding shredded ECM covalently to the glass surface, which served as substrate for embryonic kidney rudiments. In doing so it could be shown that the applied matrix had a positive influence on kidney size, branching and nephron formation after four days of culture.

Additionally the ECMs were tested for their potential to influence stem cell differentiation by combining the use of paraffin slices and two-dimensional cell culture. It could be shown that slices of formerly paraffin-embedded decellularized ECM can provide signals to influence differentiation of mESCs. This newly developed approach (decellularization in agitating tubes, paraffin embedding and sectioning, applying de-paraffinized matrices in cell culture) has the potential to be used as a quick and easy test for decellularized ECMs, derived according to different protocols, on a larger scale. Many protocols can be tested in parallel, without the necessity of having multiple perfusion systems or bioreactors.
5 Summary and Outlook

5.1 Summary

The aim of this work was the search for naturally derived biomaterials, which have the potential to be used for renal tissue engineering and regeneration. The focus was thereby on components of the extracellular matrix, which are known to act as important regulators for stem and progenitor cell fate within the tissue.

Towards this goal, four complementary studies were carried out to address the following issues:

1. The development of a platform to test the influence of exogenous signals on renal structure formation.
2. The application of a broad range of ECM components and growth factors to investigate their impact on early kidney development.
3. The isolation of whole kidney ECMs from adult murine organs by different chemical treatments to decellularize the tissue.
4. The investigation of mouse embryonic stem cell differentiation and renal tissue behaviour on whole kidney extracellular matrices.

The first task was successfully accomplished by the initial project, whereby a novel method for culturing embryonic tissue in vitro was established (Chapter 4.1, Appendix A). Using an easy set up composed of a silicon ring and a glass cover slip forming the culture chamber, isolated embryonic kidney rudiments are able to be cultured for several days. The new technique allows the growing of embryonic renal tissue in very low volumes of medium (around 85µl), which makes it ideal for testing additives. Because kidneys are cultured directly on glass, the observability is considerably improved. Ad-
ditionally, rudiments grow larger than in conventional culture and develop a clear anatomical cortico-medullary zonation with extended loops of Henle. In particular, this novel culture set up provides an ideal opportunity to investigate renal development and structure formation.

The second task was conducted by studying the role of ECM constituents on renal structure formation through the use of the novel low volume tissue culture system (Chapter 4.2, Appendix B). More than 20 different ECM components were tested for their impact on kidney development under 116 different culture conditions, including different concentrations of ECM, either bound to the substrate or dissolved in the culture medium. Aside from that, organ culture was similarly performed on immobilized adult kidney ECM extracts and on reactive polymer films, without any additionally added biomolecular components. As a result, ECM components and conditions were identified that exert either supportive or inhibitory effects on specific features associated with kidney development. ECM biopolymers were found to determine the fate of developing explants in a concentration and application dependent manner (bound to surface versus dissolved in medium). In particular, these exogenous factors provide an opportunity to direct and control renal tissue formation and development for a wide range of applications including basic research as well as bioengineering approaches up to clinical use.

In the third study, new strategies for decellularization of whole murine adult kidneys were explored (Chapter 4.3, Appendix C). The obtained whole matrices were analysed for their degree of decellularization and their ECM composition, using histological and immunohistological methods. Three different protocols using different decellularization solutions were developed, which showed distinguishable micro-structures of the resulting acellular matrices.

Based on the third task, within the scope of the fourth project, the potential of the resulting whole renal matrices to modulate stem cell differentiation (Chapter 4.4, Appendix C) was tested in a new straightforward approach. Decellularized matrices were paraffin embedded, sectioned and used as ES cell substrate after being deparaffinized.
Beside analysing cell attachment by counting cell numbers after 4 days of culture, gene expression levels of two genes associated with renal development (Pax2 and Pou3f3) were determined using quantitative Real-Time PCR (qRT PCR) after 13 days of culture. The study was able to show a dependence between the preparation of decellularized ECM and the biological response of ES cells to such scaffolds. Generally these significant results proved the capability of acellular ECM, generated by decellularization of adult kidneys, to modulate stem cell and tissue fate. Moreover, nephrogenesis and outgrowth of cultured embryonic kidney rudiments was positively affected when exposed to processed decellularized adult kidney ECM extracts (Chapter 4.2, Appendix B).

In general, the following conclusions can be drawn from the tasks performed within this thesis:

- Embryonic tissue can be cultivated in a low volume of culture medium on modifiable planar surfaces, fixed by the surface tension and showing organotypic development.
- Exogenous signals can be easily applied and their impact can be simply analysed using the recently introduced tissue culture system.
- ECM components and conditions that exert either supportive, inhibitory or ambivalent effects on specific features associated with kidney development can be identified.
- Whole extra cellular matrices of adult kidney can be easily obtained by decellularization.
- Agents used for decellularization can determine the composition and architecture of the remaining ECM extract.
- Decellularized ECM can impact behaviour of reseeded cells and tissue.

Together these results prove the applicability and versatility of two different novel techniques, which provide several advantages and new usage possibilities compared to conventional systems (Chapter 2.4.1, Chapter 2.3.2.1). The tissue culture system allows the easy application of exogenous signals, while the decellularized ECM slide
system allows a straightforward evaluation of decellularization efficiency. Both approaches can be used to investigate and modulate behaviour of biological systems and represent novel interesting tools for tissue engineering.

5.2 Outlook

Although the results gained within this thesis are promising, they also demonstrate important areas where further research is required in order to generate functional renal tissue for therapeutic applications. To reach this goal, developmental processes and the formation of tissue have to be understood.

Organ culture is thereby a powerful technique which enables studying these processes in vitro. Moreover it serves as an important tool in bioengineering experiments. Since the newly established organ culture system is adequate to culture embryonic kidney and lung rudiments (chapter 4.3.1), this method may have potential to be applied to other embryonic or even adult tissues. In first preliminary trials a broad variety of mouse embryonic tissues (E 11.5; eyes with lachrymal gland, brain, nose placode, jaws, tongue with salivary glands, ear, thyroid, heart, lung, liver, stomach with pancreas, intestines, testis and extremities; Fig. 19) were tested for culturing. All mentioned embryonic tissues could be cultured with this innovative technique for a period of at least four days. For example, in vitro cultured hearts were beating for at least five days, limb buds (extremities) were forming “fingers” with visible limb skeleton, brain tissues were starting to fold and pancreatic tissue was starting to elongate. The opportunities offered by the novel system could lead to novel insights in organ development and new approaches for tissue engineering.
Figure 19. Examples for cultured embryonic tissues and organs by using the new tissue and organ culture technique. This figure demonstrates a selection of embryonic organs and tissues (bright light images), which were cultivated for at least 4 days by the novel culture set up. The easy handling and the broad field of application implies the possibility to develop an automatic organ array system, which is based on this culture concept.

Because of its defined culture chamber and functionalized planar and transparent carrier as surface, the established set up is well suited to investigate tissue formation under various different stimuli and constraints in situ. For instance it is predestined for manipulating whole explants in gene silencing experiments (RNAi) or testing expensive drugs on early organ development.

The influence of a large set of either surface-immobilized or soluble ECM molecules and growth factors on renal tissue were analysed. This resulted in a collection of manifold phenotypes (reported at Chapter 4.2, Appendix B), but most of the mechanisms and pathways by which the additives are able to act on tissue formation remain unclear. In order to apply these factors to engineer complex kidney structures, like glomeruli or whole nephrons, their modes of action would have to be elucidated.

ECM components and conditions that exerted either promotive or inhibitory effects on specific features associated with kidney development were identified. For example collagen I, laminin 1, decellularized whole kidney ECM, pleiotrophin and neph-
ronectin all fostered the formation of renal structure. These factors could be tested in *in vivo* kidney disease model systems to push research towards clinical applications. In this context also components which act in a concentration (e.g. collagen IV, vitronectin) or molecular weight dependent manner (as shown for hyaluronic acid) should be considered, which could be applied as a gradient system. Also apparent inhibitors of development such as BMP-7, laminin 8, heparin or meteorin might be used to direct the formation of complex nephronal structures like glomeruli or whole nephrons. Additionally, other ECM components and growth factors, like collagen III and V, flexilin, nidogen protein family members and osteonectin could be tested to intensify the existing knowledge of their role in renal development.

To accelerate the screening process for candidates suitable for regenerative applications, further modifications of the organ culture technique could be undertaken. The principle behind the set up could be translated into an array like system, which would allow automatized analysis of the experiments (Fig. 19).

It has been demonstrated that different forms of applying the same additive (solved in medium versus surface bound) can result in divergent effects. This phenomenon may help to analyse how the developing loops of Henle (nephronal substructure, Chapter 2.1) are directed, as they seem to grow in the direction of surface-bound aligned collagen I fibrils [181]. In experiments where embryonic kidney rudiments (E11.5) were placed on aligned collagen strips and cultured for a period of 17 days, a majority of the already elongated loops of Henle were oriented along the aligned collagen I fibrils (observed under bright light and fluorescent microscopy after fixation and immunostaining, Fig. 20). These first results might indicate an involvement of collagen I in the orientation of developing nephronal structures, which could help to design a tool for replacing single injured nephrons *in vivo*. 
5. Summary and Outlook

Figure 20. Example of a kidney rudiment cultured for 17 days on an aligned collagen I fibril strip.
Under (A) an immunostaining for collagen I of an adult kidney section is shown. Bundles of Collagen I seem to occur along the nephronal lumina. (B) demonstrates immobilized, aligned collagen I fibrils generated by the novel culture method [181]. Pictures were kindly provided by Dr. Babette Lanfer. When cultivated on aligned collagen I fibrils developing nephrons develop along the fibrils direction (C).

With the objective to broaden the possibilities of applying promising biomolecules to renal tissue in vitro and in vivo, star-PEG-heparin hydrogels [182] could serve as innovative and multiple modifiable scaffolds. Especially with regard to future clinical applications, biofunctional hydrogels could be used as an ideal carrier system to integrate and release promising bioactive (ECM) molecules and also to combine those with specific cells or tissue. First experiments with embryonic renal tissue and stem cells have shown interesting results, as they seem to be kept in an undifferentiated state (Fig. 21, 22). This may open exciting opportunities for tissue engineering. Surprisingly, this effect also occurs when cells or tissue are not in direct physical contact with the hydrogel, which is filled in insert (Fig. 22) or applied as a ring (not shown).
Figure 21. Example of embryonic organs cultured on star-PEG-heparin hydrogels.

Star-PEG-heparin hydrogels (picture in the upper right corner kindly provided by Dr. Mikhail Tsurkan) seem to keep embryonic kidneys undifferentiated. Control kidneys (green boxes) develop normally during the quoted culture period. Organs on gel seem to stay in an undifferentiated state (red box) with a similar appearance as of freshly isolated organ rudiments (control, 0 day). After isolating the organ from the gel and culturing under normal conditions, the organ starts to develop normally (orange box), comparable to the 2 days control kidney (light green box).
These findings bring us back to the initial idea of this project, namely the ability of the environment to instruct cellular differentiation. Many aspects have to be considered to design artificial environments that orchestrate stem cell or tissue behavior. In contrast to apply single ECM components, decellularized ECMs hold the advantage of...
comprising the entire framework of matrix factors in the right ratio, at least assuming an optimal decellularization process. The establishment of decellularized matrices and their detailed characterisation is a possible starting point for manifold investigations. In this perspective especially the identification of specific bioreactive features within their complex composition is an important task. By mimicking these natural properties, intelligent artificial scaffolds could be designed that support tissue regeneration.
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7 Appendices

7.1 Appendix A: A Novel, Low-Volume Method for Organ Culture of Embryonic Kidneys That Allows Development of Cortico-Medullary Anatomical Organization

The following paper was published in *PloS One* in May 2010:

**A Novel, Low-Volume Method for Organ Culture of Embryonic Kidneys That Allows Development of Cortico-Medullary Anatomical Organization**

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**Abstract**

Here, we present a novel method for culturing kidneys in low volumes of medium that offers more organotypic development compared to conventional methods. Organ culture is a powerful technique for studying renal development. It recapitulates many aspects of early development very well, but the established techniques have some disadvantages: in particular, they require relatively large volumes (1–3 mls) of culture medium, which can make high-throughput screens expensive, they require porous (filter) substrates which are difficult to modify chemically, and the organs produced do not achieve good cortico-medullary zonation. Here, we present a technique of growing kidney rudiments in very low volumes of medium–around 85 microliters–using silicone chambers. In this system, kidneys grow directly on glass, grow larger than in conventional culture and develop a clear anatomical cortico-medullary zonation with extended loops of Henle.
Introduction

This paper describes a method for organ culture of developing kidneys that improves on conventional methods in terms of both economy and organotypic realism. Organ culture of embryonic kidney rudiments has been established for almost ninety years. The earliest methods suspended renal rudiments in Carrell flasks in semi-solid media, such as clotted owl plasma [1–6]. The clot system, which yielded good though variable development, was replaced in the 1960s by a simpler system in which kidney rudiments are grown on filters supported on a metal grid at a gas-medium interface [7–9]. This is the system generally used to this day, with the occasional variation of using filter inserts designed for multiwell plates, still at a gas-medium interface, in place of pieces of filter supported on steel grids. The filter supports are used because culture in simple liquid hanging drops, which works well for other organs such as salivary glands, does not work well for kidneys and their development in such systems is very poor.

Organ culture of kidney rudiments has been, and continues to be, very valuable in the study of renal development [3]; [10–13]. The system has been used to study the dynamics of normal development by time-lapse photography, initially by brightfield microscopy and more recently with fluorescent reporter proteins [8]; [14]. It has been used to study the developmental functions of specific molecules by experimental addition of exogenous growth factors [15], function-blocking antibodies [16], vitamins [17], oligosaccharides [18], drugs [19], antisense oligonucleotides [20] and short interfering RNAs [21]; [22]. It has also been used to test the cell autonomy of mutations by production of chimaeric recombinant kidneys [23].

Useful as it is, the established culture system suffers from a number of limitations. It requires volumes of media of the order of millilitres, which limits its use in high-throughput screens that require high concentrations of expensive reagents such as siRNAs, and it requires supporting filters that are significantly harder to modify with custom substrates than is glass. Also, while cultured kidneys show good development of the branched collecting duct system and of nephrons to the S-shaped stage and beyond, including differentiation of specific regions such as proximal tubule, distal tubule etc, they do not show development of a distinct renal medulla into which Loops of Henle extend. In conventional culture, the loops of Henle do not form [24] while in culture systems that optimize the maturation of nephrons, such as those using hyalur-
onic acid, loops of Henle form but are arranged haphazardly rather than extending into the medulla [25].

In this paper, we describe a simple culture system that allows kidney rudiments to be cultured directly on glass coverslips in just 85 µl of medium. The development of these kidneys is superior to traditional methods when compared by any of the usual metrics (overall size, nephron number and the extent of ureteric bud branching) and they show correct cortico-medullary zonation. This new technique therefore offers considerable advantages, of economy and realism of development, over the established method.

