IN VITRO CELLULAR & DEVELOPMENTAL BIOLOGY
Volume 25, Number 5, May 1989

THE USE OF RETINOIDS AS PROBES FOR ANALYZING MORPHOGENESIS OF GLANDS FROM EPITHELIAL TISSUES

MARGARET H. HARDY

Department of Biomedical Sciences, University of Guelph, Guelph, Ontario, Canada N1G 2W1

(Received 10 January 1989; accepted 18 January 1989)

SUMMARY

Thirty-five years ago Honor Fell and Edward Mellanby were studying effects of high doses of vitamin A on skeletal development in chick embryos when they noticed that a piece of epidermis, accidentally included in an organ culture, had undergone mucous metaplasia. Further studies by Fell and others eventually led to an understanding of the important role of vitamin A in modulating epithelia in vivo. Fifteen years later another organ culture experiment showed me that excess vitamin A could also initiate the morphogenesis of branching and mucus-secreting glands from developing vibrissa follicles in upper lip skin of embryonic mice. Since then our group has shown that induction of this novel structure by naturally occurring retinoids resembles a normal embryonic induction in that it is stage-dependent, time-dependent, and irreversible. Tissue separation and recombination studies showed that isolated upper lip epidermis can form these glands when combined with retinoid-treated upper lip dermis. Untreated mouse epidermis can form similar glands after combination with chick dermis containing higher retinoid levels. The hamster cheek pouch, normally devoid of glandular structures, can also form mucous glands when treated with a retinoid, either in vivo or in vitro. Recombination studies in organ culture have now shown that mesenchyme exposed to retinoid is essential for gland morphogenesis from pouch epithelium. Evidence is accumulating that retinoic acid may even be the active morphogen in some normally developing systems.

Key words: retinoids; morphogenesis; mucous glands; hair follicles; hamster cheek pouch; mesenchymal-epithelial interactions.

INTRODUCTION

The word "serendipity," according to the Oxford Dictionary, was invented by Horace Walpole for his fairytale, "The Three Princes of Serendip," the heroes of which "were always making discoveries, by accidents and sagacity, of things they were not in quest of." The story of the role of vitamin A in cell and developmental biology has always seemed to be a true story of serendipities.

Honor B. Fell was certainly the first Princess of Serendip in this story when, with Edward Mellanby, she was studying the effects of very high doses of vitamin A on limb bones of chick embryos in organ culture (10). One day they noticed that a piece of chick skin, an accidental contaminant in the preparation, had developed a mucus-secreting epithelium instead of a keratinizing one. They reported this mucous metaplasia in a classic paper in 1953 (11). Fell and her collaborators at the Strangeways Research Laboratory in Cambridge followed this up by showing, among other things, that the metaplasia could be reversed after transfer to a standard culture medium (8,13). Although Fell continued to write insightfully on the roles of vitamin A in cell and tissue differentiation in skin at least until 1965 (12), her earlier interest in skeletal tissues came to predominate in her own research. But in other parts of the Strangeways Laboratory and elsewhere, new discoveries were made about the actions on various epithelial tissues of vitamin A (or retinol), its aldehyde and esters, and other structural analogues collectively known as the retinoids. The naturally occurring retinoids with vitamin A activity are now known to play an important role in the healthy maintenance of all epithelia, of mammals as well as birds.

In 1961 Fell concluded her address to the Annual Meeting of the British Society of Dermatology (9) with these words:

> I feel apologetic at having inflicted such very academic research upon a clinical audience, but recent work in our laboratory suggests that these studies may prove to be less remote from dermatological interests than we originally thought, and something of practical value may yet emerge from the ivory tower of the cell biologist.

Today, dermatologists around the world have retinoids to prescribe for severe acne and several other skin disorders. Furthermore, retinoids are very effective in treating some epithelial cancers, and the value of dietary carotenoids and retinoids for cancer prevention has been widely recognized (27). "Something of practical value" has indeed emerged.

