Uloga čimbenika apoptoze i rasta stanica te intermedijarnih filamenata u ranom razvoju ljudskog bubrega

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POPIS KRATICA

Ki-67	University of Kiel antigen 67
bcl-2	B-cell lymphoma protein 2
p53	protein 53
EGF	epidermal growth factor
TGF-α	transforming growth factor alpha
FGF	fibroblast growth factor
BMP	bone morphogenetic protein
VEGF	vascular endothelial growth factor

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1. UVOD

Ova disertacija temelji se na objedinjenju slijedećih znanstvenih radova:

Dominko Carev, Dragan Krnić, Marijan Saraga, Damir Sapunar, Mirna Saraga-Babić
Role of mitotic, pro-apoptotic and anti-apoptotic factors in human kidney development
Pediatric Nephrology 2006 May; 21(5):627-36.

Dominko Carev, Marijan Saraga, Mirna Saraga-Babić
Expression of intermediate filaments, EGF and TGF-alpha in early human kidney development
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Involvement of FGF and BMP family proteins and VEGF in early human kidney development
Histology and Histopathology (2008) 23: (in press)

1.1. Uvod objedinjenih radova

Tijekom embrionalnog razvoja čovjeka, razvijaju se tri zasebna bubrežna sustava: pronefros, mezonefros i metanefros. Pronefros je nefunkcionalna i prolazna struktura koja nastaje od nefrotoma u vratnom području embrija početkom 4. razvojnog tjedna. Do početka 5. razvojnog tjedna, pronefros gotovo u potpunosti propada.[1,2]

Mezonefros se počinje razvijati kasno u četvrtom razvojnom tjednu iz nefrogenog tračka kaudalno od pronefrosa. Sastoji se od velikog broja bubrežnih mjehurića koji se izdužuju u petlje s-oblika. Lateralni krajevi petlji ulijevaju se u primarni ureter (mezonefrički ili Wolffov kanal), dok medijalni krajevi petlji čine Bowmanovu čahuru oko glomerula oblikujući bubrežna tjelešca. Mezonefros je privremen, ali funkcionalan bubreg, koji postepeno propada između 8. i 16. razvojnog tjedna. Dio mezonefričkih kanalića i Wolffov kanal ostaju kao dijelovi muških spolnih kanalića.[1,2]

Metanefros (trajni ili konačni bubreg), pojavljuje se u 5. razvojnom tjednu kada iz Wolffovog kanala izraste mokraćovodni pupoljak te prodire u metanefrički mezenhim (krstačni dio nefrogenog tračka) inducirajući pojavu bubrežnih mjehurića-osnova nefrona. Postupno se razvijaju zreliji oblici nefrona koji se sastoje od Bowmanove čahure, glomerula, proksimalnih i distalnih zavijenih kanalića te Henleove petlje. Od mokraćovodnog pupoljka nastaju sabirni kanalići, bubrežni vrčevi i bubrežna nakapnica s mokraćovodom.[1,2]

Razvoj sva tri sustava izlučivanja ovisi o tkivnim interakcijama i mnogim čimbenicima transkripcije, rasta, glasničkim molekulama, njihovim receptorima te različitim staničnim proteinima. Različite malformacije bubrega kao što su odsustvo razvoja bubrega, poremećaji epitelne transformacije mezenhima i smanjenje broja nefrona, mogu se u transgeničnih životinja povezati s pojedinim razvojnim čimbenicima.[3,4]

Raspored i intenzitet programirane stanične smrti (apoptoze) i stanične proliferacije važan je i usklađen proces u nefrogenezi sisavaca. Apoptoza ima važnu ulogu u razvoju i regresiji embrionalnih bubrega jer se njom uklanjaju nepotrebne i suvišne stanice i strukture, te pronefros i najveći dio mezonefrosa, a odvija se po preciznom vremenskom i prostornom rasporedu.[5-7]

Apoptoza se može dokazati praćenjem enzima iz obitelji kaspaza, preko čije kaskadne aktivacije sam proces apoptoze i započinje.[8,9] Kaspaza-3 je ključna efektorska kaspaza jer je ishodište i vanjskog (*receptorskog*) i unutrašnjeg (*mitohondrijskog*) puta aktivacije.[10,11] U kulturi metanefrosa iz mišjih embrija, pokazano je da blokada aktivnosti kaspaze-3 sa Z-D-CH_2DCB sprječava grananje mokraćovodnog pupoljka i daljni razvoj metanefrosa.[12]

Bcl-2 je bjelančevina u citoplazmatskim membranama čija povećana prisutnost štiti stanice od apoptoze,[13,14] dok u *knockout* miševa bez bjelančevine bcl-2 dolazi do hipoplazije bubrega, smanjenog broja nefrona i do formiranja bubrežnih cista.[13,15-18] U ljudskim embrijima, bcl-2 pozitivne stanice nađene su u metanefrosu najranije u 5. razvojnom tjednu, a broj im se povećava tijekom daljnje diferencijacije. U derivatima mokraćovodnog pupoljka ljudskog embrionalnog metanefrosa, bcl-2 pozitivne stanice uglavnom se ne nalaze.[14,19-23]

Ki-67 je bjelančevina jezgre koja se koristi kao pokazatelj stanične poliferacije, budući da nije izražena u fazi mirovanja stanice (G0).[24,25] U nefrogenezi čovjeka, najjači izražaj gena za Ki-67 primjećen je u ranim stadijima razvoja glomerula u fetalnom

metanefrosu (starosti od 9 do 15 tjedana), dok se sazrijevanjem glomerula smanjivao broj stanica pozitivnih na Ki-67.[26]

Bjelančevina p53 sprječava prijelaz iz G1 u S fazu staničnog ciklusa. U slučaju oštećenja DNK, inducira prekid staničnog ciklusa ili apoptozu u toj stanici.[13] U mišjih transgeničnih embrija, zbog pojačanog izražaja gena za p53, bubrezi su manji s hipertrofičnim nefronima.[27] Bjelančevina p53 je u ljudskim embrijima imunohistokemijskim bojanjem dokazana u mezonefrosu, gdje vjerojatno upravlja uklanjanjem stanica sa oštećenjem DNK i tako sudjeluje u procesu regresije kanalića.[13,20] U ljudskom embrionalnom i fetalnom metanefrosu, p53 bi mogao sudjelovati u morfogenezi nefrona i odvodnih kanalića i to također uklanjanjem stanica s oštećenom DNK.[20,28]

Morfološko i funkcionalno sazrijevanje pojedinih dijelova bubrežnih sustava u ljudskom embrionalnom i fetalnom razvoju praćeno je pojavom i nestankom pojedinih intermedijarnih filamenata u stanicama, prvenstveno citokeratina (karakterističan za epitelne stanice) i vimentina (mezenhimske, tj. vezivne stanice).[29-34] Tijekom razvoja, intermedijarni filamenti ukazuju na različite puteve diferencijacije mezenhima u smjeru nefrona (epitela) s jedne strane ili intersticija (vezivnih stanica) s druge strane. Citokeratini su u stanicama prisutni kao parovi citokeratina tipa 1 i tipa 2. U ranom embrionalnom razvoju prvi se javlja par CK8(tip 2)/CK18(tip 1), kasnije prisutan u jednostavnim epitelima. CK19 je citokeratin tipa 1 koji se također javlja sa CK8 u jednostavnim epitelima odraslih jedinki, kao i u epitelnim stanicama ljudskih fetalnih mezonefrosa i metanefrosa, gdje je često prisutan i vimentin, karakterističan za stanice neorganiziranog mezenhima.[29,31-37]

Transformirajući čimbenik rasta alfa (TGF-α) pripada obitelji epidermalnog čimbenika rasta (EGF), a oba se čimbenika vežu na isti receptor.[38,39] TGF-α je nađen u mezonefrosu i metanefrosu pilića,[40] štakora,[41,42] kao i u fetalnom metanefrosu čovjeka.[43] Oba čimbenika *in vitro*, a osobito TGF-α, mogu potaknuti grananje odvodnog sustava metanefrosa, dok blokada njihovog receptora u kulturi ljudskog metanefrosa dovodi do smanjenog stvaranja DNK.[39,44,45] U mezonefrosu i metanefrosu ljudskog embrija nađena je različita raspodjela EGF-a, TGF-α i EGF/TGF receptora. Pretpostavlja se da u okviru uzajamnih indukcija tijekom razvoja odvodnog sustava i nefrona u ljudskom metanefrosu, TGF-α i EGF, nađeni u stanicama nefrona i glomerula, djeluju na EGF/TGF receptor nađen na derivatima mokraćovodnog pupoljka.[38,41]

Fibroblastni čimbenici rasta (FGF) su molekule za koje se smatra kako sudjeluju u proliferaciji, migraciji i diferencijaciji stanica tijekom razvoja sisavaca.[46-48] FGF-8 se u istraživanjima uglavnom povezuje s razvojem udova u miševa i pilića,[49,50] a njegov izražaj je dokazan i u glomerulima embrionalnog bubrega miša.[51] Grieshammer i sur.[52] pokazali su da kod djelomičnog i potpunog nedostatka FGF-8 dolazi do poremećenog stvaranja nefrona. FGF-10 potiče grananje mokraćovodnog pupoljka izoliranog iz embrija štakora,[53] a miševi bez izražaja gena za FGF-10 imaju manje i displastične bubrege.[47] U literaturi nema podataka o istraživanjima ovih čimbenika na mesonefrosu i metanefrosu čovjeka.

Obitelj koštanih morfogenetskih bjelančevina (BMP) pripada obitelji transformirajućih čimbenika rasta β (TGF- β) i sudjeluje u procesima proliferacije i programirane smrti stanica u kralježnjaka.[54,55] Članovi te obitelji pokazuju dinamičan prostorni i vremenski slijed genskog izražaja u razvoju mišjeg bubrega i mokraćnih puteva, osobito BMP-2, BMP-4 i BMP-7 [54,56-61], dok u miševa bez ili sa djelomičnim izražajima gena za navedene čimbenike, postnatalno dolazi do različitih poremećaja građe bubrega.[55,62,63] Tijekom razvoja bubrega u miša, BMP-7 sprječava apoptozu u metanefričkom mezenhimu i nastanak odvodnih kanalića, te na taj način osigurava prisutnost nediferenciranih mezenhimskih stanica nužnih za daljnji rast bubrega.[56,58,59,63,64] Do sada su provedena istraživanja samo o ulozi BMP-7 u razvoju ljudskog bubrega, iako su Marshall i sur.[65] pronašli i BMP-4 u ljudskom mezonefrosu, povezujući ga s hematopoezom. Glasnička RNK za BMP-7 nađena je u metanefričkom mezenhimu oko mokraćovodnog pupoljka ljudskog zametka u 6. razvojnom tjednu, dok se do 14. razvojnog tjedna nalazi uglavnom u glomerulima. Suprotno tome, izražaj bjelančevine BMP-7 u tom je razdoblju najjači u zavijenim kanalićima, što bi upućivalo na otpuštanje BMP-7 iz glomerula i njegovo nakupljanje u bazalnim membranama epitela kanalića.[66,67]

Vaskularni endotelni čimbenik rasta (VEGF) ključan je u grananju krvnih žila jer potiče pupanje novih kapilara iz postojećih krvnih žila. U mišjih embrija bez jednog ili oba alela ovog gena, dolazi do poremećenog razvoja krvne mreže i smrti.[68-70] U kulturi metanefrosa štakora, dokazano je da VEGF potiče diferencijaciju endotelnih stanica, oblikovanje kapilara, kao i proliferaciju epitela bubrežnih kanalića.[71,72] U ljudskim zamecima, izražaj gena za VEGF (mRNK i/ili protein) pronađen je u mezonefrosu i metanefrosu, i to u glomerulima, nefronima i sabirnim kanalićima, dok je glasnička RNK njegovog receptora pronađena u glomerulima i u endotelu krvnih žila u

blizini i nefrona i sabirnih kanalića. To bi upućivalo na zaključak kako strukture bubrega utječu na razvoj pripadajuće im kapilarne mreže.[73-75]

Do sada su rađena brojna istraživanja na embrijima i fetusima pokusnih životinja, kojih je cilj bila analiza rasporeda i izražaja čimbenika proliferacije i apoptoze u stanicama bubrega, te ostalih čimbenika koji utječu na razvoj bubrega.[8-18,54-63,66,67] Određen broj istraživanja proveden je na ljudskom fetalnom tkivu,[19,21,26,28,31,33,34,39,43,73-75] dok je broj istraživanja na embrionalnim bubrezima čovjeka malen.[13,14,20,21,28,38,41] U razvoju bubrega čovjeka, neki od navedenih čimbenika (kaspaza-3, BMP-2, BMP-4, FGF-8, FGF-10) nisu do danas uopće istraživani. Do sada ne postoji sustavna studija koja bi obuhvatila istraživanje većeg broja razvojnih čimbenika u tkivu ljudskih bubrega, koji utječu na normalno oblikovanje i diferencijaciju bubrežnih struktura tijekom ranog (kritičnog) razdoblja njegovog razvoja.

2. PREGLED METODOLOGIJE OBJEDINJENIH RADOVA

Istraživanje se provelo na 8 normalnih ljudskih zametaka (ukupno 16 mezonefrosa i 16 metanefrosa) starih od 5 do 9 tjedana. Tkivo je potjecalo od postojeće arhivske zbirke Zavoda za anatomiju, histologiju i embriologiju i s Kliničkog zavoda za patologiju, sudsku medicinu i citologiju (tkivo spontano pobačenih zametaka i zametaka iz tubarnih trudnoća). Svi zametci su makroskopski pregledani kako bi se isključili oni sa znakovima abnormalnosti ili maceracije, a zatim izmjereni. Za istraživanje je dobivena suglasnost Etičkog povjerenstva za biomedicinska istraživanja Medicinskog fakulteta Sveučilišta u Splitu.

Starost zametaka određena je na temelju podataka o ovulacijskoj dobi, mjerenjem dužine tjeme-zadak (CRL), te usporedbom sa stadijima Carnegie Instituta.[76]

2.1. Imunohistokemijski postupci

Izrezani su kaudalni dijelovi zametaka koji su sadržavali bubrege u razvoju. Komadići tkiva su fiksirani u 4% formaldehidu i fosfatnom puferu tijekom 24 sata, te nakon ispiranja u fosfatnom puferu, dehidrirani u sve višim koncentracijama alkohola. Nakon ispiranja u ksilolu, uklopljeni su u parafin na 56 °C. Serijski su izrezani na rezove debljine 4-6 μm, te prilijepljeni na predmetna stakla prethodno obrađena 0.01%-tnom vodenom otopinom poli-L-lizina (Sigma Chemical Co., St.Louis, USA).

Imunohistokemijski postupak je započet deparafiniranjem tkivnih rezova u parafinskim blokovima, prvo u ksilolu, a zatim nastavljen rehidracijom u alkoholima sve niže koncentracije. Inkubacijom u 0.1-3% H_2O_2 (10-30 minuta pri sobnoj temperaturi) inaktivirala se endogena peroksidaza. Daljnji postupci su ovisili o uputama proizvođača za imunohistokemijsku uporabu protutijela i o referentnim metodama našeg laboratorija.

Korištena protutijela bila su razrijeđena u diluensu (S2022, Dako REAL™ Antybody Diluent, DAKO, Glostrup, Denmark) i to: bcl-2, p53 i Ki-67 1:50, kaspaza-3 1:250, vimentin 1:3000, citokeratin 1:2200, citokeratin 8 1:50, citokeratin 19 1:75; te razrijeđena u PBS-u i to u koncentracijama: FGF-10 i FGF-8 15 μg/ml, VEGF 5 μg/ml, TGF-α i BMP-2/4 10 μg/ml, EGF 1 μg/ml i BMP-7 25 μg/ml.

Nakon ispiranja u PBS-u, korištena su sekundarna biotinizirana protutijela za vizualizaciju vezanja primarnih protutijela i to prema životinjskom podrijetlu primarnih protutijela.

Rezovi su se zatim isprali u PBS-u, obojali diaminobenzidinom (DAB) na sobnoj temperaturi i isprali u destiliranoj vodi. Nakon toga su obojani hematoksilinom i pregledani svjetlosnim mikroskopom Olympus BX 40.

Rezovi namijenjeni negativnoj kontroli prošli su zajedno s ostalim rezovima identični postupak imunohistokemijskog bojanja pojedinog antigena, osim što su za vrijeme inkubacije primarnim protutijelom bili u PBS-u.

Pozitivna kontrola u istraživanim rezovima bilo je smeđe obojenje pojedinih citoplazmi ili jezgara stanica unutar struktura mezonefrosa i metanefrosa, te pozitivnost nekih struktura i organa prisutnih na tim rezovima za koje je iz literature poznato da reagiraju s navedenim protutijelima (jetra, probavna cijev, neuralna cijev sa živcima i ganglijima, gušterača itd.) Nereaktivne stanice imale su plavo obojene jezgre i svijetlu citoplazmu, a nalazile su se unutar rezova obojanih specifičnim protutijelima, kao i na rezovima negativne kontrole.

2.2. Detekcija apoptotskih stanica metodom TUNEL

DNA-fragmentacija u apoptotskim stanicama dokazana je uporabom deoksinukleotidil-transferaza (TdT) metode označavanja hidroksilnih skupina na krajevima fragmenata DNK (TUNEL-metoda; QIA39-1EA, Flourescein FragEL™ DNA Fragmentation Detection Kit, Calbiochem®, USA). Deparafinirani i rehidrirani rezovi prvo su se inkubirali u proteinazi K 20 minuta na sobnoj temperaturi, a zatim 30 minuta na sobnoj temperaturi u izjednačavajućem puferu. Rezovi su kasnije pokriveni sa TdT otopinom i inkubirani u vlažnoj komori tijekom 1.5 sati na 37°C. Nakon inkubacije, rezovi su obojani hemalaunom i pokriveni pokrovnim stakalcem. Označene stanice su analizirane koristeći standardni fluoresceinski filter (465-495 nm) na Olympus BX51 mikroskopu. Mikrofotografije su slikane sa Spot Insight QE kamerom (Diagnostic Instruments, Inc., USA) montiranoj na Olympus BX51 mikroskop koristeći SOFT *software*.

2.3. Kvantifikacija Ki-67 pozitivnih stanica

Broj proliferacijskih stanica u pojedinim bubrežnim strukturama istražen je na 8 zametaka starosti od 5 do 9 razvojnih tjedana. Ki-67 pozitivne i negativne stanice izbrojane su u tri neprileže reza uzeta iz svakog zametka. Bilo koji intezitet bojanja jezgre na Ki-67 smatrao se pozitivnim bojanjem. Analiza je provedena na tkivu mezonefrosa i metanefosa i to na tri područja površine od 50×50µm (povećanje 200×) na odabranim strukturama u svakom rezu. Tako su u mezonefrosu izbrojane stanice u tri područja nefrona i tri područja mezenhima. U metanefrosu su u svakom rezu izbrojane Ki-67 pozitivne i negativne stanice u nefronima, sabirnim kanalićima i mezenhimu. Za ovu analizu je korišten Olympus BX51 mikroskop sa DMP digitalnom kamerom kao i DP-SOFT Version 3.1 *softwar*e. U svakom području (50×50µm), izračunao se udio Ki-67 pozitivnih stanica.

Kvantitativna analiza je izvršena za tri starosne skupine embrija (5-6, 7, 8-9 tjedana). Udio Ki-67 pozitivnih stanica izražen je kao mean ± SD. Podaci su analizirani Kruskal-Wallis ANOVA testom i Dunnovim post hoc testom.

3. SAŽETI PREGLED REZULTATA OBJEDINJENIH RADOVA

3.1. Nomenklatura i prikaz razvoja struktura

Tijekom istraživanog razdoblja (5.-9. tjedan), u tijelu embrija istovremeno su prisutna dva bubrežna sustava: mezonefros (prabubreg) i metanefros (konačni bubreg). Dok se mezonefros proteže kroz torako-lumbalno područje tijela, metanefros nastaje kaudalno od njega i postepeno se pomiče prema gore dok ne budu djelomično usporedni.

U 5. i 6. tjednu razvoja, mezonefros se sastoji od mezonefričkih kanalića na čijim se medijalnim krajevima nalazi Bowmanova čahura koja obavija glomerul, dok se lateralno ulijevaju u mezonefritički (Wolffov) kanal. Ove strukture okružene su rahlim mezenhimom, a pokrivene celomskim epitelom.

Metanefros sadrži mokraćovodni pupoljak, čiji se prednji rastući prošireni kraj naziva ampula. Nasuprot ampule nalazi se zgusnuće induciranih stanica mezenhima koje se naziva kapa metanefrogenog tkiva. Neinducirane rahle stanice koje okružuju začetke odvodnog sustava i nefrona čine metanefrički mezoderm.

Za vrijeme 7., 8. i 9. razvojnog tjedna, mezonefros zadržava istu građu, dok se u metanefrosu mokraćovodni pupoljak grana u odvodne kanaliće koji završavaju ampulama. Sada brojne nove ampule induciraju stanice metanefričkog mezenhima te nastaju brojne nove kape metanefrogenog tkiva (budući nefroni). Od njih nastaju bubrežni mjehurići koji su osnova nastanka s-oblika nefrona, te još zrelijih nefrona koji sadrže bubrežna tjelešca (glomerul i Bowmanova čahura). Ostatak metanefričkog mezenhima čini intersticijsko vezivo.