Material & Methods

Organ culture

Organ rudiments were microdissected from E11.5 NMRI or CD1 mouse embryos; they were pooled and assigned randomly to control or experimental groups. For conventional culture, the rudiments were placed on 5 µm pore-size polycarbonate filters at the bottom of a well insert in a six well plate (Corning, Costar), or on top of a stainless steel Trowell grid in a 3.5 cm culture dish in kidney culture medium (KCM: Eagle's minimal essential medium with Earle's salts and non-essential amino acids (GIBCO), 10% foetal bovine serum (Biochrom/Biosera) and 1% penicillin/streptomycin (Sigma)). For some experiments, 1 nM TGF-β (Sigma T7039) or 100 ng/ml GDNF (Sigma G1777) were added.

Low-volume cultures used sterilized silicone rings (flexiPERM Cone shape A, Greiner BioOne), on 22×22 mm coverslips (Menzel Gläser, Germany), cleaned in 5:1:1 H₂O:H₂O₂:NH₄OH (10 min, 70°C), in tissue culture dishes (35×10 mm, Sarstedt/Greiner). Kidney rudiments were placed close to the middle of the circle; medium carried over in this pipetting operation was removed and replaced by the final KCM culture medium (70–200 µl), the complete enclosed area of the coverslip being wetted. The dish surrounding the silicone ring was filled with PBS containing 1% penicillin/streptomycin). All cultures were incubated at 5% CO2 at 37°C, medium being changed every 2 days.

For time lapse movies, cultures were placed in the incubation chamber of a Zeiss Axiovert 200 microscope and images were taken every 10 or 15 min. NIH ImageJ
1.37v. was used to create time lapse movies. Phase contrast images of living cultures were taken every 12 h using an Olympus IX50 microscope.

**Fixation and immuno/lectin fluorescence**

Kidney rudiments were fixed in methanol at −20°C, washed in PBS 10 min, then incubated in primary antibody in PBS, overnight at 4°C. Primary antibodies were anti-laminin (1:100 Sigma L9393), anti-calbindin D28k (1:100 Sigma C9848), anti-megalin (1:150, obtained from Thomas Wilnow), anti-human-Wilms' Tumour 1 (1:50, Dako M3561), anti-Pax-2 (1:100, Covance PRB-276P) and anti-E-cadherin (1:100, BD Transduction Laboratories 610181). Samples were washed for at least 30 min in PBS then incubated with appropriate secondary antibodies overnight at 4°C. The secondary antibodies used were: anti-rabbit IgG - TRITC (1:100, Sigma T6778), anti-mouse IgG–TRITC (1:100, Sigma T5393) and anti-mouse IgG - FITC (1:100, Sigma F6257). For antibody/lectin co-stainings, all solutions contained 1% milk powder in PBS and 10 ng/ml lectin from *Dolichos biflorus*-FITC (Sigma L9142) was included with the secondary antibody. Finally the samples were washed in PBS and mounted on slides.

**Cell proliferation and apoptosis detection assay**

BrdU (5-bromo-2-deoxy-uridine) was added to the medium of kidney rudiments in culture 4 hours before fixation to a final concentration of 100 µM. Detection was performed as described by [26] except that cell death detection master mix (*In Situ* Cell Death Detection Kit, TMR red, Roche 12156792910) was added along with primary antibodies. Samples were washed for 30 min in PBS and incubated in secondary antibodies overnight at 4°C. After a PBS wash, 1 µg/ml DAPI (Sigma) in PBS was added for 20 min. Finally the samples were washed in PBS and mounted on slides.

**Morphometric quantification**

Immunostained samples were examined on a confocal laser scanning microscope (TCS SP5, Leica Microsystems, Wetzlar, Germany). Serial 5-µm optical sections of each kidney were acquired. FITC, TRITC and DAPI emissions were acquired sequentially. Ureteric bud tips and nephrons were counted manually. Area was defined manually and measured using NIH ImageJ 1.37v (http://rsb.info.nih.gov/nih-image/).
**Measurement of cytokines**

Right and left kidneys of the same embryo were dissected and allocated randomly to either conventional culture or the new method. Supernatants conditioned from 0–2 d 2–4 d were collected and diluted in fresh medium to a standard total of 3 ml. Expression of 40 cytokines was assessed using R&D systems' mouse cytokine array panel according to the manufacturer's directions. Experiments were performed in triplicate.

**Surface tension measurements**

DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine; Sigma P6354) was added to and dispersed at 1 mg/ml in KCM by 30 min. of sonication on ice. Surface tension was determined using the pendant drop-method based on the Young-Laplace equation [27]. After calibrating the system (Contact Angle System OCA 30, DataPhysics Instruments, Germany) with degassed water, the surface tensions of KCM and KCM with DOPC were measured.

**Animals**

The animals from which tissue samples were obtained, bred and kept according to relevant UK Home Office guidance (http://scienceandresearch.homeoffice.gov.uk/animal-research/legislation/index.html) and the Animals (Scientific Procedures) Act, 1986, available from the same website: they were killed by trained technical staff according to a method listed in Schedule 1 of that Act. Ethical approval for keeping the animals and obtaining these tissue samples was approved by the University of Edinburgh's local ethics approvals process.

**Results**

We began this work with the aim of growing embryonic kidney rudiments on glass coverslips that could be coated easily with defined matrix components. To define a small culture area on the glass, we used FlexiPERM silicone rings, each with the approximate shape of a decapitated cone, the smallest end of which defined a 1 cm$^2$ (i.e. 5.6 mm radius) circle (Fig 1A). It was at once clear that kidney rudiments did not grow well when supplied with large volumes of media in this system. Smaller volumes, of 85, 90, 120 or 200 µl were therefore tried (volumes of 70 µl and less were found to be insufficient to wet the whole circle permanently). Development of the organ rudiments after four days in each condition was quantified by measuring rudiment area, number
of ureteric bud tips (2 at the time of isolation) and number of nephrons (0 at the time of isolation).

Control kidneys, cultured by the conventional Trowell method (Fig 1B) developed normally, showing a calbindin-positive ureteric bud that was well-branched (mean 23.4, $\sigma = 16$, $n = 49$) and many developing nephrons (mean 20.2, $\sigma = 16$, $n = 49$), identifiable by their shapes and by their laminin-rich basement membranes and absence of calbindin staining (Fig 2D, D'). In 200 $\mu$l medium, many times deeper than the height of the kidney, kidneys remained rounded and developed poorly (Fig 2C, C'), showing less than half the ureteric bud branches and nephrons of control kidneys grown on conventional Trowell filters. This was expected from previous reports (see Introduction). With lower volumes of medium, however (Fig 2A, A', B, B'), the extent of development improved (Fig 2E). In the optimum volume - 85 $\mu$l - kidney rudiments covered significantly more area (42%) than did filter-grown controls and they produced 46% more ureteric bud tips and 81% more nephrons. The variability between kidneys was also reduced (standard deviations in measurements of area, branch number and nephron number were all only about four fifths as large, as a proportion of the means to which they applied, as they were for filter-grown controls). Adding 500 $\mu$l of culture medium to kidney rudiments that had already been cultured in 85 $\mu$l for 1 day of culture caused the kidneys to round up and to cease developing well: they therefore require low volumes continuously, and not just to promote initial settling on the glass.

**Figure 1. Methods for embryonic kidney culture.**

(A) The low-volume culture method described in this paper, drawn from the side and photographed from above. (B) Conventional, high volume culture either on a membrane on a Trowell grid (above) or on the membrane at the bottom of a well insert (below). The blue in (A) depicts PBS, the red in all diagrams depicts culture medium, yellow in (A) symbolizes the silicone ring and dark grey the embryonic kidney rudiments. The numbered divisions on the ruler are centimetres.
Figure 2. Development of mouse kidney rudiments in conventional culture and on glass inside silicone rings. (A–C) show phase contrast views of kidney rudiments grown for 4 days in silicone rings on glass in 85 µl, 120 µl and 200 µl respectively, while (D) shows a kidney grown in the conventional system (on a filter on a Trowell grid: the 'noise' in the background is the filter). On glass, the lowest volume, 85 µl, shows the best development, resulting in a larger kidney than the conventional system. (A'–D') show kidneys grown in the same conditions as (A–D) but stained for basement membrane marker laminin (red) and the ureteric bud marker calbindin-D_{28k} (green). (E) shows a quantitative analysis of area, nephron and bud tip numbers for each of these culture conditions. Error bars depict standard errors of the mean and are derived from at least 49 kidneys in total, from six different runs of the experiment, each run using between 6 and 18 kidneys. Scale bars = 500 µm.

The nephrons and ureteric bud branches of kidneys grown in conventional organ culture showed patterns of gene expression similar to those observed in vivo, as has been described before [28–31]. The ureteric bud, for example, expressed calbindin D_{28K} (Fig 3A) and was divided into a stalk region that bound Dolichos biflorus lectin and a tip region that did not [32,33] (Fig 3C). The nephrons showed correctly-restricted expression of markers; for example, the most proximal ends of the nephrons differentiated into podocytes that expressed high levels of WT1 (Fig 3E: the weak expres-
sion of WT1 in condensing mesenchyme is normal and reflects an earlier role for that protein in nephron development [21]), the proximal tubules expressed megalin and the distal tubules and ureteric bud expressed E-cadherin (Fig 3G). Nephrons and ureteric buds of kidneys grown using our low volume culture system showed the same organotypic expression of segment marker genes. Ureteric buds were divided correctly into tip and stalk zones (Fig 3B, D), developing podocytes expressed WT1 (Fig 3F), proximal tubules expressed megalin and distal tubule expressed E-cadherin (Fig 3H). Rates of cell proliferation and apoptosis in nephrons, measured by BrdU incorporation and Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) respectively, were not significantly different in the two culture systems after two days (Fig 4).

Figure 3. Normal segmentation of nephrons grown on filters (A, C, E, G) and in low volumes on glass (B, D, F, H).

(A, B) show kidneys stained for calbindin, which stains the whole ureteric bud; (C, D) show kidneys stained for Dolichos biflorus agglutinin (DBA), which stains only the bud stalk—the 'missing' tips visible in A, B but not in C, D are marked with yellow stars; (E, F) show kidneys stained for the WT1, which is expressed strongly in crescents that consist of developing podocytes: in these images, staining for Pax2, expressed in bud, condensates and early nephrons, is used to reveal the general structure of the rudiment and to place the developing podocytes in their anatomical context: higher power views are shown in E′ and F′, revealing the nuclear location of WT1. (G, H) show higher power views of kidneys stained for the ureteric bud and distal tubule marker, E-cadherin, and the proximal tubule marker megalin; the expression of each of these markers is similar in both culture systems. Scale bars = 50 µm; the scale bar shown in F applies to A–F, that shown in F′ to E′ and F′, and that shown in H applies to G and H.
Kidney rudiments grown in the low volume system show typical responses to positive and negative morphogens

Conventional kidney organ culture has been used to identify various diffusible regulators of renal development, by applying suspected regulators to the medium and observing any changes to subsequent morphogenesis. We have tested kidneys growing in the low volume system for their response to two known modulators of morphogenesis, one positive and one negative. This had two purposes; (a) to verify the normality of development and (b) to check that the culture volume used is not so small that these regulators become quickly exhausted or inactivated by cellular secretions. The factors we used were TGF-β, a known inhibitor of ureteric bud branching [34] and GDNF, a known activator [35].
Exogenous TGF-β, applied at 1 nM for four days [36], had similar effects on the development of kidneys in conventional and low volume culture, although the inhibitory effects were a little less dramatic in the low volume method (Fig 5). Exogenous GDNF, added at a final concentration of 100 ng/ml again for four days [36] significantly increased branching morphogenesis in both systems so that more than a doubling of the amount of nephrons and ureteric bud branches was achieved.

Kidney rudiments develop cortico-medullary zonation when cultured in this system

Kidney rudiments developing in conventional organ culture show excellent recapitulation of in vivo development at a fine scale but one important large-scale aspect of development is typically lost, or at least seriously under-developed. In vivo, growth and extension of stalks of the ureteric bud and the Loops of Henle divides the kidney into
two broad zones, an outer cortex containing the glomeruli and ureteric bud tips, and an inner medulla that is dominated by straight tubules of the collecting duct system and the loops of Henle. This organization is critical to the urine-concentrating activity of the metanephros, so lack of a good culture model to allow the development of cortico-medullary differences to be studied is a serious limitation to the field. Long-term (10-day) culture in our low volume system resulted in a substantial increase in area of the organ rudiment and the formation of distinct cortical and medullary zones (Fig 6A). The forming glomeruli were restricted to the cortical zone and the medullary zone contained ureteric bud/collecting duct tubules and also loops of Henle (Fig 6B). The new culture system therefore has the substantial advantage, beyond economy with reagents, that it shows more anatomically realistic renal development.

Figure 6. Kidneys grown on glass develop organotypic features including cortico-medially zonation.

(A) Shows a time course of development to 240 h (10 d). From about 108 h (4.5 d), the kidney spreads out enough that it begins to divide into two zones, an outer medullary zone that features many nephrons and an inner medulla that contains more elongated tubules (mainly collecting ducts at this stage). By 240 h (10 d), this effect has become more marked. The last panel of (A) shows a kidney rudiment cultured on a filter for 240 h; the organ occupies less area and there is less evidence of corticomedullary zonation. The ‘grain’ in the photograph is an optical effect of the filter pores, which are beneath the kidney; their absence in bright field imaging is another advantage of the glass system.

(B) A high-power view of part of a 240 h kidney shows that nephrons (red) are arranged organotypically, with the glomerulus (‘Glom’) and proximal convoluted tube (‘PCT’) in the cortex and a loop of Henle (‘LoH’) extending down into the medulla, forming a tight hair-slide shape parallel to the collecting ducts (green). Green = calbindin, red = laminin.
Kidney rudiments cultured in the low volume system show less evidence for stress than those grown in conventional organ culture

Tissues subject to stress secrete specific cytokines that interact with cells of the immune system to initiate an inflammatory response [37]. These cytokines include tissue inhibitor of metalloproteinase I (TIMP1), monocyte chemotactic protein 1 (MCP1, also called JE), the neutrophil chemokine CXCL1 (also called KC) and interferon gamma (IFNγ) [38–41]. Release of these proteins into medium can therefore be used as an indicator for how stressed cells are in culture [42–46].

Medium from kidneys cultured conventionally in 3 mls of medium contained significant amounts of TIMP1 and MCP1 and smaller but still detectable amounts of CXCL1 (not shown) and IFNγ (Fig 7). Medium from the low volume glass culture system showed lower amounts of TIMP1 and less or equal of the other pro-inflammatory cytokines, measured over either first 48 h or the subsequent 48 h of culture (Fig 7), though the effects were stronger by the second 48 h. These results suggest that the cells in this system were significantly less stressed than those in conventional culture.