A New Role for Retinoids

Several years before the original discovery by Fell and Mellanby, I had the privilege of being a PhD student at Cambridge under Honor Fell's supervision. I had come from sheep and wool research in Australia with the aim of growing hair follicles in organ culture, and, with her guidance, success came quickly (16). But I was back in Australia, continuing research on mouse hair and sheep's wool in organ culture when Fell and Mellanby reported their discovery. A few years later I resigned from my position for family reasons.

Eight years and three children later, I arrived with my family in the eastern United States, and was interested in returning to part-time research. Thanks to the encouragement of the late Dr. Margaret Murray, I became a research associate in the laboratory of Dr. Richard Bunge at Columbia University's College of Physicians and Surgeons. A survey of recent literature on skin biology suggested to me that vitamin A would be the most interesting topic to pursue. If retinoids are so effective in suppressing the keratinization of skin and other epithelia, I wondered what they would do in the developing hair follicle, which produces a structure of almost 100% pure keratin. So organ cultures were set up, and serendipity began operating again. Although the development of pelage hair follicles of mouse embryos was merely suppressed by vitamin A in the form of retinol, the vibrissa follicles on the upper lip were transformed into branching, mucus-secreting glands (17). This was not just another case of mucous metaplasia, but of morphogenesis as well, morphogenesis of a type of structure never found in mammalian skin. What did it mean? Further experiments showed that vibrissa follicles were transformed in this way only if they were exposed to high levels of retinol at a certain critical period in their development (17,18).

After I had worked for 18 mo. in New York, my sociologist husband was appointed to Johns Hopkins University, but the university could not persuade the U.S. Immigration Department to extend his visa, so we arrived rather suddenly in Canada. A year later, I was at the University of Guelph.

Although I worked on a variety of research topics with colleagues and graduate students, every few years the unanswered questions enticed me back to retinoids in developing skin. The initiation of mucous glands from vibrissa follicles was shown to be not only stage-specific, but also time-dependent, requiring 2 or 3 d of exposure to retinol or retinyl acetate. Unlike the mucous metaplasia of chick epidermis, glandular morphogenesis was not blocked by corticosteroids. Also unlike mucous metaplasia, the morphogenesis seemed to be irreversible, in that washing and transfer of explants to a standard medium after 3 or more d of exposure was followed by continued growth and function of the glands for the remainder of the culture period (20), which was up to 18 d in some experiments (Hardy and Bellows, unpublished observations). The effects of treatment reversals after 7 d *in vitro* are shown diagrammatically in Fig. 1. This figure also illustrates that, in some cases, the cells of the dermal papilla, which were dispersed when a follicle was transformed into a gland after 7 d in excess retinoid, were able to reassemble in standard medium and then proceed to induce the further differentiation of inner root sheath and hair from the epithelial hair matrix. This differentiation is a process that normally occurs in response to what is known as the "second dermal message" (4). All the evidence so far suggests that the excess retinoids interfere

