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Dopaminergic D2 Receptor Knockout Mouse: An Animal Model of Prolactinoma

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Abstract

Dopamine receptor type 2 (D2R) knockout mice (KO) have chronic hyperprolactinemia, pituitary hyperplasia, and a moderate decrease in MSH content. They are also growth retarded evidencing an alteration in the GH-IGF-I axis. In D2R KO, lactotropes do not show dense secretory granules but degranulated cells and fewer somatotropes, gonadotropes and thyrotropes. Prolactin levels are always higher in female than in male knockouts, and in accordance, pituitary hyperplasia is observed at 8 months only in females. After 16 months of age, highly vascularized adenomas develop, especially in females. Prominent vascular channels in the hyperplastic and adenomatous pituitaries, as well as extravasated red blood cells not contained in capillaries is also a common finding. Prolactin is not the factor that enhances the hyperplastic phenotype in females while estrogen is a permissive factor. VEGF-A expression is increased in pituitaries from D2R KO. VEGF-A is expressed in follicle stellate cells. Because D2R receptors are found in lactotropes and not in follicle stellate cells, it may be inferred that a paracrine-derived factor from lactotropes is acting on follicle stellate cells to increase VEGF-A expression. VEGF-A does not induce pituitary cell proliferation, even though it enhances prolactin secretion. But it may act on adjacent endothelial cells and participate in the angiogenic process that increases the availability of different growth factors and mitogens. The D2R knockout mouse represents a unique animal model to study dopamine-resistant prolactinomas, and VEGF-A may be an alternative therapeutic target in this pathology.

Animal Models of Prolactinomas

Although mouse models may differ from their human counterparts, their study provides important insights into human pituitary tumor pathogenesis. There

are several rat and mice models to study the genesis and regulation of prolactinomas, for example:

- The traditional estrogen-induced rat pituitary tumor.
- Transgenic mice with overexpression of a truncated fibroblast growth factor receptor 4 (FGFR4) isoform (driven by the prolactin promoter) [1].
- Targeted overexpression of transforming growth factor-α (TGF-α) under the control of the prolactin promoter [2].
- Transgenic nerve growth factor (NGF) overexpression under the control of the prolactin promoter [3].
- Overexpression of the high mobility group A (HMGA2) gene [4].
- Overexpression of galanin (driven by the GH or the prolactin promoter) [5, 6].
- Targeted disruption of TGFR type II (in heterozygotes) [7].
- The prolactin receptor knockout mouse [8].
- The dopamine (DA) receptor type 2 (D2R) knockout mouse [9, 10].

Each model presents variable characteristics: for example, the first three develop adenomas, while in NGF overexpression there is lactotrope hyperplasia with no adenoma formation. The overexpression of the truncated FGFR4 isoform is linked to diminished cell adhesion.

Dopaminergic Receptor Type 2 Knockout Mouse

We will focus on results found in the D2R knockout mouse. DA is the most abundant catecholamine in the brain. The involvement and importance of DA as a neurotransmitter and neuromodulator in the regulation of different physiological functions in the central nervous system is well known. Deregulation of the dopaminergic system has been linked with Parkinson disease, Tourette syndrome, schizophrenia, attention-deficit hyperactive disorder and generation of pituitary tumors.

DA exerts its action by binding to specific membrane receptors, which belong to the family of seven transmembrane domain G-protein-coupled receptors. Five distinct DA receptors have been isolated, characterized and subdivided into two subfamilies, D1- and D2-like, on the basis of their biochemical and pharmacological properties. The D1-like subfamily comprises D1- and D5-R, while the D2-like includes the D2-, D3- and D4-Rs. In brain tissues the D2R is expressed predominantly in the caudate putamen, olfactory tubercle and nucleus accumbens. It is also expressed in the substantia nigra pars compacta and in the ventral tegmental area. These are the anatomical regions that give rise to long dopaminergic fibers (A10 y A9), indicating that the D2Rs have a presynaptic location. Outside the brain D2R is localized in the retina, kidney, vascular system and in the pituitary gland, both in melanotropes and lactotropes.

