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Research Article

RESTRICTION OF TGFB2 IN DEVELOPING ORGANS IN EMBRYOS OF MICE UNDER MATERNAL EXPOSURE OF RETINOIC ACID

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ABSTRACT

The study was conducted to reveal the effect of retinoic acid (RA) on the developing embryonic organs in which the epithelia come to contact with mesenchyme in mice foetii during pregnancy at the onset of organogenesis. Histogenesis and the expression of TGFβ2 as a potent cell cytokine differentiator were evaluated in skin; lung, kidney and testis at 14, 18 days of gestation. A thinner skin with retarded hair follicles, thin alveolar septa of the lung, retarded differentiation of kidney tubules and deformed Sertoli cells of the developing testicular tubules were noted in retinoic acid-exposed embryos. Depressed fluorescence and PAS content in either the epithelia or the mesenchyme of the tissues under investigation were also noted. These alterations in the different organs were concomitant with decreased expression of TGFβ2 which indicates its importance in proper development and differentiation. From the other hand, the study concluded that successive exposure to retinoic acid at low level during pregnancy suppresses the trans-differentiation of the mesenchyme to epithelia in the organs under investigation through dowenregulation of TGFβ2 expression.

Keywords: Retinoic acid (RA), Embryonic organs, Histogenesis, Transforming Growth Factor β2 (TGFβ2).

INTRODUCTION

Retinoic acid is recognized to profoundly modulate a wide range of biological functions including pattern formation during embryogenesis, vision, reproduction and hematopoesis (Allen *et al*, 2002; Morriss *et al*., 2002; Marill *et al*, 2003; Niles, 2003). Retinoic acid (RA) is derived from the liposoluble vitamin A (retinol). Since years ago, the molecular basis of vitamin A action was elucidated when it was shown that its acidic metabolite (RA) acts as a ligand for transcription factors of the retinoic acid nuclear receptors (RARs)(Krzyzosiak *et al*., 2010), switching them from potential repressors to transcriptional activators. Possible functions of RA during embryogenesis were first inferred by studying its teratogenic effects, i.e. how the administration of excess doses of RA, either globally or by local implantation using RA-impregnated beads, interferes with normal developmental processes. Many early studies have investigated the role of RA in embryonic limb bud patterning, triggered by reports of its ability to induce mirror-image digit duplications when applied locally in chick wing buds (Tickle *et al.*, 1982) and of the measurement of differential endogenous concentrations along the limb A-P axis (Thaller *et al.*, 1993). These studies have been performed in a wide range of species including amphibians, zebrafish, chick and rodents (Durston *et al.*, 1989; Avantaggiato *et al.*, 1996). Gene knockout studies then confirmed the crucial functions of RARs in mouse development (Mark *et al.*, 2009). Eventually, the enzymatic pathways that regulate embryonic RA were characterized, and it was found that another regulatory step involved in triggering RA catabolism by a subfamily of cytochrome P450 enzymes (Abu-Abed *et al*., 2000; Xia *et al*., 2010). Retinol-binding protein 4 (RBP4) binds to retinol and delivers retinol from the liver to peripheral tissues. Maternal RBP4 cannot cross the placenta; retinol and retinyl esters was found to diffuses across the yolk sac and placenta, where zygotic RBP4 synthesis occurs (Quadro *et al.*, 2005; Rhinn, and Dollé, 2012).

Work in avian and rodent models has established that maternal vitamin A deficiency affects the embryo and foetii leading to a complex spectrum of abnormalities (Gale *et al.*, 1999; White *et al.*, 2000). Also, earlier studies showed that the treatment of pregnant mice or rats with excess RA leads to teratogenic changes in the hindbrain (Morriss, 1972). Interestingly, RA exposure at late gastrula/early neurula stages increases hindbrain size at the expense of other brain regions (Avantaggiato *et al.*, 1996), whereas RA treatment at later stages specifically leads to a posteriorisation of rhombomeres (r) 2-3 to an (r) 4-5

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identity (Marshall *et al.,* 1996). Retinoic acid (RA) is thought to exert its effects by modifying the transcriptional activity of specific genes through its binding its receptors (Mark *et al*., 2006; Altucci *et al*., 2007). The genes which are activated by RA in embryos are unknown, but in human embryonic carcinoma cells they include several genes (Simeone *et al.,* 1991; Hua *et al*., 2009). In mouse brain the morphogenesis and the expression of Hox and Krox genes were altered by excess of retinoic acid (Morriss-Kay *et al.,* 1991). In addition, several *In Vitro* studies revealed that RA was found to induce epithelial transdifferentiation with altered gene expression in various tissues of different species including humans (Foitzik *et al.,* 2005; Obinata *et al.,* 2011).

As retinoids are widely used and hold promise for future use in stem cell-based therapy. Therefore, the present investigation was conducted, from one hand, to evaluate the effect of retinoic acid (*in vivo*) on the differentiation of selected organs (skin, lung, kidney, testis) in which the epithelia come to direct interaction with mesenchyme as a potent embryonic cells and to verify the effect on transforming growth factor TGFβ2 expression gene as a multifunctional bioregulator in cell growth and differentiation.