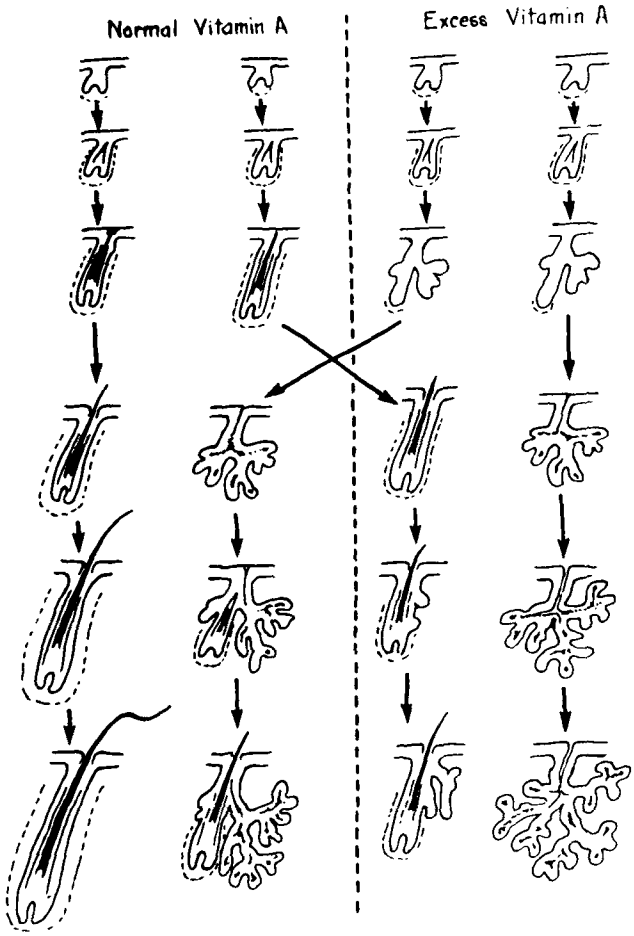

FIG. 1. Diagram showing results of a treatment reversal experiment. *Column 1* shows normal development of a vibrissa follicle as an epithelial downgrowth (*solid line*) from the epidermis (*parallel solid lines*) during 14 d in standard medium with a physiologically normal concentration of vitamin A. The hair (*solid black area*) emerged from the skin during week 2. A boundary enclosing both the aggregation of mesenchymal cells, which forms the dermal papilla, and the later aggregation, which forms the dermal root sheath, is also shown (*dashed line*). The three upper sketches in *column 2* indicate similar development in explants during the first 7 days in standard medium. These explants, after transfer to medium with excess vitamin A (*arrow* to *column 3*) continued hair growth, but a few lateral buds developed. The three upper sketches in *column 3* indicate the glandular morphogenesis and loss of dermal papilla in excess vitamin A. After transfer to standard medium (*arrow* to *column 2*), gland morphogenesis and differentiation continued. In addition, the reaggregation of scattered dermal papilla cells and resumption of hair follicle differentiation and hair growth, which was observed in a few follicles, is illustrated here. *Column 4* shows only gland morphogenesis and differentiation.

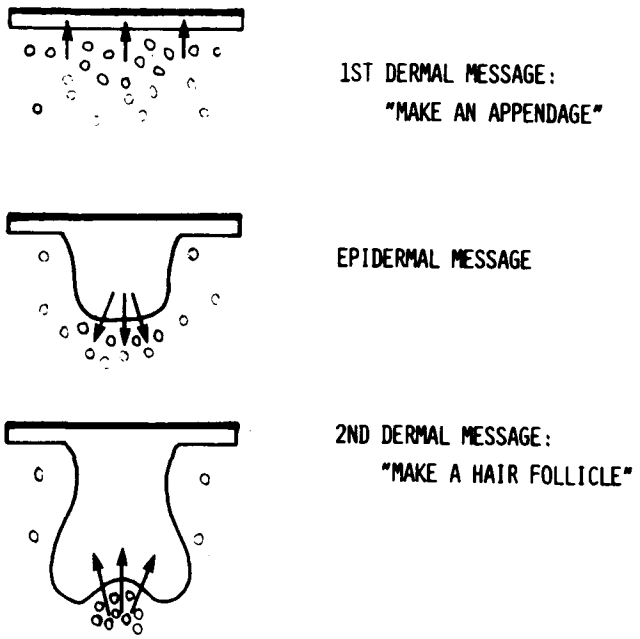

FIG. 2. Epithelial-mesenchymal interactions during hair follicle development in mammals. *Thick line* represents the upper margin of the epidermis. *Thin line* defines the lower margin of the epidermis and the "hair bud," which develops into a hair follicle. *Circles* represent the mesenchyme cells of the dermis.

with the normal sequence of tissue interactions between epithelium and mesenchyme which lead to the formation of hairs, feathers, or scales in the Amniotes (4). These interactions in mammals (Fig. 2) include a) a "first dermal message" from a cluster of mesenchymal cells which specifies the position and size of an epithelial follicle peg from the epidermis, b) an "epidermal message" from the follicle peg to the cluster of mesenchymal cells which then forms a dermal papilla, and c) a "second dermal message" from the dermal papilla to the epithelial rudiment to cause differentiation of a hair, as specified by the mammalian epithelium. In our experiments, the excess retinoid apparently leads to suppression of the second dermal message and, one may speculate, initiation of a new second dermal message at the lateral wall of the follicle (19). It may also interfere with the epidermal message, since aggregated papilla cells are frequently dispersed when they grow in retinoid medium (18,21) (Fig. 1). Retinoids may therefore be considered as probes for investigating the largely unknown mechanisms of tissue interactions.