Figure 7. Kidneys grown in the low-volume glass system show less evidence of stress.
In (A) the samples labelled ‘short’ are of medium conditioned from day 0–2 and those labelled ‘long’ are from days 2–4. Spots marked ‘C’ are positive controls (to verify the detection kit). The other spots, of TIMP1, KC, JE and IFNγ represent these markers of cellular stress (see main text). (B) shows, quantitatively, the results of these molecules on the arrays, as means of three runs. The Y axis is % relative to the signal provided by the manufacturers’ positive control. The right-most part of the graph, showing TIMP (also shown as a spot in A), is plotted to a different y axis. Although the data for each of the molecules, each individual array spot being derived from 3 pooled kidneys and 3 independent arrays being run for each condition, suggests a decrease on glass, the variation in measurement is so large that no one individual molecule has a change that can be regarded as significant at the conventional p≤0.05 (most have p≈0.1). As all molecules are markers for the same physiological state (cellular stress), however, their measurements can be pooled for a one-tailed, paired Student’s t-test that uses all 15 experiment-control measurement pairs together (the 15 pairs coming from 5 target molecules x 3 runs each). Viewed this way, the data show that glass culture does produce a significant reduction in production of these stress proteins (p = 0.05). Elements on the spot arrays not shown in this graph showed no significant differences.
Kidney rudiments may grow better in low culture volumes because of a requirement for effects of surface tension

Something that the conventional high-volume filter method of culture has in common with the low-volume method, but not with the much less effective high-volume methods (including our use of 120 and 200 µl instead of 85 µl the silicone rings), is that the organ rudiment is at the gas-medium interface. There are two reasons that this might be important. One is improved access to oxygen, although this seems unlikely since kidneys grow very well in conventional culture in low (3%) oxygen systems [47]. The other is the effect of the surface itself. Molecules of liquid are mutually attractive. Those in the bulk liquid are surrounded by other molecules on all sides so therefore experience no directional force. Those at a gas-liquid interface surface still have other attractive molecules to the side of them and below them but not above them, so they experience a net average force pulling them back into the bulk liquid. For this reason, surfaces are drawn in until balanced by compressive forces in the bulk liquid, and liquids minimize their free surface area. Actions that would expand the surface area of a liquid work against this tendency to minimize surface are therefore met with an opposing force, usually called surface tension. Where the liquid over a culture substrate is shallow compared to the height of the organ culture itself, the raised profile of the organ forces the surface of the liquid to be larger than it would otherwise be and the organ will experience a flattening force due to surface tension.

It is therefore possible that the flattening effect of surface tension is important to renal development \textit{in vitro}. We tested this idea by using a surfactant to reduce surface tension in the medium to see if this mimicked the effect of a high volume of medium. The surfactant used, 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), reduces surface tension by forming phospholipid bilayers (rather than the monolayers formed by typical lab detergents) at the air-medium interface [48]. Direct measurements of surface tension in untreated and DOPC treated medium confirmed the surface tension-lowering effects of DOPC (Fig 8A).

The presence of DOPC had no significant effect on the (already poor) development of kidneys under large volumes of medium, as would be expected since these should be relatively free from surface effects (Fig 8B, C). In the low-volume (85 µl) system, though, the presence of DOPC significantly reduced the renal area, the number of ureteric bud tips formed and the number of nephrons formed. This is compatible with the hypothesis that surface tension is important.
Discussion

In this report, we have described an improved technique for organ culture of mouse metanephric organ rudiments that is very economical of medium, shows quantitatively better development and also shows cortico-medullary zonation absent in the conventional method. It also uses a transparent substrate, useful for live imaging, that can also be coated easily with experimental custom substrates.

Conventional culture and the low-volume method described here both have the kidney supported at the air-medium interface, with only a thin film of medium covering it; larger volumes of medium, even in exactly the same system, support development significantly less well. There are two obvious a priori hypotheses for the importance of the surface: access to oxygen, or the physical compression effect of surface tension. Previous reports of normal development of cultured kidneys in just 3–5% oxygen [47,49] make the first of these unlikely. We have shown that lowering the surface tension of the medium using a surfactant results in quantitatively poorer development. This supports the surface tension hypothesis but does not prove it, for the lipid bilayer formed at the surface by the surfactant may also affect the diffusion of gases. To form-

Figure 8. Reduction of surface tension influences development of cultured kidney rudiments.

In (A) the reduction of surface tension by the addition of the surfactant DOPC to the medium is demonstrated; data represent means of a minimum of 18 measurements. (B and C) show the influence of the surfactant on ureteric bud branching and amount of nephrons, respectively, if compared to the control; each bar represents data from a minimum of 17 kidneys. The asterisk indicates significant difference (p = 3.4×10^-6 for branches and 3.3×10^-4 for nephrons) by a two-tailed Student's t-test assuming unequal variances.
ally prove the biophysical hypothesis that kidneys do better if gently squashed, it
would be necessary to vary physical forces only with no effect on chemistry [50]. A
possible, though technically difficult, way to achieve this might be to culture kidney
rudiments on glass, under large volumes of medium, in a centrifuge.

As well as producing quantitatively better development, the culture system showed
a quantitatively strongly reduced expression of markers of cellular stress. As well as ar-
guing for the superiority of the new culture system, this finding highlights a potential
but rarely measured problem in organ culture systems; the cells involved might actu-
ally be under considerable stress. Some of the molecules they produce as a result of
this (and that we measured), such as IFN-γ, will probably only be bioactive in the con-
text of an animal with an immune system but others, such as MMP9 (matrix metallo-
proteinase-9) and its antagonist Timp-1 (tissue inhibitor of metalloproteinase 1), are
important in matrix turnover during development [51] and may therefore lead to cul-
ture results not reflecting those obtained in vivo. In the kidney itself, there are instances
of this: endogenously-produced HGF is needed for collecting duct branching in cul-
ture, for example, but HGF−/− mice have normal kidneys [52]. It may be that one ex-
planation of why in vivo and in vitro results do not always agree is a reflection of cellu-
lar stress, with measureable induction of protein expression, rather than the often-as-
sumed explanation that the intact body provides some diffusible factor from elsewhere,
that can perform the same function as the molecule under study and therefore creates
redundancy in vivo, but not in vitro.

In summary, we have presented a culture method that extends the range of questions
that can be addressed in culture to include those connected to corticomedullary zona-
tion and loop of Henle formation, and have made culture conditions more economical
of medium supplements. As well as making developmental processes more easily vis-
ible, this method has the potential to significantly reduce animal use by allowing the
control of these aspects of kidney development to be studied in vitro, for example by
using siRNAs, rather than by extensive breeding of genetically-modified mice.

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Kerstin Menzer, Anke Münch-Wuttke, Patricia Murray, Lars Renner, Philipp Seib,
Thomas Wilnow and Ralph Zimmermann.
References


7.2 Appendix B: ECM modulated early kidney development in embryonic organ culture

The following publication manuscript is in preparation to be submitted to the journal *Biomaterials* (accepted for publication in May 2013):

**ECM modulated early kidney development in embryonic organ culture**

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**Abstract**

The use of exogenous signals is gaining importance in renal regenerative therapies. We wanted to explore the role of extracellular matrix (ECM) constituents on renal structure formation during renal organogenesis. We used a recently established organ culture set-up to expose embryonic kidney rudiments directly to a large set of surface-immobilized or soluble ECM molecules and growth factors. Organ culture was also performed on immobilized adult kidney ECM extracts and on reactive polymer films without any biomolecular components. The applied conditions resulted in distinct differences of organ phenotypes, underlining the multifaceted role of exogenous signals during kidney development. Specific ECM components, such as collagen I and laminin, supported nephronal and tubular structure formation of the developing organ. ECM biopolymers, e.g. hyaluronic acid, were found to determine the fate of developing explants in a concentration- and molecular weight-dependent manner. The organ culture system used was an effective and robust means to identify exogenous signals that direct kidney development. This system can provide valuable insight for future regenerative therapies of kidney diseases.
Introduction
Prospects for the treatment of severely injured kidneys, kidney failure or chronic kidney disease are currently limited. New therapies to recover renal function are an important clinical objective. A variety of cell-based therapies [1–3], tissue engineering strategies [4–6] and other regenerative medical technologies [7–9] are currently being explored. Most of these approaches rely on the use of stem and progenitor cells. Regenerative therapies require an in-depth understanding of development, maintenance, turn over and repair of the respective organ. Organ and tissue culture techniques are increasingly receiving attention and are used to cultivate material from both embryonic and adult origin [10–13]. In contrast to conventional cell culture, were only one or few cell types are cultured simultaneously, these approaches can provide additional insights into the development of organs and tissues. As a specific cluster of different cells with their specialised ECM is explanted for the use in organ or tissue culture, the in vivo situation is resembled for the most part while these approaches are still in vitro methods with the advantage of easy experimental accessibility.

Tissue-specific cellular microenvironments influence direct cellular fate and function [14,15]. Physical and (bio)chemical signals trigger different signalling pathways of cells [16–19]. These processes ultimately determine the assembly and function of tissues and organs [13,20,21]. Moreover, ECM is indispensable for the maintenance of organ functions which are critically dependent on functional basal lamina layers, e.g. gas exchange in the lung and the glomerular filtration of the kidney.

Conventional in vitro kidney organ culture based on the filter-grid method after Trowell et al. [22] has been used to test various regulators of renal development, including ECM components and growth factors. Growth factors were added to the culture media and morphological changes of the organ were observed. The ECM components hyaluronic acid [23], collagen I [13], endostatin [24], matrix metalloproteinases [25] and growth factors including BMP-2, BMP-7 [26], pleiotrophin [27] and fibroblast growth factors [28], were all shown to influence branching morphogenesis and growth of explanted embryonic renal tissue. However, organ culture set-ups vary within studies. For example, different filter inserts and well plates are used, which results in varying amounts of media (400µl to ml range). This makes it difficult to compare the studies since nominal concentrations of applied additives referred to solutions additionally added to these conventional settings (see discussion). We wanted to analyse these previously tested ECM components in a single
study and decipher the role of untested ECM components in kidney development. Our goal was to create a knowledge base for possible ECM-supported approaches in kidney tissue engineering and regeneration therapies. We used a recently established *in vitro* embryonic organ culture system [29] suitable for embryonic kidney rudiments (Fig. 1A and B) to study organ development. This system recapitulates many aspects of early *in vivo* development, like ureteric bud branching, nephron formation and clear anatomical cortico-medullary zonation combined with improved observability because of the transparent culture surface. The method uses a defined low media volume of 85µl [29], as opposed to a ml range used in conventional embryonic kidney organ culture [22]. It allows manipulation of the cultured tissue, e.g. gene silencing, micro-dissecting and drug application as well as the chemical modification of the planar culture substrate, which is in our case glass in contrast to filter or metal grip of the conventional culture set ups [22].

![Fig. 1](image)

*Fig. 1.* (A) Visualization of the low-volume culture method used for testing ECM components and other bioactive molecules. (B) Cross-section of the culture set-up. (C) Detail of the applied ECM components and other bioactive molecules as a covalently bound substrate with layer composition on the glass surface. (D) The ECM components and other bioactive molecules added as a solute in culture media.

We have tested a broad range of ECM components and growth factors, previously considered to be involved in kidney development and repair (Table 1). These
components have been reported to support nephron formation (BMP-7 [30], heparan sulphate [31,32]) and the development of glomeruli (collagen IV [33,34], laminin 8 [35,36] and laminin 10 [37,38]). Other factors that we tested have been found to interfere with branching morphogenesis and tubule formation (fibronectin [39,40], heparin [31,41], hyaluronic acid [HA] [23], laminin 1 [42], nephronectin [43] and pleiotrophin [27]). In addition, we included components which were so far not found to be involved in or essential for kidney development, e.g. aggrecan, brevican, collagen I [44], meteorin, neurocan, osteopontin [45,46], tenasin-C [47] and vitronectin [48,49]. The ECM components were presented to the developing organ either covalently immobilized on coverslips (Fig. 1C) or added to the culture medium at different concentrations (Fig. 1D), benefiting from the low volume used in our organ culture system, as we could apply high concentrations very cost effectively and ensure constant concentrations of additives during the whole culture period. Immobilized adult kidney ECM extracts obtained by decellularization [69] and reactive polymer films without any biomolecular components were included as reference controls.

Effects of the differently presented ECM components on the formation of organ phenotypes were systematically analysed using a quantification of characteristic structural parameters of the developing kidney, such as number of ureteric bud tips, nephrons and overall size (Fig. 2). The results were summarized in an array-like overview, where we used a red-green colour scheme to visualize relative differences to control kidneys.

**Fig. 2.** Three parameters of the cultured kidney rudiments were determined quantitatively.
(1) The total area enclosed by the kidney rudiments (white line). (2) The number of calbindin-positive ureteric bud tips (stained with anti-calbindin/FITC), [green arrows]. (3) The number of developing nephrons which are identified by their shape, their laminin-rich basement membrane (stained with anti-laminin/TRITC) and the absence of calbindin staining [red arrows]. Scale bar: 500 µm.
Table 1. Overview of tested ECM components and growth factors.