MATERIALS AND METHODS

Mature virgin mice, *Mus musculus,* in one round of pregnancy were housed three/cage with males at morning and checked for plugs all around the next day. The time of plug observation was designated as the start of day 0 of pregnancy. Retinoic acid (C20H28O**2**, 300.4 g/mol) in the form of 13-cis form (Isotretinoin) product of Sigma 500 mg/package was obtained.

In olive oil, stock solution was prepared and renewed as required during the experimental period. The stock solution was made up as 2.5 mg RA in 1ml absolute ethanol to which 9 ml olive oil was added (0.25 mg/ml solution). Crystalline RA was kept in the dark under argon at 4°C and the solution was newly prepared at dosing. Mice weighing up to 25 g were given 0.25 ml (2.5 mg/kg) of the solution intraperitoneally at day 7 till the 18th day of gestation. Control dams of the same stages of pregnancy were given 0.25 ml vehicle alone. Intraperitoneal injection of RA reaches near maximal levels in the embryo within 2 hrs (Creech-Kraft *et al*., 1989**).** At day 14, 18 of pregnancy, embryos of treated animals and those of the control (5 animals each) were collected. At each embryonic stage at least 30 embryo were examined morphologically for RA-exposed embryos to insure their morphological normality before fixation. Embryos were incised at the ventral abdomen, fixed in Carnoy's fluid and processed for sectioning.

For histological and histochemical study, sections of 7µ thick were stained with H&E, PAS and Acridin orange/Ethedium bromide for fluorescence examination (Drury and Wallington, 1976).

In immunohistochemical study, deparaffinized Superfrost/Plus slides-mounted sections of embryos at 14, 18 days were heat-retrived for re-antigenicity using 10 mM citrate buffer (Buchlowalow and Bocker, 2010**).** After cooling at room temperature, sections were treated for 10 minutes with 0.3% hydrogen peroxide block and then with protein block (phosphate buffer solution, pH 7.6, with 0.5% BSA, 0.5% casein and less than 0.1% sodium azide) for 10 minutes to block nonspecific background staining, then sections were incubated with primary antibody (Rabbit Anti-human TGFβ2 polyclonal antibody, Spring Bioscience, USA) and then washed using phosphate buffer and incubated with secondary antibody, Biotinylated Goat Antipolyvalent (Anti-polyvalent HRP DAB detection system, Spring Bioscience, USA) according to the manufacture protocol. In all cases, a negative control section in which the primary antibody was omitted from the sequence of reactions was conducted. Stained sections were dehydrated in ascending grades of ethanol, cleared in xylene and mounted with DPX mounting media. Sections were examined microscopically and then processed as required.

RESULTS

14 days-old embryos

Skin: HE and Acridin Orange/Ethedium Bromide stained sections revealed the effect of retinoic acid on the developing skin. A thin epidermis with poor primordial hair follicles and a little mesenchyme of the dermis were noted (Plate 1 B, D) compared to the control (Plate 1 A, C). Primordium of hair follicles were regular that invaginate into the dermis in control (Plate 1 A). These epidermal invaginations are surrounded by dense mesencyme of the dermis while the epithelial sheet of the epidermis rests upon a continuous sheet of flattened dark stained cells that represent the line of demarcation between the skin components at 14th day of gestation (Plate 1 E). In the corresponding field of skin in retinoic acid-exposed embryos, a little mesenchyme was noted in the dermis facing the poor developed primordia of the hair follicles in addition to the discontinuous cells basal to the developing epidermis (Plate 1 F). In PAS-stained sections, a well PAS-positive- basement membrane,-granules in the epidermal cells and positive matrix among the mesenchyme of the dermis (Plate 1 G). Diminished epidermal PAS-positive granules and inhibition of basement membrane and matrix stainability in retinoic acid-exposed embryos were noted (Plate H). The histological and histochemical alterations induced by retinoic acid exposure were concomitant with the decrease in TGF β2 expression both in the epidermis and the mesenchyme (Plate 1 J) as compared to control (Plate 1).