How Do Retinoids Influence Tissue Interactions?

The next question we sought to answer was "Do retinoids modify the mesenchyme so that it produces or transmits a new inductive message, or is a retinoid molecule itself the inducer of the epithelial cells when mucous glands are formed? The first part of this question was approached by asking "Do retinoids modify the mesenchyme surrounding developing vibrissa follicles?" Several ultrastructural studies in our laboratory have shown that this is so (21,28). Of particular interest is the fact that gaps appear in the basal lamina, and allow direct contacts between mesenchymal and epithelial cells only at the sites of, and just before, gland outgrowth. During normal development in vivo, similar gaps and contacts occur only between the dermal papilla cells and the surrounding epithelial cells, and only around the time of transmission of the second dermal message at that site (15). Are these gaps necessary for transmission of the messages? We do not yet know.

To pursue the question of whether the retinoids alone can induce the epithelium without acting through mesenchyme, it was necessary to combine tissues with different histories of exposure to retinoid. The remainder of this paper will summarize the results of these latter investigations, which, apart from an abstract (7) and a Ph.D. thesis (2), are still unpublished (2 manuscripts submitted).

In the laboratory of Philippe Sengel, a pioneer in epithelial-mesenchymal analysis at the University of Grenoble, Danielle Dhouailly and I began to experiment with retinoids and embryonic mice. We found that upper lip skin, cultured for 3 d with retinol (5 μg/ml), produced vibrissa follicles when grafted to the chick chorioallantoic membrane (CAM), and two-thirds of those grafts showed some glandular morphogenesis of developing follicles. However, after 3 d in culture with retinol, followed by separation from the epidermis with trypsin, the dermis became very fragile, and there was only a low yield of successful grafts when this dermis was recombined with untreated epidermis. We tried culturing younger dermis for 2 d with retinoic acid (RA) before combining it with 12.5-d untreated epidermis (Fig. 3). Even with these modifications, only 26% of the successful recombinants of epidermis with RA-treated dermis showed glands (Fig. 3). To improve the yield of glands, the more robust chick dermis was then used instead of mouse dermis. Chick embryos in ovo received either 125 μg of RA in ethanol, or ethanol alone, at Days 10 and 11; then, at 12.2 d, the trypsin-isolated tarsometatarsal

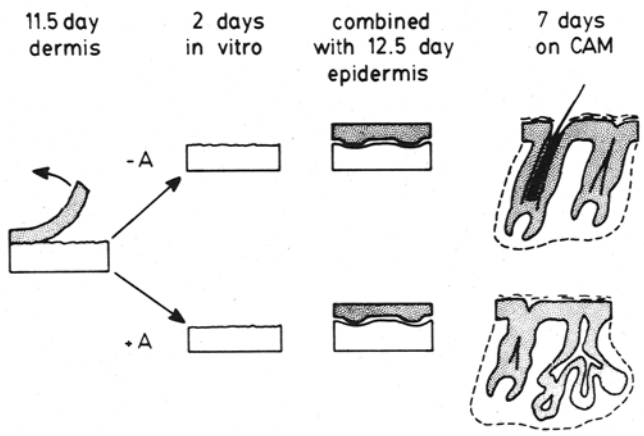

FIG. 3. Diagram showing procedure for separating mouse embryo dermis from skin grown in standard medium (−A) or retinoic acid medium (+A) and combining it with untreated epidermis. With −A dermis, normal hair follicles only were produced. With +A dermis, some early stage hair follicles and a few branching glands were formed.

