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COMPARATIVE LYMPHATIC, OCULAR, AND METABOLIC PHENOTYPES OF FOXC2 HAPLOINSUFFICIENT AND AP2-FOXC2 TRANSGENIC MICE

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ABSTRACT

FOXC2 *mutations cause the lymphatic/ocular disorder Lymphedema-Distichiasis (LD), and* Foxc2 *haploinsufficient mice mimic this disorder. To determine if* FOXC2 *overexpression might also cause lymphatic and/or ocular abnormalities, we performed dynamic lymphatic imaging (Evans blue dye), ocular tissue examination, and metabolic profiles in mice: transgenic for* FOXC2 *with an adipocyte (aP2) promoter* (aP2-FOXC2 *Tg), heterozygous for targeted disruption of* Foxc2 *(*$Foxc2^{+/-}$*), or compound heterozygous and transgenic (*$Foxc2^{+/-}$*, Tg) compared to wild-type controls (*WT*).* $Foxc2^{+/-}$*;* aP2-FOXC2 *Tg; and* $Foxc2^{+/-}$*, Tg, exhibited LD's distinctive hyperplastic lymphatic phenotype characterized by increased number of lymphatic channels and lymph nodes as well as retrograde lymph reflux.* $Foxc2^{+/-}$*, and* $Foxc2^{+/-}$*, Tg but not* aP2-FOXC2 Tg *or* WT *showed an abnormal ocular phenotype. Previously described alterations in brown/white fat distribution and lean phenotype in* aP2-FOXC2 *transgenics were confirmed.* AP2-FOXC2 *Tg immunohistochemistry disclosed aberrant FOXC2 expression in ectopic sites, especially embryonic heart. Lymphatic system links with fat metabolism are discussed.*

Keywords: FOXC2, Foxc2, lymphatic hyperplasia, haploinsufficient, transgenic, Lymphedema-Distichiasis, ocular, adipocyte

Forkhead genes (FOX) represent a family which encode transcription factors with multiple roles (1). Thirty-nine FOX genes have been identified in *Homo*, including a number involved in cardiac development and vasculogenesis. We have been interested in the role of *Foxc2* in lymphangiogenesis, and through positional cloning strategies identified *FOXC2* haploinsufficiency as causative of Lymphedema-Distichiasis [LD (2)]. LD is a dominantly inherited disorder with lower limb lymphedema of variable age onset, but typically at puberty, a second row of eyelashes arising from the Meibomian glands, and other birth defects among which cardiac and cleft palate are most prominent (3-6). Most mutations in *FOXC2* have been nonsense mutations, with only four missense mutations in 54 LD families, and three of them mutate conserved amino acids in the forkhead domain leading to predicted loss of function of these alleles (3-7). This is in marked contrast to the findings with *FOXC1* mutations and Axenfeld-Rieger anomaly (8).

Foxc2, a master gene involved in vascular (both blood and lymph) and eye

development, has different roles in the embryo and adult. In the embryo it is primarily expressed in the early mesoderm, including somites, and mesodermal derived structures including skeleton, eye, and vessels (9-12). In the post-natal period, high level expression of Foxc2 is found mostly in adipocytes (13) but also in lymphatic collectors (11,12). Haploinsufficiency of *FOXC2* causes Lymphedema-Distichiasis in man (6), and similar hyperplastic refluxing lymphatic vessels and eye abnormalities (including a double row of eyelashes or distichiasis) are found in *Foxc2* haploinsufficient mice (14). *Foxc2* knock-out mice die *in utero* or at birth of cardiovascular and lymphatic abnormalities, and therefore post-embryonic mice are not available for study (9,10).

The lymphatic circulation is crucial in fat absorption and lipid transport, and lymph stasis is associated with fat deposition in tissues. Moreover, genes involved in lymphatic development have now been implicated in metabolic disturbances and obesity. Overexpression in mice of human FOXC2 in adipocytes (aP2-*FOXC2* Tg) has been shown to produce a lean phenotype with increased brown, and decreased white fat (13). In addition, genetic variation in *FOXC2* may (15,16) or may not (17-20) be related to insulin resistance. Obesity and metabolic disturbances have also been linked with polymorphisms in *FOXC2* (21,22). Finally, mice with haploinsufficiency of the lymphatic developmental gene, *Prox1*, exhibit prominent obesity and metabolic abnormalities (23). For the aforementioned reasons, the metabolic phenotypes of mice with lymphatic gene imbalances are of particular interest.

MATERIALS AND METHODS

Mice

Foxc2 haploinsufficient mice (*Foxc2*$^{+/-}$) on the C57BL/6J background have been previously described (9,14). The aP2-*FOXC2* Tg mice were provided by S. Enerback, and their metabolic phenotype has been characterized, but without lymphatic or ocular phenotyping (13).

