From Minu3D cultural system to aldosterone-induced tubulogenesis***☆◆

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Abstract: The existing technical disadvantages of culture systems so far developed have limited the molecular biological study of tubulogenesis. Recently, a newly developed Minu3D system creates controllable growth conditions and allows the generation of tubules from renal stem/progenitor cells. It is for the first time indicated that aldosterone is able to induce the formation of tubules. Aldosterone may trigger some of mineralocorticoid receptor-mediated signaling pathways for the initiation and the regulation of the tubulogenesis. The implication of these studies toward unveiling tubulogenesis is discussed.

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INTRODUCTION

In the growing mammalian embryonic kidney, ureteric bud (UB) that extends out from a pre-existing epithelial tube, gives rise to the branched collecting duct system while the surrounding metanephric mesenchyme undergoes mesenchymal-epithelial transition to form the proximal parts of the nephron. Tubulogenesis, a process by which disorganised mesenchymal cells become a highly organised epithelial tubule, can be summarily divided into four developmental stages [1]. ①Condensation, in which groups of undifferentiated mesenchymal cells condense tightly together to form a distinct mass; ② Epithelisation, in which condensed cells lose their mesenchymal characteristics and gain epithelial ones. At the end of this period they have formed a small epithelial cyst complete with a basement membrane, cell-cell tight junctions and a defined apico-basal polarity; ③Early morphogenesis, in which the cyst invaginates twice to form a comma and then an S-shaped body, one of these invagination sites later becoming the glomerular cleft. ④Tubule maturation, in which the specialized transporting segments of nephron differentiate. In this stage, tubules grow and extend in a straightforward, a convoluting or a branching manner to form the three-dimensional structures.

Observing the events of tubulogenesis within the growing kidney, one has to admit that despite intense investigations in the past decades, little information on the underlying mechanism is available. A major obstacle is the absence of a suitable in vitro culture model, upon which the absolutely controllable conditions are created to ensure not only the formation of tubules but also the maintenance of highly differentiated structures over a relatively long cultural period so that one can investigate the progression of tubule development. The present review focuses on two aspects: ①experimental systems now already available to analyze the tubulogenesis and their existing technical limitations. An innovative culture technique is recently established by us [2-4] and its advantages are briefly introduced. ②Applying this unique *in vitro* culture model, it was for the first time indicated that the steroid hormone aldosterone exerts a significantly inductive action on the tubular development of renal stem/progenitor cells [5-8]. Basing upon the so far achieved data, the author described the process of the aldosterone-mediated action and discussed the implication of these studies toward unveiling tubologenesis.

DISADVANTAGES OF CULTURE SYSTEMS SO FAR DEVELOPED TO STUDY TUBULOGENESIS

To study tubulogenesis of metanephros, genetically manipulated mice are proven to be a useful *in vivo* model. However, how a particular phenotype associates with action of a specific gene is a complex event because of influence of environment. Some routine *in vitro* biochemical approaches to access molecular mechanisms become to be infeasible in the whole animal.

Embryonic kidney organ culture has been one of the often applied approaches for many years [9]. A piece of embryonic tissue directly dissected from animal embryos is isolated and cultured by an *in vitro* transfilter experiment. However, lifespan and developmental capacity of the isolated tissue utilizing this kind of culture technique look limited. Liking as the situation seen in animal experiment, complexity of tubulogenesis hampers the detailed analysis of the happening process. In addition, the delivery of reagents to a specific structure is difficult in many cases. An alternative approach later broadly used is the culture of renal cells at bottom of a culture dish or on a filter. However, the spreading of cells in serum-containing medium results in a flat outgrowth [10].

An exciting technical improvement is the introduction of 3D culture [2, 11]. Embedding of certain kidney-derived cells in extracellular matrix (ECM) allows the generation of tubules. The used cells include isolated renal cells [12] and various kinds of cell lines such as Madin-Darby canine kidney (MDCK), murine inner medullary collecting duct 3 (miMCD3) and UB cells [13-15]. However, all these in vitro systems have several obvious disadvantages for studying tubulogenesis: *e.g.*, the application of serum-containing media, which contain plenty of

unidentified compounds that may interfere with the performed studies, the limited amount of materials available for biochemical analysis and the low degree of nephron-specific differentiation.