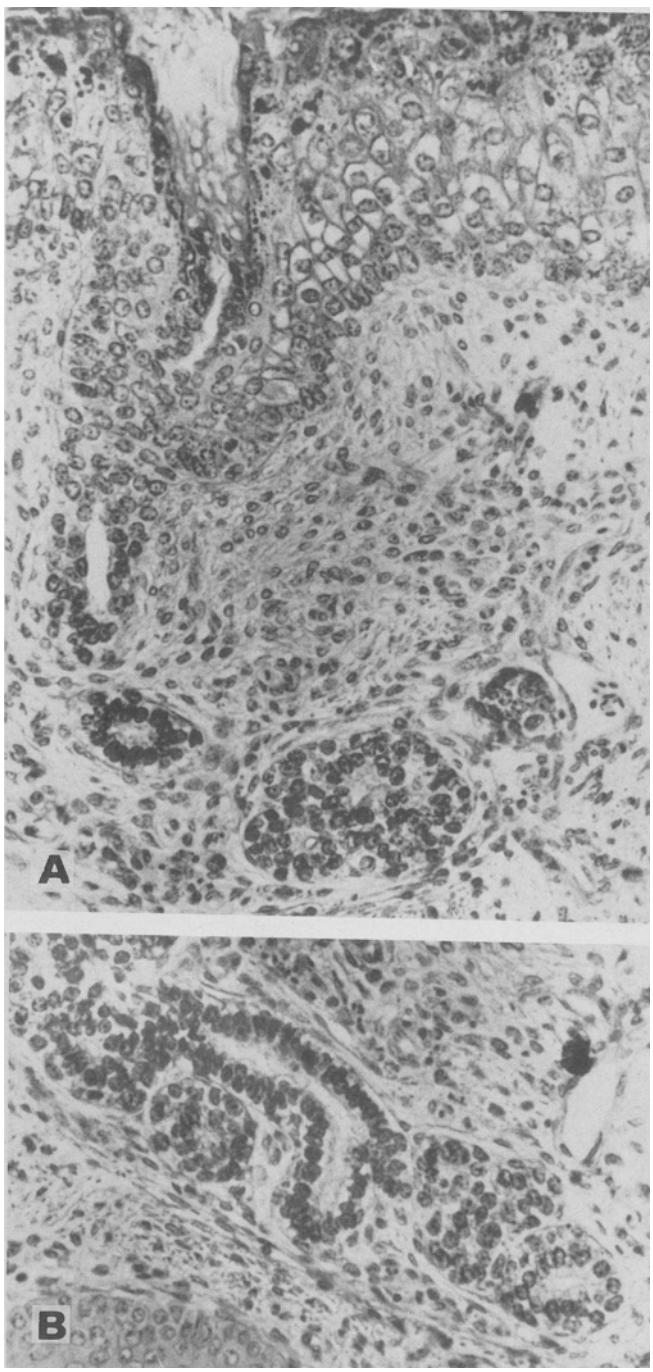

FIG. 4. Histologic sections of a recombinant graft of 12.5-d mouse embryo epidermis with 12.2-d dermis from a chick embryo that had been injected with retinoic acid. *A*, epidermis keratinizing and showing developing of a normal hair canal. Hair follicle bud is arrested, but a main gland duct extends downward from it, and some terminal buds of the gland are shown. *B*, a section through another part of the same gland shows a long section of secondary duct and several lighter-staining terminal buds. Hematoxylin, eosin.

dermis of the chick was removed and recombined with 12.5-d untreated mouse epidermis on the CAM. The results were at the same time satisfying, surprising, and serendipitous. The satisfaction was due to the higher yield of successful grafts and to the fact that RA-treated chick dermis induced advanced mucous gland morphogenesis from early vibrissa follicle epithelial pegs in 75% of the successful grafts (Fig. 4). The dermis, being avian, could induce follicle pegs but could not induce vibrissae. The surprising and puzzling finding was that dermis from chicks receiving nothing more than 100 μl of ethanol could also induce glands in 29% of "control" grafts. We later discovered, in collaboration with Dr. Anders Vahlquist of the University of Uppsala, that the level of retinoids (determined by high pressure liquid chromatography) was much higher in both the dermis and epidermis of chick than in the corresponding tissues of mouse, and this may account for the gland formation. The serendipity began with the observation that the chickens injected with retinoic acid grew feathers on their feet where there should have been only scales. This led to other series of experiments and eventually some understanding of a different type of effect of retinoids on mesenchymal-epithelial interactions (1,5,6), in which the retinoid weakens the second dermal message for scale development and thus permits an earlier instruction for feather formation to be expressed.