Oznake u tablicama: + + + jaka reaktivnost; + + umjerena reaktivnost; + slaba reaktivnost; - bez reaktivnosti; / struktura odsutna na rezovima

3.2. Rezultati prvog rada

Tablica 1. Imunoreaktivnost specifičnih protutijela u mezonefrosu i metanefrosu za vrijeme 5. i 6. razvojnog tjedna

	Protutijela				
	Ki-67	**p53**	**bcl-2**	**casp-3**	
Wolffov kanal	+ +	-	-	+ +	**MEZONEFROS**
Mezonefrički mezenhim	+ +	-	-	+ +	
Glomerul	+ +	-	-	+ +	
Bowmanova čahura	+ +	-	-	+ +	
Mezonefrički kanalić	+ +	-	-	+ +	
Celomski epitel	+ +	+	-	+ +	
Mokraćovodni pupoljak	+ +	-	-	-	**METANEFROS**
Ampula	+ + +	-	-	-	
Metanefrički mezenhim	+ +	-	+	+	
Kapa metanefrogenog tkiva	+ + +	-	+ +	+	

Tablica 2. Imunoreaktivnost specifičnih protutijela u mezonefrosu i metanefrosu od 7. do 9. razvojnog tjedna

	Protutijela								
	Ki-67		**p53**		**bcl-2**		**casp-3**		
Weeks of development	*7*	*8 i 9*	*7*	*8 i 9*	*7*	*8 i 9*	*7*	*8 i 9*	
Wolffov kanal	++	+++	-	-	+	-	++	+	**MEZONEFROS**
Mezonefrički mezenhim	++	++	+	+	+	+	+	+	
Glomerul	++	++	+	+	+	+	++	+	
Bowmanova čahura	++	++	+	+	+	++	++	+	
Mezonefrički kanalić	++	++	+	+	+	++	++	+	
Celomski epitel	++	++	+	+	-	+	++	-	
Odvodni kanalić	++	++	-	+	-	-	-	++	**METANEFROS**
Ampula	+++	+++	-	++	-	-	-	++	
Mokraćovod	++	++	-	/	-	/	-	/	
Intersticij	++	++	-	+	+	+	++	++	
Bubrežni mjehurić	++	+++	-	+	++	+++	+	+	
S-oblik nefrona	+++	+++	-	+	+	++	+	+++	
Bubrežno tjelešce	/	+++	-	+	/	++	/	+++	

Kvantitativna analiza proliferacijske aktivnosti

Kvantifikacija je rađena samo za imunohistokemijsku reakciju na Ki-67 bjelančevinu koja daje čisto smeđe obojenje jezgre stanica i omogućava točno razlikovanje i brojanje pozitivnih i negativnih stanica.

Postotak stanica pozitivnih na Ki-67 značajno je porastao u mezonefričkom mezenhimu i nefronima od razdoblja 5.-6. tjedna do razdoblja 8.-9. tjedna.

Imunoreaktivnost pokazatelja proliferacije Ki-67 u metanefričkom mezenhimu značajno je opala u 7. tjednu u odnosu na 5.-6. tjedan. Značajan porast izražaja Ki-67 nađen je u razvojnim oblicima nefrona između embrija starih 5.-6. tjedna i zametaka u 8. i 9. tjednu. Statistički je značajno i opadanje postotka Ki-67 pozitivnih stanica u metanefričkim odvodnim kanalićima i to između najranijeg razvojnog razdoblja (5.-6. tjedan) i 7. tjedna, kao i između 5.-6. tjedna i 8.-9. tjedna.

3.3. Objedinjeni rezultati drugog i trećeg rada

Tablica 1. Imunoreaktivnost specifičnih protutijela u mezonefrosu od 5. do 9. razvojnog tjedna

Protutijela	*Razv. tjedni*	**Wolffov kanal**	**Mezonef. mezenhim**	**Glomerul**	**Bowman. čahura**	**Mezonef. kanalić**	**Celomski epitel**
Vim	*5 i 6*	-	+ +	+ +	+ +	+	+
	7	-	+ +	+ +	+ +	+	+
	8 i 9	+ +	+ +	+ +	+ +	+	+
CK	*5 i 6*	+	-	-	+	+	+
	7	-	-	-	+ +	+	+
	8 i 9	-	-	-	+ +	+ +	+
CK8	*5 i 6*	+	-	+	+	+ +	+
	7	-	-	+	+ +	+ +	+
	8 i 9	+	-	+	+ +	+ + +	+
CK19	*5 i 6*	+ +	-	+	+ +	+ +	+
	7	-	-	+	+	+ +	+
	8 i 9	+	-	+	+ +	+ + +	+ +
EGF	*5 i 6*	+	+ + +	+ +	+ +	+	-
	7	+	+	+ +	+ +	+ +	+ +
	8 i 9	-	+	+	+	+	+
TGF-α	*5 i 6*	+ +	+ +	+ +	+ +	+ +	-
	7	+	+	+ +	+ +	+ +	+
	8 i 9	-	+ +	+	+	+ +	+
FGF-8	*5 i 6*	+	+ +	+ +	+ +	+	+
	7	+	+ +	+ +	+ +	+ +	+
	8 i 9	+	+ +	+	+	+	+
FGF-10	*5 i 6*	+ +	+ +	+ +	+	+	+
	7	+	+	+	+	+ +	+ +
	8 i 9	-	+ +	+	+	+	+
BMP-2/4	*5 i 6*	+ +	+ +	+ +	+ +	+ +	+
	7	+	+	+ +	+ +	+ +	+ +
	8 i 9	-	+ +	+	+	+	+
BMP-7	*5 i 6*	+	+ +	+ +	+ +	+	+
	7	+	+	+ +	+	+ +	+ +
	8 i 9	+	+ +	+	+	+ +	+
VEGF	*5 i 6*	+ +	+	+	+	+ +	+
	7	+	+ +	+	+	+ +	+
	8 i 9	+ +	+	+ +	+ +	+ + +	+

Tablica 2. Imunoreaktivnost specifičnih protutijela u metanefrosu za vrijeme 5. i 6. razvojnog tjedna

Protutijela	**Mokraćovodni pupoljak**	**Ampula**	**Metanefrički mezenhim**	**Kapa metanefrogenog tkiva**
Vim	+	+	+	+
CK	-	-	-	-
CK8	+	+	-	-
CK19	+	+	-	-
EGF	+ +	+	+ +	+
TGF-α	+ +	+ +	+ + +	+ +
FGF-8	-	-	+	-
FGF-10	+ +	+	+ +	+
BMP-2/4	+ +	+	+ +	+
BMP-7	+	+	+ + +	+
VEGF	+ +	+ +	+	+ +

Tablica 3. Imunoreaktivnost specifičnih protutijela u metanefrosu od 7. do 9. razvojnog tjedna

Protutijela	*Razvojni tjedni*	**Odvodni kanalić**	**Ampula**	**Intersticij**	**Kapa metanefrogenog tkiva**	**Bubrežni mjehurić**	**S-nefron**	**Bubrežno tjelešce**
Vim	*7*	++	++	++	++	++	+	/
	8 i 9	+++	+++	+++	++	+	+	+
CK	*7*	+	-	-	-	-	-	-
	8 i 9	++	+	-	-	-	-	-
CK8	*7*	++	++	-	+	+	/	/
	8 i 9	++	+	-	+	++	++	++
CK19	*7*	++	+	-	-	-	/	/
	8 i 9	++	+	-	-	+	+	++
EGF	*7*	++	++	++	+	+	/	/
	8 i 9	+	+	++	+	+	+	+
TGF-α	*7*	++	+	++	+	-	/	/
	8 i 9	++	+	++	+	+	+	+
FGF-8	*7*	++	-	++	+	+	+	+
	8 i 9	++	++	+++	++	++	++	++
FGF-10	*7*	++	+	++	+	+	+	/
	8 i 9	++	+	++	+	+	+	+
BMP-2/4	*7*	+	+	+	+	+	/	/
	8 i 9	+	+	++	+	+	+	+
BMP-7	*7*	++	++	+	+	+	++	/
	8 i 9	++	++	++	++	+	+	+
VEGF	*7*	++	+	+	+	+	/	/
	8 i 9	++	+	+	+	+	+	+

4. RASPRAVA

4.1. Mezonefros

Stanice pozitivne na kaspazu-3 bile su stalno prisutne u svim dijelovima mezonefrosa u embrionalnom razdoblju (5.-8. tjedan), dok je njihov broj bio nešto manji u ranom fetalnom razdoblju. Pole i sur.[6] naveli su prisustvo apoptoze koja prvo počinje u srednjem i kaudalnom dijelu ljudskog mezonefrosa. Temeljem naše analize, bjelančevina p53 prvi je put pronađena u svim mezonefričkim strukturama, osim u Wolffovom kanalu u 7. razvojnom tjednu. Naši rezultati suprotni su onima iz studije koju su na ljudskim zamecima proveli Lichnovsky i sur.,[14] gdje je bjelančevina p53 nađena već u 4. tjednu razvoja, dok joj se izražaj značajno smanjio u 7. razvojnom tjednu. Prema vremenskom i prostornom slijedu pojavnosti ovih bjelančevina u našem istraživanju, može se prepostaviti da kaspaza-3 sudjeluje u morfogenezi mezonefrosa, a da se p53 javlja kasnije u sklopu početne regresije privremenog bubrega.

Ki-67 je bio prisutan u svim mezonefričkim strukturama, a broj pozitivnih stanica je u kasnijem razdoblju značajno porastao u mezenhimu i kanalićima, što je pokazala i kvantifikacijska analiza. To potvrđuje da je vrhunac razvoja mezonefrosa u 8. razvojnom tjednu, iako se već tada u nekim dijelovima pokazuju znaci regresije.[6]

Antiapoptotska bcl-2 bjelančevina pronađena je tek u 7. razvojnom tjednu, a porast njenog izražaja samo u nekim mezonefričkim kanalićima u 8 i 9 tjednu. To se može pripisati zaštiti od apoptoze tijekom diferencijacije tih kanalića u eferentne kanaliće testisa. Istovremena prisutnost s jedne strane proliferativnog markera Ki-67, a s druge strane apoptotske bjelančevine kaspaze-3 u svim strukturama mezonefrosa do 7. razvojnog tjedna, može se objasniti početnim rastom mezonefrosa uslijed brojnih mitoza i istovremenom kontrolom oblikovanja mezonefričkih kanalića apoptozom viška stanica. Od 7. tjedna nadalje, dodatna prisutnost stanica pozitivnih na bjelančevine p53 i bcl-2 najvjerojatnije prati početne regresivne promjene u mezonefrrosu, odnosno paralelno selektivno preživljavanje nekih kanalića kao budućih struktura muškog spolnog sustava.

Prostorna i vremenska pojava vimentina uglavnom se nije mijenjala u ispitivanom razdoblju, dok se izražaj citokeratina blago povećao. Imunoreaktivnost citokeratina 8 i 19 imala je sličan obrazac pojavljivanja kao i reaktivnost na

polivalentno citokeratinsko protutijelo, s manjim razlikama u intezitetu i dodatnoj prisutnosti u glomerulu. U studiji koju su proveli Magro i sur.[34] na fetalnim ljudskim mezonefrosima, vimentin je nađen samo u glomerulu, Bowmanovoj čahuri i mezenhimu, ali ne i u kanalićima, dok su rezultati imunohistokemijskog bojanja citokeratinima bili slični našim. Zajednička prisutnost vimentina i citokeratina opisana u našoj studiji potvrđuje raniju zrelost mezonefritičkih nefrona od onih u metanefrosu u istom razvojnom periodu, što je u skladu s ranijom pojavom mezonefrosa tijekom razvoja u odnosu na metanefros.

Stanice pozitivne na EGF i TGF-α nađene su u svim strukturama mezonefrosa, a raspored i intezitet pojavljivanja ovih čimbenika nije se značajnije mijenjao u ispitivanom razdoblju. Lagani pad u intenzitetu obojenja oba čimbenika u tkivu bio je primijećen u kasnijem razvojnom stadiju, što se podudara s rezultatima istraživanja Bernardinija i sur.,[38] a mogao bi predstavljati jedan od znakova početnog propadanja mezonefrosa.

Sve su se strukture u mezonefrosu obojale imunohistokemijski na FGF-8, FGF-10, BMP-2/4 i BMP-7. Primijećen je pad u intenzitetu obojenja FGF-8, FGF-10 i BMP-2/4 tijekom razvoja, što bi se također moglo smatrati znakom početnog propadanja mezonefrosa.

VEGF je bio prisutan također u svim strukturama mezonefrosa, a povećavao se u ranom fetalnom periodu. To bi moglo upućivati na ulogu ovog čimbenika u oblikovanju krvne mreže u mezonefrosu, kao što je prethodno predloženo za metanefros.[71-75,77]

4.2. Metanefros

U ranim stadijima razvoja metanefrosa, stanice pozitivne na kaspazu-3 nađene su u metanefričkom mezenhimu i kapi metanefrogenog tkiva, dok u ograncima mokraćovodnog pupoljka nisu bile prisutne. U kasnijim stadijim embrionalnog i ranog fetalnog razvoja povećao se broj apoptotskih stanica u intersticiju i zrelijim oblicima nefrona, a u 9. su tjednu nađene po prvi put i u strukturama odvodnog sustava. U istraživanjima razvoja metanefrosa štakora, apoptotske stanice činile su 60% stanica metanefričkog mezenhima i 40% stanica nefrona u razvoju, dok su iste rijetko nađene u ograncima mokraćovodnog pupoljka.[78,79] Ovi podaci upućuju na važnost uloge apoptoze posredovane kaspazom-3 u ranom razvoju nefrona i odvodnog sustava u

ljudskom bubregu. Isto što je potvrđeno u rezultatima istraživanja na pokusnim životinjama s poremećenom nefogenezom i pojavom policističnih bubrega nakon inhibicije odnosno pojačavanja kaspazne aktivnosti.[9,11]

Stanice pozitivne na p53 prvi su put pronađene u ranom fetalnom razdoblju (8-9 tjedana) u odvodnom sustavu i nefronima, dok su ih dosadašnje studije o razvoju ljudskog bubrega opisivale u kasnijem razdoblju (10-15 tjedana), a nađene su samo sporadično i to ne u svim bubrežnim strukturama.[19,28] Pojava bjelančevine p53 u svim dijelovima metanefrosa vremenski se podudarala s intenzivnom diferencijacijom odvodnog sustava, što upućuje na moguću važnost apoptoze posredovane sa p53 u oblikovanju odvodnog sustava metanefrosa. U *knockout* miševa bez izražaja gena za p53, dolazi do smanjenog broja nefrona i smanjenja bubrega uslijed povećane apoptoze u nediferenciranom mezehimu metanefrosa.[27] Pokazano je da povećanje izražaja p53 može uzrokovati poremećaje i u razvoju ljudskih bubrega.[14]

Unutarstanični čimbenik koji štiti stanicu od programirane smrti jest bjelančevina bcl-2, koja je u našem istraživanju prvi put nađena već u 5. tjednu u stanicama metanefričkog mezenhima i kape metanefrogenog tkiva. U kasnijim je stadijima razvoja bcl-2 bio osobito intezivan u nezrelim oblicima nefrona, dok stanice pozitivne na bcl-2 nisu nađene u ograncima mokraćovodnog pupoljka, što se uglavnom podudaralo s podacima istraživanja ljudskog metanefrosa.[19,21]

Kod miševa s poremećenim izražajem gena za bcl-2, došlo je do hipoplazije bubrega uslijed fulminantne apoptoze u metanefričkom mezenhimu, te pojave teške policistične bolesti s posljedičnim zatajenjem funkcije bubrega.[15-18]

Pokazatelj stanične proliferacije Ki-67 bio je prisutan u svim strukturama metanefrosa kroz cijelo razdoblje razvoja bubrega obuhvaćeno našim istraživanjem. Kvantitativna analiza je pokazala značajan pad proliferacije u metanefričkom mezenhimu i odvodnom sustavu tijekom razvoja, dok je značajan porast zabilježen u razvojnim oblicima nefrona. U prijašnjim istraživanjima provedenim na ljudskim bubrezima tijekom kasnijih fetalnih razdoblja, nađeno je smanjenje biljega Ki-67 tijekom sazrijevanja glomerula, koji je kasnije u zrelim glomerulima bio odsutan.[26]

U ranom ljudskom metanefrosu (do 7. tjedna) samo su stanice pozitivne na Ki-67 bile prisutne u svim metanefričkim strukturama, što upućuje na važnost rane proliferacije stanica, kako za grananje mokraćovodnog pupoljka, tako i za nastanak nefrona. U tom istom vremenskom razdoblju, bjelančevine bcl-2 i kaspaza-3 bile su prisutne samo u mezenhimu i razvojnim oblicima nefrona, štiteći tako nefrone od

apoptoze odnosno omogućavajući selektivnu staničnu smrt u mezenhimu. U kasnijim istraženim stadijima razvoja bubrega, porast proliferacije pratio je daljnji razvoj nefrona dok su brojnije apoptotske stanice doprinosile oblikovanju kako nefrona, tako i odvodnog sustava. Stanice pozitivne na bcl-2 ostale su prisutne samo u nefronima, štiteći ih od apoptoze. Prvo pojavljivanje p53 bjelančevine u metanefrosu na kraju embrionalnog razdoblja može se povezati s finim oblikovanjem nefrona i odvodnog sustava.

Zajednički izražaj citokeratina i vimentina do 7. tjedna primijećen je samo u budućem odvodnom sustavu. Rani citokeratin 8 se od 7. tjedna javljao i u kapama metanefrogenog tkiva, dok su se od 8. tjedna oba citokeratina (CK8 i CK19) javljala zajedno s vimentinom u nezrelim nefronima. Metanefrički mezenhim (intersticij) bio je jedini dio metanefrosa u kojem su nađene stanice pozitivne isključivo na vimentin. Zajednički izražaj intermedijarnih filamenata već je opisivan u metanefrosima starijih ljudskih zametaka,[29,31,33,34] a općenito se smatra pojavom koja tijekom razvoja odražava sazrijevanje struktura unutar nekog organa.[24,80,81] Magro i sur.[24] navode da je pojava ovih intermedijarnih filamenata slična i u mezonefrosu i u metanefrosu, a posljedica je sličnog redoslijeda izražaja gena. Također smatraju da je prisutnost vimentina u epitelnim stanicama bubrežnih kanalića odraz njihovog mezenhimskog podrijetla, a da je pojava citokeratina jedan od znakova razvoja osobina epitelnih stanica. U istraživanju Oosterwijka i sur.[31] provedenom na ljudskim fetalnim metanefrosima, stanice bubrežnih mjehurića bile su prve pozitivne na rane citokeratine CK8 i CK19. Prema našim istraživanjima, CK8 se javljao već u stanicama kape metanefrogenog tkiva. Poznato je da se kao posljedica zgušnjavanja stanica metanefričkog mezenhima u kapu metanefrogenog tkiva, nekoliko vezivnih bjelančevina zamjenjuje bjelančevinama epitelnog tipa.[82] Taj se znak epitelne transformacije mezenhimskih stanica može povezati i s pojavom citokeratina, što bi značilo da uz promjenu sastava izvanstaničnih proteina u stanicama budućih nefrona, dolazi i do aktivacije gena za unutarstanične bjelančevine intermedijarnih filamenata u smjeru stvaranja citokeratina. Daljnji porast citokeratinske imunoreaktivnosti prati sazrijevanje epitelnih stanica nefrona. Istovremena pojava mezenhimskih i epitelnih intermedijarnih filamenata jest prolazna osobitost stanica nefrona, koja se gubi tijekom razvoja.[29] Tako se u odraslim bubrezima paralelna pojava vimentina uz postojeće citokeratinske intermedijarne filamenate u epitelu bubrežnih kanalića, kao i potpuna zamjena citokeratina vimentinom u istim stanicama, smatra znakom prijelaza normalnih

epitelnih stanica bubrega u mezenhimske stanice u sklopu patološke fibroze ili nastanka karcinoma.[33,83-85]

U našem istraživanju, bjelančevine EGF i TGF-α bile su prisutne u svim metanefričkim strukturama, dok im je najjači izražaj bio u mezenhimu (intersticiju) i ograncima mokraćovodnog pupoljka. To je u suprotnosti s rezultatima istraživanja Bernardinija i sur.[38] koji su pretpostavljali da su budući nefroni glavni izvor oba čimbenika, a stanice odvodnog sustava uglavnom sadrže njihov zajednički receptor. Međutim, ista grupa autora je u kasnijem istraživanju pronašla jednaki raspored i intenzitet izražaja i glasničke RNK i bjelančevine za TGF-α u stanicama i odvodnog sustava i nefona u razvoju.[41] Značajna imunoreaktivnost oba čimbenika, osobito u mitotskim stanicama, potvrđuje ulogu ovih čimbenika u ranom rastu i razvoju svih struktura metanefrosa, kako je već u opisano u literaturi.[39,41] U našem istraživanju, lagani pad intenziteta ovih čimbenika karakteristika je najkasnijeg razvojnog razdoblja i može se povezati s većom zrelošću metanefričkih struktura. Unatoč manjim razlikama u rezultatima i metodologiji, sve studije rađene na ljudskim bubrezima u razvoju slažu se oko uloge oba čimbenika u podržavanju proliferacije kao i u opadanju njihove aktivnosti tijekom sazrijevanja bubrega.[38,39,41] Sva su važna zbivanja u razvoju životinjskog bubrega pod utjecajem TGF-α, uključujući rast metanefrosa, grananje mokraćovodnog pupoljka i nastanak kanalića,[42] dok EGF ima dodatnu ulogu u sprječavanju stanične smrti.[86]

U ranom embrionalnom razvoju metanefrosa, pojava FGF-8 bila je ograničena na metanefrički mezenhim, dok mu je tijekom kasnijeg razvoja intenzitet rastao u odvodnom sustavu i u nefronima. Poremećeno oblikovanje nefrona javlja se u miševa s mutiranim genom za FGF-8 zbog pojačane apoptoze.[52] Promijenjeni izražaj FGF-8 u čovjeka bi također mogao dovesti do poremećaja u razvoju nefrona, s posljedicama u vidu ozbiljnih malformacija bubrega pri rođenju.

Za razliku od FGF-8, FGF-10 je bio prisutan u svim ranim metanefričkim strukturama, dok je tijekom kasnijeg razvoja, intenzitet izražaja ovog čimbenika opadao. Rezultati istraživanja razvoja mokraćovodnog pupoljka štakora,[53] zajedno s našim podacima, upućuju na stanice intersticija i autokrinu sekreciju FGF-10 kao glavni izvor ovog čimbenika u razvoju odvodnog sustava bubrega. Moguće je da FGF-10, preko poticanja rasta i grananja mokraćovodnog pupoljka upravlja i brojem novonastalih nefrona. Kao što je već pokazano u miševa,[47] nedostatnost ovog čimbenika

u opisivanom razvojnom razdoblju mogla bi dovesti do poremećenog razvoja i u ljudskom bubregu, u prvom redu zbog smanjenja broja nefrona.