Foxc2$^{+/-}$ and aP2-*FOXC2* Tg mice were bred at the University of Arizona to obtain Foxc2 transgenic-haploinsufficient compound mice (*Foxc2*$^{+/-}$, Tg). Littermate and non-littermate wild-type (WT) C57BL/6J mice served as controls. Mice of both sexes and ages ranging from 5 to 53 weeks were included. They were maintained on a 6% fat diet with water *ad libetum* in a room with a 14 hr light/10 hr dark cycle. All mouse husbandry and experimental studies were approved by the University of Arizona's Institutional Animal Care and Use Committee.

Lymphatic Studies

Mice were weighed and examined for evidence of edema or serous effusions as well as other gross phenotypic abnormalities. Mice were anesthetized with an IM injection of 20:1:79 (ketamine:xylazine:sterile saline) at ~0.1ml/10g body weight and examined clinically. Direct dynamic lymphatic visualization was performed using Evans blue dye (EBD) (~50 µl of 1% w/v), which binds to large tissue proteins that are exclusively absorbed by the initial lymphatic vessels. EBD was injected intradermally into the left ear, the dorsum of all four paws and the snout serially to sequentially highlight the peripheral lymphatic system and the central collection system. Under a dissecting microscope (Weck, Evergreen, CO, USA), five different regions were examined: the iliac, popliteal, sacral, axillary, and jugular. In addition, the thoracic duct was visualized traveling alongside the azygous vein until its final entry into the left subclavian vein. Since there was occasional overlap between WT, *Foxc2*$^{+/-}$, and aP2-*FOXC2* Tg lymphatic phenotypes, a Lymphatic Vessel/Node Score was devised to quantify the severity of these phenotypic findings. Node scores were based on the number of nodes that contained EBD, extra nodes that did not contain EBD and extra

lobes within the node sac. Vessel scores were based on extra numbers of afferent or efferent lymphatic vessels and vessels that branched or split. These two scores, summed together, constituted each animal's Lymphatic Vessel/ Node Score. The genotypes were blinded at the time of the anatomical descriptions that provided the basis of the scoring.

Statistical Analysis

The Lymphatic Vessel/Node Scores were combined for each group (WT, *Foxc2*$^{+/-}$, aP2-*FOXC2* Tg, and *Foxc2*$^{+/-}$, Tg) to yield means and standard deviations. Group scores were compared to WT using unpaired T-tests for significance. Incidence of limb edema in each group was compared to WT using X^2 to test for significance, and both aP2-*FOXC2* Tg, and *Foxc2*$^{+/-}$, Tg lymph reflux values were compared to WT using the Fisher Exact test for significance.

Histology and Immunohistochemistry

For histologic studies, samples were fixed in 4% paraformaldehyde, paraffin embedded, and stained with hematoxylin and eosin by standard protocols.Visualization of human FOXC2 and endogenous mouse Foxc2 expression was performed using goat anti-human FOXC2 reacting with both mouse and human FOXC2/Foxc2 (Abcam, Cambridge, MA, USA) as previously described (11). We used Biotinylated donkey anti-goat as secondary (Jackson Immuno Research Lab, Inc., West Grove, PA, USA), followed by Streptavidin-Horseradish Peroxidase and TSATM Flourescence Systems (Perkin Elmer, Boston, MA, USA).

Ocular Evaluation

Eyes were examined under the dissecting microscope for distichiasis and other ocular abnormalities. Selected eyes were subsequently removed and processed as above for histological examination.

Metabolic Profile

Routine clinical chemistries were performed by standard techniques in the University Animal Care Diagnostic Laboratory of the Arizona Health Sciences Center. Commercial kits (Waco NEFAC© for non-esterified fatty acids in serum and ThermoTrace Infinity Triglycerides© for triglycerides) were used to determine free fatty acids and triglycerides. Leptin and insulin (with simultaneous glucose) were determined at the University of Cincinnati Mouse Metabolic Phenotyping Center. Weights for growth curves were recorded weekly.