In the Guelph laboratory, attention was turned also to the hamster cheek pouch. The lining of the adult pouch is a thin, keratinizing epithelium, very much like the outer epidermis of a hairy mammal, but is totally lacking in hair follicles and glands. Mock and Main (23) showed that the pouch of the newborn in vitro, like the adult pouch in vivo (22), responds to high doses of retinoids by mucous

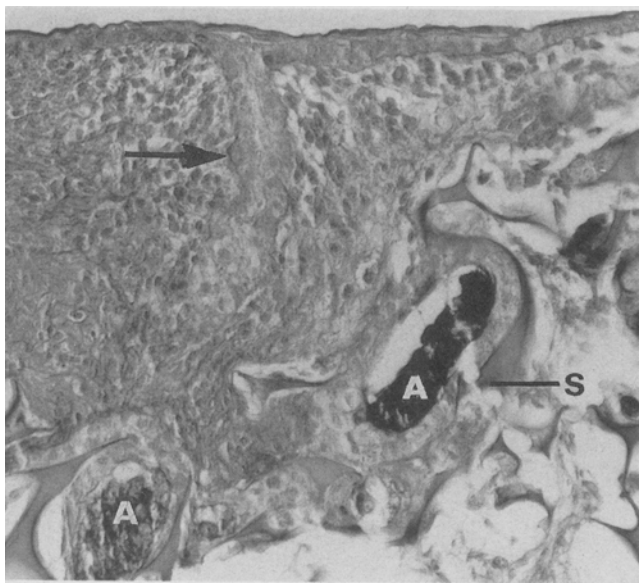

FIG. 5. Section of an explant of cheek pouch of newborn hamster after 7 d in medium with excess retinyl acetate followed by 14 d in standard medium, showing a well-differentiated gland. From the epithelial pouch lining (*top*), a discrete epithelial downgrowth (*arrow*) invades the mesenchymal stroma and has been observed through serial sections to connect with the two glandular acini (*A*) shown here. Each acinus has a cuboidal epithelium lining a central lumen that contains periodic-acid-Schiff (PAS)-positive, diastase-resistant material, indicating glycoprotein secretion. *S*, pieces of the gelatin sponge raft on which the explant was cultured. PAS-hematoxylin. ×237.

TABLE 1

INCIDENCE OF GLANDS IN EXPLANTS OF NEWBORN HAMSTER CHEEK POUCH WITH DIFFERING HISTORIES OF EXPOSURE TO RETINYL ACETATE*

Stroma Exposed to Control Medium (C), Days 1–7			Stroma Exposed to Retinyl Acetate Medium (R), Days 1–7		
Composition of Explants (Days 8–21)	No. of Explants	No. with Glands	Composition of Explants (Days 8–21)	No. of Explants	No. with Glands
Full thickness of pouch (C)	17	0	Full thickness of pouch (R)	18	4
Recombinant Epithelium (C) / Stroma (C)	14	0	Recombinant Epithelium (R) / Stroma (R)	7	1
Recombinant Epithelium (R) / Stroma (C)	16	0	Recombinant Epithelium (C) / Stroma (R)	18	3
Total	47	0	Total	43	8

*Explants of full thickness pouch were grown for 7 d in control medium (C) or medium containing 1.8×10^{-5} *M* retinyl acetate (R). They were then subjected to controlled trypsinization. Some were not separated, but most were separated into epithelium and stroma, and the tissues recombined in various ways for a further 7 or 14 d of culture in control medium.

metaplasia and occasional gland morphogenesis. This seemed to be a promising system for a further test of the roles of epithelium and mesenchyme in mucous gland initiation and morphogenesis. Covant and Hardy (3) showed that the development of glands from segments of intact cheek pouch was limited to a proportion of those in which the semisynthetic medium was supplemented with a retinoid [6 μg/ml of retinyl acetate (RAc) or RA in acetone]. They showed that 7 d of exposure to retinoid was sufficient for irreversible gland morphogenesis (Fig. 5) in a majority of RA-treated explants and a minority of RAc-treated explants. In another series of experiments by Covant (2), all possible recombinations of RAc-treated or untreated epithelium with RAc-treated or untreated stroma were compared after 14 or 21 d of culture. Glands were never formed unless the stroma had been pretreated with the retinoid (Table 1). Glands formed in one or more members of every group of explants that had retinoid-treated stroma, but the proportion of explants with glands did not rise appreciably when the epidermis also had been pretreated. We concluded that action through the stromal mesenchyme was obligatory for retinoids to initiate or support mucous gland development, or both, in this region.