<table>
<thead>
<tr>
<th>ECM component or growth factor (GF)</th>
<th>Family</th>
<th>Main Localization/ Expression</th>
<th>Role/Function in Kidney (Development)</th>
</tr>
</thead>
<tbody>
<tr>
<td>aggrecan</td>
<td>chondroitin sulfate proteoglycan</td>
<td>(articular) cartilage</td>
<td>so far unknown</td>
</tr>
<tr>
<td>aggrecan + HA</td>
<td>proteoglycan-aggregates</td>
<td>hyaline cartilage</td>
<td>so far unknown</td>
</tr>
<tr>
<td>BMP-7 (=GF)</td>
<td>TGF-β superfamily</td>
<td>brain, kidney, bladder</td>
<td>critical for kidney development, particularly nephron formation [30]</td>
</tr>
<tr>
<td>brevican</td>
<td>proteoglycan of lectican family</td>
<td>predominantly in central nervous system</td>
<td>so far unknown</td>
</tr>
<tr>
<td>collagen I</td>
<td>fibril forming protein</td>
<td>connective tissues</td>
<td>so far unknown; involvement in mesenchymal cell maintenance [44]</td>
</tr>
<tr>
<td>collagen IV</td>
<td>network forming protein</td>
<td>glomerular basement membranes</td>
<td>indispensable for kidney development and function, especially for glomeruli formation [33,34]</td>
</tr>
<tr>
<td>endostatin</td>
<td>fragment of collagen XVIII</td>
<td>basement membrane of liver, kidney, lung, skeletal muscle and testis</td>
<td>regulates branching morphogenesis of ureteric bud [24]</td>
</tr>
<tr>
<td>fibronectin</td>
<td>dimeric glycoprotein</td>
<td>liver, placenta, lung, smooth muscle, cardiomyocytes, adipocytes and olfactory bulb</td>
<td>required for branching morphogenesis and induces tubule formation [39,40]</td>
</tr>
<tr>
<td>heparan sulfate</td>
<td>sulfated glycosaminoglycan</td>
<td>basal lamina, cell surfaces and blood serum</td>
<td>important for kidney development, particularly nephron formation [31,32]</td>
</tr>
<tr>
<td>heparin</td>
<td>highly sulfated glycosaminoglycan</td>
<td>mast cells in the arteries of lung, liver and skin</td>
<td>limits nephron development in cultured renal rudiments [31] and blocks tubule formation [41]</td>
</tr>
<tr>
<td>hyaluronic acid (HA)</td>
<td>nonsulfated glycosaminoglycan</td>
<td>ECM of loose connective tissue, synovial fluid and vitreous humour</td>
<td>affects ureteric bud branching morphogenesis and differentiation of tubules [23]</td>
</tr>
<tr>
<td>laminin 1 (111)</td>
<td>trimeric glycoprotein</td>
<td>epithelial basement membrane</td>
<td>necessary for normal kidney development, in particular for tubule formation and branching morphogenesis [42]</td>
</tr>
<tr>
<td>laminin 8 (411)</td>
<td>trimeric glycoprotein</td>
<td>basement membrane of all endothelial cells</td>
<td>essential for normal kidney development [35] involved in glomerular mesangial cell migration [36]</td>
</tr>
<tr>
<td>laminin 10 (511)</td>
<td>trimeric glycoprotein</td>
<td>endothelial cell basement membranes of capillaries and venules</td>
<td>essential role during kidney development [37], required for glomerular filtration barrier of kidneys [38]</td>
</tr>
<tr>
<td>meteorin</td>
<td>monomeric growth factor</td>
<td>brain, heart, kidney, skeletal muscle, spleen, testis, gut and lung</td>
<td>so far unknown</td>
</tr>
<tr>
<td>nephronectin</td>
<td>nephronectin family</td>
<td>fetal ear, eye, heart, lung and kidney, also adult lung, kidney, brain, uterus, placenta, thyroid gland and blood vessels</td>
<td>involvement in branching morphogenesis, promotes kidney development via integrin mediated stimulation of GDNF expression [43]</td>
</tr>
<tr>
<td>neurocan</td>
<td>chondroitin sulfate proteoglycan</td>
<td>brain</td>
<td>so far unknown</td>
</tr>
<tr>
<td>osteopontin</td>
<td>small integrin-binding ligand, N-linked glycoprotein (SIBLING)</td>
<td>bone marrow and matrix, osteoblasts, osteocytes, inner ear, brain, kidney, placenta, odontoblasts, chondrocytes, macrophages, smooth muscle and endothelial cell</td>
<td>not essential for renal development [45] but may play a role in macrophage-mediated renal injury [46]</td>
</tr>
<tr>
<td>pleiotrophin (=GF)</td>
<td>neurite growth promoting factor (NEGf) family</td>
<td>developing central nervous system, peripheral nervous system, lung, kidney, gut and bone, also adult CNS, tumour cells</td>
<td>interference with ureteric bud branching morphogenesis [27]</td>
</tr>
<tr>
<td>tenascin-C</td>
<td>hexameric glycoprotein</td>
<td>lung, brain, thymus, kidney, tendons, bone and cartilage</td>
<td>not crucial for kidney development [47]</td>
</tr>
<tr>
<td>vitronectin</td>
<td>glycoprotein of pexin family</td>
<td>serum, fetal lung, fetal liver and adult liver</td>
<td>negligible for kidney development [48,49]</td>
</tr>
</tbody>
</table>
Materials and Methods

**ECM components and growth factors (GF)**
For the experiments, aggrecan (Sigma-Aldrich A1960), BMP-7 (=GF, R&D Systems 5666-BP-010/CF), brevican (R&D Systems 4009-BC-050), collagen I (AbD Serotec 2150-1425), collagen IV (Becton Dickinson 354233 and LGC Standards 30-2511), endostatin (Sigma-Aldrich E8279), fibronectin (BioPur AG 11-50-1105), heparan sulfate (Sigma-Aldrich H7640), heparin (AMS Biotechnology 24590.01), hyaluronic acid (HA) of MWs: 1.5MDa (HA7), 724kDa (HA6), 234.4kDa (HA5), 132.3kDa (HA4), 64.0kDa (HA3), 17.0kDa (HA2), 4.7kDa (HA1) (LifeCore Biomedical), laminin 1 (Becton Dickinson 354239 and LGC Standards 30-2505), laminin 8 (Biolamina AB 50411), laminin 10 (Biolamina AB 50511), decellularized matrix [69], meteorin (R&D Systems 3475-MN-025/CF), nephropectin (R&D Systems 4298-NP-050), neurocan (R&D Systems 5800-NC-050), osteopontin (R&D Systems 441-OP-050/CF), pleiotrophin (=GF, R&D Systems 252-PL-050), tenascin-C (Millipore CC065 and Chemicon CC065) and vitronectin (BioPur AG 11-51-1108) were added to the culture medium and/or covalently bound to the glass surface (see coating preparation below) at the designated conditions and concentrations presented in Table 2.

**Decellularized matrix processing**
Adult kidneys were decellularized as described by Ofenbauer et al. ([69]; Protocol I) to receive the extracellular matrix. To obtain an immobilizable solution, whole decellularized kidney matrices were weighed and shredded in PBS. The volume was adjusted to get a concentration of 7mg/ml and mixed in GentleMACS M tubes with a strainer (Miltenyi Biotec) using the GentleMACS dissociator (Miltenyi Biotec). Program RNA_1 was run three times to achieve maximal dissociation of the matrix.

**Coating preparation**
Protein immobilization onto maleic anhydride copolymer thin films was performed as described [50,51]. Briefly, coverslips (Menzel Gläser, Germany) were sonicated in water and ethanol for 30min each, then oxidized in a mixture of water:hydrogen peroxide:ammonia (volume ratio 5:1:1) at 70°C for 10min, dried for 1h at 120°C and subsequently modified in a solution of 3-aminopropyl(dimethyl)oxysilane. The aminosilane-modified coverslips were rinsed in toluene, dried for 1h at 120°C and
spin-coated with poly[ethylene-alt-(maleic anhydride)] (PEMA, Sigma-Aldrich; 0.15 wt% in THF:acetone [weight ratio 2:1]) or poly[octadecene-alt-(maleic anhydride)] (POMA, Polysciences Inc.; 0.08 wt% in THF) solutions. Stable covalent binding of the maleic anhydride copolymer films was achieved by annealing for 2h at 120°C to generate imide bonds with the underlying aminosilane layer. Covalent immobilization of proteins and other factors onto freshly prepared POMA and PEMA copolymer films was carried out by exposing the polymer layers to protein solutions of defined concentrations overnight at RT to reach monolayer coverage. In case of immobilizing the whole decellularized matrix, the processed matrix solution (see “Decellularized matrix processing”) was spread onto freshly prepared POMA/PEMA copolymer films, kept overnight at room temperature (RT) and briefly rinsed with PBS before use.

**Ethics**

All organ and embryo harvesting was conducted in a licensed animal facility in accordance with the German Animal Welfare Act, following the guidelines of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes. The experiments were approved by the regional council.

**Organ culture**

Kidney rudiments were microdissected from E11.5 NMRI mouse (Janvier, FR) embryos. They were pooled and assigned randomly to control or experimental groups. For conventional culture (HA Filter control), the rudiments were placed on polycarbonate filters (pore-size of 5µm) at the bottom of a well insert in a six-well plate (Corning, Costar). They were cultured in kidney culture medium (KCM) consisting of Eagle's minimal essential medium with Earle's salts and non-essential amino acids (GIBCO), 15% fetal bovine serum (Biochrom/Biosera) and 1% penicillin/streptomycin (Sigma). Low-volume organ cultures were performed as described by Sebinger et al. [29] (Fig. 1A and B): Sterilized silicone rings (flexiPERM Cone shape A, SARSTEDT) on 22×22 mm coverslips (Menzel Gläser, Germany) were used in tissue culture dishes (35×10 mm, Sarstedt/Greiner). Kidney rudiments were placed close to the middle of the circle. Medium carried over in the pipetting was removed and replaced by the final volume of KCM (85 µl), the complete enclosed area of the coverslip being wetted and the explants being fixed between the surface tension
of the medium and the substrate. The dish surrounding the silicone ring was filled with PBS (containing 1% penicillin/streptomycin). All cultures were incubated at 5% CO₂ at 37°C, for 4 days, with a medium change on day 2.

**Fixation and immunofluorescence**

Kidney rudiments were fixed in 100% methanol at -20°C, washed two times in PBS for 10 min each and incubated with the primary antibody solution in PBS overnight at 4°C. Primary antibodies were anti-laminin (1:100, Sigma L9393) and anti-calbindin D28k (1:100 Sigma C9848). Samples were washed two times for at least 30 min in PBS and incubated with appropriate secondary antibodies overnight at 4°C. The secondary antibodies used were: anti-rabbit IgG - TRITC (1:100, Sigma T6778) and anti-mouse IgG - FITC (1:100, Sigma F6257). Finally, the samples were washed in PBS two times for at least 30 min and mounted on slides with SlowFade Gold (Invitrogen).

**Morphometric quantification**

Immunostained samples were examined with a confocal laser scanning microscope (TCS SP5, Leica Microsystems, Wetzlar, Germany). Serial 5-µm optical sections of each kidney were acquired using a 5x objective. FITC, TRITC and DAPI emissions were excited and acquired sequentially. Calbindin-positive ureteric bud tips and nephrons were counted manually (Fig. 2). The total area covered by kidney rudiments was defined by hand and measured using NIH ImageJ 1.37v (http://rsb.info.nih.gov/nih-image/).

**Data analysis and statistics**

All data is represented as a relative difference to control kidneys. Control kidneys were cultured over the same time period and derived from E11.5 embryos of the same mouse as kidneys of experimental conditions. No ECM or GF components were added to control kidneys. Having distinct controls for every single experiment allowed us to transform our absolute data to relative numbers, making all conditions comparable amongst each other. Values greater than 1 indicate more and values smaller than 1 indicate less bud tips (B.), nephrons (N.) and area (A.) compared to control kidneys. For some conditions we observed a complete termination of development, which we defined as >95% less bud tips and nephrons compared to controls. Each condition
represents data from at least 10 kidneys which were compared to at least 10 control kidneys. Significance levels were calculated by a two-tailed Student's t-test assuming unequal variances between experimental groups and their respective controls. p-Values < 0.05 were considered significant and are represented as asterisks as followed: p < 0.05 (*); p < 0.01 (**); p < 0.001 (***) p < 0.0001 (****). Additionally tendencies with p < 0.1 are represented as plus (+).

**Results**

For testing the influence of ECM components and growth factors on early kidney development in organ culture experiments we started to culture kidney explants from E11.5 embryonic stage. The dissected rudiments consisted of the T-shaped ureteric bud surrounded by a cap of condensed metanephric mesenchyme. After the culture period of four days usually the ureteric bud branched, early nephrons formed and the overall size of the tissue expanded.

In order to compare the effect of the exogenous ECM signals, three parameters were determined quantitatively: the total area occupied by the kidney rudiments, the number of calbindin-positive ureteric bud tips and the number of developing nephrons which were identified by their characteristic shape, their laminin-rich basement membranes and the absence of calbindin staining (Fig. 2). Images of the immunostained kidney rudiments served as examples showing the related phenotype. All factors and substrate modifications we tested are listed in Table 1.

Our easy to dismantle, clean and resemble set-up allowed modifications beyond conventionally used organ culture arrangements [22] and enabled covalent immobilization of ECM components to the glass surface which clearly advantages filter based culture set ups.

**Kidney ECM components and growth factors**

Several ECM components promoted early in vitro kidney development as shown in green in Table 2. In these cases, the rudiments had more ureteric bud tips (B.) and/or nephrons (N.) than control kidneys and/or were larger in size (A.).

Factors with a promoting influence on development were found to influence either all measured parameters or individual characteristics. Examples which promoted all measured factors include reconstituted collagen I fibrils, immobilized on POMA
(100µg/ml), which formed a dense network resulting in a significantly increased number of bud tips (B. +34%, ***) and nephrons (N. +47%, ****) as well as significantly increased total explant area (A. +31%, ***) compared to control kidneys (Table 2, Fig. 3). Collagen I fibrils are involved in adhesion, morphology, growth, migration and differentiation of a variety of cultured cell types [52].

Table 2. Overview of results as array like colour code.

<table>
<thead>
<tr>
<th>ECM Compound / CF GF</th>
<th>Conditions</th>
<th>Bud tips</th>
<th>Nephrons</th>
<th>Area</th>
<th>ECM Compound / CF GF</th>
<th>Conditions</th>
<th>Bud tips</th>
<th>Nephrons</th>
<th>Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>aggrecan</td>
<td>200µg/ml in media</td>
<td>0.35</td>
<td>0.31</td>
<td>0.79</td>
<td>heparan sulfate</td>
<td>10µg/ml in media</td>
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<td>0.79</td>
<td>0.54</td>
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<td>1.25</td>
<td>1.12</td>
<td></td>
<td>100µg/ml in media</td>
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<td>1.18</td>
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<tr>
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<td>1000µg/ml in media</td>
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<td>brevican</td>
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</tr>
<tr>
<td></td>
<td>100µg/ml on POMA</td>
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<td>1.47</td>
<td>1.31</td>
<td></td>
<td>H42% on filter</td>
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</tr>
<tr>
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<td>0.85</td>
<td>1.03</td>
<td></td>
<td>H45% on filter</td>
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<td>10.0µg/ml in media</td>
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<td>0.61</td>
<td>0.78</td>
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<td>H46% on filter</td>
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<tr>
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<td>1.04</td>
<td>1.11</td>
<td></td>
<td>H4A 0.1% in media</td>
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<td></td>
<td>100µg/ml in media</td>
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<td>0.70</td>
<td>0.75</td>
<td></td>
<td>H4A 0.1% in media</td>
<td>1.16</td>
<td>1.24</td>
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<tr>
<td></td>
<td>10.0µg/ml on POMA</td>
<td>0.34</td>
<td>0.90</td>
<td>1.06</td>
<td></td>
<td>H4A 0.1% in media</td>
<td>1.08</td>
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<td>fibronectin</td>
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<td>H46 0.1% in media</td>
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<td></td>
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<td>0.33</td>
<td>0.84</td>
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<td>H46 0.1% in media</td>
<td>1.09</td>
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<td>0.63</td>
<td>1.22</td>
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<td>H42% on filter</td>
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<td>H43% on filter</td>
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<td>heparan sulfate</td>
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<td></td>
<td>100µg/ml on POMA</td>
<td>0.87</td>
<td>0.81</td>
<td>0.97</td>
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<td>H46% on filter</td>
<td>0.22</td>
<td>0.16</td>
<td>0.51</td>
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</tbody>
</table>

XXVIII
Data for each tested ECM component / maleic anhydride copolymer film (CF) / growth factor (GF) at various concentrations and different application methods (bound, unbound) are listed in alphabetical order. Each condition represents data from at least 10 kidneys and was compared to at least 10 control kidneys which had been cultured in parallel. Quantification of ureteric bud tip (branches) numbers, nephrons numbers and area for each culture condition is represented as a relative difference to control kidneys. The numbers are highlighted with a colour code, which is illustrated in the figure legend. Values greater than 1 indicate more and values smaller than 1 indicate less bud tips/nephrons/area compared to control kidneys.