U našem je istraživanju po prvi put opisan izražaj BMP-2/4 podgrupe ovih bjelančevina u ljudskom metanefrosu. Iako možemo samo nagađati o rasporedu pojedinog čimbenika (BMP-2 ili BMP-4), izražaj ove podgrupe bio je veći i rasprostranjeniji od ukupnih dosad opisanih izražaja oba čimbenika u miša.[57] U studiji Dudleya i Robertsona,[57] glasnička RNK BMP-2 bila je ograničena na kapu metanefrogenog tkiva i kasnije privremeno na nefrone, dok je BMP-4 bio prisutan u mezenhimu oko odvodnog sustava i u bubrežnom tjelešcu. Naši rezultati upućuju na metanefrički mezenhim i razvojne oblike nefrona kao mogući izvor oba čimbenika ove podgrupe, kao i na ulogu oba čimbenika u razvoju odvodnog sustava, što je već predloženo u istraživanjima na životinjama.[58,59,61,87] Malformacije bubrega i mokraćovodnog sustava opisane su u nekoliko istraživanja na heterozigotnim miševima za BMP-4 gen.[55,87-89] Stoga je moguć nastanak sličnih razvojnih poremećaja u ljudi uslijed poremećenog izražaja gena za BMP-4.

Bjelančevina BMP-7 je bila prisutna u svim metanefričkim strukturama kroz cijelo istraživano razdoblje razvoja. Tijekom ranog razvoja, najjači je izražaj nađen u mezenhimu, dok se u kasnijem razvoju smanjivao u razvojnim oblicima nefrona, a povećavao u odvodnom sustavu. U prijašnjim se istraživanjima razmatralo da se bjelančevina BMP-7 uglavnom otpušta iz glomerula i postepeno nakuplja na bazalnim membranama epitela nefrona.[66,67] Iako je u tim istraživanjima najjači izražaj glasničke RNK za BMP-7 bio u glomerulima, njezina prisutnost u stanicama nefrona i odvodnog sustava upućuje također i na ove strukture kao mogući izvor BMP-7. Moguća uloga ovog čimbenika je i u diferencijaciji epitelnih struktura iz mezenhima (epitelno-mezenhimska transformacija) i u nastanku bubrežnih struktura.[90] Rezultati našeg istraživanja, zajedno s rezultatima studija o izražaju BMP-7 tijekom metanefričkog razvoja u miša, upućuju na ulogu ovog čimbenika u održavanju stalnog rasta ljudskog metanefrosa, kao i na nastanak različitih malformacija (npr. displazije bubrega), uslijed poremećenog izražaja ovog čimbenika.[56-59,62-64,91,92]

U 5. i 6. tjednu razvoja, prisutnost VEGF bjelančevine bila je najizraženija u mokraćovodnom pupoljku, ampulama i kapama metanefrogenog tkiva, dok je u kasnijem razdoblju najviše stanica pozitivnih na VEGF bilo u odvodnim kanalićima. To bi značilo da već rane strukture metanefrosa počinju utjecati na razvoj buduće krvne mreže bubrega. Naši rezultati slažu se s onima iz prethodnih istraživanja VEGF-a u

ljudskom fetalnom metanefrosu.[73-75] Kod pokusnih životinja, smanjeni izražaj gena za VEGF dovodi do poremećaja u nastanku i grananju krvnih žila.[68] Promjene u točnom vremenskom slijedu i rasporedu pojave ovog čimbenika, mogle bi biti povezane s poremećenim razvojem krvnih žila, važnim za funkciju bubrega.

5. ZAKLJUČCI

U našoj studiji, obrazac pojavljivanja istraživanih bjelančevina u ljudskom mezonefrosu je sukladan prvo razvoju, rastu i diferencijaciji, a zatim postepenom propadanju toga organa.

Sve navedene mitotske (Ki-67), proapoptotske (kaspaza-3, p53) i antiapoptotske (bcl-2) bjelančevine imale su točno određeni vremenski i prostorni slijed pojavljanja u ranom razvoju ljudskog trajnog bubrega (metanefrosa). Promjene u tom obrascu pojavljivanja mogle bi uzrokovati ozbiljne poremećaje u oblikovanju i funkciji bubrega nakon rođenja, s posljedičnim zatajenjem bubrega.

Istovremena prisutnost mezenhimskih (vimentin) i epitelnih bjelančevina (citokeratini) intermedijarnih filamenata u stanicama metanefrosa prolazna je pojava, a odraz je mezenhimalnog podrijetla epitelnih struktura i njihove diferencijacije u smjeru epitela (mezenhimsko-epitelne transformacije). Odstupanje od navedenog procesa tijekom ranog razvoja ljudskog bubrega moglo bi se povezati s poremećajima u razvoju bubrega.

Čimbenici rasta EGF and TGF-α bili su najizraženiji u strukturama bubrega koje su pokazivale intezivan rast. Njihova bi uloga stoga bila u grananju i razvoju odvodnog sustava, kao i u razvoju nefrona. Smanjenje rasta i veličine bubrega (hipoplazija) i poremećaji u funkciji bubrega moguće su posljedice poremećaja pojavnosti ovih čimbenika u razvoju ljudskog bubrega.

Nastanak i grananje mokraćovodnog pupoljka, te indukcija i neprestano stvaranje novih nefrona mogli bi ovisti o prisutnosti bjelančevina iz obitelji FGF-a i BMP-a. Promjene u njihovom obrascu pojavljivanja tijekom razvoja ljudskog metanefrosa mogle bi biti povezane s nastankom cista te manjih i displastičnih bubrega.

Prisutnost bjelančevine VEGF u mezonefrosu i metanefrosu upućuje na rani početak oblikovanja krvožilne mreže tijekom razvoja obaju bubrega. Poremećena aktivnost ovog čimbenika mogla bi dovesti do promjena u nastanku i rasporedu krvnih žila u bubregu.

6. SAŽETAK

U ovoj se studiji istraživao vremenski i prostorni obrazac pojavljivanja bjelančevina Ki-67, bcl-2, kaspaze-3, p53, citokeratina, vimentina, EGF, TGF-α, FGF-8, FGF-10, BMP-2/4 podgrupe, BMP-7 i VEGF u mezonefrosu i metanefrosu ljudskih zametaka starosti od 5 do 9 tjedana. Prisutnost ovih bjelančevina dokazivala se imunohistokemijskim metodama u parafinskim rezovima ljudskih zametaka.

Između 5. i 7. razvojnog tjedna, prisutnost stanica pozitivnih na Ki-67 i na kaspazu-3 u svim mezonefričkim strukturama upućuje na važnost stanične proliferacije tijekom rasta mezonefrosa, kao i na ulogu apoptoze u oblikovanju nefrona. Od 7. tjedna nadalje, u tkivu mezonefrosa bile su prisutne i stanice pozitivne na p53 i bcl-2. Bjelančevina p53 vjerojatno sudjeluje u regresivnim promjenama u mezonefrosu, dok bi bjelančevina bcl-2 mogla doprinositi selektivnom preživljenju pojedinih kanalića iz kojih se dijelom razvijaju strukture muškog spolnog sustava u odraslih. Prisutnost bjelančevine Ki-67 u stanicama svih metanefričkih struktura između 5. i 7. razvojnog tjedna, ukazuje na važnost stanične proliferacije u grananju mokraćovodnog pupoljka i oblikovanju nefrona. Tijekom istog razdoblja, bjelančevine bcl-2 i kaspaza-3 bile su prisutne samo u nefronima i mezenhimu (intersticiju). Bjelančevina bcl-2 vjerojatno je štitila nefrone od apoptoze, dok je kaspaza-3 u mezenhimu sudjelovala u procesu stanične smrti. Pojava bjelančevine p53 u tijekom kasnijeg razvoja (7-9 tjedana) mogući je pokazatelj njezinog sudjelovanja u daljnjem oblikovanju odvodnog sustava u metanefrosu.

U mezonefrosu, vimentin je bio nađen u svim strukturama, dok su stanice pozitivne na citokeratine bile prisutne samo u mezonefričkim kanalićima. Imunoreaktivnost vimentina bila je prisutna u svim strukturama metanefrosa od 5. razvojnog tjedna, a tijekom kasnijeg razvoja povećavala se u odvodnom sustavu i intersticiju. Citokeratini 8 i 19 pojavili su se u mokraćovodnom pupoljku i ampuli u 5. tjednu, a kasnije su pokazivali porast imunoreaktivnosti u odvodnom sustavu i nefronima. Zajednička prisutnost bjelančevina intermedijarnih filamenata u stanicama metanefrosa tijekom razvoja prolazna je pojava i može se povezati s prijelazom mezenhimskih svojstava stanica nefrona u epitelna svojstva. U odraslim se bubrezima takva koekspresija u stanicama bubrega povezuje s fibroznim i karcinomatoznim promjenama.

Imunoreaktivnost čimbenika rasta EGF i TGF-α tijekom razvoja je opadala u svim strukturama mezonefrosa. U metanefrosu je imunoreaktivnost na EGF i TGF-α bila prisutna u svim strukturama, a kasnije se smanjivala u nefronima. Obrazac pojavljivanja ovih čimbenika ukazuje na njihovu ulogu u indukciji, proliferaciji i rastu struktura metanefrosa. Sve strukture mezonefrosa sadržavale su stanice pozitivne na FGF-8, FGF-10, BMP-2/4 i BMP-7, a intenzitet njihove imunoreaktivnosti blago se smanjivao krajem ispitivanog razvojnog razdoblja. Imunoreaktivnost čimbenika rasta VEGF također je bila prisutna u svim mezonefričkim strukturama, povećavajući se u kasnijem razvoju zajedno s razvojem mezonefričkih krvnih žila. Čimbenik rasta FGF-8 u ranom embrionalnom metanefrosu prvi se put pojavio samo u mezenhimu, a od 7. razvojnog tjedna njegova se imunoreaktivnost pojavila u ostalim bubrežnim strukturama. Već u 5. tjednu pojavile su se stanice pozitivne na FGF-10 u svim metanefričkim strukturama, a tijekom daljnjeg razvoja blago se povećavala imunoreaktivnost ovog čimbenika rasta. Oba čimbenika rasta mogla bi utjecati na preživljenje stanica i oblikovanje nefrona u bubregu. Svi čimbenici rasta iz obitelji BMP-a imali su sličnu imunoreaktivnost u svim metanefričkim strukturama, koja se pojačavala tijekom daljnjeg razvoja, što upućuje na njihovu ulogu u preživljenju stanica i grananju mokraćovodnog pupoljka. Bjelančevina VEGF pojavila se već u najranijem razvoju metanefrosa u svim strukturama, a njezina se imunoreaktivnost tijekom razvoja povećavala u odvodnom sustavu više nego u intersticiju i nefronima. Prisutnost ovog čimbenika rasta može se povezati s utjecajem struktura metanefrosa u stvaranju pripadajuće im krvne mreže.

Promjene u obrascima pojavnosti opisanih bjelančevina mogle bi dovesti do različitih promjena u građi i funkciji bubrega u ranom djetinjstvu.

7. SUMMARY

The involvement of apoptotic factors, growth factors and intermediate filament proteins in early human kidney development

The spatial and temporal patterns of appearance of proteins Ki-67, bcl-2, caspase-3, p53, cytokeratins, vimentin, EGF, TGF-α, FGF-8, FGF-10, BMP-2/4 subfamily, BMP-7 and VEGF were investigated in the mesonephros and metanephros of 5-9 –weeks old human conceptuses using immunohistochemical methods. Between the 5th and 7th developmental weeks, Ki-67 and caspase-3 positive cells characterized all mesonephric structures indicating importance of cell proliferation in the growth of the mesonephros, and role of apoptosis in the nephrogenesis. From the 7th week on, p53 and bcl-2 positive cells appeared in the mesonephros as well. Regressive changes in the mesonephros could be regulated by activation of p53, while bcl-2 could contribute to selective survival of some tubules giving rise to adult structures. In the early human metanephros (5-7-weeks), Ki-67 positive cells characterized all metanephric structures, indicating a role of cell proliferation in branching of the ureteric bud and in nephron formation. During the same period, bcl-2 and caspase-3 reacting cells were found only in the metanephric mesenchyme and nephrons. Bcl-2 protein probably protected nephrons from apoptosis, while caspase-3 protein controlled cell death in the mesenchyme. At later stages (7-9-weeks), appearance of p53 positive cells could participate in further morphogenesis of the metanephric collecting system.

Vimentin was found in all mesonephric structures, while cytokeratins were seen only in the mesonephric tubules. In the 5-6-week metanephros, vimentin immunoreactivity was found in all structures and later increased in the collecting system and interstitium. In the 5th week, cytokeratins 8 and 19 appeared in the ureteric bud and ampullae, and later showed increasing immunoreactivity in the collecting system and nephrons. The coexpression of intermediate filament proteins in metanephric development is a temporary feature and might be associated with mesenchymal to epithelial transformation of developing nephrons. In adult kidneys, such coexpression is associated with fibrogenesis or carcinomatous changes.

EGF and TGF-α were detected early in all mesonephric structures, and immunoreactivity to both factors decreased in later stages. At early stages,

immunoreactivity to EGF and TGF-α was detected in all metanephric structures and from the 7th week onward, it decreased in differentiating nephrons. EGF and TGF-α patterns of appearance indicate their role in induction, proliferation and growth of metanephric structures.

In the mesonephros, cells positive to both FGF's and BMP's were found in all structures and their immunoreactivity slightly decreased in the early fetal period. VEGF positivity appeared in all mesonephric structures, and increased in the fetal period coincidently with formation of the mesonephric blood vessel network. In the metanephros, FGF-8 first appeared only in the metanephric mesenchyme, but from the 7th week on, its reactivity increased and spread to other metanephric structures. FGF-10 positive cells appeared in all metanephric structures already in the 5th week, and slightly intensified with progression of development. Cell survival and nephrogenesis in the permanent kidney might be associated with the appearance of both growth factors. Both BMP-2/4 and BMP-7 displayed a similar pattern of reactivity in all metanephric structures, which intensified with advancing development, indicating their involvement in cell survival and ureteric bud branching. Already in the earliest developmental stages, VEGF protein appeared in all metanephric structures. At later stages, VEGF showed more intense reaction in the collecting system than in the differentiating nephrons and interstitium. The presence of this growth factor might be associated with the influence of metanephric structures on formation of adherent blood vessels.

Changes in the described patterns of appearance might lead to different disturbances of kidney formation and function in the early childhood.

8. ŽIVOTOPIS

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ORIGINAL ARTICLE

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Role of mitotic, pro-apoptotic and anti-apoptotic factors in human kidney development

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Abstract The expression pattern of mitotic Ki-67 and anti-apoptotic bcl-2 proteins, as well as apoptotic caspase-3 and p53 proteins, were investigated in the human mesonephros and metanephros of 5–9 week-old human conceptuses. Apoptotic cells were additionally detected using the terminal deoxynucleotidyl transferase (TdT) nick-end labelling (TUNEL) method. Between the 5th and 7th developmental weeks Ki-67, caspase-3 and TUNEL-positive cells characterized all mesonephric structures, indicating importance of cell proliferation in the growth of the mesonephros and role of apoptosis in nephrogenesis. From the 7th week on, p53 and bcl-2 positive cells appeared in the mesonephros as well. Regressive changes in the mesonephros could be regulated by activation of p53, while bcl-2 could contribute to selective survival of some tubules giving rise to adult structures. In the early human metanephros (5–7 weeks), Ki-67 positive cells characterized all metanephric structures, indicating a role of cell proliferation in branching of the ureteric bud and in nephron formation. During the same period bcl-2, caspase-3 and TUNEL-positive cells were found only in the metanephric mesenchyme and nephrons. Bcl-2 protein probably protected nephrons from apoptosis, while caspase-3 protein controlled cell death in the mesenchyme. At later stages (7–9-weeks), appearance of p53-expressing cells could participate in further morphogenesis of the metanephric collecting system. The factors investigated had a spatially and temporally restricted pattern of appearance in developing kidneys. Changes in that pattern might lead to serious disturbances of kidney formation and function in early childhood.

Keywords Human embryo · Kidney development · Mesonephros · Metanephros

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Introduction

During the human embryonic period, three excretory kidney systems develop: the pronephros, the mesonephros and the metanephros. The pronephros is a non-functional and transitory structure by the end of the fourth week of development; it degenerates leaving behind only its caudal part, which will become a primary ureter used by mesonephros. The mesonephros develops from nephrogenic cord caudal to the pronephros late in the fourth developmental week. It is a large ovoid organ, situated laterally to the developing gonad and formed of large numbers of vesicles that differentiate into S-shaped loops. The lateral ends of these loops enter the primary ureter, now called the mesonephric (Wolffian) duct, while the medial end forms the Bowman's capsule surrounding the glomerulus, thus forming the mesonephric corpuscle. The mesonephros is a temporary functional kidney, which subsequently degenerates. However, in the male embryo, some parts of the Wolffian duct and mesonephric tubules persist as genital ducts. Formation of the permanent kidney, the metanephros, begins in the fifth week of development, when a dorsal outgrowth of Wolffian duct called the ureteric bud penetrates the metanephric mesenchyme. The metanephric mesenchyme develops from the sacral portion of the nephrogenic cord and differentiates into renal vesicles and S-shaped tubules (nephrons) under inductive influence of the terminal branches (ampullae) of the ureteric bud. Later on, more mature nephrons develop, consisting of the Bowman's capsule and glomerulus, proximal and distal convoluted tubule, and the loop of Henle. Collecting tubules, renal calyces, renal pelvis and ureter are derivates of the ureteric bud [1, 2].

Development of all three excretory systems depends on reciprocal tissue interactions and many transcription factors, growth factors, signalling molecules, their receptors and other proteins. Knowledge about the role of those factors and genes is based on investigations on transgenic animals showing different kidney abnormalities, such as absence of kidney morphogenesis (Pax-2 and Lim-1 knockout), defects in epithelial transformation of mesenchyme (Wnt-4 and Bmp-7 knockout) and reduced numbers of nephrons (FGF-7 knockout) [3, 4].

Among those factors, distribution and intensity of programmed cell death and cell proliferation are considered as important and often combined processes in nephrogenesis. Apoptosis, a programmed cell death, occurs at precise stages of kidney development and has an important role in the normal development and regression of all three forms of embryonic kidneys [5]. During degeneration of the pronephros and mesonephros in rat embryos, cell death follows a strict temporo-spatial pattern [6]. In metanephric development of the rat embryo, evidence of apoptosis, such as condensed nuclei, fragmented cytoplasm and cell shrinking, were found in cells surrounding the new epithelium induced by the ureteric bud [7].

Apoptosis may be executed through the caspase protein family. Over a dozen caspases have been found to operate in humans [8]. Caspases have an important function in cell cycle regulation [9], especially caspase-3 which is the key effector caspase in apoptotic process [10, 11]. In mouse embryos, general caspase inhibition was shown to prevent ureteric bud branching and further development of the metanephros [12].

Bcl-2 is an integral mitochondrial, nuclear and endoplasmic reticulum membrane protein. It was shown that bcl-2 over-expression protects cell from apoptotic death [13, 14]. During human kidney development, *bcl-2* gene expression may be necessary for the differentiation of uninduced mesenchyme into the mature nephrons [13]. In bcl-2 deficient mice, renal hypoplasia was found associated with abnormal renal growth, cyst formation and decrease in number of nephrons [13, 15–18].

Proliferation is another important factor involved in kidney development and differentiation. Ki-67 nuclear protein is often used as a proliferation marker because of its expression in all phases of the cell cycle except the resting phase (G0) [19, 20]. In human nephrogenesis, Ki-67 expression was found to be the most pronounced in the early stages of metanephric glomeruli differentiation, in the differentiating vesicles and folding glomeruli. Ki-67 expression was found to decrease with glomerular maturation [21].

p53 is a negative regulator of the G1-S phase transition in the cell cycle. In the case of DNA damage it induces growth arrest or apoptosis in the damaged cells [14]. In p53 transgenic mouse, the kidneys are smaller and have lower numbers of compensatory hypertrophic glomeruli, owing to altered differentiation of the ureteric bud [22]. In human embryos aged 4 to 8 weeks, p53 is expressed in the nuclei of the mesonephric secretory canal epithelium and focally in the cells of the mesonephric glomeruli [14]. In the metanephros, p53 expression is found only during the foetal period [23].

Numerous studies on kidney development, dealing with cell proliferation, apoptosis and their genetic background and control, have been done on experimental animals [3, 12, 13, 15–18, 22]. Only few studies of their expression pattern in the mesonephros and metanephros have been performed in human embryos [13, 14], some of them done on developmental stages different from ours [24–26].

Aberrations of proliferation and cell death in the human metanephros often appear in association with lower urinary tract obstructions. Even changes in environment, such as altered maternal diet, cause nephron defects accompanied by enhanced apoptosis [27].

Therefore, the aim of this study was to analyse the spatial and temporal expression pattern of factors involved in cell proliferation and programmed cell death during the mesonephric and early metanephric development of the human kidney. The consequences of disturbed cell proliferation and cell death are associated with several kidney abnormalities (agenesis, dysplasia, hypoplasia), thus accounting for the great number of young children with chronic renal failure.

Materials and methods

Human material

A total of six normal human conceptuses between their 5th and 9th week of development was collected after spontaneous abortions from the Department of Gynaecology and Obstetrics, Clinical Hospital of Split, Croatia, and after tubal pregnancies from the Department of Pathology, Clinical Hospital of Split. The embryos and foetuses were examined macroscopically and measured. Only normal conceptuses, without any sign of abnormality, signs of intrauterine death or maceration, were used in our study. The embryonic tissues were treated as postmortem material with permission of the Ethics and Drug Committee of the Clinical Hospital of Split in accordance with the 1964 Helsinki Declaration. The post-ovulatory age was estimated on the basis of menstrual data and correlated with crown–rump length (CRL) and Carnegie stages [28].