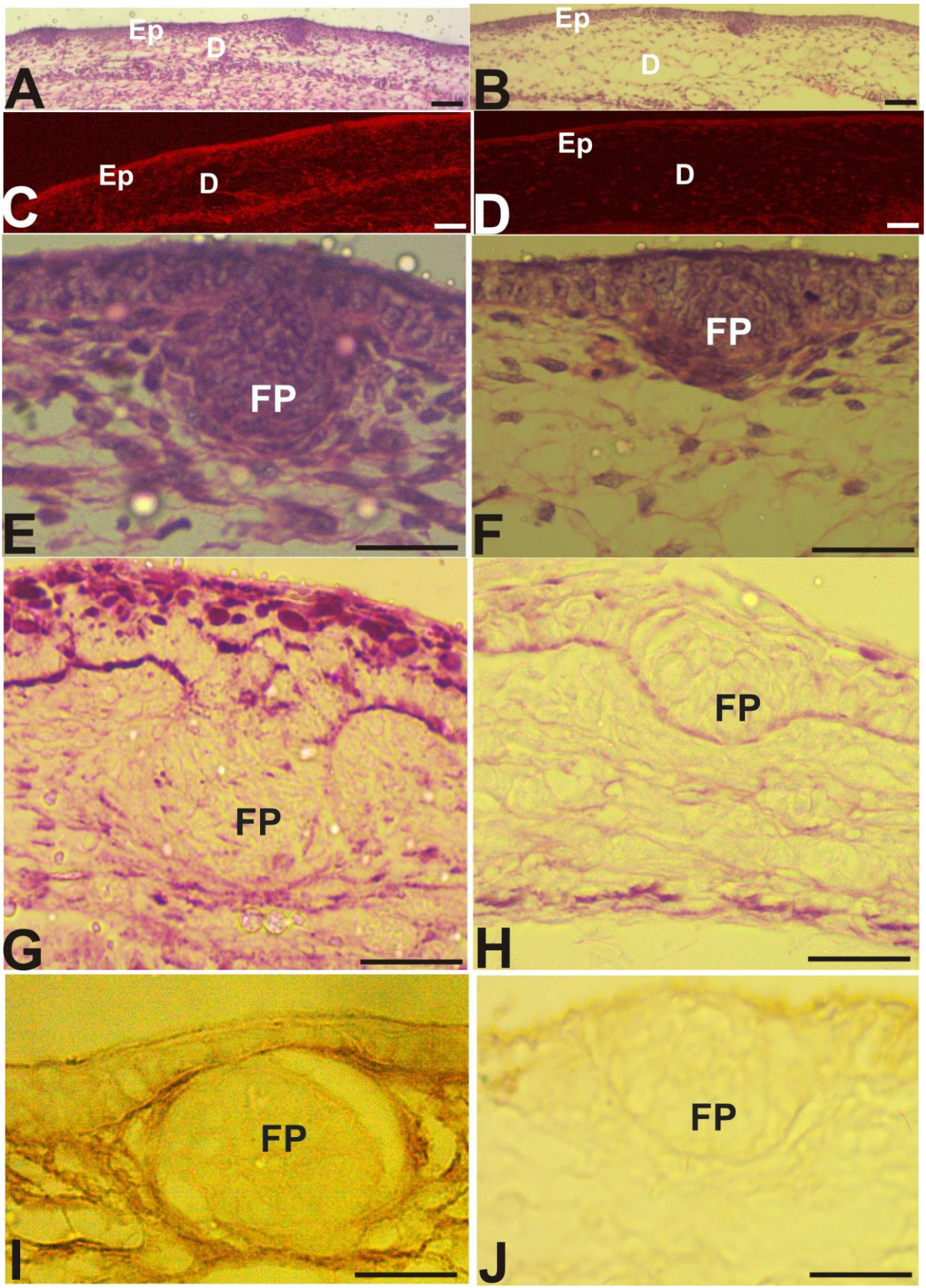


Plate 1. Photomicrographs of the developing skin at 14 days of gestation showing a thinner epidermis; little mesenchyme of the dermis; depressed flourescense; less invasive follicle primordium; diminished PAS-positive contents and dowenregulated expression of TGFβ2 in retinoic acid-exposed embryos B, D, F, H, J, respectively as compared to the corresponding control as shown A, C, E, G, I, respectively. Scale bar 50 µm.

Lung: A gland-like appearance of the lung tissue was observed in either the control (Plate 2 A) or retinoic acid-exposed embryos (Plate 2 B) at 14th day of development. In control, conducting air ways and the alveoli are prominent that embedded in stromal background of mesenchyme. A less differentiated alveoli with increased saccular diameter and necrotic cells were observed in retinoic acid-exposed embryos as compared to control. Acridin orange/ethidium bromide-stained sections revealed a lesser flourescence of cell nuclei with pale central areas in either the alveolar or the mesenchyme cells of retinoic acid-exposed embryos (Plate 2 D) as compared to control (Plate 2 C). PAS-stained sections revealed the prenatal secretory functions of the lung in which the developing alveoli has PAS-positive intracellular inclusions and interalveolar staining of the ground mesenchyme in control embryos (Plate 2 E). Inhibition of PAS-stained alveoli and the mesenchyme with concomitant dowenregulation of the transforming growth factor were noted in the developing lung of retinoic acid-exposed embryos (Plate 2 G, H) compared to control (Plate E, F).