MINU3D: A PROMISING TOOL

Our aim is to construct a more effective *in vitro* cultural model aiming at minimizing or eliminating disadvantages that so far available culture systems have. Recently, Minuth's group developed an innovative Minu3D system through which a perfusion culture container is introduced [2-3]. No ECM is coated in the culture container. Instead, renal stem/progenitor cells are mounted at the interface of artificial interstitium made of polyester. Only chemically defined IMDM medium is applied. Therefore, a totally controllable growth environment is established. In addition, due to the limited size of embryonic mouse or rat kidneys, the neonatal rabbits are selected as study materials since the embryonic cortex contains significantly more stem cell niches in their original extracellular environment. Thus, the embryonic tissue layer is easily accessible for isolation and can be harvested in sufficient amounts for tissue culture or cell biological investigation.

ALDOSTERONE-INDUCED TUBULOGENIC ACTION: A REVOLUTIONARY FINDING

Applying this innovative *in vitro* perfusion culture system, embryonic renal tissue that is isolated from neonatal rabbit kidney by microdissection and without enzymatic disintegration is exposed to the interface of an artificial interstitium. Over a 14-day culture period the fresh, chemically defined Iscove's Modified Dulbecco's Medium (IMDM, I) supplemented with 50 mmol/L HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, HEPES) is constantly perfused (IH). To get new insight into the process of the development of tubules, multiple growth factors and hormones described in literatures to promote tubulogenesis were tested. Surprisingly, none of them induced recognizable development. During the further experiments it was discovered that the steroid hormone aldosterone exerts a clearly tubulogenic action on the development of renal stem/progenitor cells. The developed tubules are able to be recognized by immunohistochemical label with soybean agglutinin (SBA) and epithelial cell-specific antibodies. The newly generated tubules exhibited polar differentiation with a continuously developed basal lamina consisting of a lamina rara interna, lamina densa, and lamina rara externa. Collagen type III was found to be the linking molecule between the basal lamina and the surrounding polyester fibers [8, 16]. The effect of aldosterone is shown to be specific since the antagonists of aldosterone such as spirolactone and canrenoate abolish the tubular development but the glucocorticoid hormone dexamethasone does not demonstrate any comparable tubulogenic action [5-6]. The subsequent results indicated that the mineralocorticoid receptor (MCR) is involved in the developmental process [17-18]. MCR belongs to a member of steroid receptor family and the Aldosterone-MCR complex regulates electrolyte and water homeostasis in adult kidney [19-21]. Interestingly, in comparison with its counterpart in adult renal tissues, two isoforms of MCR with slightly different molecular weights were identified and they exist only in embryonic period [18].

How both of MCR isoforms are involved in tubulogenesis is so far still speculatively. However, the morphological induction actions of receptor-mediated steroid hormones during the embryonic phase in different tissues are increasingly reported. Recently, Fujioka's group studied the morphological development of hippocampal neurons in prenatal rats. MCR is found to stimulate the process of neurogenesis, whereas GCR (glucocorticoid receptor) is involved in the suppression of neuronal differentiation [22]. The experiment by another group demonstrated that both MCR and GCR are able to be detected in the circulation from the 10th day of embryonic development [23]. They induce and regulate the differentiation of growth hormone cells in the chicken embryos. Furthermore, blocking MCR by the antagonist ZK 91587 leads to significant inhibition of organogenesis in cultured rat embryos [24]. The pronounced adverse effects on the total length, the somite number and the embryo vascularization are observed in a ZK 91587 dose-dependent fashion.

SIGNALING PATHWAYS INVOLVED IN INITIATION AND REGULATION OF TUBULOGENESIS

A central question is how to understand that aldosterone can induce the tubulogenesis. It was noticed that aldosterone exerts its pleiotropic actions by influencing expression of a broad pool of genes or by a non-genomic way. In fact, aldosterone-mediated signaling is quite complex with much convergence and divergence onto key signaling factors that program the intended cellular response. Furthermore, during the tubulogenesis of a growing mammalian embryo the mesenchymal-to-epithelial transition and the tubular organization are driven by a series of regulatory factors that act in a cooperative fashion to stimulate or suppress tubule formation. Among the growing list of such molecules are the members of growth factors, transcription factors, kinases and regulatory proteins (Table 1). It appears that the balance between stimulating growth factors such as hepatocyte growth factor (HGF), epidermal growth factor (EGF), EGF receptor ligands (EGFR) and negative regulators such as transforming growth factor-β (TGF-β) contributes to control the exact tubule development. Impressively, intracellular signaling pathways mediated by phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) are required not only for tubulogenesis in embryos but also for aldosterone-induced electrolyte transport in adult kidneys.