Immunohistochemical staining

Caudal parts of embryos containing developing kidneys were dissected. Tissue samples were fixed in 4% paraformaldehyde in phosphate buffer and dehydrated in 100% ethanol. They were embedded in paraffin wax, serially sectioned at 4–6 μm, mounted on glass slides, and examined with an Olympus BX-40 light microscope (Olympus, Tokyo, Japan). The paraffin was removed with xylene, and the sections were rehydrated in ethanol and water. In order to quench endogenous peroxidase activity, we incubated the sections for 30 min in 0.1% H_2O_2.

For Ki-67 staining, after incubation in H_2O_2, paraffin-embedded sections were washed with phosphate-buffered saline (PBS) and then incubated in EDTA (pH 8.0) for 10 min at 95°C. They were than cooled to room temperature and incubated with monoclonal mouse anti-human Ki-67 antigen (M 7240, DAKO, Glostrup, Denmark) for 30 min. After being washed with PBS, the binding was visualized by incubating the sections with Envision+ single reagent visualization system, which contains peroxidase-conjugated anti-mouse secondary an-

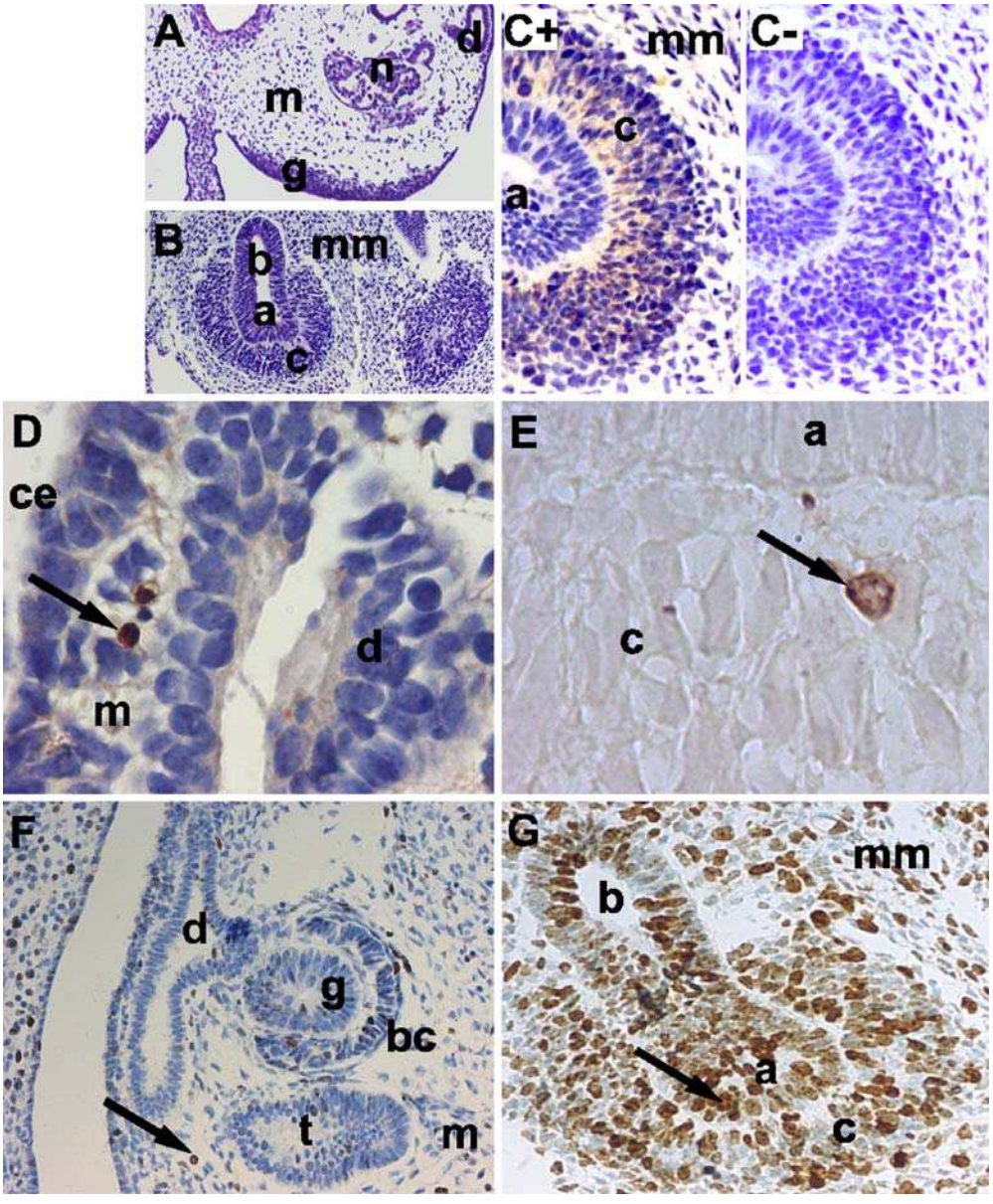

Fig. 1 Transverse sections through the developing mesonephros and metanephros in the 5th and 6th developmental weeks. **A** Mesonephros (5 weeks): primitive nephrons (*n*), mesonephric duct (*d*), mesonephric mesenchyme (*m*), the gonad (*g*) primordium. Haematoxylin and eosin, ×10. **B** Metanephros (5 weeks): the ureteric bud (*b*), ampullae (*a*), metanephric mesenchyme (*mm*), the mesenchymal metanephric cup (*c*). Haematoxylin and eosin, ×10. **C+** Metanephros (5 weeks): note moderately bcl-2 positive cells in the metanephric cup (*c*), very mildly positive cells in the metanephric mesenchyme (*mm*) and non-reactive cells in the ampullae (*a*). Immunostaining to bcl-2 protein, ×40. **C−** Absence of bcl-2 positivity in all metanephric structures. Immunostaining to bcl-2 protein pre-incubated with immunizing peptide, ×40. **D** Mesonephros (5 weeks): three caspase-3 positive cells with dark brown-stained nuclei (*arrow*) are seen among the vital euchromatic nuclei of mesenchymal cells (*m*). Mesonephric duct (*d*) and coelomic epithelium (*ce*) are seen as well. Immunostaining to active caspase-3, ×40. **E** Metanephros (5 weeks): only one caspase-3 positive cell nucleus (*arrow*) is seen among the euchromatic cell nuclei of the metanephric cup mesenchyme (*c*). Ampulla (*a*) is missing caspase-3 positive cells. Immunostaining to active caspase-3, ×100. **F** Mesonephros (6 weeks): nuclei of Ki-67 positive cells (*arrow*) are seen in the mesonephric duct (*d*), glomeruli (*g*) and Bowman's capsule (*bc*), mesonephric tubules (*t*) and mesonephric mesenchyme (*m*). Immunostaining to Ki-67 protein, ×20. **G** Metanephros (5 weeks): numerous brown-stained nuclei of Ki-67 positive cells (*arrow*) are seen in the metanephric cup (*c*), adjacent ureteric bud (*b*) and ampullae (*a*), and in the metanephric mesenchyme (*mm*) Immunostaining to Ki-67 protein, ×10

tibody (K 4001, DAKO) for 30 min. Afterwards, sections were washed with PBS and then stained with diaminobenzidine tetrahydrochloride (DAB) solution. Finally, the sections were rinsed in distilled water, counter-stained with haematoxylin, and dehydrated in ethanol and xylol [29].

Sections for immunohistochemical staining of bcl-2, p53 and caspase-3 antigens were treated with H_2O_2, washed in PBS and then incubated in sodium citrate buffer for 10 min at 95°C. After being cooled to room temperature, they were incubated with rabbit anti-human/mouse active caspase-3 primary antibody (AF835, R&D Systems, Minneapolis, Minn., USA) and mouse anti-human Bcl-2 oncoprotein primary antibody (M 0887, DAKO) overnight at 4°C in a humidified chamber. The rest of the paraffin sections were incubated with mouse anti-human p53 protein (M 7001, DAKO) for 45 min. After being washed with PBS, the bcl-2 and caspase-3 sections were incubated with biotinylated secondary antibody (mouse and rabbit UniTect ABC Kit, Oncogene, Boston, Mass., USA) for 30 min at room temperature. They were then washed again in PBS and incubated with avidin biotinylated horseradish peroxidase complex (ABC) for 30 min, washed again with PBS, and then stained with DAB. Finally, the sections were rinsed in distilled water, counter-stained with haematoxylin, and dehydrated in ethanol and xylol.

Additionally, we pre-incubated some sections with immunizing peptide for 30 min in order to remove the bcl-2 signal.

For the p53 sections, primary antibodies were detected using a streptavidin–biotin peroxidase system (K0690, DakoCytomation, Carpinteria, Calif., USA) as recommended by the manufacturer. The p53 sections were later washed with PBS, stained, counter-stained and dehydrated as described above [30, 31].

Detection of apoptotic cells by the terminal deoxynucleotidyl transferase-mediated nick-end labelling method

DNA fragmentation in apoptotic cells was examined using the terminal deoxynucleotidyl transferase (TdT)-mediated nick-end labelling (TUNEL) method. DNA fragmentation in apoptotic cells was detected with TdT-mediated nick-end binding of fluorescein-labelled and unlabelled deoxynucleotides using the Fluorescein FragEL DNA Fragmentation Detection Kit (Calbiochem, USA). Sections with the paraffin removed were rehydrated and pretreated with proteinase K for 20 min and then treated with equilibration buffer for 30 min, all at room temperature. The sections were later covered with working TdT-labelling reaction mixture and incubated in a humidified chamber at 37°C for 1.5 h. After the incubation, the sections were briefly stained with haematoxylin and mounted with the mounting media provided in the TUNEL kit. Labelled nuclei were examined with a standard fluorescein filter (465–495 nm). Microphotographs were captured with a SPOT Insight QE camera (Diagnostic Instruments, USA) mounted on an Olympus BX51 microscope using the SPOT software.

Quantification of Ki-67 positive cells

Six conceptuses of different developmental ages (5–9 weeks) were examined. The positively labelled and unlabelled cells were counted in three non-adjacent sections taken from each conceptus. For Ki-67 positivity, any level of nuclear positivity was considered, regardless of the intensity of the staining. Counts were made over the total area of the kidney (mesonephric or metanephric). In the mesonephros, as well as in the metanephros, three distinct areas of 50 μm×50 μm at 200× magnification were counted over each chosen structure. Thus, in the mesonephros, three collecting nephrons and three mesenchymal areas were counted in each section. In the metanephros, three nephrons, three tubules and three mesenchymal

Table 1 Immunoreactivity to specific antibodies in the human mesonephros and metanephros during the 5th and 6th weeks of development

Location	Antibodies				Structure
	Ki-67	p53	bcl-2	caspase-3	
Wolffian duct	+ +	−	−	+ +	MESONEPHROS
Mesonephric mesenchyme	+ +	−	−	+ +	
Glomeruli	+ +	−	−	+ +	
Bowman's capsule	+ +	−	−	+ +	
Mesonephric tubules	+ +	−	−	+ +	
Coelomic epithelium	+ +	+	−	+ +	
Ureteric bud	+ +	−	−	−	METANEPHROS
Ampulla	+ + +	−	−	−	
Metanephric mesenchyme	+ +	−	+	+	
Metanephric cup	+ + +	−	+ +	+	

+ + + strong reactivity, + + moderate reactivity, + mild reactivity, − no reactivity

(interstitial) areas were counted per section. The examination was performed on an Olympus BX-51 microscope equipped with a DMP digital camera and using DP-SOFT version 3.1 software. In each area (50 μm×50 μm), the percentage of Ki-67-positive cells was calculated.

Quantitative analysis was performed for three groups (5 and 6, 7, 8 and 9 weeks) according to the corresponding age of the embryo. The percentage of Ki-67 positive cells was expressed as mean ± SD. Data were analysed by the Kruskal–Wallis analysis of variance (ANOVA) test followed by Dunn's post-hoc test.

Results

During the 5th and 6th week of development, both the mesonephros and the metanephros are present in the human embryo. The mesonephros forms the large ovoid organ on each side of the midline, on the lateral side of the developing gonad. It consists of renal glomerules and tubules opening into the mesonephritic duct (Wolffian duct) at the lateral side and forming the Bowman's capsule at its medial extremity (Fig. 1a). Behind the lower end of the mesonephros, the metanephros develops as well. The collecting system of the mesonephros develops from the ureteric duct undergoing multiple divisions. The anterior, actively growing, portion of the bud is the ampulla, which induces surrounding cells of the metanephric mesoderm to proliferate, condense and form the metanephric cup (Fig. 1b).

During the developmental period described, bcl-2 positive cells are not present in the human mesonephros, while, in the metanephros, mildly expressing bcl-2 cells can be seen in the metanephric mesenchyme, and moderately expressing bcl-2 cells in the metanephric cup. Ampullae do not contain bcl-2 positive cells. In the control sections, preincubated with immunizing peptide, the bcl-2 signal was gone from metanephric mesenchyme and metanephric cup. (Table 1, Fig. 1c+,c-).

Caspase-3 positive cells are present in all structures forming the human mesonephros: the Wolffian duct, mesonephric mesenchyme, glomeruli and Bowman's capsule, mesonephric tubules and coelomic epithelium (Table 1). In comparison with the euchromatic nuclei of vital cells, caspase-3 positive cells have characteristic condensed nuclei or dark-stained nuclear fragments (Fig. 1d). Contrary to the mesonephric structures, in the metanephros rare caspase-3 positive cells can be seen only in the metanephric mesenchyme and in its condensation forming the metanephric cup. Uretheric bud and ampullae are completely devoid of caspase-3 positive cells (Table 1, Fig. 1e).

In the 5th and 6th weeks of development, both mesonephros and metanephros are missing p53 positive cells, but they can be found in the coelomic epithelium (Table 1).

During the same developmental period, all structures of the human mesonephros show Ki-67 positive cells: the Wolffian duct and surrounding mesenchyme, mesonephric glomeruli and Bowman's capsule and mesonephric tubules. Ki-67 positive cells are also seen among the cells of the coelomic epithelium (Table 1, Fig. 1f).

In the metanephros, the number of Ki-67 positive cells is even higher than in the mesonephros, particularly in the ampullae and metanephric cup mesenchyme (Table 1, Fig. 1g).

During the 7th, 8th and 9th weeks of development, the caudal parts of the mesonephros still differentiate, while its cranial parts already show signs of degenerative changes.

Table 2 Immunoreactivity to specific antibodies in the human mesonephros and metanephros during the 7th, 8th and 9th developmental weeks

	Antibodies								Structure
	Ki-67		p53		bcl-2		casp-3		
Weeks of development	7	8 and 9	7	8 and 9	7	8 and 9	7	8 and 9	
Wolffian duct	+ +	+ + +	−	−	+	−	+ +	+	MESONEPHROS
Mesonephric mesenchyme	+ +	+ +	+	+	+	+	+	+	
Glomeruli	+ +	+ +	+	+	+	+	+ +	+	
Bowman's capsule	+ +	+ +	+	+	+	+ +	+ +	+	
Mesonephric tubules	+ +	+ +	+	+	+	+ +	+ +	+	
Coelomic epithelium	+ +	+ +	+	+	−	+	+ +	−	
Collecting tubules	+ +	+ +	−	+	−	−	−	+ +	METANEPHROS
Ampulla	+ + +	+ + +	−	+ +	−	−	−	+ +	
Ureter	+ +	+ +	−	/	−	/	−	/	
Interstitium	+ +	+ +	−	+	+	+	+ +	+ +	
Renal vesicle	+ +	+ + +	−	+	+ +	+ + +	+	+	
S-shaped nephrons	+ + +	+ + +	−	+	+	+ +	+	+ + +	
Renal corpuscle	/	+ + +	−	+	/	+ +	/	+ + +	

+ + + strong reactivity, + + moderate reactivity, + mild reactivity, − no reactivity, / structure absent in the tissue section

In the same developmental period, the metanephros undergoes characteristic developmental changes: the ureteric bud dilates to form the primordium of the collecting system, including collecting tubules and developing ureters. Further differentiation of metanephric cup mesenchyme leads to formation of metanephric vesicles, S-shaped nephrons and more mature nephrons, consisting of Bowman's capsule and renal glomerulus. Other parts of the metanephric mesenchyme between the developing

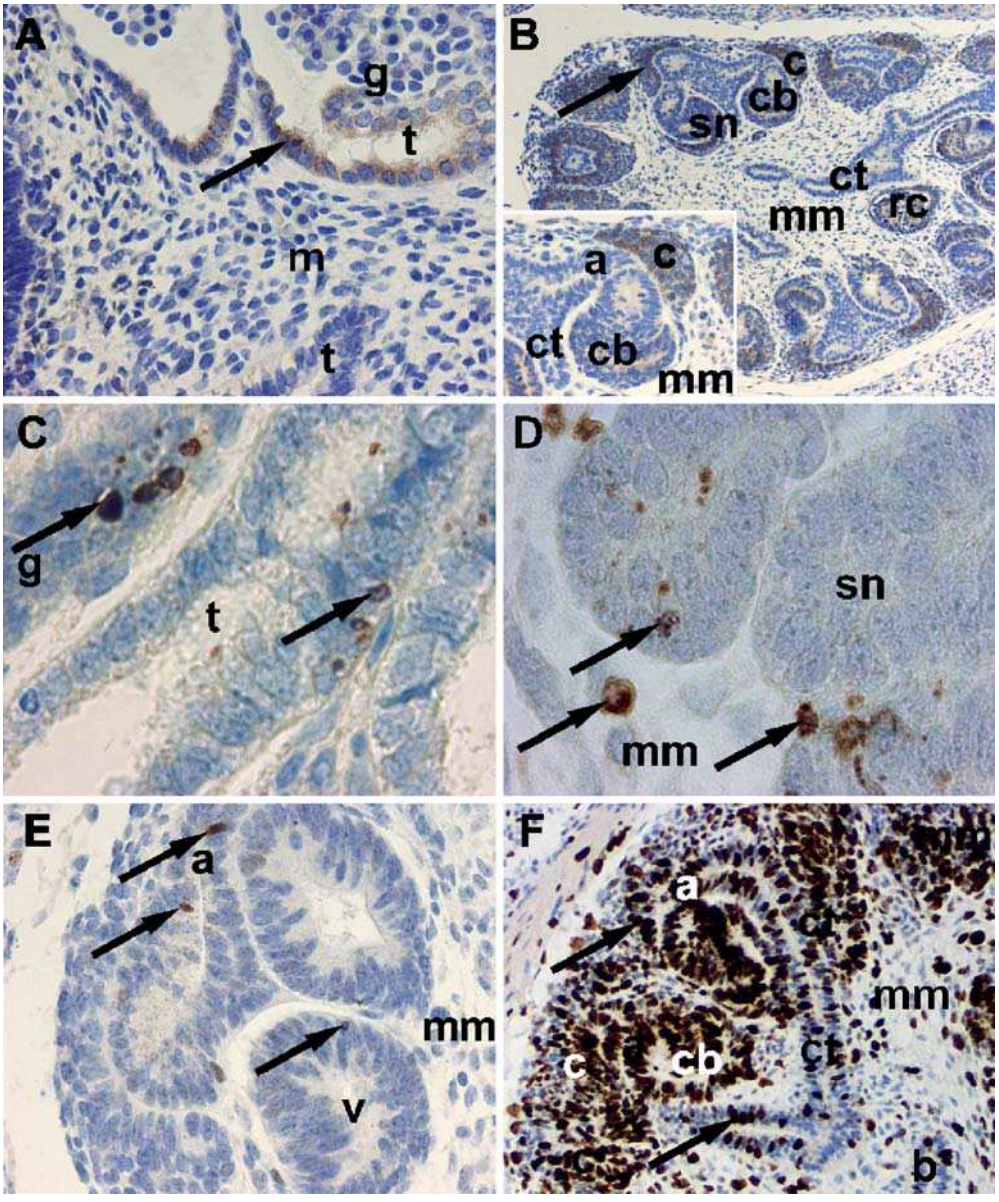

Fig. 2 Transverse sections through the 7th and 8th week human mesonephros. **A** Mesonephros (7 weeks): bcl-2 positive cells (*arrow*) are seen in metanephric tubules (*t*), glomeruli (*g*) and mesonephric mesenchyme (*m*). Immunostaining to bcl-2 protein, ×40. **B** Metanephros (8 weeks): bcl-2 positive cells (*arrow*) are seen in the metanephric cup (*c*), comma-shaped bodies (*cb*), S-shaped nephrons (*sn*) and renal corpuscles (*rc*), while they are missing in the collecting tubules (*ct*). Some metanephric mesenchymal cells (*mm*) are bcl-2 positive as well. Immunostaining to bcl-2 protein, ×10. Insert: detail showing the strongest bcl-2 positivity in the metanephric cup, less strong positivity in the comma-shaped bodies (*cb*) and mesenchyme (*mm*) and absence of positivity in the collecting tubuli and ampullae (*a*), ×40. **C** Mesonephros (7 weeks): caspase-3 positive cells (*arrows*) are seen in the mesonephric tubuli (*t*) and glomeruli (*g*) as brown-stained nuclei among the blue-stained nuclei of vital cells. Immunostaining to active caspase-3, ×40. **D** Metanephros (8 weeks): nuclei of caspase-3 positive cells (*arrows*) are seen in the developing S-shaped nephrons (*sn*) and in the nearby cells of the metanephric mesenchyme (*mm*). Immunostaining to active caspase-3, ×40. **E** Metanephros (8 weeks): p53-positive cells (*arrows*) are found in the ampullae (*a*), renal vesicles (*v*) and metanephric mesenchyme (*mm*). Immunostaining to p53 nuclear marker, ×40. **F** Metanephros (8 weeks): numerous Ki-67 positive cells (*arrows*) are seen in the collecting tubuli (*ct*) and ampullae (*a*), and they are less numerous in the proximal divisions of the ureteric bud (*b*). The metanephric cup (*c*) and comma-shaped bodies (*cb*) contain extensive numbers of Ki-67 positive cells, while their numbers are lower in the metanephric mesenchyme (*mm*). Immunostaining to Ki-67 protein, ×10

nephrons give rise to interstital connective tissue (Table 2, Fig. 2b).