<table>
<thead>
<tr>
<th>ECM Compound / CF: GF</th>
<th>Conditions</th>
<th>Bud tips</th>
<th>Nephrons</th>
<th>Area</th>
<th>ECM Compound / CF: GF</th>
<th>Conditions</th>
<th>Bud tips</th>
<th>Nephrons</th>
<th>Area</th>
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<tbody>
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<td>HA 1% media</td>
<td>HA6 1% in media</td>
<td>0.14</td>
<td>0.13</td>
<td>0.21</td>
<td>matrix on PEMA</td>
<td>7000ug/ml</td>
<td>0.72</td>
<td>0.83</td>
<td>1.22</td>
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<td></td>
<td>HA7 1% in media</td>
<td>0.37</td>
<td>0.33</td>
<td>0.42</td>
<td>matrix on POMA</td>
<td>7000ug/ml</td>
<td>1.12</td>
<td>1.42</td>
<td>1.16</td>
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<td>HA4 1% in media</td>
<td>0.02</td>
<td>0.02</td>
<td>0.06</td>
<td>melanin 20ug/ml in media</td>
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<td>0.06</td>
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<td>HA2 4% in media</td>
<td>0.02</td>
<td>0.02</td>
<td>0.06</td>
<td>nephrocin 1ug/ml in media</td>
<td>1.05</td>
<td>1.08</td>
<td>1.09</td>
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</tr>
<tr>
<td></td>
<td>HA4 4% in media</td>
<td>0.02</td>
<td>0.02</td>
<td>0.06</td>
<td>neurocan 10ug/ml in media</td>
<td>1.27</td>
<td>1.18</td>
<td>1.16</td>
<td></td>
</tr>
</tbody>
</table>

| HA 4% media           | HA1 1% on POMA | 1.11     | 0.95     | 1.02 | osteopontin 100ug/ml on POMA | 0.72     | 0.83     | 1.22     |
|                       | HA2 1% on POMA | 1.04     | 0.99     | 1.06 | osteopontin 1ug/ml in media | 1.03     | 0.95     | 0.90     |
|                       | HA3 1% on POMA | 1.06     | 0.98     | 1.03 | osteopontin 10ug/ml in media | 0.91     | 0.86     | 0.71     |
|                       | HA4 1% on POMA | 0.94     | 0.87     | 0.84 | PHEMA (CF) | 10ug/ml in media | 1.04     | 1.22     | 1.02     |
|                       | HA5 1% on POMA | 0.83     | 0.82     | 0.83 | PHEMA (CF) | 10ug/ml in media | 0.95     | 1.08     | 0.91     |
|                       | HA6 1% on POMA | 0.79     | 0.80     | 0.76 | PHEMA (CF) | 10ug/ml in media | 1.18     | 1.41     | 0.94     |

| HA 1% POMA            | HA1 4% on POMA | 1.10     | 1.10     | 1.40 | POMA-C 100ug/ml on POMA | 1.12     | 1.28     | 2.31     |
|                       | HA2 4% on POMA | 1.13     | 1.23     | 1.10 | tenacin-C 20ug/ml on POMA | 0.72     | 0.91     | 1.09     |
|                       | HA3 4% on POMA | 1.16     | 1.20     | 1.24 | tenacin-C 10ug/ml on POMA | 0.85     | 1.01     | 1.27     |
|                       | HA4 4% on POMA | 0.65     | 0.75     | 0.71 | tenacin-C 10ug/ml in media | 0.91     | 0.86     | 0.75     |
|                       | HA5 4% on POMA | 0.38     | 0.40     | 0.46 | vitronectin 10ug/ml on POMA | 0.07     | 0.08     | 0.20     |
|                       | HA6 4% on POMA | 0.27     | 0.37     | 0.47 | vitronectin 10ug/ml in media | 0.02     | 0.02     | 0.20     |
|                       | HA7 4% on POMA | 0.14     | 0.14     | 0.28 | vitronectin 10ug/ml in media | 0.14     | 0.09     | 0.22     |

| laminin 1 (111)       | laminin 1 (111) | 10ug/ml in media | 1.16     | 1.26     | 1.03 | laminin 1 (111) | 10ug/ml in media | 1.26     | 1.58     | 1.31 |
|                       | laminin 1 (111) | 100ug/ml in media | 1.26     | 1.58     | 1.31 | laminin 1 (111) | 100ug/ml in media | 0.95     | 1.29     | 1.20 |

<table>
<thead>
<tr>
<th>Colour code</th>
<th>Statistical significance</th>
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<td>***</td>
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<td>+++</td>
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</table>

Data for each tested ECM component / maleic anhydride copolymer film (CF) / growth factor (GF) at various concentrations and different application methods (bound, unbound) are listed in alphabetical order. Each condition represents data from at least 10 kidneys and was compared to at least 10 control kidneys which had been cultured in parallel. Quantification of ureteric bud tip (branches) numbers, nephrons numbers and area for each culture condition is represented as a relative difference to control kidneys. The numbers are highlighted with a colour code, which is illustrated in the figure legend. Values greater than 1 indicate more and values smaller than 1 indicate less bud tips/nephrons/area compared to control kidneys.
100µg/ml laminin I immobilized on POMA, which significantly promoted rudiment development when added as a surface-bound layer (B. +26%,**, N. +58%,****, A. +31%,**). Laminin 1 is a basal lamina element and important for tubule formation and branching morphogenesis [42]. POMA surface modification without any additional coating also promoted development (see “Maleic anhydride copolymer films”; Table 2, Fig. 3). Examples which promoted single factors include 100µg/ml collagen IV immobilized on POMA, which significantly promoted the outgrowth (A. +30%,**), but significantly reduced the number of bud tips formed (B. -19%,*), and 10µg/ml pleiotrophin immobilized on POMA, which had a significant impact only on nephron formation, (N. +41%,**; Table 2). Pleiotrophin is involved in the epithelial-mesenchymal communication during development [27].

Several ECM components had an inhibitory influence on the developing organs as shown in red in Table 2. In these cases, the rudiments had fewer ureteric bud tips and/or nephrons than the control kidneys and/or were smaller in size. Examples include dissolved collagen IV at a concentration of 50µg/ml (B. -25%,+, N. -39%,**, A. -22%,+), dissolved endostatin at a concentration of 100µg/ml (B. -38%,****, N. -30%,**, A. -25%,*) and dissolved osteopontin at a concentration of 10µg/ml (B. -39%,***, N. -34%,***, A. -29%,**; Table 2, Fig. 3). The results for endostatin are in line with Karihaloo et al. [24], indicating a possible role in ureteric bud arborization.
Beside a general promoting or inhibiting effect on the developmental parameters we defined, we could also observe varying phenotypes for some of the tested components, such as malformations. BMP-7 is expressed by cells of the ureteric bud and the mesenchyme during kidney development [53]. However, at a concentration of 0.5µg/ml dissolved in the medium it stunted the developing branches (B. -66%,****) and nephrons (N. -73%,****), underlining the dose-dependent effect of BMP-7 during kidney development (Table 2, Fig. 4A). Other examples include 1000µg/ml heparan sulfate in media (B. -49%,****, N. -41%,****, A. -10%) or 20µg/ml meteorin in media (B. -72%,****, N. -74%,****, A. -58%,****; Table 2, Fig. 4A). A complete termination of development could be observed for example with 10µg/ml laminin 8 in media (B. -99%,****, N. -98%,****, A. -98%,****), 10µg/ml laminin 10 in media (B. -94%,****, N. -94%,****, A. -93%,****) and 100µg/ml tenascin-c in media (B. -98%,**, N. -94%,****, A. -98%,**, Table 2, Fig. 4B).

**Fig. 4. Selection of kidney explant phenotypes caused by different biomolecules.**

(A) Different malformations of embryonic kidneys under the influence of BMP-7, heparan sulfate and meteorin are exhibited. (B) Rudiments cultured with laminin 8, laminin 10 or tenascin-C show a complete lack of growth and development with varying appearances. Fibronectin, heparin and vitronectin applied as indicated show abnormal phenotypes (C) – (E). The severity of the effect is dose and application dependent (bound or unbound). All illustrated rudiments were immunostained for basement membrane marker laminin (red) and the ureteric bud marker calbindin-D128k (green). The embryonic kidneys were cultured for four days. Scale bar: 500µm.
We could show dose-dependence for many factors. For example, fibronectin dissolved in culture media showed an increasing phenotype and caused an increasing inhibitory effect on the developing organs with rising concentrations from 1µg/ml (B. -6%, N. -20%,*, A. -11%) to 100µg/ml (B. -99%,****, N. -99%,****, A. -92%,****; Table 2, Fig. 4C). These results are not in line with the common opinion that fibronectin facilitates branching morphogenesis, even though our results imply a role in kidney development. A reason for our divergent results from the published ones could be the difference in applied concentrations of fibronectin and the way of cultivation. Another example is laminin 10, which dissolved in culture media had no significant effect at a concentration of 0.5µg/ml but had a substantial inhibiting effect on development at 10µg/ml (B. -94%, N. -94%, A. -93%, Table 2).

Not only the concentration, but also the mode of presentation was often crucial for the impact of a given molecule. For instance, bound heparin at a very low and high concentration had a strong inhibiting and branching distorting effect (10µg/ml on POMA: B. -70%,****, N. -51%,**, A. -47%,***; 4000µg/ml on POMA: B. -91%,****, N. -84%,****, A. -60%,****). On the other hand, a concentration of 100µg/ml heparin added directly to the medium had a less inhibiting effect, which was not significant due to several kidneys that developed rather normally (B. -31%,+, N. -27%, A. -23%). A tenfold increase of the concentration (1000µg/ml) caused a complete termination of development (B. -97%,****, N. -99%,****, A. -43%,**; Table 2, Fig. 4D). Another example is vitronectin, where we observed completely different results dependant on the mode of application. When dissolved in the media, it had severe inhibiting effects. At a concentration of 10µg/ml it caused branching malformations and nephrons which were less developed (B. -86%,****, N. -91%,****, A. -78%,****). A tenfold increase of the concentration (100µg/ml) caused a complete termination of development (B. -98%,***, N. -98%,****, A. -41%,*). Interestingly, when bound to the surface, vitronectin significantly increased nephron formation (e.g. 10µg/ml on POMA: B. -5%, N. +39%,***, A. +20%,*; Table 2, Fig. 4E). Also the basal lamina element laminin 1 promoted rudiment outgrowth and development when added as a surface-bound layer (e.g. 100µg/ml on POMA: B. +26%,**, N. +58%,****, A. +31%,**), but when dissolved in culture media did either less so (100µg/ml: B. +21%,+, N. +29%,*, A. +3%) or had non significant inhibiting effects (e.g. 10µg/ml: B. -21%, N. -33%, A. -26%; Table 2).
Some factors showed no obvious effects on development in our culture system at the concentrations used, e.g. 20µg/ml dissolved nephronectin (B. -10%, N. -5%, A. -1%) and 10µg/ml dissolved brevican (B. +6%, N. -8%, A. -4%; Table 2).

**Glycosaminoglycans**

The results obtained from the organ culture with different glycosaminoglycans confirmed their important regulatory role in organ development. We found that the effect of dissolved HA varied from promoting kidney development (e.g. HA3, 0.1% dissolved in media: B. +16%+, N. +24%*, A. +39%,***) to completely terminating development (e.g. HA3, 4% dissolved in media: B. -98%****, N. -99%****, A. -96%****), depending on the concentration and molecular weight (MW) used (Table 2, Fig. 5). At a concentration of 0.1%, HAs of all MWs tested supported development. In contrast, higher concentrations reduced the growth of the isolated rudiments. The impact of higher concentrations was less pronounced for lower molecular weight HAs, but had an increasingly severe effect for higher molecular weight HAs. Immobilized HA with a concentration of 1% had no significant (lower MWs) or minor inhibiting effects (HA5-7). Bound HA with a concentration of 4% had promoting effects for HA1 (MW of 4.7 kDa), HA2 (17.0 kDa) and HA3 (64.0 kDa), but increasingly inhibiting effects for HA4 (132.3 kDa), HA5 (234.4 kDa), HA6 (724kDa) and HA7 (1.5MDa, Table 2, Fig. 5). Concentration dependent effects were observed while comparing the number of HA subunits, i.e. considering chain lengths (MWs) and concentrations. Concentration levels corresponding to similar numbers of HA repeating units (increasing concentration from HA1-7) resulted in negative effects for HA1 and HA2, which were not present for HA3-7 (Table 2). This finding can be explained by the fact that the higher quantity of HA molecules contained in the solutions with HA of lower molecular weight can bind more cellular receptors than lower concentrations of HAs of bigger size [54].

Our results for the highly sulfated heparin confirmed published findings [31]. An inhibition of development of the whole organ anlagen occurred in a dose-dependent manner for both bound and dissolved heparin (see “Kidney ECM components and growth factors”, Table 2, Fig. 4D). The severe lack of growth and development of the whole rudiment is possibly related to a deleterious effect of 1µg/ml heparin on human embryonic stem cells shown by Furue et al. [55]. Unbound heparan sulfate with a concentration of 100µg/ml resulted in non significantly slightly higher values for the
tested parameters (B. +31%, N. +14%, A. +18%) while both a ten-fold increase and decrease resulted in less bud tips, nephrons and explant area (e.g. 1000µg/ml: B. -49%,****, N. -41%,****, A. -10%) as compared to control kidneys (Table 2). Interestingly, in combination with aggrecan, the effect of glycosaminoglycans (heparan sulfate, hyaluronan) on developing kidney rudiments was generally amplified (e.g. 1000µg/ml heparan sulfate without aggrecan: B. -49%,****, N. -41%,****, A. -10%; 1000µg/ml heparan sulfate with 200µg/ml aggrecan: B. -69%,****, N. -46%,***, A. -21%; Table 2).

Interestingly, in combination with aggrecan, the effect of glycosaminoglycans (heparan sulfate, hyaluronan) on developing kidney rudiments was generally amplified (e.g. 1000µg/ml heparan sulfate without aggrecan: B. -49%,****, N. -41%,****, A. -10%; 1000µg/ml heparan sulfate with 200µg/ml aggrecan: B. -69%,****, N. -46%,***, A. -21%; Table 2).