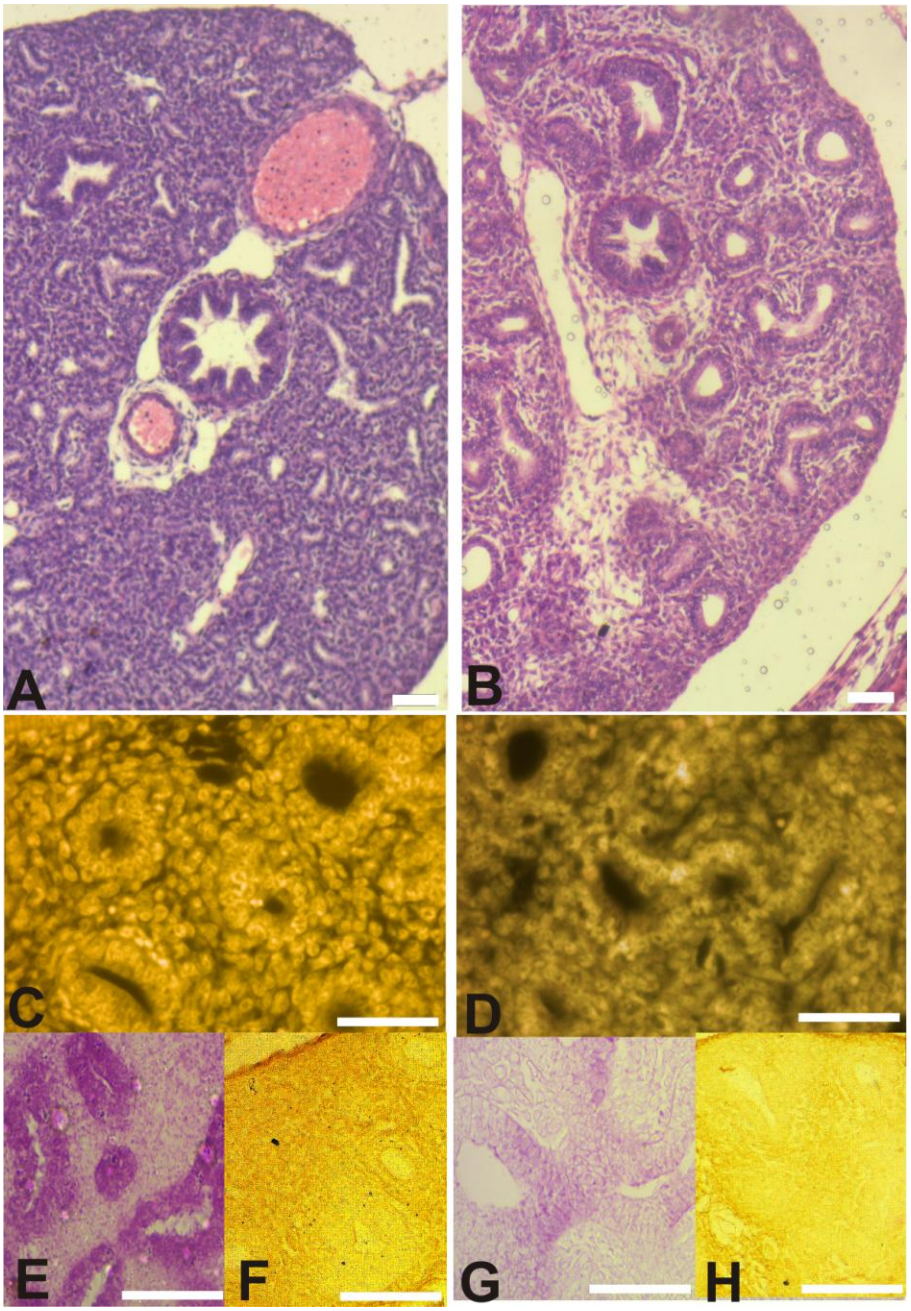


Plate 2. Photomicrographs of the developing lung at 14 days of gestation showing a less differentiated alveoli, depressed flourescense; diminished PAS-positive contents and dowenregulated expression of TGFβ2 in retinoic acid-exposed embryos B, D, G, H, respectively, as compared to the corresponding control as shown A, C, E, F, respectively. Scale bar 50 µm.

Kidney: In the developing metanephros at 14 days-old embryo, a well differentiated cortical and medullary region were noted in control embryos (Plate 3 A). The nephrogenic zone has increased tubules interspersed with mesenchyme that penetrate the medullary region of the developing kidney. Retinoic acid-exposed embryos show a less differentiated tubules and a thin nephrogenic zone (Plate 3 B) as compared to control. Immuno-stained sections of the developing metanephros revealed intense expression of TGFβ2 among both the epithelia of the developing tubules and the mesenchyme (Plate 3 C). Stained less kidney tubules and dowenregulated expression of TGFβ2 in the mesenchyme were noted in retinoic acid-exposed embryos (Plate 3 D) compared to control.