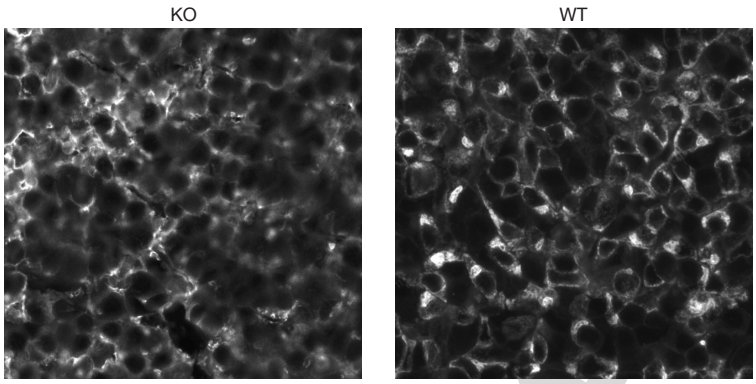

Fig. 1. Confocal microscopy of pituitaries from a D2R female knockout (left) and a wildtype mouse (right), using anti-PRL coupled to FITC.

The D2R gene is composed of eight exons, seven of which are coding. It encodes two splice variants, a short and a long D2R. The signal transduction pathways activated by D2Rs are numerous. They are coupled to pertussis toxin-sensitive Gi/Go proteins, and the best-described effects mediated by DA are the inhibition of the cAMP pathway and modulation of Ca^{2+} signaling.

Drs. Malcolm Low and Marcelo Rubinstein generated a D2R knockout mouse by targeted mutagenesis [10]. The study of locomotor behavior of D2R knockout mice revealed a motor impairment in mutant mice, even though mice did not show a compelling parkinsonian locomotor phenotype. Results predicted that locomotor activity is a polygenic trait that varies widely among inbred strains of mice [11]. On the other hand, this knockout mouse model yielded important results on the participation of the D2R in pituitary function. It has been well settled that the D2R is the principal receptor involved in prolactin inhibition at the pituitary level, and in MSH regulation at the intermediate pituitary.

Therefore, as expected D2R knockout mice had chronic hyperprolactinemia, pituitary hyperplasia, and a moderate decrease in MSH content [10]. They were also growth retarded evidencing an alteration in the GH-IGF-I axis [12].

In D2R knockout mice, lactotropes did not show the dense secretory granules characteristically found in wild types but degranulated cells, indicating constitutive secretion (fig. 1). Furthermore, we observed a decrease in somatotropes, gonadotropes and thyrotropes (fig. 2).

In knockout animals prolactin levels increased on the third month of life, and a chronic hyperprolactinemic state ensued throughout life [12]. Prolactin levels were always higher in female than in male knockout mice, and in accordance, pituitary hyperplasia was observed at 8 months of age only in females [10].