Fig. 5. Effect of HA concentration and molecular weight on in vitro kidney growth and development. Whole embryonic kidneys (immunostained for basement membrane marker laminin (red) and the ureteric bud marker calbindin-D_{28k} (green)) after four days in vitro culture with 0.1%, 1% and 4% HA in media and 4% HA immobilized on POMA of the following molecular weights: HA1 (4.7 kDa), HA2 (17.0 kDa), HA3 (64.0 kDa), HA4 (132.4 kDa), HA5 (234.4 kDa), HA6 (724 kDa) and HA7 (1.5 MDa) compared to control rudiment are shown. Scale bar: 500µm.

**Neural ECM components**

We tested the effect of brevican, meteorin and neurocan, three important factors in central nervous system (CNS) development and turnover [56–63]. These factors have not been considered to play a role in kidney development. We found that 10µg/ml brevican dissolved in media had no significant effect on kidney development (B. +6%, N. -8%, A. -4%). 20 µg/ml meteorin dissolved in media had a significant strong inhibiting impact (B. -72%,****, N. -74%,****, A. -58%,****) on the *in vitro* kidney development and generated star shaped phenotypes with strongly reduced and malformed branching of the ureteric bud and delayed nephrogenesis (Table 2, Fig. 4A).
In contrast, 10µg/ml neurocan dissolved in media supported especially the branching (+27%, **) and also the growth of the rudiments (N. +18%,*, A. +16%,+). These effects might be due to neurocan’s involvement in the modulation of cell adhesion and migration [62]. We suggest that these results point to an as-yet undescribed involvement of meteorin and/or neurocan in renal development or to a structural or functional similarity to factors that are involved in kidney organogenesis.

**Maleic anhydride copolymer films**

Kidney rudiments were grown on maleic anhydride copolymer films (POMA and PEMA, used for the immobilization of the ECM components given above), directly after regeneration of the anhydride groups of the copolymers, but without pre-coating with biomolecules. The use of the reactive copolymer thin film substrates had a remarkably beneficial influence on the cultured rudiments and had a significant impact on nephrogenesis and rudiment outgrowth (Table 2 and Fig. 3). The effects of the more hydrophobic POMA were superior (B. +12%, N. +28%, ***, A. +31%,****) to that of the more hydrophilic PEMA (B. -2%, N. +19%,**, A. +27%,***). This may be due to enhanced anchoring of the rudiments to the reactive surface [50,51] and the resulting tight connection of the embryonic kidney to the support surface.

**Decellularized matrix of adult murine kidney**

Immobilizing processed decellularized adult kidney ECM significantly promoted nephrogenesis and rudiment outgrowth (e.g. bound to POMA: B. +12%, N. +42%,****, A. +16%,*) of the cultured rudiments (Table 2). These results suggest that the adult kidney ECM is able to support embryonic kidney development. Compositional and structural features of this multicomponent matrix remain to be characterized.

**Discussion**

In this study we applied a novel low volume organ culture method [29] to test the impact of exogenous signals on developing embryonic nephrogenic tissue. Microdissected E11.5 NMRI kidney rudiments were exposed to ECM components either as covalently bound substrate layers or as soluble culture medium additives over periods of four days *in vitro*. The culture period was followed by a quantitative
determination of the size of the rudiments, the number of their calbindin-positive ureteric bud tips and the number of the contained developing nephrons. The results provide a comprehensive overview of the influence of matrix components on embryonic kidney development and structure formation, including information on dose-response effects.

The low-volume culture system is advantageous because it maintains a constant concentration of biomolecules over the whole culture period, by directly dissolving them in the media or covalently binding to the glass surface. In earlier studies, kidney rudiments were cultivated on filters at a gas-medium interface with the need of a relatively high amount of media (1-3ml), located in a well beneath the filter. In this case only a relatively small amount of the dissolved biomolecule solution was applied directly on the kidney rudiments and distributed in the media phase underneath subsequently. This approach was taken by Rosines et al. by applying 270µl of differently concentrated hyaluronic acid solutions directly on top of the kidney rudiments for their experiments [23] which explains the differences of the results in our studies. Although in theory the filter method allows to directly dissolve bioactive molecules in the media as well, it is not practicable because of the high costs caused by the relatively high amount of medium. Nevertheless we did this for 1% HA, which we added directly to the entire media. This allowed us to compare our novel method with the conventional setup, as this assured an equal concentration of HA. The inhibiting effect of 1% HA was bigger for conventionally cultured kidneys, as for kidneys cultured with our novel setup (Table 2). This is due to the fact that the development of kidneys grown with the novel technique is in general superior to the conventional filter method when compared by any of the usual metrics (overall size, nephron number and the extent of ureteric bud branching) [29].

Another advantage of our new culture method over traditional filter methods [22] was the possibility to modify the glass surface on which the kidney rudiments were cultured. This allowed us to expose the kidney rudiments locally to immobilized ECM components which acted only on the outer tissue layer that is in direct contact with the substrate. The components were able to influence kidney attachment, spreading and development by interactions with surface receptors on the contact site. Presented immobilized, the components did not change physical properties (e.g. viscosity) of the medium. Alternatively, similar components of the ECM were dissolved directly in the whole volume of the applied media at defined concentrations. These biomolecules
were able to act on the whole surface of the tissue by directly binding to surface receptors and additionally changing the physical properties of the surrounding medium in some cases. Some soluble molecules possibly also permeate the developing tissue.

Soluble ECM components were found to cause stronger effects than surface bound ECM in most of our experiments. Interestingly, laminin 1, the main component of the basal lamina, was an exception. Bound laminin 1 supported kidney development more effectively than the dissolved molecule (Table 2). This is possibly due to improved anchoring of the kidney rudiments. Experiments with other laminins also revealed very interesting results (Table 2). By applying laminin 10 at a concentration of 10µg/ml (unbound), the development of the rudiments came to a complete halt. It is possible that the differentiation inhibiting effect of laminin 10, that has been described for embryonic stem cells [64,65], is responsible. Laminin 8 caused similar results at the same concentration (Table 2).

In general, the mode of application was crucial for the impact of some ECM components. Collagen IV, provided as a substrate coating, triggered attachment and spreading of the embryonic tissue (as it does for embryonic stem cells [66]), but negatively influenced branching. In contrast, collagen IV suspended in media reduced the outgrowth of the developing rudiments and the formation of branches and nephrons (Table 2). Another example was the glycoprotein family member vitronectin. Rudiments cultured on immobilized vitronectin showed a promoted development, especially on nephrogenesis and growth. In contrast to this, dissolved vitronectin resulted in reduced branching, nephron formation and outgrowth or even completely inhibited development at the highest concentration used (Table 2, Fig. 4E).

As a major finding, we could show that the influence of HA on early kidney development is both dose and chain-length dependent (Table 2, Fig. 5). As discussed before, one problem of the study by Rosines et al. [23], was that the concentration of the applied hyaluronans reduced over time due to diffusion into the culture medium. These difficulties were solved by our novel methodology, which uses a small volume of 85µl and glass as a surface. Our system provided additional insights and allowed us to define the applied HA concentrations more precisely. We also used covalently bound hyaluronans. The studies differed in the choice of model organism (mouse as opposed to rat) and time of culture (four days as opposed to seven days). The effect of increased media viscosity, when using higher concentrated HA solutions, should be considered as it may have a considerable inhibiting influence on organ development. To exclude
this physical property, we applied covalently bound hyaluronans. Immobilized HA1 - HA3 with a concentration of 4% promoted development (defined by number of bud tips, number of nephrons and size), in contrast to HA4 - HA7, which resulted in inhibiting effects (Table 2, Fig. 5). The combination of 200µg/ml Aggrecan with 1% unbound HA seemed to amplify the negative effects of HA on developing kidney rudiments (aggrecan alone had no significant effect, while the effect of just 1% concentrated hyaluronans was less severe). An increased viscosity caused by HA-aggrecan complexes has been described by Nishimura et al. [67] and could possibly explain this phenomenon. Our findings suggest that the tissue is not only sensitive to the concentration of hyaluronans and the media's viscosity, but is also influenced by the chain length. Bound substrates of shorter hyaluronans (HA1 – HA3) had promoting effects, but longer HAs (HA4 – HA7) had inhibiting effects on rudiments’ development.

Our results will allow more insight into the use of ECM components in new therapeutic approaches that rely on the endogenous capability of tissues to regenerate in kidney diseases. Hereby star-PEG-heparin hydrogels [68] could serve as an ideal carrier system for injecting the most promising components into injured tissue to test these factors in vivo. In particular, the observed increase in nephron formation induced by immobilized collagen I, laminin 1 (Table 2, Fig. 3A), decellularized matrix, pleitrophin and nephronectin (Table 2) might find applications in regenerative therapies. In general, stronger supportive effects on nephron formation were obtained with surface bound ECM components as compared to dissolved components.

Taken together, our approach intended to broaden the spectrum of exogenous cues to include not only proliferation enhancing factors (such as collagen I or laminin 1), but also components that restrict development (such as BMP-7, heparin or meteorin) which can together be used to direct the formation of complex tissue structures. Moreover, the use of matrix components (or combinations thereof) that affect organogenesis depending on the mode of presentation or the concentration (as shown for collagen IV, HA or vitronectin) are expected to become of particular interest in the engineering of complex kidney structures like glomeruli or whole nephrons.
Conclusions
We used a novel and facile approach to test the impact of ECM and other bioactive molecules on renal organogenesis in vitro. More than 20 different components were tested under 116 different culture conditions. These included different concentrations of ECM components and other bioactive molecules either bound to the substrate or dissolved in the culture medium. We identified ECM components and conditions that exerted either supportive or inhibitory effects on specific features associated with kidney development. These results create a base for the rational use of exogenous signals in regenerative kidney therapies. The applied low-volume organ culture set up with functionalized planar and transparent carrier as surface was well suited to study tissue formation in situ under various different stimuli and constraints.

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References


Appendix C: The dewaxed ECM: An easy method to analyze cell behaviour on decellularized extracellular matrices.

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**The dewaxed ECM: An easy method to analyze cell behavior on decellularized extracellular matrices**

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**Abstract**

Decellularization techniques have been used on a wide variety of tissues in order to create cell-seedable scaffolds for tissue engineering. Finding a suitable decellularization protocol for a certain type of tissue can be laborious, especially when organ perfusion devices are needed.

Here we report a quick and easy method to compare decellularization protocols, combining the use of paraffin slices and two-dimensional cell culture. We have developed three decellularization protocols for adult murine kidney which yielded decellularized extracellular matrices (ECMs) with varying histological properties. The resulting paraffin-embedded ECM slices were deparaffinized and reseeded with murine embryonic stem cells (mESCs). We have analyzed cell attachment 4 days post seeding via determination of cell numbers, and used quantitative Real-Time PCR 13 days post seeding to measure gene expression levels of two genes associated with renal development (Pax2 and Pou3f3). The three decellularization protocols produced kidney-matrices that showed clearly distinguishable results. We could demonstrate that formerly paraffin embedded decellularized ECMs can effectively influence differentiation of stem cells. The here presented method can be used to identify optimal decellu-
larization protocols for recellularization of three-dimensional tissue-scaffolds with embryonic stem cells or other tissue-specific cell types.

1. Introduction

Tissue engineering is a rapidly evolving field, dealing with tissue repair and the de novo engineering of tissue (Langer and Vacanti, 1993; Rustad et al., 2010). Cell types capable of supporting tissue regeneration include totipotent embryonic stem cells and various pluripotent progenitor cells. Understanding cell differentiation will be a key factor for advanced tissue engineering. Cell differentiation depends on many factors, including growth factors, cell-cell interaction, tissue elasticity, physical forces and extracellular matrix (ECM) composition. Furthermore, especially for de novo engineering of tissues, bio-functional scaffolds as primary structures for seeding the cell type(s) of choice are crucial parameters to success. One popular approach to this respect is the decellularization of allogenic or xenogenic tissue to create an acellular ECM which can be repopulated in subsequent procedures (Gilbert et al., 2006; Badylak et al., 2011).

Decellularization techniques have been used on a wide variety of tissues, ranging from blood vessels (Uchimura et al., 2003; Funamoto et al., 2010), heart valves (Ota et al., 2007; Zeltinger et al., 2001), ligaments (Woods and Gratzer, 2005; Gratzer et al., 2006) or urinary bladder (Rosario et al., 2008; Yang et al., 2010) to highly structured organs like kidneys (Ross et al., 2009; Nakayama et al., 2010), hearts (Ott et al., 2008; Akhyari et al., 2011), livers (Uygun et al., 2011; Uygun et al., 2010) or lungs (Cortiella et al., 2010; Price et al., 2010). To find a suitable and satisfying decellularization protocol for a certain type of tissue, it is necessary to compare the performance of different protocols among each other. Beside analyzing the retention of certain ECM components like fibronectin, laminin, glycosaminoglycans (GAGs) or collagen, the most abundant undertaking in analyzing decellularized ECMs is a proof of cell removal. Furthermore, decellularized ECMs can have an influence on cell proliferation and differentiation for cells seeded on such matrices (Badylak et al., 2011; Ross et al., 2009; Uygun et al., 2011; Cortiella et al., 2010). The decellularization protocol itself has a great impact on the quality of decellularized ECMs and thus further on the potential of seeded cells to proliferate and differentiate as desired. However, reseeding decellularized ECMs derived from multiple decellularization protocols can be laborious, especially when whole organ grafts are reseeded. One reason is that seeding and culturing of whole organ grafts often require organ perfusion devices (Rustad et al., 2010;
Badylak et al., 2011; Ross et al., 2009; Akhyari et al., 2011; Uygun et al., 2011; Price et al., 2010; Crapo et al., 2011), which makes cell culture more time-consuming and difficult as compared to conventional two-dimensional systems.

To address this problem, we explored in this study the recellularization of decellularized adult kidney-ECMs that had been paraffin-embedded and processed into two-dimensional slices. Such paraffin slices are easily available, because they are used for histological staining, which is a very common technique in analyzing decellularized ECMs. Subsequently, these slices were deparaffinized and reseeded with mESCs to study their adhesion and differentiation behavior on differentially processed kidney-ECM structures. To do this, we established three decellularization protocols for adult murine kidneys which yielded decellularized ECMs with varying histological properties. The resulting acellular ECM slices of 1.5µm thickness were reseeded with mESCs and compared to controls (glass and gelatine coatings). We analyzed cell attachment by counting cell numbers after 4 days of culture and determined gene expression levels of two genes associated with renal development (Pax2 and Pou3f3), using quantitative Real-Time PCR (qRT PCR) after 13 days of culture. Pax2 is a transcription factor expressed, among other tissues, in the ureteric bud, the induced metanephric mesenchyme as well as glomerular and tubular precursors and plays a key role in branching morphogenesis (Narlis et al., 2007; Rothenpieler and Dressler, 1993). Pou3f3 is a transcription factor expressed in the developing loop of Henle and distal convoluted tubule during nephrogenesis and plays an important role in distal convoluted tubule, loop of Henle and macula densa formation in the developing kidney (Nakai et al., 2003).

The study was able to show a dependence between the preparation of decellularized tissue-ECM and the biological response of mESCs to such scaffolds in a simplified two-dimensional manner.