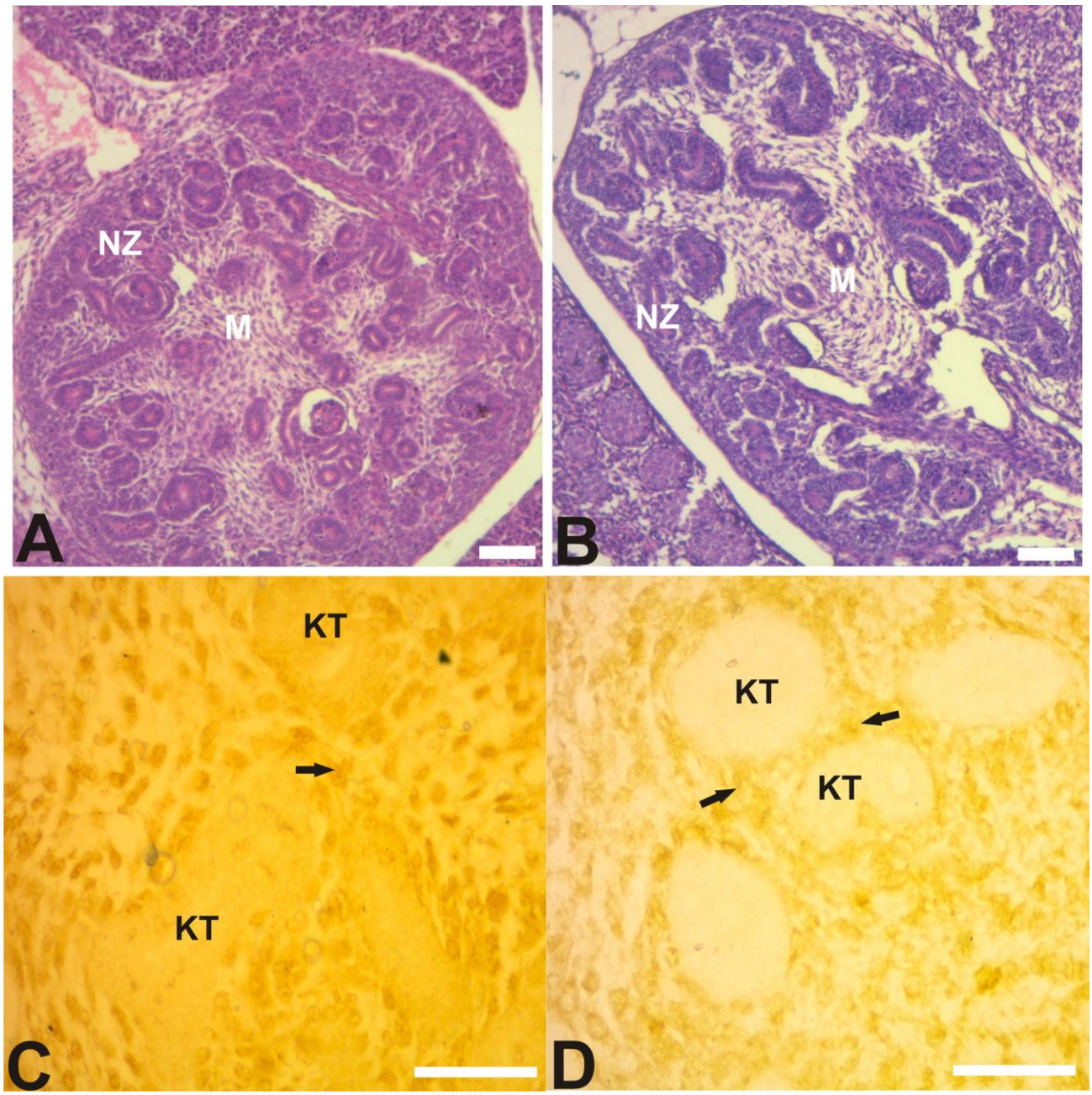


Plate 3: Photomicrographs of the developing metanephric kidney at 14 days of gestation showing a less differentiated tubules (B) and dowenregulated expression of TGFβ2 (D) in retinoic acid-exposed embryos compared to control (A, C). M (mesenchyme), KT (kidney tubules), arrows indicate TGFβ2 expresssion in the intertubular mesenchyme.Scale bar 50µm.

Testis: In either the control or retinoic acid-exposed embryos of 14 days-old, the testicular tissue composed of seminiferous tubules and intertubular tissue (Plate 4. A, B). The seminiferous tubules were observed at the gonadal periphery whereas the inner part of parenchyma consisted of network of polygonal mesenchyme cells. The seminiferous tubules were surrounded by a distinct basement membrane, a layer of peritubular cells and

rounded goncytes with rounded nuclei that fill the developing semineferous tubules (Plate 4 C). The structural organization of semineferous tubules in retinoic acid-exposed embryos look as the same observed in control except some vacuolated cells and detachement of the basement membrane (Plate 4 D) as compared to control. The effect of retinoic acid exposure was well recognized in histochemical and immunohistochemical demonstration of PAS-stained components of the developing semineferous tubules and the expression of TGF expression. In control embryos, PAS-positive pyramidal cells (Sertoli cells) were regularly encountered at the periphery of the developing tubules that rests on a well stained basement membrane (Plate 4. E). Also, the intertubular connective and the epithelia of the developing tubules showing intense expression of the transforming growth factor (Plate 4 G) tissue. In retinoic acid-exposed embryos, inhibition of PAS components and dowenregulation of the transforming growth factor expression were noted (Plate 4 F, H) as compared to control.