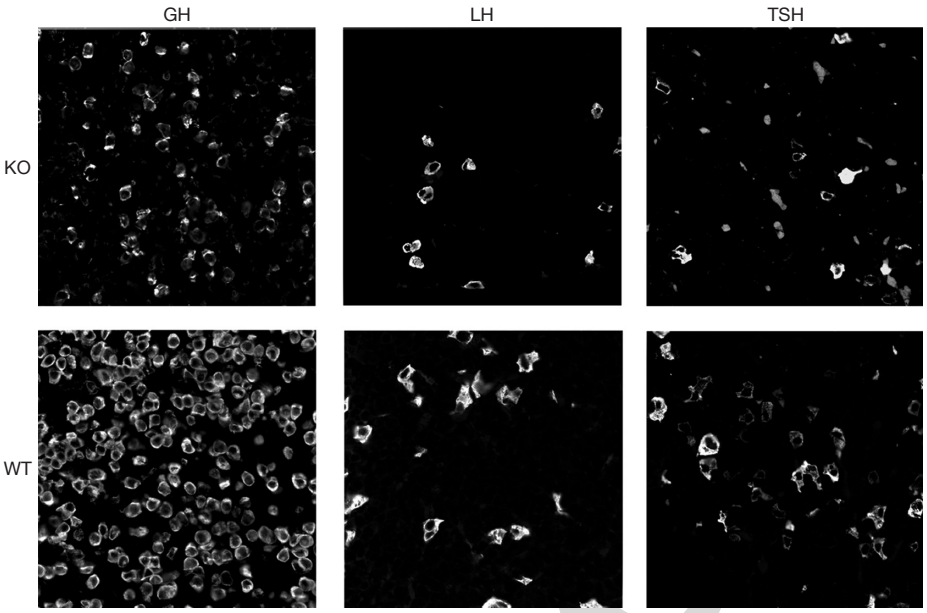

Fig. 2. Confocal microscopy of pituitaries from a D2R female knockout (upper panels: KO) and a wild-type mouse (lower panels: WT), using anti-GH, LH or TSH coupled to FITC.

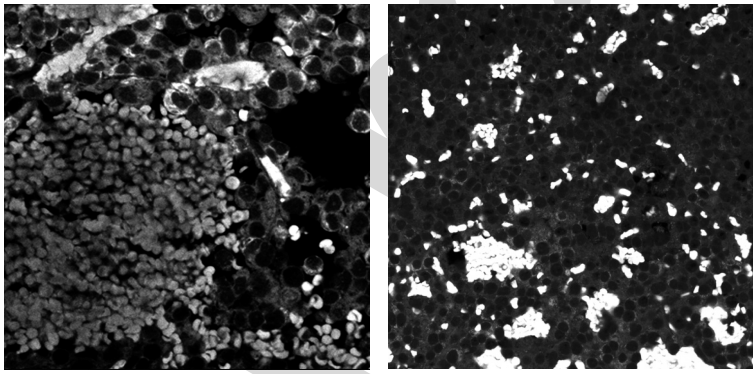

Fig. 3. Images of areas of peliosis (a) in pituitaries from D2R knockout mice.

After 16 months of age highly vascularized adenomas developed, especially in females [9]. Prominent vascular channels in the hyperplastic and adenomatous knockout pituitaries, as as extravasated red blood cells not contained in capillaries or peliosis was also a common finding (fig. 3).

Sex-Dependent Pituitary Hyperplasia in D2R Knockout Mice

Marked sexual differences in pituitary enlargement were important. As prolactin was chronically higher in females, it was possible that prolactin could perpetuate the hyperplastic phenotype by a positive feedback loop. To this regard, prolactin has been documented to be a proliferative growth factor in epithelial cells, kidney, the immune system and some tumors. A prolactin receptor (PRLR) knockout mouse was generated, and crossed with the D2R knockout. If prolactin was the proliferative factor acting at the pituitary, in the double knockout mouse hyperplasia would be reduced. But, contrary to the hypothesis PRLR knockout mice had enlarged pituitaries, and when combined with the D2R knockout pituitary hyperplasia was not decreased, and in males it was increased [8]. This clearly indicated that prolactin was not the factor involved in the sexual differences found in pituitary enlargement, and that at the pituitary level the prolactin receptor had an antiproliferative role.

A second candidate was estrogen, and indeed when female mice were ovariectomized, pituitary hyperplasia decreased. Nevertheless, estrogen replacement could not reproduce pituitary enlargement, indicating that estrogen was a permissive factor, but other factors were involved [13]. Furthermore, estrogen levels were not very different between male and female knockout mice [10].

We started to search for a factor which would participate in the generation of the hyperplasia in knockout females. Vascular endothelial growth factor-A (VEGF-A) seemed to be an interesting candidate as some reports have documented that it is regulated by DA and by estrogen [14–17], and besides, it is a potent angiogenic factor.