During the described developmental period, bcl-2 positive cells appear in all structures forming the disappearing mesonephros (Table 2, Fig. 2a).

In the methanephros, the bcl-2 positive cells are missing in the whole collecting system, while they are numerous in developing nephrons, particularly in the less mature forms (Table 2, Fig. 2b).

In the mesonephros, caspase-3 positive cells are still present in all structures forming the mesonephros, but their numbers decrease in the 8th and 9th developmental weeks. (Table 2, Fig. 2c).

Compared with earlier developmental stages, the number of caspase-3 positive cells increases in the metanephros: in the 8th and 9th developmental weeks, caspase-3 positive cells appear in ampullae and collecting tubuli for the first time. They are also present in all parts of developing nephrons, particularly in the more mature forms of renal bodies, and in interstitial cells (Table 2, Fig. 2d).

The p53-positive cells appear in the mesonephros for the first time at the end of the 6th developmental week, except in the Wolffian duct and Bowman's capsule. In the 8th and 9th weeks, some p53-positive cells are detected in the Bowman's capsule as well (Table 2).

During the same developmental period, p53-positive cells are present in all structures of the developing metanephros, being the most numerous in the ampullae (Table 2, Fig. 2e).

All structures of the mesonephros show Ki-67 positive cells from the 7th to 9th developmental week (Table 2).

In the metanephros, the collecting system displays numerous Ki-67 positive cells, particularly in its ampullar part. The interstitial cells and differentiating nephrons contain large numbers of Ki-67 positive cells; their numbers increase with maturation of nephrons and advanced developmental stage (Table 2, Fig. 2f).

In the mesonephros, TUNEL-positive cells are seen in the same areas as caspase-3 positive cells (Fig. 3a–c). In the metanephros, TUNEL-positive cells are found in all parts of the developing kidney, having the same distribution as caspase-positive cells (Fig. 3d–f).

Quantitative analysis of the proliferation activity

Quantification was done only with Ki-67 nuclear marker, because it is the only antibody used in our study that gives very clear and distinct brown nuclear staining of reactive cells.

The percentage of Ki-67 positive cells increased significantly in both the mesonephric mesenchyme and nephrons comparing 5- and 6-week with 8- and 9-week old conceptuses (Fig. 4a).

The activity of the proliferation marker Ki-67 in the metanephric mesenchyme (Fig. 4b) decreased significantly in embryos aged 7 weeks compared with embryos aged 5 and 6 weeks. Significant increase of Ki-67 expression in the nephrons was found between 5- and 6-week and 8- and 9-week-old conceptuses (Fig. 4b). In the metanephric collecting tubules, significant decrease of Ki-67 activity was found between 5- and 6-week and 7-week-old embryos as well as between 5- and 6-week and 8- and 9-week-old embryos (Fig. 4b).

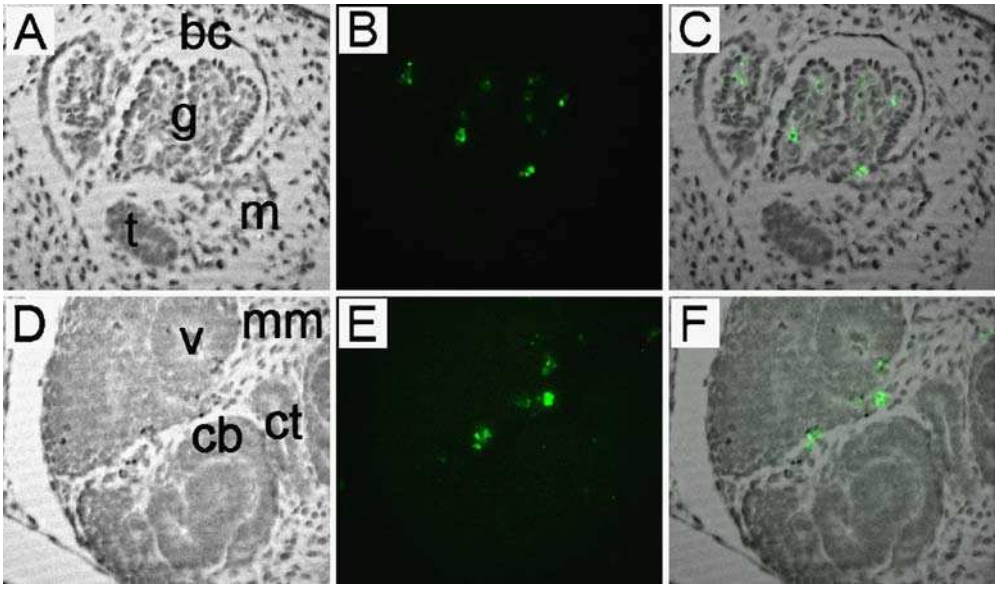

Fig. 3 Transverse sections through the 5th week mesonephros and 8th week metanephros. **A** Mesonephros: mesonephric glomerulus (*g*), Bowman's capsule (*bc*), mesonephric tubule (*t*), and mesonephric mesenchyme (*m*). Haematoxylin, ×40. **B** Several fluorescent TUNEL-positive cells are seen. TUNEL method, ×40. **C** TUNEL-positive cells are seen in areas corresponding to the glomerulus and mesenchyme. Merging of A+B, ×40. **D** Metanephros: renal vesicle (*v*), comma-shaped bodies (*cb*), collecting tubuli (*ct*) and metanephric mesenchyme (*mm*). Haematoxylin, ×40. **E** Several fluorescent TUNEL-positive cells are seen. TUNEL method, ×40. **F** Position of TUNEL-positive cells corresponds to areas of renal vesicle and metanephric mesenchyme. Merging of D+E, ×40

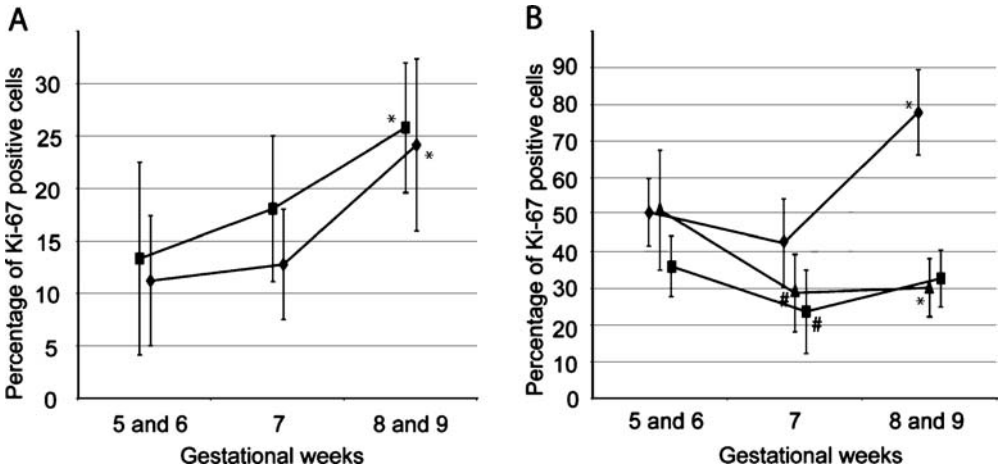

Fig. 4 Percentage of Ki-67 positive cells in **A** the mesonephros and **B** the metanephros from the 5th to 9th gestational weeks. *Diamonds* nephrons, *squares* mesenchyme, *triangles* collecting tubuli. *Significant difference ($P<0.05$, Dunn's post-hoc test) between the first and the third group, #significant difference ($P<0.05$, Dunn's post-hoc test) between the first and the second group

Discussion

The mesonephros

The mesonephric human kidney consists of transient glomeruli and tubules, which mostly degenerate except for the part that becomes the efferent ductules of the testes [32, 33].

Apoptosis seems to be important in the normal regression of this primitive kidney system that usually ends in the 16th developmental week [6, 27]. Indeed, in our study, caspase-3 and TUNEL-positive cells were already permanently present in all parts of the developing mesonephros in 5–8 week embryos, while their number slightly decreased with the beginning of the foetal period. According to Pole et al. [6], apoptosis commences in the mid-to-caudal region of the human mesonephros. We found cells expressing the p53 gene in all mesonephric structures, except for the mesonephric duct, for the first time in the 7th developmental week, while Lichnovsky et al. [24] found the same expression already in the 4-week human embryos.

Owing to the temporal and spatial appearance of those two factors in our study, caspase-3 mediated apoptosis might be associated with early morphogenesis of the mesonephros, while p53 expression appeared later in development, parallel to regressive processes in the primitive kidneys. Proliferation marker Ki-67 was expressed in all parts of the mesonephros and increased significantly at later embryonic stages, both in the mesenchymal and in the epithelial tissue components (nephrons). This is in line with the peak of mesonephric size and development being in the 8th developmental week, following the described intense cell proliferation and differentiation of all tissue components [6]. The anti-apoptotic bcl-2 protein appeared for the first time in the 7th developmental week. Its expression increased in some of the metanephric tubules in 8–9 week conceptuses, probably due to their survival and transformation into efferent ductules of a male adult.

The metanephros

During the developmental period investigated in our study, the human metanephros underwent several characteristic developmental steps, from invasion of the ureteric bud into the metanephric mesenchyme to induction and gradual differentiation of nephrons.

In the early metanephric development, we found caspase-3 positive and TUNEL-cells only in the metanephric mesenchyme and its condensation forming the metanephric cup. At later developmental stages, the number of apoptotic cells increased in the developing nephrons, particularly in the more mature forms and in the part of the metanephric mesenchyme giving rise to the interstitium. Apoptotic cells were detected in all parts of the metanephric collecting system for the first time in the 9th developmental week. These data indicate the important involvement of caspase-3 mediated apoptosis in early nephrogenesis and, later on, in development of the collecting system as well. This is in line with experimental data showing that administration of caspase-3 and caspase-9 inhibitors led to disturbances of both ureteric branching and nephrogenesis [11], while excessive apoptosis occurred in polycystic kidney disease where expression of various caspases, bax and bcl-2 was upregulated [9]. Similar to our results, in rat kidneys, dying cells formed 60% of metanephric mesenchymal cells and 40% of developing nephrons, while they were rarely found in branches of the ureteric bud [34]. The peak of apoptotic index was 3% in rat kidneys [34, 35]. In the early foetal metanephros (8–9th week), p53-positive cells appeared for the first time in both collecting systems and developing nephrons, being more numerous in the ampullae. Miosge et al. [23] found the first p53 expression later in development (10th–15th week) than we did in our study, while Prochazkova et al. [36] found p53-positive cells only sporadically and not in all metanephric structures. The appearance of p53-positive cells in all parts of the metanephros temporally coincided with the intense differentiation of the kidney collecting system. This might indicate importance of both apoptotic pathways, caspase-3 and p53 mediated, for the formation of the metanephric

collecting system. Experiments with p53 transgenic mice showed defective differentiation of the ureteric bud and hypoplastic kidneys due to increased apoptosis in the undifferentiated mesenchyme [22]. It was also shown that p53 over-expression could cause defects in human kidney development [14]. The process of apoptosis might be regulated through extrinsic factors, such as trophic hormones and growth factors [13]. Thus, administration of epidermal growth factor can reduce the number of apoptotic cell to 50% [35]. An intrinsic regulatory factor that can protect cells from apoptosis is bcl-2 protein. In the 5th developmental week, bcl-2 positive cells appeared in the metanephros, later on increasing in the nephrons, particularly in less mature forms, as previously shown by Prochazkova et al. [36]. Throughout the developmental stages investigated, bcl-2 positive cells were hardly or not at all detectable in derivatives of the ureteric bud, as earlier described in human tissue [24–26, 36]. Experiments with bcl-2 deficient mice showed kidney abnormalities, including hypoplasia with fulminant apoptosis within the metanephric mesenchyme [15, 16]. In the early development, only metanephric mesenchymal cells expressing bcl-2 differentiate into renal epithelium [13], as bcl-2 inhibits apoptosis in those cells [16]. Most of the remaining mesenchymal cells differentiate into stromal (interstitial) cells, some (cortical cells) of them giving rise to renin-producing cells, and some (medullary cells) differentiating into nephrons or undergoing apoptosis [7, 37]. In bcl-2 deficient mice renal failure results from severe polycystic kidney disease [17, 18].

A proliferation marker, Ki-67, was detected in all structures of the developing human metanephros throughout the developmental period investigated. Our quantitative analysis showed a significant decrease of proliferation of the metanephric mesenchyme between the 5th and 9th developmental weeks and a decrease of proliferation activity in the collecting system (50% – 30%) with advancing development. On the other hand, intense nephrogenesis was accompanied by significantly increased proliferation activity (from 50% to 80%). Previous investigations on human foetal kidneys of older developmental stages found a decrease of Ki-67 expression with progressive glomerular maturation, while Ki-67 expression was undetectable in terminally differentiated glomeruli [21].

In conclusion, in the human mesonephros, up to the 7th developmental week, the parallel presence of proliferation Ki-67 and apoptotic caspase-3 and TUNEL-positive cells in all mesonephric structures might be explained by initial growth of the mesonephros by mitosis and simultaneous nephron formation due to apoptosis. From the 7th week on, the appearance of p53 expression coincided with the regressive changes in the mesonephros, while bcl-2 positive cells enabled selective survival of some tubules giving rise to adult efferent ductules. In the early human metanephros (5–7 weeks), only Ki-67 positive cells were present in all metanephric structures, indicating the importance of this gene for the intense branching of the ureteric bud and induction of distinct numbers of nephrons. During the same period, both bcl-2 and caspase-3 expressing and TUNEL-positive cells were seen only in the metanephric mesenchyme and in differentiating nephrons, protecting nephrons from apoptosis on one hand and enabling selective cell death in the metanephric mesenchymal cells on the other hand. At later stages (from the 7th week on), increased proliferation activity accompanied advancing nephrogenesis. More numerous apoptotic cells, and the appearance of p53-expressing cells, probably contributed to the morphogenesis of both the collecting system and the nephrons. Cells expressing bcl-2 remained present only in the developing nephrons, thus saving them from apoptosis. All the mitotic, pro-apoptotic and anti-apoptotic factors described had spatially and temporally restricted patterns of appearance in developing human kidneys. Their balance and strict time course seem to be important for normal kidney development. Changes in their expression pattern might be associated with serious disturbances of kidney morphogenesis and function, often leading to chronic renal failure.

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ORIGINAL PAPER

Expression of intermediate filaments, EGF and TGF-α in early human kidney development

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Abstract The spatial and temporal expression patterns of cytokeratins, vimentin, epithelial growth factor (EGF) and transforming growth factor alpha (TGF-α), were investigated in the 5–9-week old human mesonephros and metanephros. Vimentin was found in all mesonephric structures, while cytokeratins were seen only in the mesonephric tubules. EGF and TGF-α were detected early in all mesonephric structures, and immunoreactivity to both factors decreased in later stages. In the 5–6-week metanephros, vimentin immunoreactivity was found in all structures and later increased in the collecting system and interstitium. In the 5th week, cytokeratins 8 and 19 appeared in the ureteric bud and ampullae, and later showed increasing immunoreactivity in the collecting system and nephrons. The coexpression of intermediate filament proteins in metanephric development is a temporary feature and might be associated with mesenchymal to epithelial transformation of developing nephrons. In adult kidneys, such coexpression is associated with fibrosis or carcinomatous changes. At early stages, immunoreactivity to EGF and TGF-α was detected in all metanephric structures and from the 7th week onward, it decreased in differentiating nephrons. EGF and TGF-α patterns of appearance indicate their role in induction, proliferation and growth of metanephric structures. Disturbances in that pattern might cause reduction in kidney growth.

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Keywords Human embryo · Mesonephros · Metanephros · Cytokeratins · Vimentin · EGF · TGF-α

Introduction

The mesonephros is a temporary functional kidney, which is formed of large number of vesicles that differentiate into s-shaped loops, which enter the mesonephric (Wolffian) duct laterally and form the mesonephric corpuscle medially. During development, it subsequently degenerates from the 8th to the 16th week. In the male, some parts of the Wolffian duct and mesonephric tubules persist as the adult genital ducts (Saxen 1987; Carlson 2004). Formation of the metanephros, or the permanent kidney, begins in the fifth week of development when a dorsal outgrowth of the Wolffian duct called the ureteric bud penetrates the metanephric mesoderm. The metanephric mesoderm gradually differentiates into metanephric cups, renal vesicles, the s-shaped nephrons and the more mature nephrons under inductive influence of the terminal branches (ampullae) of the ureteric bud. The collecting tubules, the renal calyces, the renal pelvis and the ureters are derivates of the ureteric bud (Saxen 1987; Carlson 2004).

The expression of intermediate filament proteins is different among the structures forming both the mesonephros and the metanephros. The cytokeratins are proteins characteristic for epithelial cells, while vimentin filaments occur in mesenchymally derived cells. The cytokeratins are coexpressed as type 1/type 2 pairs in the epithelial cells. In the early embryonic development, the first cytokeratins to be expressed are type 2 cytokeratin 8 and type 1 cytokeratin 18, later coexpressed as a pair in the adult simple epithelia. Cytokeratin 19, a type 1 cytokeratin, can also be coexpressed with CK8 in adult simple epithelia and

epithelial cells of the human fetal mesonephros and metanephros (Moll et al. 1982; Godsave et al. 1986; Magro et al. 2001; Lane 2007). During human kidney development, the appearance of intermediate filament proteins is associated with different pathways of the mesenchymal cell differentiation, one leading to formation of the epithelial cells (nephrons) and another to the connective tissue (interstitial) cells. In the human fetal mesonephros, the cytokeratins (mostly CK8, CK18 and CK19) are found in the Bowman's capsule and in the mesonephric tubules, while vimentin is expressed in the Bowman's capsule, glomeruli and in the mesenchyme (Magro et al. 2001). During human fetal metanephric nephrogenesis, the coexpression of the cytokeratins and the vimentin is found in Bowman's capsule, glomeruli, and partially in the other parts of the nephrons (Magro et al. 2001; Holthöfer et al. 1984; Oosterwijk et al. 1990; Moll et al. 1991; Nagata et al. 1993). Systematic data on the appearance of intermediate filaments in the successive stages of the early human kidney development are missing.

Transforming growth factor α (TGF-α) is a member of the epidermal growth factor family (EGF). Both the TGF-α and the EGF bind to the same EGF/TGF-α receptor (Bernardini et al. 1996; Chailler and Briere 1998). TGF-α expression is detected in the mesonephros and the metanephros of the chick (Diaz-Ruiz et al. 1993) and rat embryos (Bernardini et al. 2001; Rogers et al. 1992), as well as in the human fetal metanephros (Goodyer et al. 1991). Both factors, especially TGF-α, are capable of inducing tubulogenesis and branching morphogenesis in vitro, although they are found to be less potent than hepatocyte growth factor (HGF) and exhibit different patterns of inductive activity (Barros et al. 1995; Sakurai et al. 1997). Addition of the anti-TGF-α antibodies to the organ culture medium causes inhibition of the ureteric bud branching and tubulogenesis in the rat metanephros, thus inhibiting metanephric growth in general (Rogers et al. 1992). In the human fetal metanephric organ culture, the addition of the anti-EGF/TGF-α receptor antibody decreased the exogenous EGF and TGF-α effect on the basal DNA-synthesis rate in the subcapsular mesenchymal cells, peritubular cells, glomeruli and in the tubules (Chailler and Briere 1998). It is believed that the TGF-α and EGF, both present in different stages of the nephron development, might have an important role in the mutual induction of the human embryonic metanephric structures by binding to EGF/TGF-α receptor found on the derivates of the ureteric bud (Bernardini et al. 1996, 2001).

The studies of developing human kidneys involving the cytokeratin and vimentin intermediate filaments expression were performed only during fetal period, while early embryonic stages were missing (Moll et al. 1982; Godsave et al. 1986; Magro et al. 2001; Holthöfer et al. 1984; Oosterwijk et al. 1990; Moll et al. 1991; Nagata et al. 1993). Based on studies performed on animal models, it is believed that "severe" mutations of genes encoding cytokeratins have lethal consequences, while "mild" mutations might lead to multifactorial disorders (Lane 2007). In adult human kidneys, expression of intermediate filaments differs from their expression pattern in early development. Additionally, the return to embryonic type of expression found in adult kidneys is associated with injuries or carcinomatous transformation of kidney tissue (Moll et al. 1991).

Despite several studies on the TGF-α and EGF expression in human kidney development, only two investigations were partially done on the embryonic tissues (Bernardini et al. 1996, 2001). Disturbances in the TGF-α and EGF expression might lead to reduction in kidney growth (Chailler and Briere 1998; Rogers et al. 1992).

The aim of this study was to analyze the temporal and spatial pattern of appearance of the cytokeratins, the vimentin, and the TGF-α and EGF during the embryonic and early fetal stages of the human mesonephric and metanephric development. Their possible contribution to normal differentiation of structures enabling normal kidney development and function is discussed.

Materials and methods

Human material

A total of 8 normal human conceptuses between the 5th and the 9th developmental week were collected after spontaneous abortions from the Department of Gynecology and Obstetrics, Clinical Hospital Split, Croatia, or after tubal pregnancies from the Department of Pathology, Clinical Hospital Split. The embryos and fetuses were examined macroscopically and measured. Only normal conceptuses, without any sign of abnormality, intrauterine death or macerations, were used in our study. The embryonic tissues were treated as postmortem material with permission of the Ethical and Drug Committee of the Clinical Hospital Split, in accordance with the 1964 Helsinki Declaration. The postovulatory age was estimated on the basis of the menstrual data and correlated with the crown-rump length (CRL) and Carnegie stages (O'Rahilly and Gardner 1971).