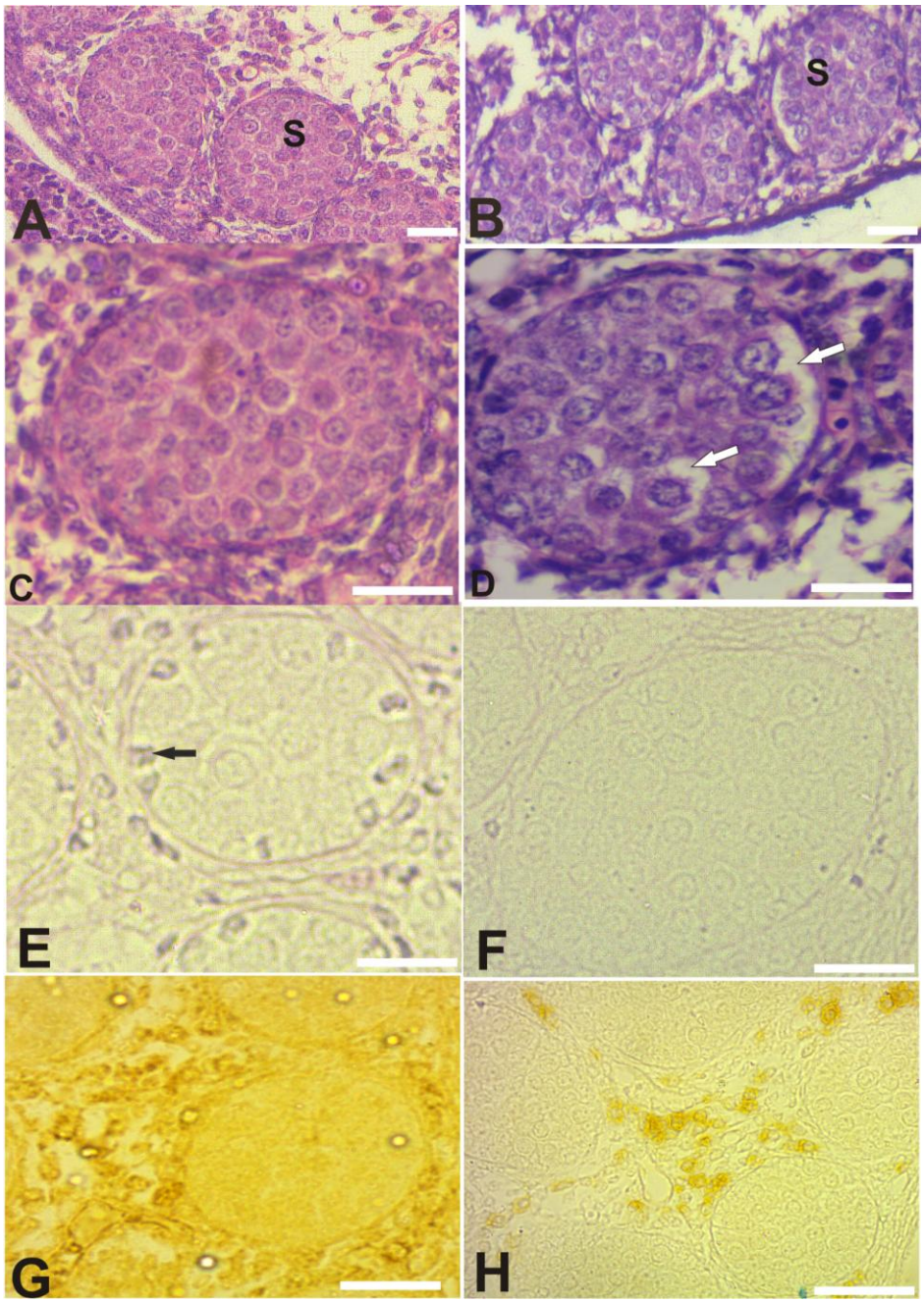
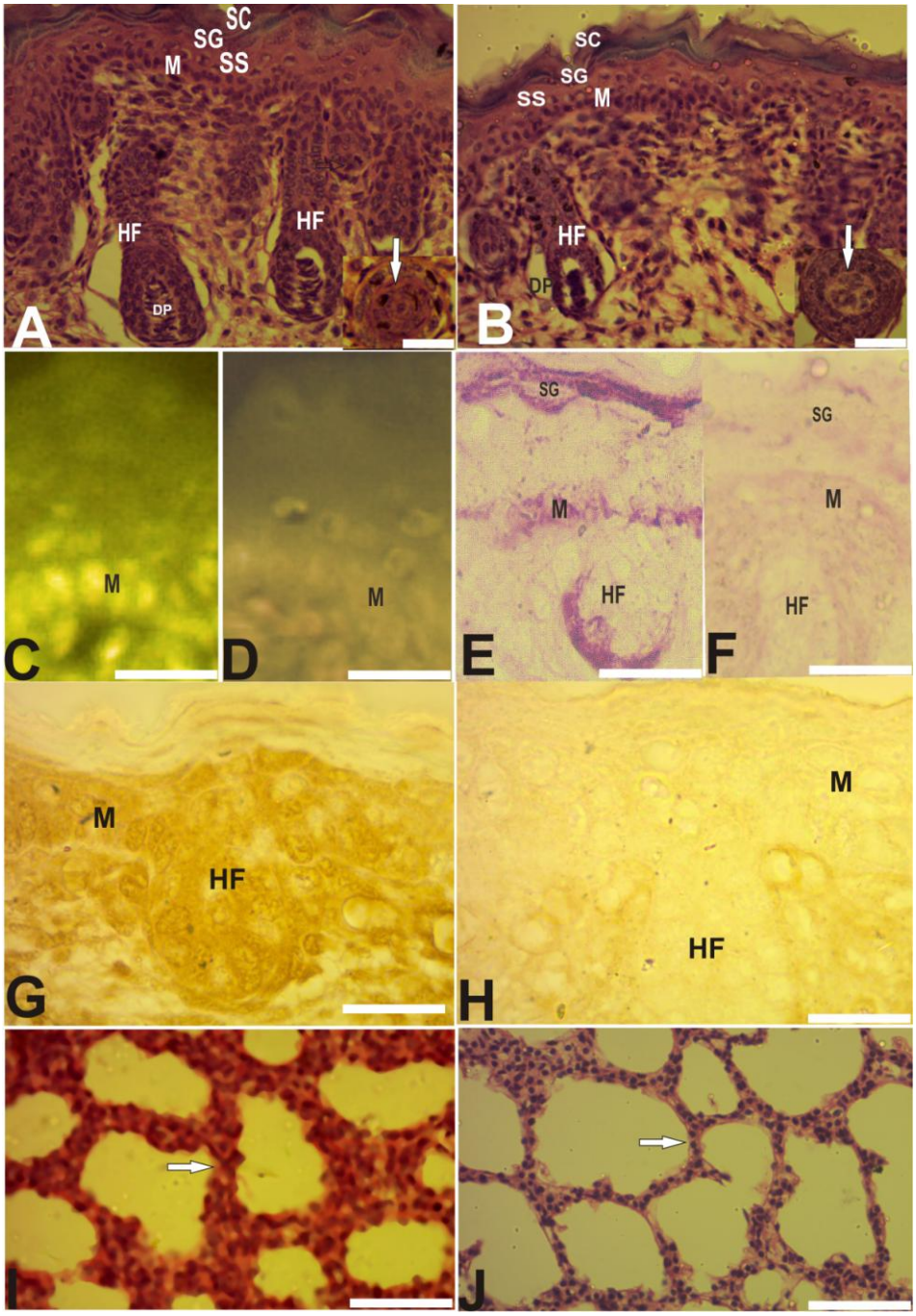