RESULTS

Lymphatic Phenotype

Whereas three *Foxc2*$^{+/-}$ mice exhibited limb edema (*Table 1*), aP2-*FOXC2* transgenic and *Foxc2*$^{+/-}$, Tg mice displayed no effusions or peripheral edema. Lymphatic phenotypic features are illustrated in *Figs. 1* and *2*. EBD visual lymphography documented, to a varying degree in all three groups of mice, an increase in the number and caliber of the peripheral and central deep lymphatic collectors and trunks (*Fig. 1*). The number and size of lymph nodes were also generally increased throughout the body or in one or more of the regions examined, resulting in a heightened Lymphatic Vessel/Node Score when compared to WT controls (*Fig. 3).* Occasionally, a light blue halo surrounded isolated segments of the lymphatic trunks and/or nodes, suggesting localized areas of heightened permeability to the protein-bound dye, a phenomenon not seen in wild-type littermates (*Fig. 1B*). The incidence of retrograde lymph reflux was increased in the aP2-*FOXC2* transgenic mice: EBD-stained lymph was seen refluxing retrograde from the cisterna chyli into dilated lymphatic channels within the hepatic hilum, mesentery (intrinsic lymphatic contractions intact), mesenteric

TABLE 1
Incidence of Limb Edema and Retrograde Lymph Reflux in *Foxc2* Haploinsufficient (+/-), aP2-*Foxc2* Transgenics (+/+, Tg and +/-, +Tg) and Wild-type (+/+) Controls

Foxc2 Genotype	Incidence of Limb Edema	Incidence of Lymph Reflux
+/+ (n=41)	0/41	3/41
+/- (n=60)	3/60	26/60 *
+/+, +Tg (n=19)	0/19	9/19 *
+/-, +Tg (n=13)	0/13	3/13

*p ≤ 0.001 against +/+

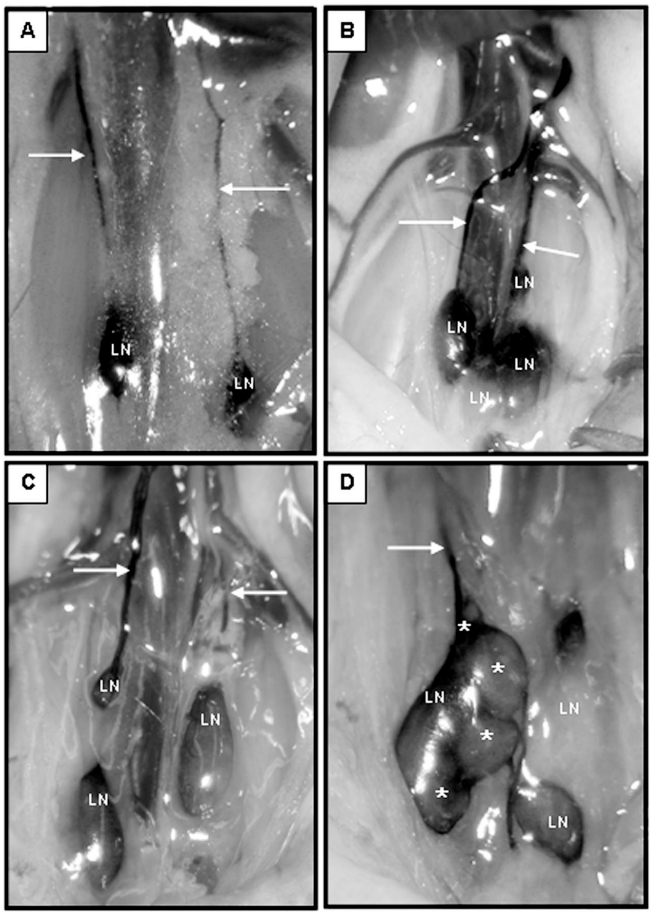

Fig. 1. Lymphatic phenotypes (after EBD injection) in retroperitoneum displaying lymphangiodysplasia/hyperplasia in Foxc2$^{+/-}$ *(B),* Foxc2$^{+/+}$*, Tg (C), and* Foxc2$^{+/-}$*, Tg (D) compared with wild-type control* Foxc2$^{+/+}$ *mice (A). LN=lymph node, arrows designate lymphatic vessels. *= designates separate lobes within lymph node.*

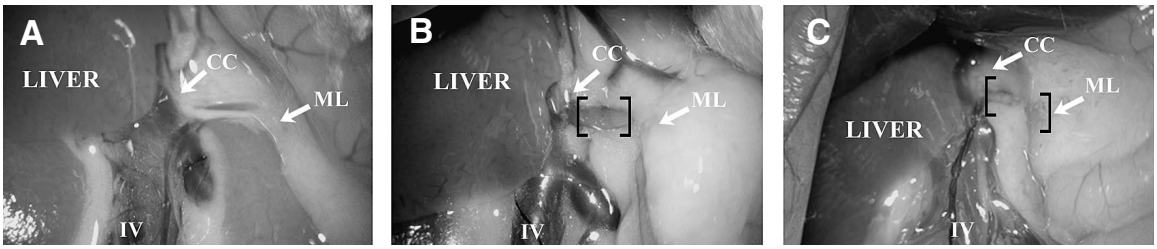

Fig. 2. Visceral lymph retrograde reflux of EBD (within brackets) after lower limb injection in Foxc2$^{+/-}$ *(B),* Foxc2$^{+/+}$*, Tg (C) also in* Foxc2$^{+/-}$*, Tg (not depicted) but not in* Foxc2$^{+/+}$ *mice (A). CC= cisterna chyli, IV= inferior vena cava, ML= mesenteric lymph node.*