Immunohistochemical staining

Caudal parts of embryos containing developing kidneys were dissected. Tissue samples were fixed in 4% paraformaldehyde in phosphate buffer and dehydrated in 100%

ethanol. They were embedded in paraffin wax, serially sectioned at 4–6 μm, mounted on glass slides, and analyzed using an Olympus BX-40 light microscope (Olympus, Tokyo, Japan).

Sections were deparaffinized in xylene and rehydrated in ethanol and water. In order to quench endogenous peroxidase activity, sections were incubated for 30 min in 0.1% H_2O_2.

After washing in PBS, sections for pancytokeratin (reacting with cytokeratins 5, 6, 8, 17 and 19), cytokeratin 8, cytokeratin 19 and vimentin staining, were incubated in EDTA for 10 min at 95°C. After cooling to room temperature, sections were incubated with primary antibodies: monoclonal mouse anti-human cytokeratin (1:2200 dilution; M 0821, DAKO, Glostrup, Denmark), Monoclonal Mouse Anti-Human Cytokeratin 8 (1:50 dilution; M631, DAKO), Monoclonal Mouse Anti-Human Cytokeratin 19 (1:75 dilution; M 0722, DAKO) and mouse anti-vimentin/HRP (1:3000 dilution; U 7034, DAKO), for 45 min in the dark.

Sections for immunohistochemical staining of EGF and TGF-α antigen, after washing in PBS, were incubated in 0.05% saponin for 30 min. Then they were washed in PBS and incubated with rabbit anti-human EGF (1:100 dilution; PC08, Calbiochem, San Diego, CA) or mouse anti-TGF-α (1:10 dilution; GF10, Calbiochem) primary antibodies for 45 min in the dark.

Pancytokeratin and vimentin primary antibody binding was visualized by incubating the sections with Envision+ single reagent visualization system, which contains peroxydase-conjugated anti mouse secondary antibody (K 4001, DAKO) for 30 min.

Binding of CK8 and CK19 primary antibodies was detected using streptavidin-biotin peroxidase system (K0690, DAKO) as recommended by the manufacturer.

Afterwards, sections were washed with PBS and then incubated in DAB system (K3468, DAKO), as recommended by the manufacturer.

Rabbit ABC Staining System (sc-2018, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used for detection of EGF primary antibody binding and mouse ABC Staining System (sc-2017, Santa Cruz Biotechnology, Inc.) was used for visualization of TGF-α primary antibody binding.

Finally, sections were rinsed in distillated water, counter-stained with hematoxylin, and dehydrated in ethanol and xylol.

Sections without primary antibodies incubation were used as negative controls. Positive controls were developing kidney structures or other tissues in the same sections (as each section contained various types of tissues and organs) that were known to label specifically with primary antibodies.

Analysis was performed on Olympus BX-51 microscope equipped with DMP digital camera and using DP-SOFT Version 3.1 software.

Results

During the 5th and 6th week of development, both the mesonephros and the metanephros are present in the human embryos. The mesonephros already consists of renal glomeruli and tubules opening into the mesonephric duct (Wolffian duct) at the lateral side, and forming Bowman's capsule at its medial extremity. The metanephros develops behind the lower end of the mesonephros and contains less developed structures than the mesonephros. The anterior actively growing portion of the ureteric bud is the ampulla, which induces surrounding cells of the metanephric mesoderm to proliferate and form the metanephric cup. The remaining cells of the metanephric mesoderm form the metanephric mesenchyme.

Vimentin positive cells were found in all mesonephric structures, except the Wolffian duct (Table 1, Fig. 1A). In the metanephros, weakly vimentin positive cells were present in all structures: ureteric bud, ampullae, metanephric mesenchyme and metanephric cup (Table 2, Fig. 1B+).

During the described developmental period, cells stained with pancytokeratin, CK8 and CK19 antibodies were present in the Wollfian duct, Bowman's capsule, mesonephric tubules and in the coelomic epithelium. CK8 and CK19 positive cells were also detected in the glomeruli, while the reaction to all three antibodies was absent in the mesenchyme (Table 1, Fig. 1C). The metanephros showed no reaction to pancytokeratin antibody, while mild positivity to both CK8 and CK19 was found in the ureteric bud and in the ampullae (Table 2, Fig. 1D).

In the same developmental period, both the EGF and TGF-α positive cells were present in all structures forming the mesonephros, with the exception of coelomic epithelium. Strong EGF positivity was detected in the mesonephric mesenchyme (Table 1), while moderate TGF-α reactivity was present in the Wolffian duct and in the mesonephric tubules (Table 1). In the metanephros, moderate EGF reactivity was found in the mesenchyme and in the ureteric bud, while mild reactivity was present in the metanephric cup and in the ampulla (Table 2, Fig. 1E). TGF-α positivity had the same distribution pattern, but with stronger intensity in all structures except the ureteric bud (Table 2, Fig. 1F).

During the 7th, 8th and 9th week of development, the caudal parts of the mesonephros still differentiate, while its cranial parts already show signs of regressive changes. In the same period, the metanephros undergoes several

Table 1 Immunoreactivity to specific antibodies in the human mesonephros during the 5th–9th week of development

Antibodies	Developmental weeks	Wolffian duct	Mesonephric mesenchyme	Glomerulus	Bowman's capsule	Mesonephric tubule	Coelomic epithelium
Vim	5 and 6	−	+ +	+ +	+ +	+	+
	7	−	+ +	+ +	+ +	+	+
	8 and 9	+ +	+ +	+ +	+ +	+	+
CK	5 and 6	+	−	−	+	+	+
	7	−	−	−	+ +	+	+
	8 and 9	−	−	−	+ +	+ +	+
CK8	5 and 6	+	−	+	+	+ +	+
	7	−	−	+	+ +	+ +	+
	8 and 9	+	−	+	+ +	+ + +	+
CK19	5 and 6	+ +	−	+	+ +	+ +	+
	7	−	−	+	+	+ +	+
	8 and 9	+	−	+	+ +	+ + +	+ +
EGF	5 and 6	+	+ + +	+ +	+ +	+	−
	7	+	+	+ +	+ +	+ +	+ +
	8 and 9	−	+	+	+	+	+
TGF-α	5 and 6	+ +	+ +	+ +	+ +	+ +	−
	7	+	+	+ +	+ +	+ +	+
	8 and 9	−	+ +	+	+	+ +	+

+ + +, Strong reactivity; + +, Moderate reactivity; +, Mild reactivity; −, No reactivity

developmental changes: ureteric bud dilates and branches to form the primordium of collecting system, including collecting tubules ending with ampullae, and developing ureters. Continuous branching of the ureteric bud induces further differentiation of the metanephric mesenchyme into numerous metanephric cups, giving rise to renal vesicles, s-shaped nephrons and more mature forms of nephrons containing renal corpuscles (Bowman's capsule and glomerulus). Other parts of the metanephric mesenchyme give rise to interstitial connective tissue cells.

In the 7th developmental week, vimentin showed the same immunostaining pattern as at earlier stages: moderate positivity could be found in the mesonephric mesenchyme, glomeruli and in the Bowman's capsule, while slight vimentin reactivity was detected in the mesonephric tubules and in the coelomic epithelium. During the later developmental period (8–9 weeks), mesonephric structures retained the same reactivity, except the Wolffian duct, which showed moderate vimentin immunoreactivity (Table 1). In the 7-week old metanephros, slight vimentin immunoreactivity was detected in the s-shaped nephrons, while other metanephric structures had moderate vimentin positivity. Between 8th and 9th developmental week, strong vimentin positivity was present in the collecting tubules and ampullae and in the interstitium (former metanephric mesenchyme), while moderate reactivity could be found in the metanephric cup. Mild vimentin positivity was detected in some cells of the renal vesicle, the s-shaped nephrons and in the renal corpuscles (Table 3, Fig. 2A).

In the 7-week mesonephros, pancytokeratin positive cells were present in the Wolffian duct and the surrounding mesenchyme, in the Bowman's capsule, mesonephric tubules and in the coelomic epithelium. CK8 and CK19 were detected in all mesonephric structures, except the mesenchyme and the Wolffian duct. The mesenchyme surrounding the Wolfian duct showed CK8 positivity. The distal part of the mesonephric tubules was stained more intense with both specific cytokeratins (Table 1). In the 7-week metanephros, cells positive for pancytokeratin antibody were found in the collecting tubules, but not in the ampulla, which was stained with both CK8 and CK19. Additionally, metanephric cup and renal vesicle showed clear CK8 positivity (Table 3). Between the 8th and 9th developmental week, pancytokeratin positive cells were present in the mesonephric mesenchyme surrounding the Wollfian duct, in the mesonephric tubules, in the coelomic epithelium and in the Bowman's capsule. CK8 and CK19 positive cells had similar distribution pattern, with additional positivity in the glomeruli and clear positivity of both cytokeratins in the epithelia of the Wolffian duct (Table 1). In the metanephros, moderate pancytokeratin reactivity was detected in the collecting tubules, while mild reactivity was found in the ampulla. Both specific cytokeratin antibodies were present in all metanephric structures except in the interstitium. Compared to CK8,

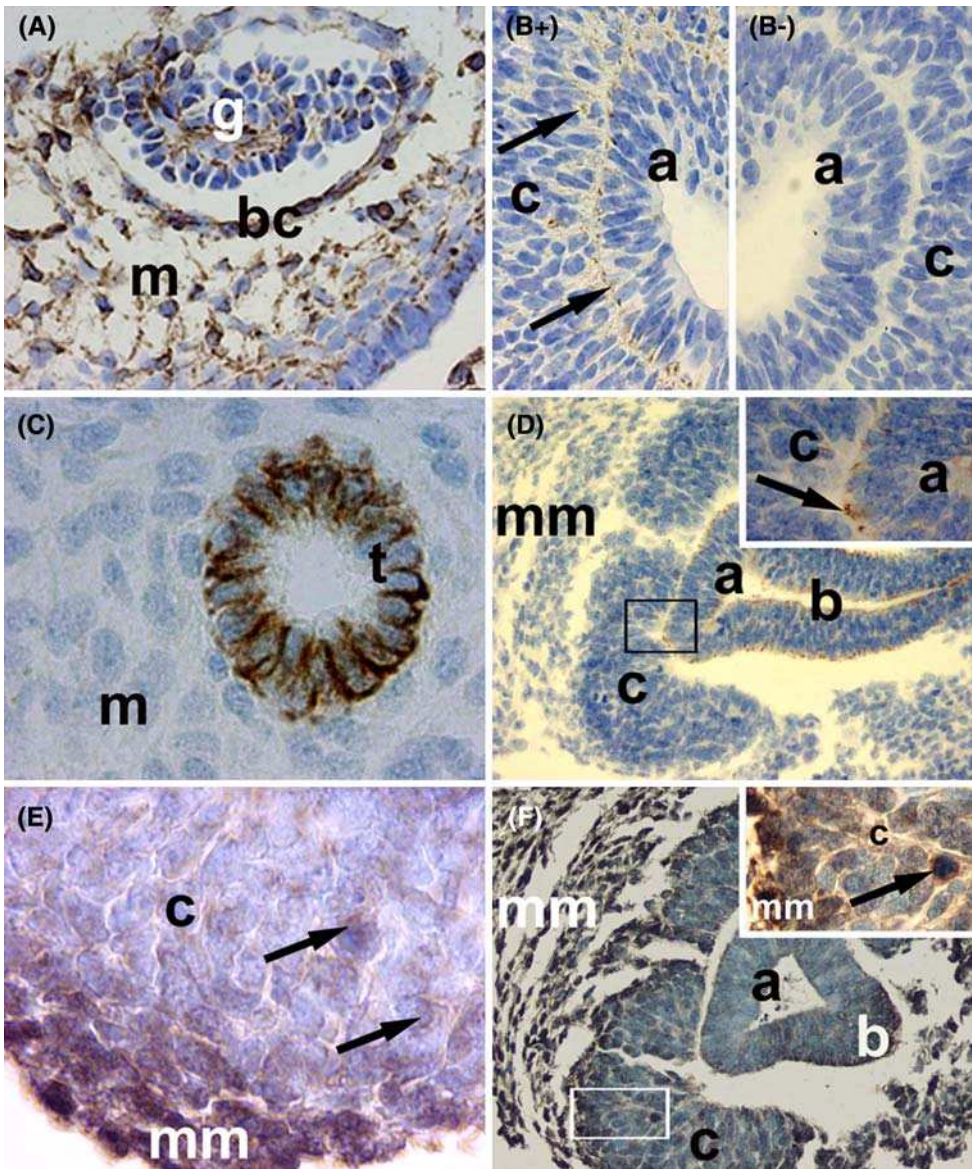

Fig. 1 Transversal sections through the human mesonephros and metanephros in the 5th and 6th developmental week: (**A**) Mesonephros (5 weeks): note positive staining to vimentin in the glomerulus (g), Bowman's capsule (bc) and the surrounding mesenchyme (m). Immunostaining to vimentin, ×40. (**B+**) Metanephros (5 weeks): weak vimentin positivity (arrows) seen both in the ampulla (a) and metanephric cup (c). Immunostaining to vimentin, ×40. (**B−**) Metanephros (5 weeks): Absence of immunohistochemical reaction (vimentin positivity) in metanephric cup (c) and ampulla (a). Negative control, ×40. (**C**) Mesonephros (6 weeks): strong cytokeratin positivity characterizes mesonephric tubules (t), while surrounding mesenchyme (m) shows no reactivity. Immunostaining to cytokeratins, ×100. (**D**) Metanephros (5 weeks): mild positivity to CK8 is seen in the ureteric bud (b) and ampulla (a), while it is absent in the metanephric cup (c) and mesenchyme (mm). Immunostaining to CK8, ×20. Insert: enlarged area of apposed ampulla (a) and metanephric cup (c). CK8 positivity is present in the ampulla (arrow). Immunostaining to CK8, ×100. (**E**) Metanephros (5 weeks): reactivity to EGF protein is moderate in the metanephric mesenchyme (mm), while some cells of the metanephric cup (c) display mild EGF positivity (arrows). Immunostaining to EGF protein, ×100. (**F**) Metanephros (5 weeks): note moderate reactivity to TGF-α in the ureteric bud (b), ampulla (a) and metanephric cup (c), and strong reaction in the metanephric mesenchyme (mm). Immunostaining to TGF-α protein, ×20. Insert: enlarged area of metanephric cup (c) and nearby metanephric mesenchyme (mm). Mitotic cells show particularly strong reaction (arrow). Immunostaining to TGF-α protein, × 100

CK19 was absent in the metanephric cup (Table 3, Fig. 2B).

EGF and TGF-α positive cells were present in all mesonephric structures in the 7th developmental week, with stronger EGF reactivity in the coelomic epithelium. Some of the mesonephric tubules (proximal parts) had stronger immunoreactivity to both EGF and TGF-α than the others (Table 1, Fig. 2C). In the metanephros, only some cells of

Table 2 Immunoreactivity to specific antibodies in the human metanephros during the 5th–6th week of development

Antibodies	Ureteric bud	Ampulla	Metanephric mesenchyme	Metanephric cup
Vimentin	+	+	+	+
CK	−	−	−	−
CK8	+	+	−	−
CK19	+	+	−	−
EGF	+ +	+	+ +	+
TGF-α	+ +	+ +	+ + +	+ +

+ + +, Strong reactivity; + +, Moderate reactivity; +, Mild reactivity; −, No reactivity

the collecting tubules, ampullae, interstitium and the metanephric cup displayed both EGF and TGF-α positivity, with stronger EGF positivity in the ampullae. Additionally, EGF positive cells were detected in the renal vesicles (Table 3). Between 8th and 9th developmental week, all mesonephric structures except the Wolffian duct, contained both EGF and TGF-α positive cells. TGF-α reactivity was stronger in the mesonephric mesenchyme and tubules (Table 1). During the same period of metanephric development, EGF and TGF-α positive cells had the same distribution pattern as described for the 7th week, with stronger TGF-α reactivity in the collecting tubules (Table 3, Fig. 2D).

Discussion

The mesonephros

According to the results of the present study, expression pattern of vimentin intermediate filament protein did not change in the mesonephros between the 5th and 9th week of development, with the exception of the Wolffian duct, which showed vimentin reactivity only in the 8th and 9th week. Similar to vimentin, pancytokeratin immunoreactivity only slightly increased during the same developmental period. The distribution of immunostaining to CKs 8 and 19, representing cytokeratins of simple epithelia, mostly coincided with the distribution of immunostaining to pancytokeratin, with only slight differences in staining intensity and additional reactivity of both CK8 and CK19 in the glomeruli. Magro et al. (2001) performed similar study on the older human mesonephros in the 8–12 week old conceptuses: they found vimentin positivity only in the glomeruli, Bowman's capsule and mesenchyme, but not in the mesonephric tubules, as we did. However, their results on CK8 and CK19 immunoreactivity in the early fetal mesonephros were similar to ours. Coexpression of CK8 and CK19 with vimentin found in our study confirmed earlier maturity of mesonephric nephrons in comparison to metanephric nephrons, and was in line with the fate of the mesonephros to appear earlier in the development than the metanephros, and than to subsequently regress.

The EGF and TGF-α positive cells were found in all mesonephric structures and the distribution pattern of both factors was basically the same from the 5th to 9th developmental week, with slight differences in staining intensity. Bernardini et al. (1996) found weak EGF and TGF-α positivity in human mesonephros already in the 4th week. We also noted slight decrease in their expression with advancing development, what might be associated with initial regression of the mesonephros during the late embryonic and the early fetal period.

The metanephros

During the developmental period investigated in our study, all metanephric structures contained vimentin positive

Table 3 Immunoreactivity to specific antibodies in human metanephros during the 7th–9th week of development

Antibodies	Dev. weeks	Collecting tubule	Ampulla	Interstitium	Metanephric cup	Renal vesicle	S-nephron	Renal corpuscle
Vim	7	+ +	+ +	+ +	+ +	+ +	+	/
	8 and 9	+ + +	+ + +	+ + +	+ +	+	+	+
CK	7	+	−	−	−	−	−	−
	8 and 9	+ +	+	−	−	−	−	−
CK8	7	+ +	+ +	−	+	+	/	/
	8 and 9	+ +	+	−	+	+ +	+ +	+ +
CK19	7	+ +	+	−	−	−	/	/
	8 and 9	+ +	+	−	−	+	+	+ +
EGF	7	+ +	+ +	+ +	+	+	/	/
	8 and 9	+	+	+ +	+	+	+	+
TGF-α	7	+ +	+	+ +	+	−	/	/
	8 and 9	+ +	+	+ +	+	+	+	+

+ + +, Strong reactivity; + +, Moderate reactivity; +, Mild reactivity; −, No reactivity; /, Structure absent in the tissue section

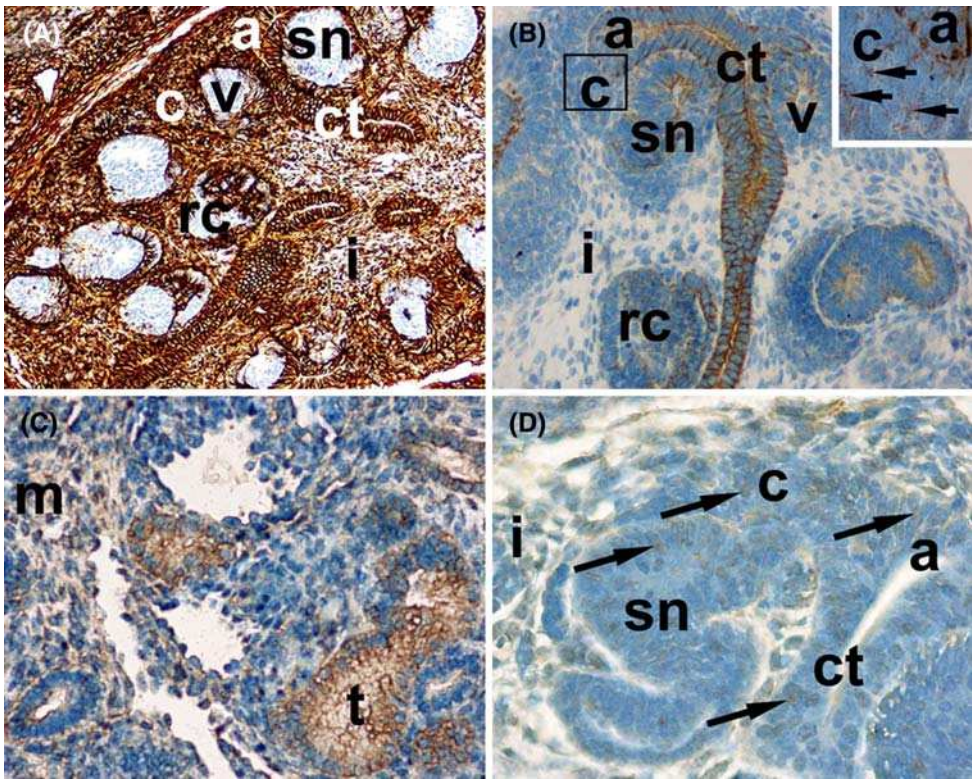

Fig. 2 Transversal sections through the human mesonephros and metanephros in the 7th, 8th and 9th developmental week: (**A**) Metanephros (8 weeks): strong vimentin reactivity is seen in collecting tubules (ct), ampullae (a) and interstitium (i), while moderate reactivity is present in the metanephric cup (c). Note mild reactivity of some cells forming the renal vesicles (v), s-shaped nephrons (sn) and renal corpuscles (rc). Immunostaining to vimentin, ×10. (**B**) Metanephros (9 weeks): note moderate CK8 reactivity in collecting tubules (ct) and mild in the ampullae (a). Moderate to mild reactivity is seen in metanephric cup (c), renal vesicles (v), s-shaped nephrons (sn) and renal corpuscles (rc), while interstitium (i) shows no reaction. Immunostaining to CK8, ×20. Insert: enlarged area of apposed metanephric cup and ampulla. Note several CK8 positive cells in the metanephric cup (arrows). Immunostaining to CK8, ×100. (**C**) Mesonephros (7 weeks): note stronger reaction of some mesonephric tubules (t) to TGF-α, and mild reaction in the mesenchyme (m). Immunostaining to TGF-α protein, ×20. (**D**) Metanephros (8–9 weeks): some cells (arrows) in the collecting tubules (ct), ampullae (a), metanephric cup (c) and s-shaped nephrons (sn) show mild reactivity to EGF. Interstitium is characterized by moderate EGF positivity. Immunostaining to EGF protein, ×40

cells: while in the 5–6-week embryos the vimentin immunoreactivity was mild, later in development it increased, particularly in the collecting system. At the earliest investigated developmental stages, CKs 8 and 19 appeared parallel to vimentin, but only in the ureteric bud and its branches. From the 7th week onward, coexpression of vimentin and CK8 additionally appeared in the metanephric cups, while from the 8th week on, both cytokeratins coexpressed with vimentin also in the differentiating nephrons with increasing intensity. Coexpression of the two intermediate filaments was previously described in the human 7–20 week metanephros (Magro et al. 2001) and at later stages of the human fetal development (Holthöfer et al. 1984; Oosterwijk et al. 1990; Moll et al. 1991). Among all the metanephric structures, the metanephric mesenchyme was the only part of metanephric kidney to show only vimentin expression, without signs of coexpression with cytokeratins at any investigated developmental stage.