Plate 4: Photomicrographs of the developing testis at 14 days of gestation showing the differentiated leumenless testicular tubules in both the control (A) and retinoic acid-exposed embryos (B). Intact gonocyte, differentiated Sertoli (arrow) and TGFβ2 expression in control (C, E, G) respectively are shown against gonocyte detachement and vaculation (D), undifferentiated Sertoli cells (F) and a restricted expression of TGF β2 expression (H) compared to control. Scale bar 50µm.

18 days-old embryos: In 18 days-old embryos, the effect of retinoic acid is best manifested in skin and lung. In control embryos, the germ, spinos, granular and corneal layers of skin strata are well differentiated (Plate 5 A). Also, abundant and a well developed hair follicles that penetrate the dermal layer were noted. In retinoic acid-exposed embryos a thin wrinkled epidermis and a less abundant and disorganized hair follicles with shrinkage of dermal papilla (Pl. 5 B) were noted as compared to control. Also, Acridin orange/ethedium bromide-; PAS- and TGFβ2-stained skin sections revealed a depressed stainability and immunoexpresssion (Pls. 5 D, F, H) compared to the corresponding stainability and immunoexpression of control (Plate 5 C,E,G) respectively. In lung, thin and necrotic alveolar septa were noted (Plate 5 J) compared to the differentiated alveolar septa of control (Plate 5 I).



Pl. 5: Photomicrographs of skin and lung at 18 days of gestation showing a thin wrinkled with little and disorganized follicles (arrow of insert) (B), depressed fluorescence (D). negatively stained PAS (F), dowenregulated expression of TGFβ2 (H) and a thin alveolar septa (arrow) (J) in retinoic acid-exposed embryos compared to the corresponded in control (A, C, E, G, I) respectively. Malpighaian layer (M), stratum spinosum (SS), stratum granulosum (SG), stratum cornium (SC), hair follicle (HF). Scale bar 50 µm.