In addition, the abnormal modulation of PI3K, MAPK pathways leads to the disease-related tissue remodelling. Thus, it is reasonable to assume that aldosterone may trigger some common signal transduction pathways that are responsible for cellular growth, differentiation and tubule formation (Figure 1). In the different developmental phases, it seems that the different isoforms of MCR bind aldosterone to form a functionally distinct complex.

Table 1 The components so far reported involved in the signaling pathways of tubulogenesis

Signaling components	Functions during tubular formation	References
Growth factors and their receptors		
Hepatocyte growth factor (HGF)	+	[25-26]
Epidermal growth factor (EGF)	+	[27]
EGF receptor ligands (EGFR)	+	[27-28]
Transforming growth factor-α (TGF-α)	+	[28]
Glial-derived neurotrophic factor (GDNF)	+	[29]
Bone morphogenetic protein 7 (BMP7)	+	[30]
Insulin-like growth factor (IGF)	+	[31]
Transforming growth factor-β (TGF-β)	–	[32]
Bone morphogenetic protein 2 (BMP2)	–	[30]
Transcription factors		
Wnt family	+	[33]
Lim1	+	[34-35]
Pax2/8	+	[36]
Odd1	+	[37]
Protein Kinases		
PI3k	+	[38-39]
MAPK	+	[39-40]
Extracellular signal-related kinase (ERK)	+	[40]
Ras	+	[38]
EphA	–	[41]
Regulatory proteins		
integrins	+	[42]
E-cadherin and ZO-1	+	[43]
β-catenin	+	[44]
Matrix metalloproteinase (MMP) family	+	[45-46]
Collagen Ⅰ, Ⅲ	+	[16, 47-48]
Fibronectin	+	[47]
Collagen Ⅳ	–	[47]
Vitronectin	–	[47]

+: positive stimulators; –: negative regulators

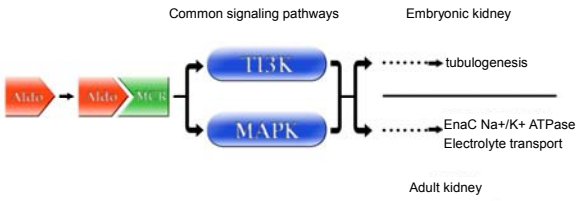

Figure 1 MCR-aldosterone complex activates some of common signaling transduction pathways such as TI3K and MAPK. The consequences of these signaling cascades may be either triggering tubulogenesis in embryonic kideneys or stimulating electrolyte transport in adult kidneys by up-regulation of expression of epithelial sodium channel and Na^+/K^+ ATPase

Recently, Kellner *et al*. applied DNA microarray to analyse mRNA expression profile of human renal epithelial cells in response to aldosterone [49]. Among altered expression of all 1 430 genes following aldosterone treatment, significant up-regulation of *sgk*, *p*21/*waf*1, *gadd*45 and *gadd*153 was found within 1 hour while long-term treatment (> 4 hours) with aldosterone induced the mRNA *de novo* expression of transcription factors *ppara* and *pura*. Moreover, transcription factor *gadd*153 is reported to be involved in cell growth and differentiation and can be phosphorylated by MAPK pathway, leading to expression of a series of cytoskeletal structural proteins [50].

However, although some data have been provided in the past, numerous investigations remain to be performed so that we understand the implication of differential expression of these genes and begin to construct an elaborate signaling network involved in the initiation and regulation of the tubulogenesis.