Coexpression of cytokeratins and vimentin was shown to be relatively frequent in development (Magro et al. 2001) and usually reflected the advancement of developmental steps and maturation of the organ. Thus, similar coexpression was found in certain stages of human notochord and central nervous system development (Lehtonen et al. 1995; Saraga-Babic et al. 2002). Due to Magro et al. (2001), overlapping expression of cytokeratins and vimentin suggested similar genetic program of the two intermediate filaments in both the mesonephros and the metanephros. While presence of vimentin in the tubular cells might be explained by their mesenchymal origin (Magro et al. 2001), the cytokeratin expression coincided with subsequent development of their epithelial characteristics. Due to Oosterwijk et al. (1990), in human fetal kidneys, first cells to express CK8 and CK19 were the cells of renal vesicles. In contrast to those findings, we detected CK8 protein already in the metanephric cup, the earliest condensations of metanephric mesenchyme induced by the

ampullae. As a consequence of cell condensation in the metanephric cup, several interstitial proteins were replaced with the epithelial-type proteins (Carlson 2004). In our study, this epithelial transformation was additionally associated with first signs of cytokeratin immunoreactivity. This might mean that inside the cells of the future nephrons, in addition to the change in the composition of extracellular proteins, genetic program controlling the type of intracellular intermediate filament proteins also switched towards more epithelial characteristics. Further increase of cytokeratin immunoreactivity followed the advancement of mesenchymal to epithelial transformation, as well as maturation of the cells forming the nephrons.

Thus, as shown in our study, expression of different intermediate filament proteins changed during early human metanephric development. Coexpression of vimentin and cytokeratins was only transitional feature of nephron cells and appeared at clearly defined developmental stages. At later fetal development, their coexpression gradually ceased and was replaced by more specialized intermediate filament expression pattern, characteristic for adult kidney tissue (Holthöfer et al. 1984). In adult kidneys, conversion from cytokeratin expression to cytokeratin-vimentin coexpression and later to exclusively vimentin expression, is one of the signs of epithelial to mesenchymal transition of epithelial cells in renal fibrosis or carcinomatous transformation (Moll et al. 1991; Zoltan-Jones et al. 2003; Liu 2004; Brandal et al. 2006).

EGF and TGF-α are considered as mitogenic factors and seem to be related to proliferation status of the cell (Chailler and Briere 1998). In our previous study, we stressed the role of cell proliferation in both the branching of the ureteric bud and in the nephron formation (Carev et al. 2006). In our present study, both growth factors were detected in all metanephric structures, but their immunoreactivity was particularly strong in the mesenchyme (later interstitum) and in the branches of the ureteric bud (collecting system). Bernardini et al. (1996) suggested that the developing nephrons were the main source of EGF and TGF-α, while the collecting system was predominantly expressing their receptor. However, in the later investigation of the same authors, both TGF-α mRNA and protein were equally expressed in the cells of the developing collecting system, as well as in the cells of the developing nephrons (Bernardini et al. 2001). Significant immunoreactivity to both factors, especially in mitotic cells, confirmed the role of those growth factors in the early growth and intense development of all metanephric structures, as previously suggested by other authors (Chailler and Briere 1998; Bernardini et al. 2001). A slight decrease in their expression characterized advanced developmental stages, and was coincidental with progression in maturation of nephrons. Despite differences in the results, conclusions resulting from studies provided on developing human kidneys agreed on the role of both factors in supporting proliferation, and on their progressive downregulation during nephrogenesis (Bernardini et al. 1996; Chailler and Briere 1998). All important developmental processes including growth of the metanephros, branching of the ureteric bud and tubulogenesis seemed to be influenced by TGF-α (Rogers et al. 1992), while for EGF was additionally suggested to inhibit cell death (Coles et al. 1993).

In conclusion, our investigation on expression of vimentin and cytokeratin intermediate filament proteins during early human kidney development disclosed their precise spatial and temporal pattern of appearance in both the mesonephros and the metanephros. Changes in this developmental pattern might lead to disturbances in the mesenchymal to epithelial transformation of developing nephron cells e.g. to disturbed nephrogenesis.

EGF and TGF-α proteins also showed characteristic pattern of expression in developing human kidneys, being more intensely expressed in all structures that showed intense growth. Therefore, the suggested role of those two growth factors is in both branching and development of the collecting system, as well as in the development of nephrons. Consequently, the failure in their expression pattern might lead to the reduction of kidney size and function.

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Involvement of FGF and BMP family proteins and VEGF in early human kidney development

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Summary. The spatial and temporal pattern of the appearance of the fibroblast growth factor proteins (FGF-8 and FGF-10), the bone morphogenetic proteins (BMP-2/4 subfamily and BMP-7) and the vascular endothelial growth factor protein (VEGF) was investigated in the human mesonephros and metanephros of the 5-9 week-old conceptuses. In the mesonephros, both FGF's and BMP's were found in all structures and their expression slightly decreased in the early fetal period. VEGF positivity appeared in all mesonephric structures, and increased in the fetal period coincidently with formation of the mesonephric blood vessel network. In the metanephros, FGF-8 first appeared only in the metanephric mesenchyme, but from the 7th week on, its reactivity increased and spread to other metanephric structures. FGF-10 positive cells appeared in all metanephric structures already in the 5th week, and slightly intensified with progression of development. Cell survival and nephrogenesis in the permanent kidney might be associated with the appearance of both growth factors. Both BMP-2/4 and BMP-7 displayed a similar pattern of reactivity in all metanephric structures, and their reactivity intensified with advancing development. Alterations in their pattern of appearance might lead to the formation of small and dysplastic kidneys. Already in the earliest developmental stages, VEGF protein appeared in all metanephric structures. At later stages, VEGF showed more intense reaction in the collecting system than in the differentiating nephrons and interstitium. Due to VEGF involvement in vasculogenesis and angiogenesis, abnormal VEGF appearance might lead to impaired formation of the blood vessel network in the human permanent kidney.

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Introduction

The mesonephros is a transitional, but functional kidney that develops late in the 4th developmental week and is formed of a large number of s-shaped loops. The lateral ends of these loops enter the mesonephric (Wolffian) duct, while the medial end forms the Bowman's capsule surrounding the glomerulus. The mesonephros undergoes gradual degeneration from 8th to 16th developmental week

The metanephros begins to develop in the fifth week of development when the ureteric bud penetrates the metanephric mesoderm. Under the inductive influence of the terminal branches (ampullae) of the ureteric bud, metanephric mesoderm condenses and covers the ampullae to form metanephric cups. During further development, metanephric cups give rise to the renal vesicles, s-shaped tubules (nephrons) and renal corpuscles (consisting of Bowman's capsule and glomerulus). The metanephros is a permanent kidney, which later in development transforms into more mature nephrons and a collecting system consisting of the collecting tubules, renal calyces, renal pelvis and ureter (Saxen, 1987; Sadler, 2004).

Fibroblast growth factors (FGF) are involved in cell proliferation, migration and differentiation of different structures and organs during the mammalian development (Ornitz et al., 1996; Celli et al., 1998; Ohuchi et al., 2000). Previous studies on FGF-8 mostly emphasized its involvement in chick and mouse limb development (Heikinheimo et al., 1994; Mahmood et al., 1995; Vogel et al., 1996). However, the expression of FGF-8 was found in the metanephric glomeruli of the mouse embryo (Mahmood et al., 1995), while FGF-8 mRNA was detected in the chick mesonephros as well

(Vogel et al., 1996). In the recent study performed by Grieshammer et al. (2005) on developing mice kidney, severely reduced FGF-8 signaling resulted in abnormal nephron formation, while in the complete absence of FGF-8, the nephron progenitor cells underwent apoptosis and no s-shaped nephrons were formed.

In the isolated rat ureteric bud culture, FGF-10 (and FGF-1) stimulates ureteric bud cells to form long, branching tubular structures with clearly formed ampullae (Qiao et al., 2001). Ohuci et al. (2000) reported smaller kidneys with dysplastic outer medulla in the FGF-10 null mice, indicating the importance of FGF-10 in the kidney growth.

Bone morphogenetic proteins (BMP) are the largest subfamily of the transforming growth factor ß (TGF-ß) superfamily of secreted proteins (Martinez et al., 2002). There are three BMP subgroups, with approximately 90% amino acid identity within the subgroup (Hogan, 1996; Raatikainen-Ahokas et al., 2000). BMP-2, BMP-4 and BMP-7 show dynamic expression patterns during the development of mouse kidney and urinary tract (Dudley and Robertson, 1997; Piscione et al., 1997, 2001; Godin et al., 1999; Raatikainen-Ahokas et al., 2000; Myazaki et al., 2003; Nakano et al., 2003). BMP-2 and BMP-4 form one of the BMP subgroups (Hogan, 1996) and have different expression patterns in the developing mouse metanephros (Dudley and Robertson, 1997). Piscione et al. (1997, 2001) suggested that in mice, BMP-2 controls ureteric branch formation by inhibiting the formation of cellular processes. Miyazaki et al. (2000, 2003) proposed that BMP-4 prevents cell death and inhibits cell condensation in the mice metanephric mesenchyme, regulates the site of initial ureteral budding on the Wolffian duct and promotes the growth and the elongation of the ureter. Both BMP-2 and BMP-4 homozygous null mutant mice died prior to metanephric development, whereas only BMP-4 heterozygous mice had renal abnormalities such as cortical cysts, hydronephrosis and other urinary tract abnormalities (Zhang and Bradley, 1996; Dunn et al., 1997; Miyazaki et al., 2000; Martinez et al., 2002). In the study on human embryonic aorta-gonad-mesonephric region, BMP-4 was found in the mesonephros and was suggested to have a role in human hematopoesis (Marshall et al., 2000).

BMP-7, a member of another BMP subgroup, was detected in developing mouse metanephros (Dudley et al., 1995; Luo et al., 1995; Vukicevic et al., 1996; Dudley and Robertson, 1997). BMP-7 signaling might be required for survival and for maintaining of the undifferentiated cell population in the metanephric mesenchyme by suppressing apoptosis and inhibiting tubulogenesis, thus enabling further kidney growth (Dudley et al., 1995, 1999; Piscione et al., 1997, 2001; Godin et al., 1999). BMP-7 defficient mice had small dysgenic kidneys with hydroureters (Luo et al., 1995; Jena et al., 1997; Dudley and Robertson, 1997). During human metanephric kidney development, BMP-7 mRNA was first detected at 6 weeks of gestation in the metanephric mesenchyme surrounding the ureteric bud. At later developmental stages, the highest levels of BMP-7 mRNA were detected in the developing glomeruli, whereas BMP-7 protein expression was found mostly in convoluted tubules (Vukicevic et al., 1994; Helder et al., 1995).

Vascular endothelial growth factor (VEGF) is essential for vasculogenesis and for the sprouting of new capillaries from preexisting vessels (angiogenesis) (Kim and Goligorsky, 2003; Makanya et al., 2005). VEGF was detected in mice and rat metanephros, where it was suggested to be involved in providing capillary formation and spatial direction toward forming nephrons, as well as in proliferation of tubular epithelia (Loughna et al., 1997; Tufro et al., 1999; Tufro, 2000). Disrupted vasculogenesis, angiogenesis and vascular spatial organization were found in VEGF deficient mice (Carmeliet et al., 1996; Ferrara et al., 1996; Ferrara, 1999). In human fetal kidneys, VEGF receptors (KDR and Flt-1) mRNA and protein were detected in the glomeruli and in capillaries and veins. VEGF mRNA and protein found in both mesonephric and metanephric glomeruli, were detected also in the collecting ducts, while only VEGF mRNA was present in the S-shape nephrons (Simon et al., 1995, 1998; Kaipainen et al., 1993).

The studies on the FGF-8, FGF-10, BMP-2 and BMP-4 involvement in kidney development were performed only on experimental animals, while the investigations on the role of VEGF and BMP-7 in the human developing kidney were done mostly on the human fetal tissue. In this study we investigated the spatial and temporal distribution pattern of FGF-8, FGF-10, BMP-2/4 subgroup, BMP-7 and VEGF in human embryonic and early fetal mesonephros and metanephros. We speculate on the possible role of these growth factors in the formation of nephrons, collecting system and renal vasculature in early human kidney development.

Materials and methods

Human material

A total of 8 normal human conceptuses between the 5th and the 9th developmental week were collected after spontaneous abortions from the Department of Gynecology and Obstetrics, Clinical Hospital Split, Croatia, and after the tubal pregnancies from the Department of Pathology, Clinical Hospital Split. The embryos and fetuses were examined macroscopically and measured. Only normal conceptuses, without any sign of abnormality, signs of intrauterine death or macerations were used in our study. The embryonic tissues were treated as postmortem material with permission of the Ethical and Drug Committee of the Clinical Hospital Split, in accordance with the 1964 Helsinki Declaration. The postovulatory age was estimated on the basis of the menstrual data and

correlated with the crown-rump length (CRL) and Carnegie stages (O'Rahilly and Gardner, 1971) (Table 1).

Immunohistochemical staining

Caudal parts of embryos containing developing kidneys were dissected. Tissue samples were fixed in 4% paraformaldehyde in phosphate buffer and dehydrated in 100% ethanol. They were embedded in paraffin wax, serially sectioned at 4-6 µm, mounted on glass slides, and analyzed using an Olympus BX-40 light microscope (Olympus, Tokyo, Japan). The shape of each developing structure in the mesonephric or metanephric tissue was analyzed using successive serial sections. This method allowed us to distinguish between part of the collecting system and different stages of nephron formation.

Sections were deparaffinized in xylene and rehydrated in ethanol and water. In order to quench endogenous peroxidase activity, sections were incubated for 10 minutes in 1% H_2O_2.

Sections for FGF-8, FGF-10 and BMP-2/4 antigen staining were incubated with anti-goat serum (X0907, DakoCytomation, Glostrup, Denmark) for 20 minutes in the dark. Sections were then washed in PBS and incubated with goat anti-FGF-8b primary antibody (AF-423-NA, R&D Systems, Minneapolis, MI, USA; concentration 15 µg/mL), anti-human FGF-10 antibody (AF345, R&D Systems, Minneapolis, MI, USA; concentration 15 µg/mL) and goat anti-BMP-2/4 antibody (AF355, R&D Systems, Minneapolis, MI, USA; concentration 10 µg/mL) for 45 minutes in the dark.

For BMP-7 staining, after washing in PBS, sections were immediately incubated with mouse monoclonal anti-human BMP-7 antibody (MAB3541, R&D Systems, Minneapolis, MI, USA; dilution 1:10) for 45 minutes in the dark.

After washing in PBS, sections for VEGF staining were incubated in EDTA for 10 minutes at 95°C. After cooling to room temperature, sections were incubated with rabbit anti-VEGF primary antibody (PC37, Calbiochem, USA; dilution 1:20), for 45 minutes in the dark.

Binding of FGF-8, FGF-10, BMP-2/4 and BMP-7 primary antibodies was detected using streptavidin-biotin peroxidase system (K0690, DakoCytomation, Carpinteria, CA, USA) as recommended by the manufacturer. Rabbit ABC Staining System (sc-2018, Santa Cruz Biotechnology, Inc., Santa Cruz, California, USA) was used for detection of VEGF primary antibody binding.

Afterwards, all sections were washed with PBS and then stained with diaminobenzidine solution (DAB). Finally, sections were rinsed in destillated water, counter-stained with hematoxylin, and dehydrated in ethanol and xylol. Analysis was performed on an Olympus BX-51 microscope equipped with DMP digital camera and using DP-SOFT Version 3.1 software. Three observers with consideration of inter-observer variation analyzed the labeling.

Controls

Sections without primary antibodies incubation were used as negative controls. Positive controls were developing kidney structures or other tissues in the same sections (as each section contained various types of tissues and organs) that were known to label specifically with primary antibodies. All antibodies used were obtained from respected commercial brands and when antibody data sheets were checked, no specific cross-reactivity was ever reported.

Semi-quantification

The intensity of labeling of the kidney tissue was semi-quantitatively selected into 4 categories according to the staining reactivity: absence of any reactivity at high magnifications (x100) = - ; mild reactivity - clearly seen at higher magnifications (x40) = + ; moderate reactivity - seen already at lower magnifications (x20, x 10) = + + ; and strong reactivity seen as a clear signal at the lowest magnification (x4) = + + +.

Results

Between the 5th and 6th week of development, the mesonephros consists of renal glomeruli and tubules opening into the mesonephritic duct (Wolffian duct) at the lateral side and forming the Bowman's capsule at its medial extremity. In the metanephros, the anterior actively growing portion of the ureteric bud (ampulla) induces surrounding cells of the metanephric mesoderm to proliferate and condensate to form the metanephric cup. The remaining cells of the metanephric mesoderm form the loose metanephric mesenchyme.

During the described developmental period, all mesonephric structures contain both FGF-8 and FGF-10 positive cells, with slight differences in staining intensity (Table 2, Fig. 1A). In the metanephros, FGF-8 positivity is found only in the metanephric mesenchyme, while FGF-10 positivity is present in all metanephric structures, especially in the ureteric bud and undifferentiated metanephric mesenchyme (Table 3, Fig. 1B).

During the same developmental period, BMP-2/4 and BMP-7 positive cells have almost the same distribution pattern in all mesonephric structures (Table

Table 1. Age and number of human conceptuses analyzed.

Age (weeks)	Carnegie stage	No.
5-6	15-17	3
7	17-20	3
8-9	22- /	2

Table 2. Immunoreactivity to growth factor specific antibodies in the human mesonephros during the 5th to 9th week of development.

Antibodies/ Weeks of development	Wolffian duct	Mesonephric mesenchyme	Glomerule	Bowman's capsule	Mesonephric tubule	Coelomic epithelium
FGF-8						
5 and 6	+	+ +	+ +	+ +	+	+
7	+	+ +	+ +	+ +	+ +	+
8 and 9	+	+ +	+	+	+	+
FGF-10						
5 and 6	+ +	+ +	+ +	+	+	+
7	+	+	+	+	+ +	+ +
8 and 9	-	+ +	+	+	+	+
BMP-2/4						
5 and 6	+ +	+ +	+ +	+ +	+ +	+
7	+	+	+ +	+ +	+ +	+ +
8 and 9	-	+ +	+	+	+	+
BMP-7						
5 and 6	+	+ +	+ +	+ +	+	+
7	+	+	+ +	+	+ +	+ +
8 and 9	+	+ +	+	+	+ +	+
VEGF						
5 and 6	+ +	+	+	+	+ +	+
7	+	+ +	+	+	+ +	+
8 and 9	+ +	+	+ +	+ +	+ + +	+

+ + +, strong reactivity; + +, moderate reactivity; +, mild reactivity; -, no reactivity.

2). In the metanephros, moderate BMP-2/4 positivity is found in the ureteric bud and mesenchyme. In the ampulla and in the metanephric cup mitotic cells show moderate reactivity, while the other cells show mild reaction to BMP-2/4 (Table 3, Fig. 1C+). Only a few BMP-7 positive cells are detected in the ureteric bud, ampullae and in the metanephric cup, while the metanephric mesenchyme is characterized by very intense reactivity (Table 3, Fig. 1D).

In the mesonephros, moderate VEGF positivity is found in the Wolffian duct and in the mesonephric tubules, while other mesonephric structures show mild reactivity (Table 2, Fig. 1E). In the metanephros, moderate reactivity to VEGF is found in the ampullae, ureteric bud and metanephric cup, while mild VEGF reactivity characterizes the metanephric mesenchyme (Table 3, Fig. 1F).

During the 7th, 8th and 9th week of development, the mesonephros shows signs of degenerative changes only in its cranial parts. In the metanephros, the ureteric bud dilates and branches to form the collecting tubules ending with ampullae. The metanephric mesenchyme differentiates to form more metanephric cups, giving rise to renal vesicles, s-shaped nephrons and more mature nephrons containing renal corpuscles in medullary direction (Bowman's capsule and renal glomerulus). The rest of the metanephric mesenchyme forms interstitial connective tissue.

In the 7th developmental week, moderate FGF-8 reactivity is detected in most of the mesonephric structures. Moderate FGF-10 positivity is seen in the mesonephric tubules and in the coelomic epithelium, while all other structures express mild reactivity (Table 2). In the metanephros, moderate FGF-8 and FGF-10 reactivity is found in the collecting tubules and in the interstitium, while mild positivity is found in the metanephric cup, renal vesicles and s-shaped nephrons. No FGF-8 positive cells are seen in the ampulla (Table 4).

Table 3. Immunoreactivity to growth factor specific antibodies in human metanephros during the 5th to 6th week of development.

Antibodies	Ureteric bud	Ampulla	Metanephric mesenchyme	Metanephric cup
FGF-8	-	-	+	-
FGF-10	+ +	+	+ +	+
BMP-2/4	+ +	+	+ +	+
BMP-7	+	+	+ + +	+
VEGF	+ +	+ +	+	+ +

+ + +, strong reactivity; + +, moderate reactivity; +, mild reactivity; -, no reactivity.