DISCUSSION

The present investigation revealed that intraperitoneal injection of low level of retinoic acid that didn't initiate the characteristic morphological abnormalities during pregnancy led to developmental defects in skin including thinning of the epidermal layer, degeneration of the developing hair follicles at its first anagen phase and decrease in the proliferating mesenchyme of the dermal layer. The effect of vitamin (A) on the developing embryos was manifested in deeper organs including the developing lung, kidney and testis. A thinner alveolar septae, less differentiated kidney tubules and undifferentiated Sertoli cells with epithelial detachment of the semineferous tubules were the most observed effects at the histological level of study. The effects in all studied organs of the developing embryos were accompanied with decrease in carbohydrate either in the developing epithelium or in the mesenchyme to which the epithelia come to interacte during development. Also, depressed fluorescence and dowen regulated expression of the transforming growth factor β2 ligand were recognized in retinoic acid-exposed embryos compared to control. In this concern, the metabolizing enzymes of retinoic acid in embryonic tissue were differentially expressed during murine organogenesis (Abu-Abed *et al.,* 2002). In skin, retinoids are recognized to suppress sebum production, keratinization, and proliferation, to stimulate differentiation in the epithelium, and to exert anti-inflammatory effects in doses beyond the irritation threshold were recorded (Millikan, 2000; Chapellier *et al.,* 2002). Like for other ligands of the nuclear steroid hormone receptor family with their huge repertoire of diverse biological functions. Retinoids have been implicated in the control of hair follicle morphogenesis, cycling and epithelial transdifferentiation. Treatment of upper mouse lip skin explants with retinoic acid *in vitro* resulted in development of glomerular glands instead of vibrissae hair follicles (Viallet and Dhouailly, 1994). However, conflicting data have been reported about the effects of retinoids on the growth and cycling of human and murine hair follicles (Bazzano *et al*., 1993). Hair loss during systemic retinoid therapy in humans is hypothesized to be provoked by an arrest at the onset of anagen and a defective anchoring of the hair shaft during telogen (Okano *et al.,* 2012). The importance of retinoic acid in kidney and lung development was also reported (Malpel *et al*., 2000; Batourina *et al*., 2001). However, the maternal-fetal transfer of retinoids and carotenoids, as well as the metabolism of these compounds in the developing tissues are still poorly understood as reported by Spiegler *et al.* (2012). The results of the present investigation are consistent in which systemic retinoic acid provoke the reported degenerative effects as early as during the first anagen stage of the developing hair follicles during intrauterine development. The reported degenerative effects in either the skin, lung, kidney or the developing testis can be attributed in part to the ability of retinoic acid to cross freely the placenta or conjugated with its protein carrier and in part to the disturbance in the metabolizing enzymes of retinoic acid in the embryonic tissues *In Vivo* even at low maternal dosing.

More than 500 genes are known to be regulated by RA (Balmar and Blomhoff, 2002). A great number of these genes have been shown to control embryonic development (Mark *et al.,* 2009). When RA signaling needs to be turned off, RA is degraded by members of the cytochrome P450 family of enzymes, such as Cyp26A1, to produce more polar compounds, like 4-hydroxy or 4-oxo RA, which are believed to be non-transcriptionally active (Abu-Abed *et al.,* 2001). Like retinodes, transforming growth factor TGFβ2 is a multifunctional bioregulator with the capacity to inhibit epithelial cell growth and to stimulate keratinocyte apoptosis (Soma *et al*., 2002; Wang *et al*., 2002). Retinoides are also induces TGFβ2 expression in keratinocytes, fibroblasts, and pancreatic tumor cell lines (Glick *et al*., 1989; Choudhury *et al*., 2000). TGFβ2 has been shown to exert hair growth promoting effects during morphogenesis (Foitzik *et al*., 1999). In contrast, TGFβ1 and TGFβ2 are recognized as potent catagen inducers in murine and human anagen HF *in vivo* and *in vitro* by inhibition of proliferation and induction of apoptosis in hair matrix keratinocytes (Foitzik *et al*., 2000; Soma *et al*., 2002). So far, most of the *in vivo* studies have been focused on TGFβ1 and the results are consistent with its *in vitro* growth inhibitory effects. For instance, overexpression of TGFβ1 in the epidermis results in a reduced number of hair follicles and an impaired proliferation of the epidermis. The skin of TGFβ1-deficient mice was shown to have an increased number of proliferating keratinocytes and TGFβ1 inhibited growth and thymidine incorporation of anagen hair follicles in organ culture (Philpott *et al*., 1994). More directly, treatment of skin organ culture with TGFβ1 was found to inhibit hair follicle development and keratinocyte proliferation. In striking contrast to TGF-β1, direct treatment with TGFβ2 potently stimulates hair follicle formation and at the same time produces epidermal hyperplasia. TGFβ2 null mice exhibited a profound delay of hair follicle morphogenesis, with a 50% reduced number of hair follicles. In contrast to hair follicle development, growth and differentiation of interfollicular keratinocytes proceeded unimpaired. Unlike TGFβ2-/- mice, mice with a disruption of the TGFβ1 gene showed slightly advanced hair follicle formation, while lack of the TGF-β3 gene did not have any effects (Foitzik *et al.*, 2000). Vital role of transforming growth factors family members was also reported in lung, kidney and testis either in their development or functional performance (Serra *et al*., 1994; Itman *et al*., 2006; Sims-Lucas *et al*., 2008).

CONCLUSION

Therefore, it can be concluded that dowenregulation of TGFβ2 ligand may act as a mediator of retinoids-induced not only follicular growth inhibition during intrauterine skin development but also contribute to the imperfect development of the deeper organs of the embryo as recorded in both the lung, kidney and testis through disturbed epithelial-mesenchyme interaction between the potent embryonic cells of these organs *in vivo* at low maternal low level of exposure.

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