Neovascularization is essential for pituitary tumor formation, and is the result of a delicate balance between factors that stimulate or inhibit endothelial proliferation. A number of cytokines and growth factors have been demonstrated to modulate angiogenesis with a paracrine mode of action. Among these factors VEGF is one of the most potent angiogenesis inducers.

VEGF-A

VEGF-A is the founding member of a family of closely related cytokines that exert critical functions in vasculogenesis and in both pathologic and physiologic angiogenesis and lymphangiogenesis [18]. The VEGF-A gene is located on the short arm of chromosome 6 and is differentially spliced to yield several different isoforms, the three most prominent of which encode polypeptides of

189, 165 and 121 amino acids in human cells. The protein has a hydrophobic leader sequence, typical of secreted proteins. It was discovered in the late 1970s as a tumor-secreted protein that potently increased microvascular permeability to plasma proteins. VEGF-A is a potent mitogen for micro- and macrovascular endothelial cells derived from arteries, veins and lymphatics but not for other cell types [18]. Thus, in addition to rendering microvessels hyperpermeable, VEGF-A stimulates endothelial cells to migrate and divide.

We can summarize its unique properties [19]:

(1) It is essential for normal developmental vasculogenesis and angiogenesis, as both null (VEGF-A−/−) and heterozygote (VEGF-A±) animals are embryonic lethals.
(2) It increases vascular permeability to plasma and plasma proteins, a characteristic property of the tumor microvasculature and a critical early step in tumor stroma generation.
(3) It is selective for vascular endothelium because its major tyrosine kinase receptors are selectively (though not exclusively) expressed on vascular endothelium.
(4) It is overexpressed in a variety of human cancer cells. Enhanced VEGF-A expression has been associated with several human vascular tumors including brain, colon, gastrointestinal tract, ovary, breast and others.
(5) It has a potential for evaluating prognosis in individual patients and as a therapeutic target.

Recently, it was found, quite unexpectedly, that DA and other related catecholamine neurotransmitters that interact with the D2R selectively inhibit VEGF-A-induced angiogenesis and inhibit the growth of tumors that express VEGF-A. At nontoxic doses, DA inhibited all VEGF-A activities tested including stimulation of microvascular permeability and endothelial cell proliferation and migration [14, 20]. Besides, in two outbred lines of Wistar rats, which present high and low dopaminergic reactivity, respectively, VEGF expression was reduced in the first group, which was more resistant to tumor implantation, and developed significantly fewer lung metastases [20]. These data point to a relation of VEGF-A and DA receptors.

Interesting to our experimental model was also the fact that the VEGF-A gene promoter has AP-1 and AP-2 sites. This means that phorbol esters and forskolin which activate adenylate cyclase and increase cAMP induce VEGF mRNA expression [19]. In the pituitary of the D2R knockout mice, the lack of action of DA on its receptor prevents physiological adenylate cyclase inhibition, probably leading to increased cAMP levels.

Therefore, because VEGF is thought to be the most important angiogenic cytokine in cancer and other types of pathological angiogenesis and because it has been related to the D2R in endothelial cells [14, 15] we investigated VEGF

expression, localization, and function in pre-adenomatous pituitary tumors of D2R knockout female mice.

VEGF-A in Pituitaries from D2R Knockout Female Mice

We found that VEGF expression was increased in pituitaries from D2R knockout female mice when compared to age-matched, wild-type female mice [21]. VEGF production had been demonstrated to be stimulated by estrogen in rat pituitaries [16] and the somatolactotrope cell line GH3 [22]. Nevertheless, estrogen levels are not increased in D2R knockout female mice, indicating that increased pituitary VEGF expression is mainly dependent on the lack of dopaminergic control. In experiments with wild-type female mice we found that prolonged treatment with the D2R antagonist, haloperidol, enhanced pituitary VEGF protein content and prolactin release [21], and that there was a significant correlation between pituitary VEGF levels and serum prolactin after haloperidol treatment (fig. 4). This further supports that DA acting at the D2R inhibits pituitary VEGF expression. VEGF was also increased in cultured pituitary cells from knockout mice, and in the conditioned media from these cells.

