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Redirection of renal mesenchyme to stromal and chondrocytic fates in the presence of TGF-β2

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ABSTRACT

Many members of the transforming growth factor-β (TGF-β) superfamily have been shown to be important regulators of metanephric development. In this study, we characterized the effect of TGF-β2 on metanephric development. Rat and mouse metanephroi cultured in the presence of exogenous TGF-β2 for up to 15 days were small, and contained rudimentary ureteric branches and few glomeruli. These metanephroi were mostly comprised of mesenchymal cells, with two cell populations (designated Type 1 and Type 2 cells) evident. Type 1 cells were only observed when TGF-β2 was added from the commencement of culture, they resembled chondroblasts and were Alcian Blue and Col IIB positive. Type 2 cells were observed whenever TGF-β2 was added to the media, formed a band at the periphery of the explants consisting of 5–10 layers of spindle-shaped cells, and were alpha-smooth muscle actin positive. Molecular and RNA *in situ* hybridization analysis of metanephroi cultured in the presence of TGF-β2 for 6 days demonstrated that Type 1 and 2 cells were negative for Pax2, WT1, GDNF and FoxD1. Gene expression profiling demonstrated an upregulation of chondrocyte, myogenic and stromal genes, some of which were identified as markers of Type 1 and Type 2 cells. In addition, TGF-β2 was capable of maintaining the survival of mouse isolated metanephric mesenchyme (iMM) in the absence of serum or inductive signals from the ureteric epithelium. TGF-β2 also induced the differentiation of iMM into Type 1 and 2 cells. The presence of chondrocytes and muscle in these cultures is reminiscent of the cell types found in some Wilms' tumors. These studies demonstrate that TGF-β2 is capable of differentiating metanephric mesenchyme away from a renal cell fate.

1. Introduction

The permanent kidney (metanephros) is derived from two embryonic precursor tissues, the epithelial ureteric bud (UB) and the metanephric mesenchyme (MM) both of which are derived from intermediate mesoderm. Upon invasion of the UB into the MM reciprocal interactions between these two tissues occur. The MM induces the UB to undergo several generations of branching morphogenesis which gives rise to the collecting ducts, calyces, renal pelvis and ureter. The UB induces a sub-population of MM cells to condense around the ureteric epithelial tips, forming cap mesenchyme. The cap mesenchyme contains nephron progenitors which are capable of self-renewal and also generating all the cell types of the nephron. In addition, the MM contains progenitors giving rise to the renal stroma, smooth muscle cells, and endothelial cells (Clark and Bertram, 1999; Davies and Fisher, 2002; Carroll and McMahon, 2003; Moritz et al., 2008; Kobayashi et al., 2008; Al-Awqati and Oliver, 2002).

In the absence of the UB or key genes expressed in the MM, such as *WT1, Eya1, Odd-1* and *Six 1* the MM is programmed to undergo apoptosis (Kreidberg et al., 1993; James et al., 2006; Xu et al., 1999, 2003). Several key factors secreted by the UB have been identified in the rat to be capable of maintaining the survival of the MM such as fibroblast growth factor 2 (FGF2), transforming growth factor-α (TGF-α) and ELR$^+$ CXC chemokines (Perantoni et al., 1995; Levashova et al., 2007). TGF-α is also expressed during mesonephric and metanephric development in both rats and humans (Bernardini et al., 1996; Bernardini et al., 2001; Carev et al., 2008). In combination with leukaemia inhibitory factor (LIF) and transforming growth factor-β2 (TGF-β2) these factors cooperate to induce nephrogenesis in isolated

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rat MM cultures (Plisov et al., 2001; Karavanova et al., 1996; Barasch et al., 1997).

The importance of TGF-β2 in nephron formation has also been demonstrated in the analysis of kidneys from *TGF-β2* homozygous (*TGF-β2*$^{-/-}$) and heterozygous (*TGF-β2*$^{+/-}$) null mutant mice. Although *TGF-β2*$^{-/-}$ mice present with heart, craniofacial, skeletal, eye, ear, and intestine abnormalities, they also display a range of urogenital abnormalities (Sanford et al., 1997). These include renal agenesis, dilated renal pelvis, dysplastic tubulogenesis, abnormal ureteric branching morphogenesis and reduced nephron number (Sanford et al., 1997; Sims-Lucas et al., 2008). In contrast, *TGF-β2*$^{+/-}$ mice display an increase in ureteric branching and nephron number demonstrating that the dosage of TGF-β2 plays an important role in kidney development and ultimately regulating nephron number (Sims-Lucas et al., 2008).

To further examine the role of TGF-β2 during nephrogenesis we undertook a gain-of-function approach culturing both rat and mouse metanephroi with exogenous TGF-β2. Previous reports from our laboratory and others (Martinez et al., 2001; Ritvos et al., 1995) have demonstrated that TGF-β2 added to metanephroi results in an expansion of the MM and an inhibition of ureteric branching. This current report extends these findings demonstrating that TGF-β2 is capable of inducing the differentiation of at least two distinct cell types derived from the MM. Immunohistochemistry, molecular analysis and gene expression profiling revealed that TGF-β2 induces subsets of cells within the metanephros to undergo differentiation towards chondrocyte and myofibroblast/smooth muscle cell lineages. TGF-β2 alone was capable of maintaining the survival of isolated mouse MM (iMM) in the absence of serum or any other inductive signal. In turn, TGF-β2 was capable of differentiating the iMM predominantly into chondrocyte-like and myofibroblast/smooth muscle-like cells demonstrating that these cell types are indeed derived from the mesenchyme and not dependent on signals from the ureteric epithelium. Interestingly, the presence of cartilage and muscle in these cultures is reminiscent of cell types present in some Wilms' tumors. These findings demonstrate that the MM contains progenitor cells capable of differentiating away from their renal cell fate in the presence of TGF-β2.