2. Materials and Methods

2.1. Decellularization of kidneys
Kidneys were harvested from female 4-6 weeks old NMRI mice (Janvier, FR) and transported in cold phosphate buffered saline (PBS, ~1h). They were then cut laterally into two halves, using forceps and a scalpel. Kidney halves were placed in 50ml tubes...
(6-10 halves per tube) containing 30ml of a hypotonic solution: 10mM Tris/HCl pH 8; 5mM EDTA (Sigma-Aldrich), 10U/ml penicillin and 100U/ml streptomycin (Pen/Strp, Invitrogen) in sterile dH2O. The tubes were fixed on a wave platform shaker (Heidolph Polymax 1040) which was set to 35rpm so that a consistent physical force of the moving solution could act on the kidney halves. Three different protocols were tested over a time course of approximately two weeks. Solutions were changed as followed (always 30ml):

Protocol I: 90h hypotonic solution, exchanged four times (after 4, 16, 25 and 25h) - 48h 1% sodium dodecyl sulfate solution (SDS, 1% w/v in hypotonic solution, Sigma-Aldrich), exchanged one time (after 24h) – 72h PBS with Pen/Strp (exchanged two times after 24h each). Protocol II: 70h hypotonic solution with protease inhibitors (protease inhibitor mix m, Serva, applied as suggested by the manufacturer) freshly added after each exchange, exchanged three times (after 4, 16 and 25h) – 2.5h 1% SDS – 70h 0.1% SDS, exchanged one time (after 30h) – 50h PBS with Pen/Strp, exchanged one time (after 20h) – 90h 15% fetal bovine serum (FBS, Biochrom/Biosera, 15% v/v in PBS with Pen/Strp) – 72h PBS with Pen/Strp, exchanged two times. Protocol III: same as protocol II except that between the hypotonic solution and the 1% SDS step, a 70h 1% polyethylene glycol solution 1000 (PEG, molecular weight = 1000, Sigma-Aldrich) step was included in addition. The three protocols are summarized in Figure 1. To test the effect of differential concentrated PEG 1000 solutions, kidney halves were incubated in hypotonic solution as in protocol II, followed by 70h incubation in 0.5%, 1%, 3%, 6%, 33% and 50% PEG in H2O.

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<th>Protocol I</th>
<th>Protocol II</th>
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<tr>
<td>hypotonic solution 90h, 4x</td>
<td>1% SDS 48h, 1x</td>
<td>PBS + pen/strp 72h, 2x</td>
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<td>hypotonic solution with protease inhibitors 70h, 3x</td>
<td>1% SDS 2.5h</td>
<td>0.1% SDS 70h, 1x</td>
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<tr>
<td>hypotonic solution with protease inhibitors 70h, 3x</td>
<td>1% PEG 1000 70h</td>
<td>1% SDS 2.5h</td>
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*Figure 1. Schematic comparison of the three decellularization protocols.*

Three different protocols are shown. Abbreviations: 1x-4x: number of solution exchanges; SDS...sodium dodecyl sulfate; PBS...phosphate buffered saline; pen/strp...penicillin and streptomycin; FBS...fetal bovine serum; PEG...polyethylene glycol.
In all protocols, before every change to a new type of solution, kidney halves were rinsed briefly with that solution. Additionally, between changes from SDS to other solutions, the sample tube was exchanged as well.

2.2. Paraffin-embedding and histochemistry
Decellularized kidney halves were fixed with 10% neutral buffered formalin (approx. 4% paraformaldehyde, Sigma-Aldrich) for 6h and embedded in paraffin (either directly after harvesting for native kidney or after decellularization for decellularized kidneys with a maximum storage time in PBS with Pen/Strp for up to two days at 4°C). Paraffin sections (1.5µm on super frost plus object slides, R.Langenbrinck) were stained with hematoxylin and eosin (H&E), alcian blue and van Gieson using standard protocols.

2.3. Immunohistochemistry
Kidney slices were first deparaffinized and rehydrated: heating O/N at 37°C, 2 changes of Roticlear (Carl Roth) for 5 min. each, 2 changes of 100% ethanol for 3 min. each, 2 changes of 95% ethanol for 3 min. each, 1 change of 70% ethanol for 3 min., and finally by 2 changes of H2O for at least 5 min. each.

Native slices were treated with 0.5% Triton X-100 (15 minutes, followed by washing in PBS, Sigma-Aldrich), prior the following staining procedure, which was the same for all slices. A universal polymer based blocking solution was applied (Roti-ImmuNoBlock, 1:10 in H2O, Carl Roth) for 30 minutes at room temperature (RT) prior to incubating the slices in primary antibody solution (in PBS with 1/100 Roti-ImmuNoBlock) over night (O/N) at 4°C. The next day samples were washed in PBS (2 times, at least 10 minutes each, at RT), followed by a second blocking step (30 minutes at RT) and incubation with secondary antibody solution for 30 minutes at RT. Primary Antibodies used were: anti-hyaluronic-acid (1:50, AbD Serotec), anti-laminin (1:100, Sigma-Aldrich) and anti-Pax2 (1:50, Lifespan Biosciences). Secondary Antibodies used were: anti-sheep IgG-FITC (1:100, AbD Serotec) and anti-rabbit IgG-Tritic (1:125, Sigma-Aldrich). After washing in PBS (2 times, at least 10 minutes each, at RT) samples were mounted in SlowFade Gold or SlowFade Gold with DAPI (Invitrogen).
ECM slices seeded with mESCs were formalin-fixed (6h), rinsed with PBS and treated with 0.5% Triton X-100 (15 minutes, followed by washing in PBS, Sigma-Aldrich) prior to the staining procedure described above.

Immunostained samples were examined on a confocal laser scanning microscope (TCS SP5, Leica Microsystems, Wetzlar, Germany).

2.4. Test of decellularization efficiency by determination of relative DNA levels

Decellularization efficiency was estimated histologically by H&E and DAPI stainings. DAPI-only images were used to do a semi-quantitative analysis using ImageJ software (http://rsb.info.nih.gov/ij/). To do so, we first set a threshold of 50 (threshold function) for all images to remove any false positive cross fluorescence from the other channels. 8 DAPI-only images (5 from cortex, 3 from medulla) were analyzed for each condition (native, protocols I-III) determining the mean gray value (measure function) which is proportional to the intensity of the DAPI staining.

2.5. Paraffin removal and preparation of kidney slices for mESC seeding

Kidney tissue-slices (decellularized by protocols I-III and native kidney) were deparaffinized and rehydrated as described above with the exception that the last three steps (70% ethanol and 2x H2O) were performed under sterile conditions. An autoclaved silicone ring (d= 12mm, FlexiPERM conA, Sarstedt) was applied on each dried object slice such that the kidney slice was in the middle of the so formed tissue culture well (Fig. 2). A water drop was applied on each kidney slice to ensure it was properly wetted and could not dry out during this process. The assembled object slides were put into suitable cell culture dishes (quadriPERM, Sarstedt) and the tissue culture wells were filled with PBS with Pen/Strp and stored O/N at 4°C. Glass-only controls were prepared the same way, starting at the 70% ethanol step and using plain object slides. For gelatine controls, silicone rings were applied directly in cell culture 6-well plates (TPP) and the resulting reaction chambers were treated with sterile 0.1% gelatine solution for 20 minutes at RT. After changing the gelatine solution with PBS with Pen/Strp, the well plates were stored O/N at 4°C.
2.6. **mESC culture and seeding on (decellularized) kidney slices**

mESC culture was done as described in the technical bulletin of StemCell Technologies (Stem Cell Technologies, 2010). Shortly, mESCs (R1, ATCC, SCRC-1011) were propagated on mitotically inactivated mouse primary embryonic fibroblasts (MEFs, StemCell Technologies, 00322; prepared beforehand using mitomycin C, Sigma-Aldrich). For each experiment, passage 4 mESCs were plated in gelatinized T25 cell culture flasks (TPP) without MEFs. To remove any remaining MEFs, mESCs were passaged one further time (gelatinized T25) before they were seeded onto kidney-slices and controls which were prepared the day before.

To detach cells from the surface, Accutase (Millipore) was used. For maintaining and passaging mESCs before seeding, maintenance medium was used: 15% fetal bovine serum (ATCC), 10U/ml penicillin, 100U/ml streptomycin (StemCell Technologies), 2mM glutamine (StemCell Technologies), 0,1mM non essential amino acids (StemCell Technologies), 10ng/ml mouse leukemia inhibitory factor (LIF, Millipore), 100µM MTG (Sigma-Aldrich) in high glucose DMEM including sodium pyruvate (StemCell Technologies). For all experiments on kidney-slices and control substrates, maintenance medium without LIF was used to enable differentiation of the cells. No pro-differentiation agents were added.

To analyze mESC attachment, 105 cells (counted by hemocytometer) were seeded on the (decellularized) kidney slices, glass-only and gelatine controls. The cells were cultivated for 4 days changing the medium on the second and third day. While changing the medium, cells touching the silicone ring were aspirated using a vacuum pump (Fig. 2), since the cells attached to this area easier than to glass or to the ECM slice.

![Figure 2. Setup for mESC seeding.](image)
After 4 days, cells were enzymatically detached (Accutase) and viable cells (identified with Trypan blue solution, Sigma-Aldrich) counted using a hemocytometer.

The size of all slices was determined using microscopic images with the free selection and area measurement tool of ImageJ. Viable cell numbers per square centimeter have been normalized to the respective tissue-ECM area using the following formulas: Area well (1.13 cm²) = area glass + area ECM. Viable cells per cm² = (viable cells counted in well - (mean number of viable cells of glass-only controls x area glass / area well)) / area ECM. For controls, the following formula was used: Cells per cm² = viable cells counted in well / total area.

To test mESC differentiation, 2x10⁴ cells were seeded and cultivated for 13 days, changing the media on day 2 and 4, followed by daily medium changes from day 5 onwards. After 13 days, cells were either formalin-fixed for immunofluorescent stainings or used for quantitative real-time PCR (qRT-PCR).

2.7. qRT-PCR
Cultivated mESCs were enzymatically detached (Trypsin-EDTA, Sigma-Aldrich) and total RNA was extracted using peqGOLD Total RNA Kit C-Line (Peqlab). The RNA was transcribed to cDNA using QuantiTect Reverse Transcription Kit (Qiagen) which includes a gDNA removal step. Quantitative PCR was performed using QuantiTect SYBR Green PCR Kit (Qiagen) and QuantiTect Primer Assays for Pax2, Pou3f3 and GAPDH (Qiagen) on a LightCycler 1.5. Cycling conditions were chosen as suggested by the QuantiTect Primer Assay handbook (hot start: 15 min. 95°C, denaturation: 15 sec. 94°C, annealing: 20 sec. 55°C, extension: 20 sec. 72°C, 40 cycles). The second derivative maximum method was used to determine Ct values. Relative transcript levels between glass-only control and protocols I, II, III or gelatine control were calculated by the formula 2^(-ΔΔCt), using GAPDH as housekeeping gene to normalize the values: ΔCt = (average Ct of Pax2 or Pou3f3) – (average Ct of GAPDH); ΔΔCt = (average ΔCt of gelatine or protocol I,II,III) – (average ΔCt of glass).

2.8. Statistical analysis
Results are presented as mean +/- SEM (standard error of the mean). One-way ANOVA (analysis of variance) was used test the inequality of test groups. Tukey's HSD (honestly significant difference) test was used as a post-hoc analysis to identify significances between test groups. Significances are presented with asterisks as followed: p
3. Results

3.1. Characterization and comparison of decellularization methods

Three different decellularization protocols have been established to investigate optimal cellular removal parameters, while maintaining an intact kidney-ECM. Protocols include the use of a hypotonic solution to initially lyse cells and SDS solutions to disrupt cell membranes and intercellular adhesions. Furthermore, the osmotic regulator PEG 1000 has been used to study its effect on stabilizing the remaining ECM structures during decellularization processes.

After the initial step of cellular removal via hypotonic solution (including protease inhibitors), increasing concentrations of PEG 1000 (0.5-50%) were tested to stabilize the ECM architecture prior to subsequent protocol steps. Initially, we anticipated to achieve a decellularization effect with PEG 1000, however this was not the case. Hypotonic and SDS solutions successively allowed decellularization of the kidney (traceable as the solutions took on color), whereas all PEG 1000 solutions did not trigger a release of cells from the organ throughout the whole incubation process (they remained clear). Higher concentrations of PEG 1000 (>30%) resulted in rapid (within minutes) kidney shrinkage (Fig. 3A) with predicted damage to the ECM. However, the reduction in tissue size was reversible by subsequent application of a 1% SDS solution for 48h, resulting in a more natural looking kidney matrix (Fig. 3B). Based on preliminary experiments (histological stainings of kidneys decellularized with different concentrations and incubation times of SDS and PEG 1000) we have finally chosen to proceed with a 1% concentrated PEG 1000 solution for the subsequent experiments, which is therefore used in protocol III.
Figure 3. The effect of PEG 1000 on decellularization of murine kidneys.
(A) Kidney halves after treatment with hypotonic solution for 48h following PEG 1000 treatment with different concentrations (0.5-50%) for 70h. (B) Two of the kidneys shown in (A), 1% and 50%, after further treatment with 1% SDS solution for 48h. Scale bar = 2.5mm.

To compare decellularization efficiency and ECM maintenance between the three different protocols, paraffin-embedded tissue slices were analyzed histologically and compared to a native (un-decellularized) kidney (Fig. 4). The native kidney shows a high content of glycosaminoglycans within the glomeruli (Fig. 4A). Distribution of collagen can be seen in the van Gieson staining (Fig. 4B). A high concentration of cell nuclei could be detected within glomeruli (Fig. 4C), as well as laminin, which is a major component of the basement membrane (Fig. 4C, 4D). A very fine-structured hyaluronic acid network could be detected within regions of the medulla (Fig. 4D). Preservation of the ECM differed greatly from protocol to protocol, however none of the protocols could preserve the mentioned hyaluronic acid network (Fig. 4E-4P). Protocol I results in a rather fuzzy matrix without distinct borders of the glomerular, tubular and vascular structures, which appeared to have collapsed or were disrupted (Fig. 4E-4H). It was the only protocol were a larger content of remaining nucleic acids could be detected (Fig. 4H). Protocol II resulted in a matrix with distinct but discontinuous borders of the above mentioned structures, with a lesser content of disruption (Fig. 4I-4L). Protocol III yielded the most intact ECM, preserving most glomerular, tubular and vascular micro-structures with no signs of collapse or disruption (Fig. 4M-4P).