Pituitary VEGF-A Localization

In the normal human pituitary, VEGF-A has been localized mainly in ACTH, GH and follicle stellate cells, with lower levels detected in other cell types [23]. In rats, VEGF-A has been described in follicle stellate cells [24], in a part of the total TSH cells [25], as well as in the lactosomatotrope GH3 pituitary tumor cell line [22]. Interestingly, we found that the main source of VEGF-A in the hyperplastic pituitary were follicle stellate cells and not lactotropes [21].

Follicle stellate cells represent 5–10% of pituitary cells and are an important component of paracrine communication within the pituitary. The expression of gap-junction channels in follicle stellate cells supports the hypothesis that they operate as a network of functionally interconnected cells [26]. They were originally described as non hormone secreting cells, and they are believed to function as (1) support; (2) fagocytes; (3) trophic cells; (4) stem cells; (5) transport cells, and (6) paracrine modulators. They are detected by their content of the glial protein S100, they form follicles, are star shaped and have long processes in between the secretory cells of the pituitary. They also contain FGF-2, follistatin and interleukin-6.

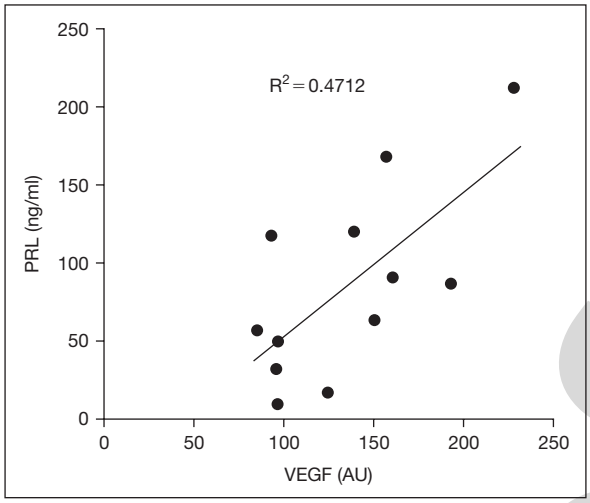

Fig. 4. Correlation between serum prolactin levels (measured by RIA), and pituitary VEGF-A content (Western blot, normalized to actin content, AU = arbitrary units), after i.p. administration of haloperidol decanote (5 mg/kg s.c. for 3 weeks, one injection per week, or 1.2 mg/kg i.p. for 7 days, one injection per day).

Because D2R receptors have been described in lactotropes and not in follicle stellate cells, it may be inferred that a paracrine-derived factor from lactotropes is acting on follicle stellate cells to increase VEGF-A expression. To this regard, it has been described that agents that increase cAMP levels increase VEGF-A in a follicle stellate cell line [27]. As mentioned, in D2R knockout lactotropes, DA-mediated inhibition of adenylate cyclase is chronically lacking, so we presume that increased production of cAMP in this cell type may modulate VEGF-A synthesis in neighboring follicle stellate cells.

VEGF-A and Peliosis

Another interesting association found was that of VEGF-A and peliosis (extravasated erythrocytes not contained in capillaries). As mentioned, in hyperplastic and in adenomatous pituitaries of female D2R knockout mice we described the occurrence of peliosis. Peliosis has been found in different tumors that secrete VEGF-A. This may be linked to the permeabilizing function of this growth factor, and to the increased fenestration induced in blood vessels stimulated by VEGF-A overexpression. Peliosis occurrence has been related to high

VEGF-A expression in hepatocarcinogenesis, spleen damage and in a lethal hepatic syndrome in mice [28–30].