DISCUSSION AND PROSPECTIVES

Then, how to explain that none of the growth factors, that have been already reported to promote tubulogenesis by applying other culture systems, is able to induce tubular formation in our Minu3D model? Recently, aldosterone synthase knockout mice were produced by Gomez *et al*. Despite abnormal electrolyte homeostasis and decreased blood pressure these mice have grossly normal kidney development and are still viable [51]. In fact, in many of the single growth factor knockout mice a defective kidney phenotype fails to demonstrate [52-53]. It is yet unknown whether all these regulatory factors are simultaneously indispensable to trigger tubulogenesis *in vivo*. It is most likely that different growth factors are needed in different developmental periods. We noticed differential expression profile of such factors. For example, transforming growth factor-α (TGF-α) is expressed in the embryonic kidney whereas EGF is expressed in the kidney mainly after birth. In addition, most of growth factors express in redundancy in developing tissues. Thus, compensatory pathways may exist *in vivo* in the case that a single factor lacks.

In our experiments, aldosterone-induced tubules can be seen only after the 5th culture day [6]. The applied embryonic explants are a mixture of different cell populations. During such a long culture period, it does not yet exclude the possibility whether other endogenous factors, which may be already over-expressed in neonatal kidneys, play a potential role to facilitate tubulogenesis as well in concert with aldosterone. Currently, by administration of aldosterone combined with different growth factors, we are searching for the possible candidates that are likely to improve or accelerate the action of aldosterone.

Finally, the innovative Minu3D model allows us under absolutely controllable culture conditions to investigate the progression of tubulogenesis in different developmental phases. To understand the specific subcellular process, numerous cell biological and molecular biological approaches such as immunohistochemistry, real-time PCR, 2D western blotting and proteomics are being employed for analysis. Among them, antibody microarray may provide a promising tool to find out key signaling molecules/regulatory proteins for insights into the underlying mechanisms of tubulogenesis.

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Minu3D 培养系统中醛固酮诱导肾小管发生的意义***☆◆

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摘要：迄今已有的细胞培养系统所存在的技术缺陷限制了对肾小管发生的分子生物学研究。最近，作者创建的 Minu3D 系统能产生完全可控制的生长条件，在此基础上，肾干细胞/祖先细胞能发育成结构清晰的肾小管。研究首次显示醛固酮能诱导形成肾小管。醛固酮可能通过启动某些盐皮质激素受体介导的胞内信号通路，从而启动和调节肾小管发生。该文讨论了近年来该领域的一些最新研究工作在揭示肾小管发生机制方面的意义。

关键词：醛固酮；肾小管发生；干细胞/祖先细胞；盐皮质激素受体
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作者对文章内容的延伸说明与观点--

现有肾小管培养系统的缺陷：

基因小鼠已被认可作为一种体内模型用于肾小管生成的研究。然而，由于环境的影响，通过一些常规的体外生化方法来获得的分子机制不太可行。

胚胎肾器官培养是近年来常采用的方法，但其分离组织的生命周期和发育能力受到了限制。如同动物实验中所见，肾小管的复杂性阻碍了对其发生过程的详细分析。另外，传送试剂到某一特定结构在大多数时候都不能完成。另一种被广泛应用的方法就是培养皿或过滤器中培养肾细胞。除此以外，一种三维培养技术取得成功。细胞外基质嵌入某些肾脏细胞能够生成肾小管。这些细胞包括分离的肾细胞和多种细胞系，如 MDCK、miMCD3 和输尿管芽细胞。但是这些系统存在明显的不足。

Minu3D：一种行之有效的方法。

作者的目的是建立一个更有效的体外培养模型，旨在最大限度地减少或消除以往培养系统的不足。近期，Miuth 团队研制出一种新型 Minu3D 系统，将连续灌注培养引入肾组织片体外培养研究中。