Semi-quantitative analysis of DAPI levels revealed a strong reduction of DNA content for all tested protocols (Fig. 5). The null-hypothesis was rejected (p < 0.001), differences between the native and all three decellularization protocols were significant (p < 0.001 each). Running an Anova test on the three protocols alone, revealed further differences, as the null-hypothesis was rejected (p < 0.01). Post-hoc analysis detected significant differences between protocol I and both other protocols (p < 0.05 each).
3.2. Recellularization of deparaffinized kidney matrices

Following decellularization, paraffin-embedding, sectioning and paraffin removal, kidney slices were applied as advanced substrates for the culture of mESCs. Adherent stem cell culture usually includes gelatine-coated substrates or feeder-layer-dependent conditions (Ulloa-Montoya et al., 2005, -), because of the cells poor attachment to normal tissue culture plastic or glass surfaces. Therefore, the standard substrate gelatine served as a positive control for cell attachment, while glass served as a negative control.
We observed that during media exchanges, cells were more adherent to gelatine or ECM slices than to glass surfaces (Fig. 6A, B). After four days of culture, viable adherent cell numbers per square centimetre were determined (Fig. 6C).
Gelatinized tissue culture plastic, which served as a positive control, had about eleven times more viable adherent cells than glass-only controls ($p < 0.001$). Native kidney slices, that have not been decellularized, have shown very little cell attachment, similar to that of glass controls (no significant difference, $p > 0.99$). All decellularized kidney-ECM slices have demonstrated an improvement in cell attachment compared to glass substrates, but only tissue-substrates that were generated via protocol III were significant ($p < 0.001$). The null-hypothesis was rejected ($p < 0.001$).

3.3. **Differentiation studies on deparaffinized kidney matrices**

Following the attachment studies on decellularized kidney-ECM, the differentiation capacity of mESCs into renal-type cells was analyzed. Cells were allowed to differentiate for 13 days and were either cultured on glass, gelatine, native kidney slices or one of the three differently decellularized matrices (Fig. 7).

**Figure 7. Differentiation of mESCs on deparaffinized matrices.**
(A) Pax2 expression of differentiated mESCs after 13 days of culture on either glass, gelatine, native kidney or decellularized matrices I, II, III as described in the material and methods part. Images show Pax2 (red) and cell nuclei (blue). Right-hand a higher magnification of protocol III is shown, clearly demonstrating nuclear Pax2 expression (a different object glass was used, so the sectional plane may differ slightly from the image on the left). (B) Gene expression levels of Pax2 and Pou3f3 for mESCs grown on gelatine, native kidney or matrices derived from protocol I, II, III as compared to mESCs grown on glass controls after 13 days. The x-axis cuts the y-axis at 1, meaning that bars below 1 indicate a lower expression level as compared to glass while bars greater than 1 indicate higher expression levels. Asterisks (***) indicate significances ($p < 0.001$) as compared to glass controls. Scale bars = 50µm.
Immunofluorescent stainings revealed areas of cells strongly expressing Pax2 only on matrices derived from protocol III (Fig. 7A). Quantitative real time PCR could verify this finding, as gene expression for Pax2 was approximately six times higher (p < 0.001) for cells grown on matrices derived from protocol III as compared to glass-only controls (Fig. 7B). As well as a higher Pax2 expression, there was increased transcription of Pou3f3 (=Brn1) for cells grown on matrices derived from protocol III (~25 times higher than glass-only controls; p <0.001; Fig. 7B). These results suggest that the decellularized ECM as processed by protocol III induces differentiation of mESCs towards renal lineages. No other substrates showed significant renal lineage differentiation. The null-hypothesis was rejected for both data sets (Pax2 and Pou3f3, p < 0.001 each).

4. Discussion
We have shown that slices of formerly paraffin-embedded decellularized ECM can provide signals to influence differentiation of mESCs. Our approach has the potential to be used as a quick and easy test for decellularized ECMs, derived according to different protocols on a larger scale. Cell seeding in this system is simple as is gathering statistically robust data, since a single decellularized organ can be used to produce large numbers of slices to reseed. Many protocols can be tested in parallel, without the necessity of having multiple perfusion systems or bioreactors. These features have great potential in effectively testing (new) chemicals and determining their optimal concentrations for a certain type of tissue, prior to establishing new protocols for three-dimensional perfusion systems. Another more general application can be seen in the use of such ECM slices as differentiation propagating substrates in two-dimensional cell culture or low volume embryonic organ culture, which we have recently developed (Sebinger et al., 2010).

Our approach is suitable for smaller organs such as murine kidneys, but larger organs and tissues would have to be sampled into adequate pieces in order to be decellularized using agitation and diffusion. Sampling should result in specimens of the same size and it may be necessary to further group them depending on the complexity of the tissue, resulting in groups with different ECM composition and/or structures (e.g. grouping in medulla and cortex region). Specimens of such groups would then be analyzed separately. Depending on the chosen size of the specimens, bigger tubes or bottles (e.g. 100ml or 200ml) may be used instead of 50ml tubes.
One question that arose during our study was how the use of a cross-linking agent such as buffered formalin would not mask important cell signaling epitopes of the ECM. Cross-linking is a two-step process: firstly formaldehyde reacts with the ε-amino group of lysine or the β-thiol group of cysteine. In a second, much slower reaction (hours to days), the resulting methylol groups then form methylene bridges (–CH2–) by attacking available nucleophiles (Kunkel et al., 1981). A second type of cross-link can occur between a secondary amine and a carbonyl compound (Sompuram et al., 2004). Such masking processes via cross-links may be intramolecular or intermolecular (including an additional protein). We think that there are several reasons why such cross-linking may play a minor role for the differentiation inducing potential of the ECMs described in this study. Firstly, the fixation time of six hours is not enough to reach complete cross-linking and binding-motifs within the ECM are presumably very common (unlike certain cell proteins that may get easier fully masked because of a low abundance). Secondly, most integrin binding motifs are relatively short (Hynes, 2002) and may therefore by less likely to get masked. Thirdly, due to de-cellularization, the remaining ECM is less dense than the former tissue which correlates to less molecular crowding. This should result in a lesser degree of intermolecular cross-linking of adjacent proteins. Another point is that independently of available cell signaling epitopes, the provided ECM serves as a specific 3D template for newly cell secreted ECM components, which may attach and increase the existing functionality.

It should be considered that seeded cells grew not only in contact with the ECM, but also next to it, directly on the glass slide within the tissue culture well that is bounded by the silicone ring (Fig. 2). Removing such cells prior cell counting / cell lysis or preparing culture wells that are fully covered with ECM may be advantageous, as the results could probably be even more distinct (e.g. levels of gene expression for qRT-PCR). However such optimizations would presume extensive testing in order to establish a reproducible work-flow and would further increase the complexity of the system. Nevertheless we could in this first step successfully demonstrate the potential of our approach in gathering reliable data with relatively little time and effort, thus making too complicating optimizations obsolete.

Protocol I was the only protocol not including nuclease activity of FBS, which has been described previously (Gui et al., 2010). Remaining DNA levels of matrices from this protocol were significantly higher than from protocol II or III (Fig. 5), suggesting that nucleases of FBS were active even at RT. Generally determination of remaining
DNA levels is important for ECM constructs that are used in vivo, as the presence of nucleic acids could result in a proinflammatory reaction of the host innate immune system after implantation. This study demonstrates the potential of formerly paraffin-embedded ECM slices to influence mESCs in vitro, therefore a semi-quantitative evaluation based on histological data is sufficient. For in vivo applications, a sensible test to quantify absolute DNA levels should be performed to guarantee sufficiently low levels of remaining DNA, such as the pico green assay (Gui et al., 2009).

Another difference between protocol I and the other two was the absence of protease inhibitors (Fig. 1). The matrices derived from protocol I showed massive alterations (Fig. 4), which cannot be attributed to the absence of protease inhibitors only, as a harsher SDS treatment was applied in this protocol as well (Fig. 1). Our intention here was to produce a damaged matrix structure to enable a cellular response being less faithful to the origin of the tissue. From our preliminary experiments and observations we conclude that protease inhibitors slow down the decellularization process, as endogenous proteases become inactive. Inhibiting nuclease activity may better preserve ECM micro-structures, but create a need for prolonged SDS treatment. This is undesired, as SDS alters the matrix which effects cell repopulation negatively (Gratzer et al., 2006).

While developing our decellularization protocols, we were not able to decellularize murine kidneys using PEG 1000 without combining it with SDS (Fig. 3; additional data not shown). However, this procedure has been reported to be effective for cardiovascular grafts (Uchimura et al., 2003) and porcine valves (Ota et al., 2007), where high concentrated PEG 1000 solutions were used (1g/ml (w/v) and 80% PEG 1000). Reasons could be structural and compositional differences of the tissues, and the fact that we did not use a glass rod for pressing the tissue as described for cardiovascular grafts or gamma irradiation as described for porcine valves. Nevertheless, we found that including a PEG 1000 step before SDS treatment (protocol III) resulted in better matrix composition (Fig. 4), which coincides with increased cell attachment (Fig. 6). These results suggest, that the use of PEG 1000 has the potential to stabilize ECM structures during decellularization processes, which could motivate further investigations. Based on our data we can only speculate upon possible mechanisms. Within protocol III the tissue is loaded with PEG molecules when the 1% SDS solution is introduced (Fig. 1). Two effects might be important: firstly a possible membrane destabilizing effect of PEG (Uchimura et al., 2003), and secondly, noncovalent intermolecular
interaction of PEG molecules with components of the ECM (Zobnina et al., 2012; Michel et al., 2005). The first effect might speed up the lysis of remaining cells, thus SDS molecules could interact with cytoplasmatic content quicker as compared to protocol II were no PEG was used. This quicker cytoplasma-SDS-interaction translates to less SDS molecules interacting with the ECM. The second effect could possibly further reduce interactions of SDS with the ECM and may stabilize the ECM during the rather short period (2h) of 1% SDS incubation.

In addition, we could clearly show that the matrix obtained by protocol III was able to influence mESC differentiation towards renal linages, as gene expression levels for Pax2 and Pou3f3 have been significantly higher as compared to the other matrices and controls (Fig. 7).

In conclusion, we developed a quick and easy method to compare decellularization protocols combining the use of paraffin slices and two-dimensional cell culture. The method holds great potential to discover and validate optimized protocols for three-dimensional cell reseeding experiments, but can also be used in conventional two-dimensional cell culture or embryonic organ culture.

**Ethics statement**

All organ explantations were conducted in a licensed animal facility (Max Planck Institute of Molecular Cell Biology and Genetics, Dresden) in accordance with the German Animal Welfare Act, following the guidelines of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes and approved by the regional council. No actual in vivo experiments were performed.

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**Author contributions:**

Andreas Ofenbauer and David Sebinger conceived, designed and performed the experiments.

Andreas Ofenbauer analyzed the data and wrote the paper.

Marina Prewitz and Petra Gruber gave substantial input in arranging the figures and presenting the data.

David Sebinger and Carsten Werner supervised Andreas Ofenbauer.

All Authors have revised the paper critically.

**Disclosure statement**

No competing financial interests exist.

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Stem Cell Technologies 2010, Maintenance of mESCs & miPSCs Using ES-Cult®.


7.4 Publications and scientific contributions

PUBLICATIONS:


PUBLICATIONS IN PROGRESS:
David D. R. Sebinger, Andreas Ofenbauer, Petra Gruber, Susann Malik, Carsten Werner. “ECM modulated early kidney development in embryonic organ culture.” Manuscript in preparation for submission in Biomaterials. (Accepted in May 2013)

CONFERENCE CONTRIBUTIONS:


Invited speaker at “The Stem Cell and the Kidney” Meeting in Genoa, Italy (Jun. 2010): David D. R. Sebinger, Andreas Ofenbauer, Mathieu Unbekandt, Mikhail Tsurkan, Jamie A. Davies & Carsten Werner. “Novel Insights in the Modulation of
Murine Embryonic Kidney Development using Interactions with Biofunctional Matrices.”


Participant at the DGM Symposium about Organ Culture. Potsdam, Germany (Sep. 2009)


Invited speaker at the Annual meeting of the KIDSTEM Project in Bergamo, Italy (Dez. 2007): David D. R. Sebinger. “Promoting Differentiation and Organisation of Stem Cells into functional Nephrons by Designing synthetic Scaffolds.”

Participant at the annual I.M.P. Recess, 1030 Vienna, Austria (1st-3rd Oct. 2003).

SCHOLARSHIPS AND AWARDS:
CRTD seed grant fellowship (fund 043_2615 E2) in the scope of the “Embryonic liver organ culture as a pre-clinical model system to bridge in vitro and in vivo liver studies” objective (since Jan. 2012).


Marie Curie PhD fellowship granted by the European Union as part of the Framework program 6 (FP6 036097-2) “KIDSTEM Research Training Network” (Apr. 2007 – Sep. 2010).

PATENTS AND UTILITY PATENTS:
Utility patent on the Cultivation of Embryonic kidneys with our novel organ culture system (Nov. 2010)
„Gebrauchsmuster Nr.: 202010015310.7“

Patent application for a universal organ/tissue culture system (since 2008)
„Nr.: DE102010043555A1 - Offenlegungsschrift 10909/DE“

EDUCATIONAL SKILLS:
Supervision of the master thesis of Andreas Ofenbauer (Sept. 2009 - May 2011)

Supervision of the practical work of the student trainee Andreas Ofenbauer (Aug. - Sept. 2008)

Organization of the KIDSTEM workshop in Dresden for international participants (May. – Jun. 2008)

COMMUNICATION AND PUBLIC WORK:
TV appearances (ZDF and MDR) within the scope of a science program introducing and explaining current scientific work to the German public (Jun. & Jul. 2010).

A submitted image has been chosen as front cover picture for the brochure “Regenerative Medicine in Germany”, biotechnologie.de, on behalf of the German Ministry of Education and Research (2010).

A submitted image has been chosen as an exhibit during the traveling exhibition “Science is beautiful”. The exhibition introduced scientific imaging to the public all across Germany, closing at the Japanese Palace in Dresden. (2008).
COLLABORATION CONTRIBUTIONS and SCIENTIFIC COMMUNICATION:

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With group of Mike Karl, MTZ, Dresden, Germany (Retina and embryonic eye culture).

With ETH Zurich, Switzerland (Combination of long term organ culture with gene silencing techniques).

With the group of Prof. Schedl, Marseille, France (Sternum culture).
7.5 Selbstständigkeitserklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe; die aus fremden Quellen direkt oder indirekt übernommenen Gedanken sind als solche kenntlich gemacht. Die Arbeit wurde bisher weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

Die vorliegende Dissertation wurde am Leibniz-Institut für Polymerforschung Dresden e.V. in der Arbeitsgruppe Biofunktionelle Polymermaterialien am Max Bergmann Zentrum für Biomaterialien unter der Betreuung von Herrn Prof. Dr. Carsten Werner angefertigt.

Bei der Auswahl und Auswertung des Materials sowie bei der Herstellung des Manuskripts habe ich Unterstützungsleistungen von folgenden Personen erhalten:

- Prof. Dr. Carsten Werner
- Prof. Jamie A. Davies, Ph.D.
- Andreas Ofenbauer
- Dr. Uwe Freudenberg
- Prof. Dr. Tilo Pompe


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