VEGF-A Action at the Pituitary Level

We also determined whether VEGF-A had any action on pituitary cell proliferation or prolactin release. VEGF-A mediates its mitogenic and vasopermeabilizing effects principally through two tyrosine kinase receptors, VEGF-R1 (or Flt 1) and VEGF-R2 (KDR, or Flk-1). Both VEGF-R1 and VEGF-R2 have seven immunoglobulin (Ig)-like domains in the extracellular domain. VEGF receptor-2 has the highest binding affinity for VEGF-A [19].

Expression of these two VEGF receptors exclusively on endothelial cells indicates that this factor should have no direct influence on endocrine cells. VEGF-A might act on the intrapituitary endothelium, maintaining vascular integrity, stimulating vascular permeability and endothelial cell proliferation. Nevertheless, there is one report of VEGF-R2 expression in pituitary endocrine cells [31].

We found that VEGF-A did not induce pituitary cellular proliferation; this result is consistent with several reports that claim that VEGF-A is a potent mitogen for vascular endothelial cells but that it is devoid of consistent and appreciable mitogenic activity for other cell types [32]. In fact, the denomination of VEGF was proposed to emphasize such narrow target cell specificity.

On the other hand, a prolactin-releasing effect could be evidenced [21]. The prolactin-releasing effect of VEGF-A could be related to VEGF-R described in the pituitary cells. This effect was evidenced only under an estrogenic environment. To this respect, it has been conclusively described that estradiol modifies lactotrope sensitivity to physiological stimulators and inhibitors of prolactin secretion.

VEGF-A and Angiogenesis

Therefore, increased pituitary VEGF-A expression may not be important for cellular proliferation of endocrine cells per se, even though it may enhance the prolactin secretory capacity of the gland. On the other hand, increased VEGF-A may act in adjacent endothelial cells and participate in the angiogenic process that increases the availability of different growth factors and mitogens.

To support this idea, we found that conditioned medium from the hyperplastic pituitaries (D2R−/−) was able to induce proliferative changes in human umbilical vein endothelial cells (HUVECs) [21]. The proliferating effect was in part evoked by secreted VEGF-A, as shown by immunoneutralization experi-

ments. This probably indicates that pituitary-secreted VEGF-A accumulates in the target endothelial cells in which it may act in a paracrine manner enhancing vessel proliferation.

Role of Cell Adhesion in Pituitary Tumorigenesis

Cell adhesion is an important determinant of organized growth and the maintenance of architectural integrity within the pituitary. Reduced adhesiveness between cells and, between cells and the extracellular matrix (ECM) is a hallmark of neoplastic growth. The ECM is a three-dimensional network of proteins, glycosaminoglycans and other macromolecules which has a structural support function as well as a role in cell adhesion, migration, proliferation, differentiation, and survival. The ECM conveys signals through membrane receptors called integrins and plays an important role in pituitary physiology and tumorigenesis.

To this regard, the pituitary contains some components within the basement membrane, such as laminin and FGF-2, which decrease in the tumorigenic process in the D2R knockout mouse [33, 34]. These reductions could reflect a more general reduction of the basement membrane by action of matrix metalloproteinases. Matrix metalloproteinase activity is very high in all types of human pituitary adenomas. These metalloproteinases secreted by pituitary cells could release growth factors from the ECM that, in turn, may control pituitary cell proliferation and hormone secretion. Therefore, we must bear in mind that FGF2 and laminin might be released in the process of ECM remodeling and participate in proliferation and angiogenesis in an early step of tumor formation. Remodeling of the existing ECM and diminished cell adhesiveness has been linked to pituitary tumorigenesis in transgenic mice with overexpression of a truncated FGFR4 isoform [35].

D2R Knockout Mouse as an Experimental Model for Dopamine-Resistant Prolactinomas

We have described that pituitary VEGF-A and not FGF2 expression is increased in female mice lacking DA D2Rs. Even though VEGF-A does not promote pituitary cellular proliferation in vitro, as it does in endothelial cells, it may be critical for effective tumor angiogenesis, which is important for pituitary hyperplasia, and, furthermore, it may participate in increased prolactin secretion.