During the 8th and 9th developmental week, FGF-8 and FGF-10 positive cells have the same distribution pattern in the mesonephros, with the exception of the Wolffian duct, which is devoid of FGF-10 positive cells (Table 2). All metanephric structures express moderate FGF-8 positivity, except for the interstitium, characterized by strong reactivity. Within those structures, some cells show clear reaction, while in others reaction is completely missing (Table 4, Fig. 1A) Moderate FGF-10 positivity is found in some cells of the interstitium and collecting tubules, while mild reactivity is present in the metanephric cup and in the developing nephrons, except for the mitotic cells which are clearly FGF-10 positive (Table 4, Fig. 2B).

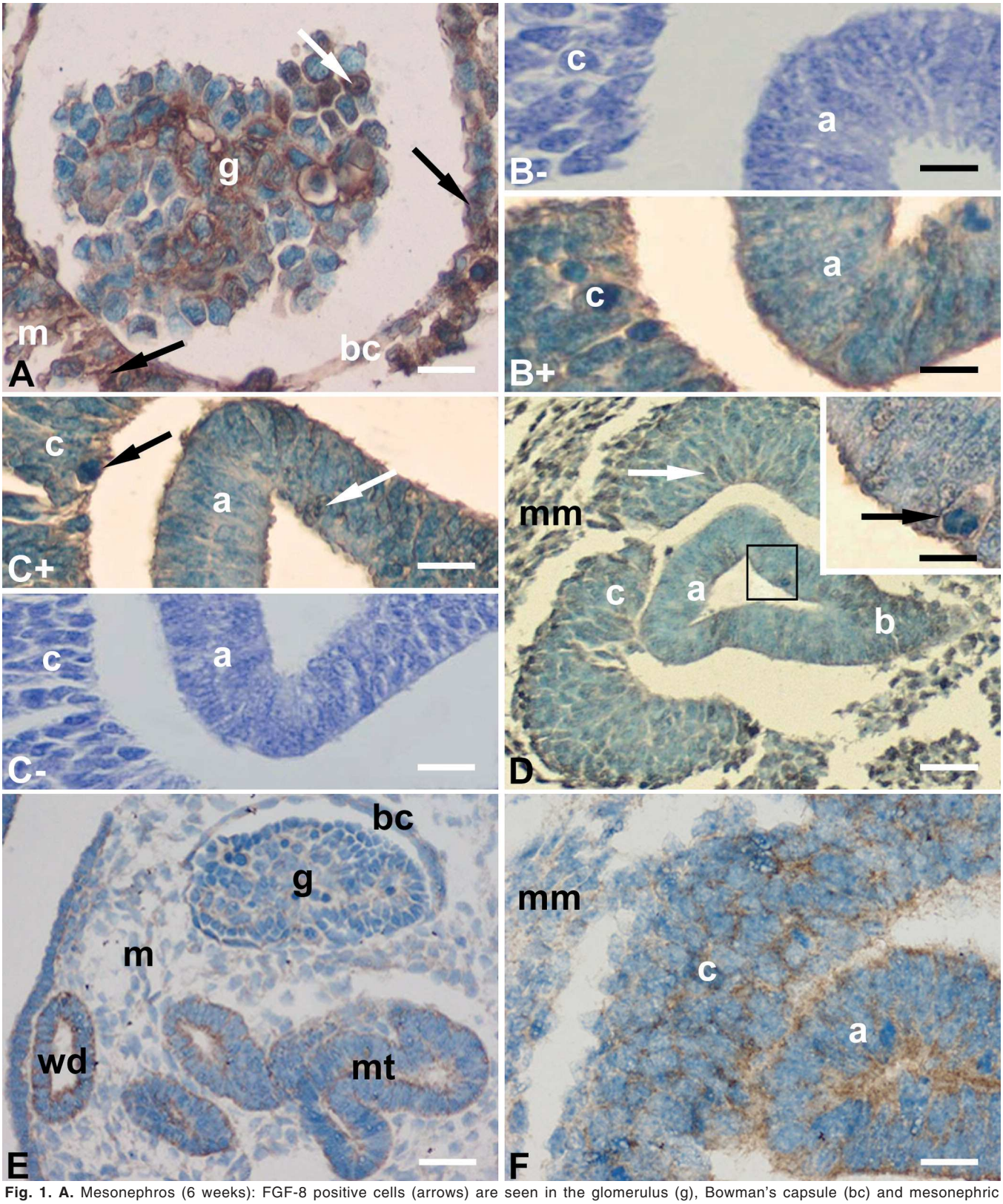

Fig. 1. A. Mesonephros (6 weeks): FGF-8 positive cells (arrows) are seen in the glomerulus (g), Bowman's capsule (bc) and mesonephric mesenchyme (m). Immunostaining to FGF-8, x 40. Scale bar 18 µm. **B+.** Metanephros (5 weeks): FGF-10 is positive in the metanephric cup (c) and ampulla (a). Immunostaining to FGF-10, x 40. Scale bar 10 µm. **B-.** Metanephros (5 weeks): Negative control to FGF-10 staining. **C+.** Metanephros (5 weeks): BMP-2/4 positivity (arrows) characterizes mitotic cells of the metanephric cup (c) and ampulla (a), while other cells show only mild reaction. Immunostaining to BMP-2/4, x 40. Scale bar 18 µm. **C-.** Metanephros (5 weeks): Negative controle to BMP-2/4 staining, x 40. Scale bar 18 µm. **D.** Metanephros (5 weeks): note strong reaction to BMP-7 antibody in the mesenchyme (mm), while only mitotic cells (arrow) in the metanephric cup (c) and ampulla (a) show mild reaction. Immunostaining to BMP-7, x 10. Scale bar 43 µm. Insert: mitotic cell (arrow) in the ampulla shows BMP-7 positivity. Immunostaining to BMP-7, x 100. Scale bar 13 µm. **E.** Mesonephros (6 weeks): VEGF positive cells characterize Wolffian duct (wd), mesonephric tubules (mt), glomerulus (g), Bowman's capsule (bc) and mesonephric mesenchyme (m). Immunostaining to VEGF, x 20. Scale bar 43 µm. **F.** Metanephros (5 weeks): VEGF moderately positive cells are seen in the ampulla (a) and metanephric cup (c), and mildly positive in the metanephric mesenchyme (mm). Immunostaining to VEGF, x 40. Scale bar 21 µm.

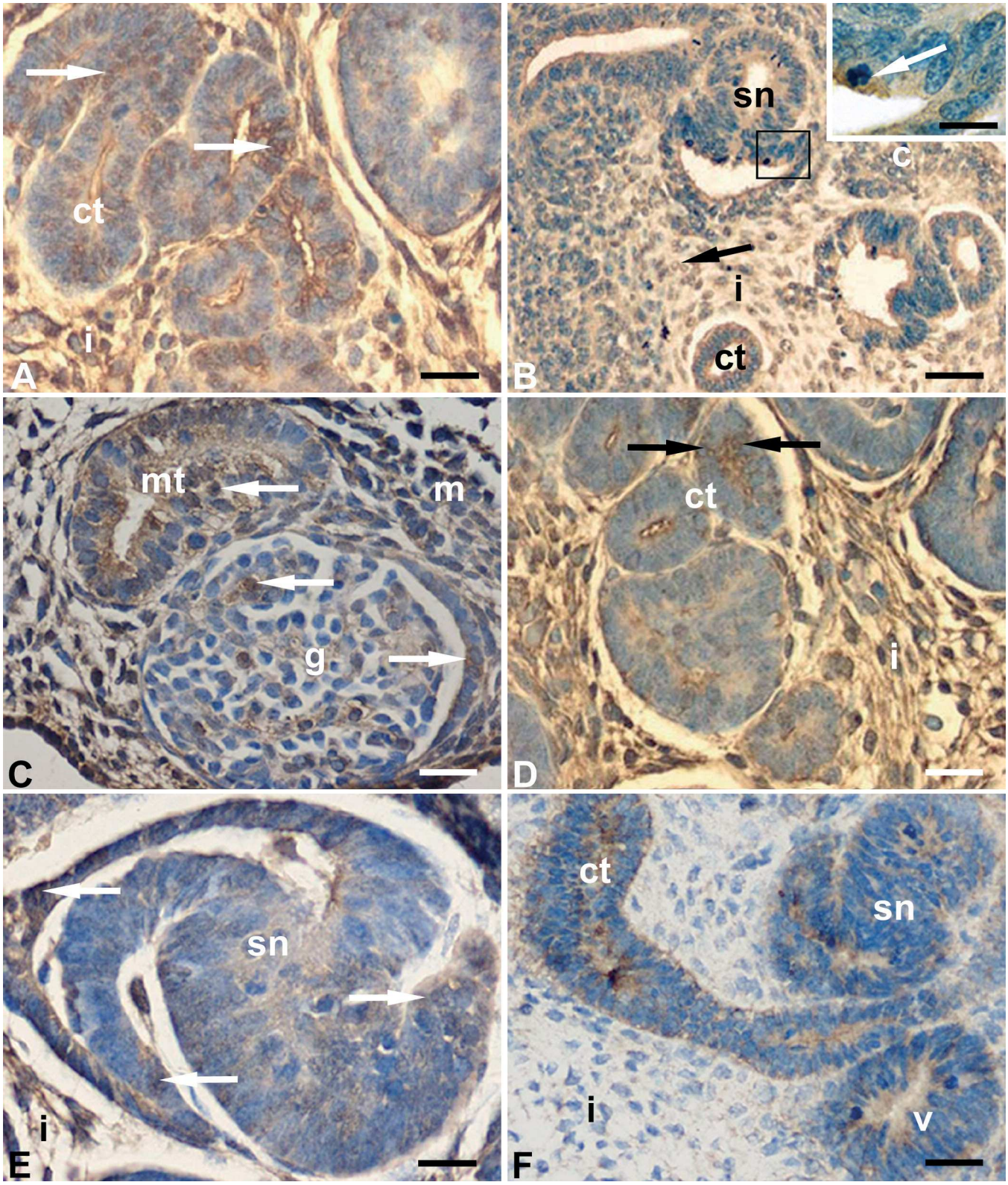

Fig. 2. A. Metanephros (9 weeks): some cells (arrows) in the collecting tubules (ct) show clear reaction to FGF-8, while stronger reaction is seen in the surrounding interstitium (i). Immunostaining to FGF-8, x 40. Scale bar 24 μm. **B.** Metanephros (8 weeks): note moderately FGF-10 positive cells (arrows) in the interstitium (i) and in the collecting tubules (ct). Some cells of the metanephric cup (c) and differentiating nephrons (sn) show clear FGF-10 positivity. Immunostaining to FGF-10, x 20. Scale bar 47 μm. Insert: Mitotic cell (arrow) of the differentiating nephron shows strong reaction to FGF-10. Immunostaining to FGF-10, x 100. Scale bar 14 μm. **C.** Mesonephros (9 weeks): some cells (arrows) of the glomerulus (g), mesonephric tubules (mt), and Bowman's capsule (bc) show clear BMP-7 reactivity, while all cells of the surrounding mesenchyme (m) are moderately positive to BMP-7. Immunostaining to BMP-7, x 40. Scale bar 20 μm. **D.** Metanephros (9 weeks): Some cells of the collecting tubules (ct) are characterized by clear BMP-2/4 reactivity (arrows), while surrounding interstitium (i) shows moderate reactivity. Immunostaining to BMP-2/4, x 40. Scale bar 24 μm. **E.** Metanephros (9 weeks): some cells of the s-shaped nephron (sn) show BMP-7 positivity (arrows), while interstitial cells (i) show stronger reaction. Immunostaining to BMP-7, x 40. Scale bar 15 μm. **F.** Metanephros (9 weeks): mild VEGF reaction is seen in the interstitium (i), renal vesicle (v) and s-shaped nephron (sn), while it is stronger in the collecting tubules (ct). Immunostaining to VEGF, x 20. Scale bar 29 μm.

Table 4. Immunoreactivity to growth factor specific antibodies in human metanephros during the 7th to 9th week of development

Antibodies/ Weeks of development	Collecting tubules	Ampulla	Interstitium	Metanephric cup	Renal vesicle	S-shaped nephrons	Renal corpuscle
FGF-8							
7	+ +	-	+ +	+	+	+	+
8 and 9	+ +	+ +	+ + +	+ +	+ +	+ +	+ +
FGF-10							
7	+ +	+	+ +	+	+	+	/
8 and 9	+ +	+	+ +	+	+	+	+
BMP-2/4							
7	+	+	+	+	+	/	/
8 and 9	+	+	+ +	+	+	+	+
BMP-7							
7	+ +	+ +	+	+	+	+ +	/
8and 9	+ +	+ +	+ +	+ +	+	+	+
VEGF							
7	+ +	+	+	+	+	/	/
8 and 9	+ +	+	+	+	+	+	+

+ + +, strong reactivity; + +, moderate reactivity; +, mild reactivity; -, no reactivity; /, structure absent in the tissue section.

During the 7th developmental week, both BMP-2/4 and BMP-7 positive cells are present in all mesonephric structures (Table 2). All metanephric structures are characterized by mild BMP-2/4 reactivity (Table 4). Moderate BMP-7 positivity is detected in the collecting tubules, ampulla and s-shaped nephrons, while mild reactivity is present in all other metanephric structures (Table 4).

Between the 8th and 9th week of development, BMP-2/4 and BMP-7 positive cells are present in all mesonephric structures and have the same distribution pattern, except for the Wolffian duct, which is devoid of BMP-2/4 positive cells (Table 2, Fig. 2C). During that developmental period, both factors are present in all the structures forming the metanephros (Table 4, Fig.2D), but now the BMP-7 expression has increased in the collecting system and in the metanephric cup (Table 4, Fig. 2E).

In the 7th developmental week, moderate VEGF positivity is present in the mesonephric mesenchyme and in the mesonephric tubules, while mild reactivity is found in all other structures (Table 2). In the metanephros, only the collecting ducts are characterized by moderate VEGF reactivity, while all other metanephric structures express mild VEGF positivity (Table 4).

In the period between 8th and 9th developmental week, VEGF reactivity has increased in all mesonephric structures, particularly in the mesonephric tubules (Table 2). Mild VEGF positivity is present in all metanephric structures except the collecting tubules, where VEGF shows moderate reaction (Table 4, Fig. 2F).

Discussion

The mesonephros

In our study, analysis FGF-8 and FGF-10 distribution pattern showed cells positive to both factors in all human mesonephric structures. All mesonephric structures contained BMP-2/4 and BMP-7 positive cells as well. The decrease in reactivity of both growth factors and of BMP-2/4 subgroup detected in the early fetal mesonephros, might be considered as a sign of gradual regression of the transitional mesonephric kidney.

All mesonephric structures were characterized by VEGF expression, whose intensity increased in the early fetal period. Simon et al. (1995) previously reported the presence of VEGF in the glomerular visceral epithelial cells of the human fetal mesonephros. Our data on VEGF expression pattern in the mesonephros might indicate the involvement of VEGF in the formation of the mesonephric blood vessel network, as a previously suggested role for the metanephric vessel network formation (Kaipainen et al., 1993; Simon et al., 1995, 1998; Tufro et al., 1999; Tufro, 2000; Kim and Goligorsky, 2003).

The metanephros

During the early embryonic period (5-6 weeks) of human metanephric development, the FGF-8 positivity was restricted only to metanephric mesenchyme. At later stages, it appeared with increasing intensity both in the collecting system and in the developing nephrons. These results are in accordance with the data on mice FGF-8 mRNA localization in the metanephros (Grieshammer et al., 2005). Thus, the possible source of FGF-8 might be the developing nephrons. Disrupted nephrogenesis in FGF-8 mutant mice due to extensive apoptosis suggested that FGF-8 signaling might be required for cell survival. When applied to the human metanephric development, FGF-8 could influence nephron formation by controlling apoptosis. A similar developmental function was addressed to bcl-2 protein in the developing human kidneys (Carev et al., 2006). Thus, the decrease in FGF-

8 expression in the human kidney development might result in severely disrupted nephrogenesis and extensive apoptosis leading to kidney malformations at birth.

Contrary to FGF-8, FGF-10 reactivity was detected in all metanephric structures at early developmental stages, particularly in the mesenchyme. During further development, FGF-10 reactivity gradually decreased. The results of our study, together with the investigation on the rat ureteric bud (Qiao et al., 2001), might indicate that the interstitium and the autocrine FGF-10 secretion are the main source of FGF-10. Through stimulation of ureteric bud growth and branching, FGF-10 seems to control the number of induced nephrons. As also shown for mice (Ohuchi et al., 2000), FGF-10 deficiency during the described developmental period might lead to abnormalities in human kidney development, primarily to reduction of the number of nephrons. Besides importance of FGF-10 in the growth of the collecting system and nephrogenesis, FGF receptors might also be important for mesenchymal-epithelial cell interactions in the developing kidney (Celli et al., 1998).

In our study, the BMP-2/4 subgroup reactivity was described for the first time in the human metanephric tissue. It was more positive in the ureteric bud and mesenchyme than in the developing nephrons. Although we could only speculate about single BMP (BMP-2 or BMP-4) distribution pattern, the BMP-2/4 pattern of appearance that we found was much more ubiquitous than both single BMP (2 and 4) expression patterns together, reported in the previous study performed in the mouse by Dudley and Robertson (1997). In that study, BMP-2 mRNA expression was first restricted to the metanephric cup and later appeared transiently in the developing nephrons, while BMP-4 was found in the mesenchyme surrounding the developing collecting system and in the renal corpuscle. The possible source of BMP2/4 might be the metanephric mesenchyme and developing nephrons. Due to our results, the involvement of both factors in the collecting system development, as also suggested in the previous studies on mammalian metanephric development (Piscione et al., 1997, 2001; Miyazaki et al., 2000, 2003), might be applicable for the developing human kidney. Renal abnormalities, as well as the urinary tract abnormalities, reported in the several studies on BMP-4 heterozygous mice (Zhang and Bradley, 1996; Dunn et al., 1997; Miyazaki et al., 2000; Martinez et al., 2002), might occur because of disrupted BMP-4 expression during human permanent kidney development as well.

In our study, the BMP-7 expression was present in all metanephric structures from 5th to 9th developmental week, being strongest in the metanephric mesenchyme during early stages. At later stages, BMP-7 decreased in the developing nephrons, but increased in the collecting tubules. This time-sequence of appearance of BMP-7 protein coincided with localization and pattern of BMP-7 mRNA expression (Vukicevic et al., 1994). This led to the suggestion that BMP-7 was released from the developing glomerules and subsequently accumulated in the basement membranes of tubular epithelium (Vukicevic et al., 1994; Helder et al., 1995). As BMP-7 mRNA was found also in the ureteric bud, both the developing nephrons (tubules) and collecting system could be the source of BMP-7 protein. Our data, together with the results on BMP-7 expression in the mouse metanephric development (Luo et al., 1995; Dudley et al., 1995, 1999; Dudley and Robertson, 1997; Jena et al., 1997; Godin et al., 1999; Piscione et al., 1997, 2001), might indicate that BMP-7 signaling is required to enable continuous growth of the human metanephros (Schedl and Hastie, 2000) and that the deficiency in that signaling might lead to kidney abnormalities such as kidney dysplasia (Dudley et al., 1995). BMP-7 seems to have a crucial role in epithelial-mesenchymal conversion and induction of the metanephros, as well as in nephrogenic differentiation (Vukicevic et al., 1996).

During the investigated developmental period, all metanephric structures were characterized by VEGF expression, especially the ureteric bud, ampullae and the metanephric cup in the early embryonic period (5-6-weeks). At a later developmental period, the strongest expression was detected in the collecting ducts. VEGF expression in the early human metanephric development indicates that the early metanephric structures are already initiating blood vessel formation. The results of our study accord with the previous studies on VEGF and its receptors performed on human fetal metanephros (Kaipainen et al., 1993; Simon et al., 1995, 1998). All those studies indicate that VEGF is mitogenic for vascular endothelial cells (Kaipainen et al., 1993; Hyink and Abrahamson, 1995). Additionally, it promotes differentiation of the endothelial cells, but also the proliferation of tubular cells, as well as capillary network formation (Tufro et al., 1999). VEGF receptors were expressed predominantly in the glomerular capillaries, while VEGF protein was found in distal tubules and glomerular epithelial cells (Tufro et al., 1999; Kim and Goligorsky, 2003). In experimental animals, VEGF deficient embryos had impaired all steps of vascular development including vasculogenesis (in situ differentiation of blood vessels) and angiogenesis (sprouting from preexisting vessels) (Carmeliet et al., 1996). As shown by Kaipainen et al. (1993), expression of VEGF receptors in human fetuses was modulated in a dynamic manner during development. The source of VEGF could be glomerular epithelial cells, but also the developing nephrons and collecting ducts. Developmental changes in expression pattern for VEGF protein described in our study could be associated with subsequent development of blood vessels in the differentiating kidney.

In conclusion, all investigated growth factors were dynamically reacting in the developing human mesonephros in the manner that accorded with growth and gradual regression of the mesonephric kidney system. VEGF presence indicated developed blood vessels in the transient kidney tissue. In the developing permanent human kidney (metanephros), several growth

factors appeared in parallel during the early kidney development, which was characterized by formation and branching of the ureteric bud. Thus, appearance of FGF-10, BMP2/4 and BMP-7 proteins could be associated with ureteric bud stimulation and branching due to their role in prevention of apoptosis. At later developmental stages, an increase of FGF-8 and BMP-7 reactivity indicated their involvement in control of nephrogenesis by the same process. Changes in the described pattern of appearance of the investigated growth factors might be associated with different kidney abnormalities, such as small and dysplastic kidneys or cyst formation. VEGF expression in the metanephros suggested early initiation of blood vessels formation, its mitogenic role in endothelial and tubular cells, and its importance in all steps of vascular development. Thus, abnormal VEGF expression might lead to impaired formation of blood vessels in the human kidneys.

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