Is Retinoic Acid More Than Just a Probe?

There remain, of course, many unanswered questions. It is not clear how the retinoids act to bring about a new program of development. Meanwhile, studies of the effects of retinoids on limb bud development in other laboratories have led to some exciting conclusions. Several authors [e.g., Summerbell (25)] had previously shown that adding RA can cause duplication of digits in the developing limb buds of chickens. The new discovery of a natural posteroanterior gradient of RA in the developing limb bud of the chick by Thaller and Eichele (26) has led them to suggest that this compound could be not merely a probe, but the natural morphogen in this region, specifying distinctive skeletal elements according to position on its concentration gradient. Furthermore, a specific, high affinity nuclear RA receptor, and its DNA-binding domain, have been discovered by two research groups (14,24). The way seems to be open for the powerful tools of molecular biology to be used in the search for mechanisms of retinoid action in development. Giguere et al. (14) wrote of their great surprise at two of their accidental findings concerning the nuclear RA receptor site and its close relationship to thyroid and steroid hormone nuclear receptors. Dame Honor Fell might not have been surprised to know of these serendipitous events. I hope that all of us here today will have not only lucky accidents in our experiments but the sagacity of that first Princess of Serendip.

References

1. Cadi, R.; Dhouailly, D.; Sengel, P. Use of retinoic acid for the analysis of dermal-epidermal interactions in the tarsometatarsal skin of the chick embryo. Dev. Biol. 100:480–495; 1983.
2. Covant, H. A. An investigation of the site of retinoid action in the induction of glands from the hamster cheek pouch. Guelph, Canada: Univ. Guelph; 1987. Thesis.
3. Covant, H. A.; Hardy, M. H. Stability of the glandular morphogenesis produced by retinoids in the newborn hamster cheek pouch in vitro. J. Exp. Zool. 246:139–149; 1988.
4. Dhouailly, D. Dermis-epidermal interactions during morphogenesis of cutaneous appendages in mammals. Front. Matrix Biol. 4:86–121; 1977.
5. Dhouailly, D.; Hardy, M. H. Retinoic acid causes the development of feathers in the scale-forming integument of the chick embryo. Wilhelm Roux's Arch. Dev. Biol. 185:195–200; 1978.
6. Dhouailly, D.; Hardy, M. H.; Sengel, P. Formation of feathers on chick foot scales: a stage-dependent morphogenetic response to retinoic acid. J. Embryol. Exp. Morphol. 58:63–78; 1980.
7. Dhouailly, D.; Hardy, M. H.; Vahlquist, A. Glandular morphogenesis of mouse epidermis associated with chick dermis is