Prolactin adenomas are common benign monoclonal neoplasms accounting for approximately 30% of intracranial tumors. They are usually benign, and can be effectively treated with dopaminergic agents. But 15% of these may

become resistant to classical pharmacological therapy, are invasive and aggressive, and require extirpation. An alternative target, such as VEGF-A, would be desired in these circumstances.

It has been shown that tumor characteristics and environment promote VEGF-A expression. For example, oncogene expression (*kRAS*, *HRAS* and *HER2*), EGF, TGF-β, or keratinocyte growth factor and hypoxia have been related to VEGF-A regulation. The description of dopaminergic control of VEGF-A expression in the pituitary may be important in the clinical action of dopaminergic agents. Furthermore, we believe that VEGF-A and its receptor may become important therapeutic tools in DA-resistant prolactinomas.

To this regard, in recent years, antiangiogenesis has been publicized as a novel alternative or supplement to conventional cancer therapy, and a variety of regimens that prevent tumor angiogenesis and/or that attack tumor blood vessels have met with remarkable success in treating mouse cancers.

Overexpression of VEGF-A by tumor cells can be targeted by:

- antibodies against VEGF (Bevacizumab);
- antibodies against VEGF receptors;
- soluble VEGF receptors (VEGF-TRAP) that bind circulating VEGF;
- catalytic RNA molecules (ribozymes), which cleave VEGF receptor mRNA;
- orally available molecules that selectively block or prevent activation of VEGF-A's receptor tyrosine kinases.

Despite the spectacular successes reported in the treatment of mouse tumors, the first clinical trials were discouragingly negative. This could be related to the fact that most of the patients treated in the beginning had advanced disease and had already failed conventional treatments. Also, antiangiogenesis therapy differs fundamentally from chemotherapy, and optimal implementation was needed.

Several agents targeting the VEGF ligand are now being developed in different clinical trials around the world to treat colon, rectal, breast, lung and other cancers [36]. Bevacizumab (Avastin™), an anti-VEGF monoclonal antibody that inhibits formation of neovasculature and tumor growth in many human cancer cell lines, has a proven survival benefit in metastatic colon rectal cancer and has now been approved by the FDA in combination with intravenous 5-FU-based chemotherapy as a treatment for patients with first-line metastatic cancer of the colon or rectum [37].

Angiogenesis and Pituitary Hyperplasia

It is important to consider that normal pituitaries are usually highly vascularized; therefore, the changes that occur during tumor development may be

somewhat different from some other tissues which require active angiogenesis. To this regard, slow angiogenesis in prolactinomas and even lower vascular densities have been described in human adenomas compared to normal pituitary tissue [38, 39]. Only carcinomas exhibit a clear-cut increase in microvascular density, and there is no information about the angiogenic process in DA-resistant prolactinomas.

Conclusions

In conclusion, we describe that mice lacking DA D2Rs present an enhanced pituitary VEGF-A expression in correlation with pituitary hyperplasia and peliosis of the gland. FGF2, on the other hand, is decreased and such a decrease may be related to the low angiogenesis in prolactinomas and may have a role in the slow pace of pituitary tumor growth, the rarity of metastases [38], and the benign nature of the tumors.

Lack of the D2R likely encourages or permits the development of tumors by increasing the population of proliferating cells that are susceptible to oncogenic factors, or mutation. Increased VEGF-A expression may participate in enhancing permeability and maintaining angiogenesis. This would be in accordance with the multistep theory of carcinogenesis, which reconciles the two proposed theories of pituitary tumorigenesis (hormonal or clonal expansion). It is likely that the majority of pituitary adenomas develop from transformed cells that are, to some extent, dependent on hormonal and/or growth factor stimulation for tumor progression. Proliferating cells would be at increased risk of genetic alteration during mitosis and manifestation of genetic alterations would be precipitated by the growth stimulus.

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