醛固酮诱导肾小管生成：一个革命性的发现。

运用这一创新的体外灌注培养系统，分离的胚胎肝组织与人工脉管结合，培养 14 d 后，用含 HEPES 的新鲜合成培养基 Iscove's Modified Dulbeccos Medium (IMDM)连续灌注培养。研究从一个新视角提示多种生长因子和激素能够促进肾小管生成。令人惊讶的是，任何因子或激素都没有诱发明显的肾小管发育。在进一步的试验中，醛固酮对诱导肾干细胞/祖细胞分化成肾小管起到明显的作用。采用免疫组化法 SBA 和上皮特异性抗体标记 生成的肾小管。新生成的肾小管呈现极分化，伴随持续性的基底膜发育，包括内透明板、致密板和外透明板。实验证明Ⅲ型胶原是联系肾小管基底面和人工聚酯纤维的分子纽带。由于醛固酮的拮抗剂螺内酯及坎利盐阻碍肾小管发育，醛固酮被认为在肾小管生成中具有特异性作用。但是糖皮质激素地塞米松并未显示形似的促肾小管发育的作用。研究结果提示盐皮质激素受体(MCR)，类固醇受体的一员，参与了肾小管发育过程。醛固酮与 MCR 的复合物能够调节成人肾组织中的电解质的体内平衡。有趣的是，与醛固酮相比较，MCR 的两种亚型通过些微分子质量进行辨别，并且他们尽存于胚胎时期。

是否 MRC 的亚型都参与肾小管的生成还存在争议。然而，在胚胎阶段的不同组织中受体介导的类固醇激素的相关报道越来越多。

肾小管发生开始与调节的信号通道：

事实上，醛固酮介导的信号相当复杂。此外，在肾小管发育过程中，间充质到上皮的转化及管状结构形成是由一系列调控因子合作完成的，这些因子促进或抑制肾小管的生成，包括生长因子、转录因子、酶及调控因子。

刺激生长因子 如 HGF,EGF,EGFR 与阴性调节因子如 TGF-β 间存在一种平衡。PI3K 和 MAPK 介导的细胞内信号通路不仅对胚胎肾小管生成起到作用，而且对醛固酮诱导成人肾脏电解质转运也具有一定意义。另外，PI3K, MAPK pathways 的异常调节作用能够引起疾病相关的组织重建。因此，醛固酮可能引发一些共同的信号转导通路，作用于细胞生长、分化及肾小管形成。

Kellner 等近来运用 DNA 微阵列技术分析对应醛固酮的人肾上皮细胞 mRNA 表达谱。接受醛固酮治疗后的所有表达改变的 1430 个基因中，sgk, p21/waf1, gadd45 和 gadd153 在 1 h 内表达显著上升，而转录因子 ppara 和 pura 治疗 4 h 后 mRNA 表达从头开始。而且，转录因子 gadd153 被报道参与细胞生长分化，能够通过 MAPK 通路被磷酸化，导致一系列细胞骨架结构蛋白的表达。

尽管过去已有了一些研究成果，但仍然要进行大量的实验研究，以使很好地了解这些基因的差异表达的意义，并且构建一个与肾小管生成开始及调节的信号网络

展望：醛固酮诱导肾小管形成，是单因子诱导作用还是多因子协同作用？

近日，Gomez A.等成功研制了醛固酮合成酶基因敲除小鼠。尽管在异常的电解质平衡及低血压的影响，这些小鼠大体上还是具有正常的肾发育。事实上，许多单生长因子基因敲除小鼠并未表现出一个有缺陷的肾型。是否所有的调节因子都对诱导肾小管生成是不可或缺？对这一问题依然未知。最有可能的是，不同的生长因子在不同发育阶段起到作用。另外，大多数生长因子在发育阶段的表达是冗余的。因此，补偿通路可能在体内单一因子缺少的情况下存在。

实验表明，醛固酮诱导生成的肾小管在培养 5 d 后可见。采用的胚胎外植物是由不同的细胞群混合而成。在长期培养过程中，是否有其他在新生儿肾脏过度表达的内源性因子，发挥潜在的作用，协同醛固酮促进肾小管生成？其可能性依未排除。现在，通过醛固酮和不同生长因子结合注射的方式，我们正在寻找有可能改善或加快醛固酮作用的有效方法。

最终，Minu3D 培养系统可以让我们在完全可控制的培养条件下研究肾小管发育的不同进程。在了解具体的细胞亚型过程中，需要大量的细胞生物学、分子生物学技术比如免疫组化、实时 PCR，2D 蛋白质印迹法及蛋白质组学。其中，抗体微阵列为找到关键的信号分子或调节蛋白，进而了解肾小管生成机制提供了有力帮助。