2. Methods

2.1. Animals

Time-mated wild-type B6xCBA mice, *Hoxb7/GFP* (B6x CBA), and *BMP4*$^{+/lacZ}$ (129/SvEV × Black Swiss) mice were sacrificed at E11.5 and E12.5 by cervical dislocation. Time-mated Sprague-Dawley rats were sacrificed at E14.5 via intraperitoneal injection of sodium pentobarbitone (5 mg/100 g body weight; Abbott Laboratories, Sydney, Australia). *Hoxb7/GFP* mice were obtained from Dr. Frank Costantini, Columbia University, USA (Srinivas et al., 1999). Mice were housed at Mouseworks, Monash University, Clayton. *BMP4*$^{+/lacZ}$ mice were obtained from Dr Brigid Hogan, Howard Hughes Medical Institute and Department of Cell Biology, Vanderbilt University Medical Center, Nashville, Tennessee, USA (Lawson et al., 1999). *BMP4*$^{+/lacZ}$ mice were housed at Monash Animal Services, Monash University, Clayton. All animal experiments were approved in advance by a Monash University animal ethics committee and were conducted in accordance with the *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes*.

2.2. Metanephric culture

Whole organ culture: whole metanephroi were dissected from E14.5 Sprague-Dawley rat embryos and E12.5 *Hoxb7/GFP* transgenic mouse embryos. Metanephroi were cultured whole on transfilter polycarbonate membranes (Costar, NY, USA) in an incubator at 37 °C with 5% CO_2. Metanephroi were cultured in Dulbecco's Modified Eagle's Medium (DMEM): Ham's F12 liquid medium (Trace Biosciences, Castle Hill, NSW, Australia) including supplements: 5 μg/ml of transferrin (Sigma-Aldrich, Castle Hill, NSW, Australia), 2.5 mM L-glutamine (Trace Bioscience), 100 μg/ml penicillin (Trace Bioscience) and 100 μg/ml streptomycin (Trace Bioscience), with or without recombinant human (rh) TGF-β2 (R & D Systems, MN, USA), 50 ng/ml or a range from 1 to 200 ng/ml. At the completion of the culture period (3, 6, 9, 12 or 15 days) whole metanephroi were either fixed in 10% buffered formalin for histological analysis or RNA was extracted from unfixed fresh tissue for gene expression studies.

iMM culture: whole metanephroi were dissected from E11.5 *Hoxb7/GFP* transgenic mouse embryos. Metanephroi were incubated for 15 min at 37 °C in 0.2% collagenase (Scimar, Templestowe, Victoria, Australia) in 10% FCS (Trace Biosciences) in media. The UB was physically separated from the MM using fine dissection needles. The iMM from several metanephroi was pooled and cultured as for whole explants (above) in media with or without rhTGF-β2. At the completion of the culture period (3, 6 or 9 days) the iMM was fixed in 10% buffered formalin for histological analysis.

2.3. RNA isolation, preparation of cDNA and RT-PCR expression analysis

Total RNA was isolated from cultured whole metanephroi grown in the presence or absence of rhTGF-β2 using a Micro-RNA isolation kit (Qiagen, Clifton Hill, Australia). cDNA was prepared from total RNA using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen Life Technologies, Victoria, Australia). RT-PCR was used to identify the expression of collagen II using the primers–5′-tct cct gcc tcc tcc tgc tc-3′ (sense) and 5′-ctc cat ctc tgc cac ggg gt-3′ (anti-sense) and HPRT 5′-cct gct gga tta cat taa agc act g-3′ (sense) and 5′-gtc aag ggc ata tcc aac aac aaa c-3′ anti-sense. All gene products were electrophoresed in 1% agarose gels and visualized under UV light. All primers were obtained from Invitrogen Life Technologies.

2.4. Histology

Following fixation in 10% buffered formalin for 30 min, cultured whole metanephroi were placed in 70% ethanol then processed for embedding in paraffin wax. 5 μm sections were cut and placed onto polylysine glass slides and incubated at 37 °C overnight for histology and immunohistochemistry. For Alcian Blue staining sections were treated with 3% acetic acid for 2 min, stained with 1% Alcian Blue in 3% acetic acid for 20 min then washed with tap water. Following staining with Alcian Blue, slides were placed into 6% H_2O_2 (in methanol) for 30 min, and then rinsed in H_2O. Slides were counterstained with Nuclear Fast Red and then mounted.

2.5. Immunohistochemistry

For Pax2 and WT1 immunohistochemistry sections were dewaxed through xylene and graded alcohols to water then washed in PBS. Antigen retrieval was performed by boiling the sections in 10 mM sodium citrate (pH 6.0) in a microwave, followed by incubation in 1% Triton-X in PBS for 10 min. Sections were blocked in 10% normal goat serum prior to incubation with rabbit anti-Pax2 (Zymed, CA, USA)(1:50) or mouse anti-WT1 (Dako Corporation, CA, USA) (1:100) for 60 min. Sections were