enhanced by retinoic acid treatment. Dermatologica 169:228-229; 1984.
8. Fell, H. B. The effect of excess vitamin A on cultures of embryonic chicken skin explanted at different stages of differentiation. Proc. R. Soc. Lond. B. Biol. Sci. 146:242-256; 1957.
9. Fell, H. B. Some effects of environment on epidermal differentiation. Br. J. Dermatol. 74:1-5; 1962.
10. Fell, H. B.; Mellanby, E. The effect of hypervitaminosis A on embryonic limb bones cultivated *in vitro*. J. Physiol. (Lond.) 116:320-349; 1952.
11. Fell, H. B.; Mellanby, E. Metaplasia produced in cultures of chick ectoderm by high vitamin A. J. Physiol., (Lond.) 119:470-488; 1953.
12. Fell, H. B.; Rinaldini, L. M. The effects of vitamins A and C on cells and tissues in culture. In: Willmer, E. N., ed. Cells and tissues in culture. Methods, biology and physiology, vol. 1. London: Academic Press; 1965:659-699.
13. Fitton Jackson, S.; Fell, H. B. Epidermal fine structure in embryonic chicken skin during atypical differentiation induced by vitamin A in culture. Dev. Biol. 7:394-419; 1963.
14. Giguere, V.; Ong, E. S.; Segui, P., et al. Identification of a receptor for the morphogen retinoic acid. Nature 330:624-629; 1987.
15. Goldberg, E. A.; Hardy, M. H. Heterotypic cell contacts and basal lamina morphology during hair follicle development in the mouse. Can. J. Zool. 61:2703-2719; 1983.
16. Hardy, M. H. The development of mouse hair *in vitro* with some observations on pigmentation. J. Anat. 83:364-384; 1949.
17. Hardy, M. H. Glandular metaplasia of hair follicles and other responses to vitamin A excess in cultures of rodent skin. J. Embryol. Exp. Morphol. 19:157-180; 1968.
18. Hardy, M. H. The differentiation of hair folliciles and hairs in organ culture. Adv. Biol. Skin 9:35-60; 1969.
19. Hardy, M. H. Vitamin A and the epithelial-mesenchymal interactions in skin differentiation. In: Sawyer, R. H.; Fallon, J. F., eds. Epithelial-mesenchymal interactions in development. New York: Praeger; 1983:163-188.
20. Hardy, M. H.; Bellows, C. G. The stability of vitamin A-induced metaplasia of mouse vibrissa follicles in vitro. J. Invest. Dermatol. 71:236-241; 1978.
21. Hardy, M. H.; Van Exan, R. J.; Sonstegard, K. S., et al. Basal lamina changes during tissue interactions in hair follicles—an in vitro study of normal dermal papillae and vitamin A-induced glandular morphogenesis. J. Invest. Dermatol. 80:27-34; 1983.
22. Lawrence, D. J.; Bern, H. A. Mucous metaplasia and mucous gland formation in keratinized adult epithelium *in situ* treated with vitamin A. Exp. Cell Res. 21:443-446; 1960.
23. Mock, D.; Main, J. H. P. The effect of vitamin A on hamster cheek pouch mucosa in organ culture. J. Dent. Res. 58:635-637; 1978.
24. Petkovich, M.; Brand, N. J.; Krust, A., et al. A human retinoic acid receptor which belongs to the family of nuclear receptors. Nature 330:444-450; 1987.
25. Summerbell, D. The effect of local application of retinoic acid to the anterior margin of the developing chick limb. J. Embryol. Exp. Morphol. 78:269-289; 1983.
26. Thaller, C.; Eichele, G. Identification and spatial distribution of retinoids in the developing chick limb bud. Nature 327:625-628; 1987.
27. Trifiates, G. P.; Prasad, K. N., eds. Nutrition, growth and cancer. Progress in clinical and biological research 259. New York: Alan R. Liss; 1988.
28. Van Exan, R. J. An in vitro study of the effects of excess vitamin A on the differentiation of the mammalian dermis. Guelph, Ontario: Univ. Guelph; 1979. Thesis.

I acknowledge the valuable collaboration of Dr. Phillip Sweeny of the University of Guelph, Dr. Danielle Dhouailly and Dr. Philippe Sengel of the University of Grenoble, and Dr. Anders Vahlquist of the University of Uppsala in some of the studies referred to in this paper. The graduate students, research assistants, and technicians who participated enthusiastically in research on retinoid effects on differentiation and development at Guelph over the years included Carl Bellows, Howard Covant, Mary Robinson, Elizabeth Roff, Karen Stonstegard, Robert Van Exan, and Olga Vrablic. The Natural Sciences and Engineering Research Council of Canada made this possible by uninterrupted funding. Finally, I am deeply indebted to my teacher, Dame Honor Fell, who, as a scientist and a person, remains a source of inspiration after 40 years.

Figures 1, 3, and 4 have been reproduced with the kind permission of Praeger Press (19). Figure 5 has been reproduced with the kind permission of Alan Liss, Inc. (3).