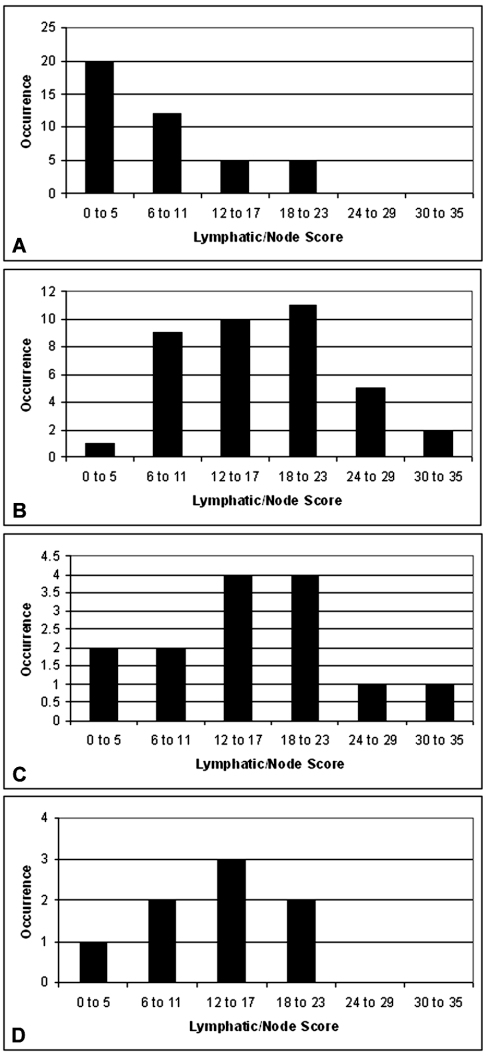

Fig. 3. Distribution of Lymphatic Vessel/Node Score. A) Foxc2$^{+/+}$*; B)* Foxc2$^{+/-}$*; C)* Foxc2$^{+/+}$*, Tg, D)* Foxc2$^{+/-}$*, Tg mice. Occurrence=number of mice*

lymph nodes and the intestinal wall through visible, yet apparently incompetent, interlymphangion valves in many of these mice *(Fig. 2, Table 1)*. There was a significant difference in the incidence of reflux between the WT and both the *Foxc2*$^{+/-}$ and aP2-*FOXC2* Tg mice (both $p<0.001$). However, the incidence of reflux in the small group of *Foxc2*$^{+/-}$, Tg mice studied did not reach significance ($p=0.096$). The thoracic duct in aP2-*FOXC2* Tg and *Foxc2*$^{+/-}$, Tg mice was generally normal in location and caliber as well as chyle-containing and EBD-stained. Although somewhat friable on dissection, the duct was patent throughout its length without obstruction, including proximal to its entry into the left subclavian vein. The findings were similar to those we previously noted in *Foxc2*$^{+/-}$ mice (14).

Lymphatic Vessel/Node Scores (LVNS)

Combined scores for the index were obtained for each group. WT mice (n=42) displayed an LVNS of 7.12 ± 6.4 (mean ± S.D.). *Foxc2*$^{+/-}$ (n=38) and aP2-*FOXC2* Tg (n=14) LVNS were substantially higher at 17.1 ± 7.8 ($p<0.001$) and 16.4 ± 7.9 ($p<0.001$), respectively. *Foxc2*$^{+/-}$, Tg (n=8) at 12.8 ± 6.4 was also increased ($p=0.027$) (*Fig. 3*).

Ocular Phenotype

While the *Foxc2*$^{+/-}$ and aP2-*FOXC2* Tg mice displayed similar hyper-dysplastic lymphatic abnormalities, ocular abnorma-

TABLE 2
Ocular Phenotypes *Foxc2* Haploinsufficient (+/-) aP2-*Foxc2* Transgenic (+/+, +Tg and +/-, +Tg) and Wildtype Control Mice

Foxc2 Genotype	Distichiasis	Ptosis	Periorbital Edema	Ocular Dysplasia	Cataracts	Corneal Abrasions
+/+ (n=41)	0/41	0/41	0/41	0/41	0/41	0/41
+/- (n=65)	65/65	3/65	8/65	7/65	6/65	4/65
+/+, +Tg (n=19)	0/19	0/19	0/19	0/19	0/19	0/19
+/-, +Tg (n=13)	13/13	1/13	2/13	2/13	1/13	3/13

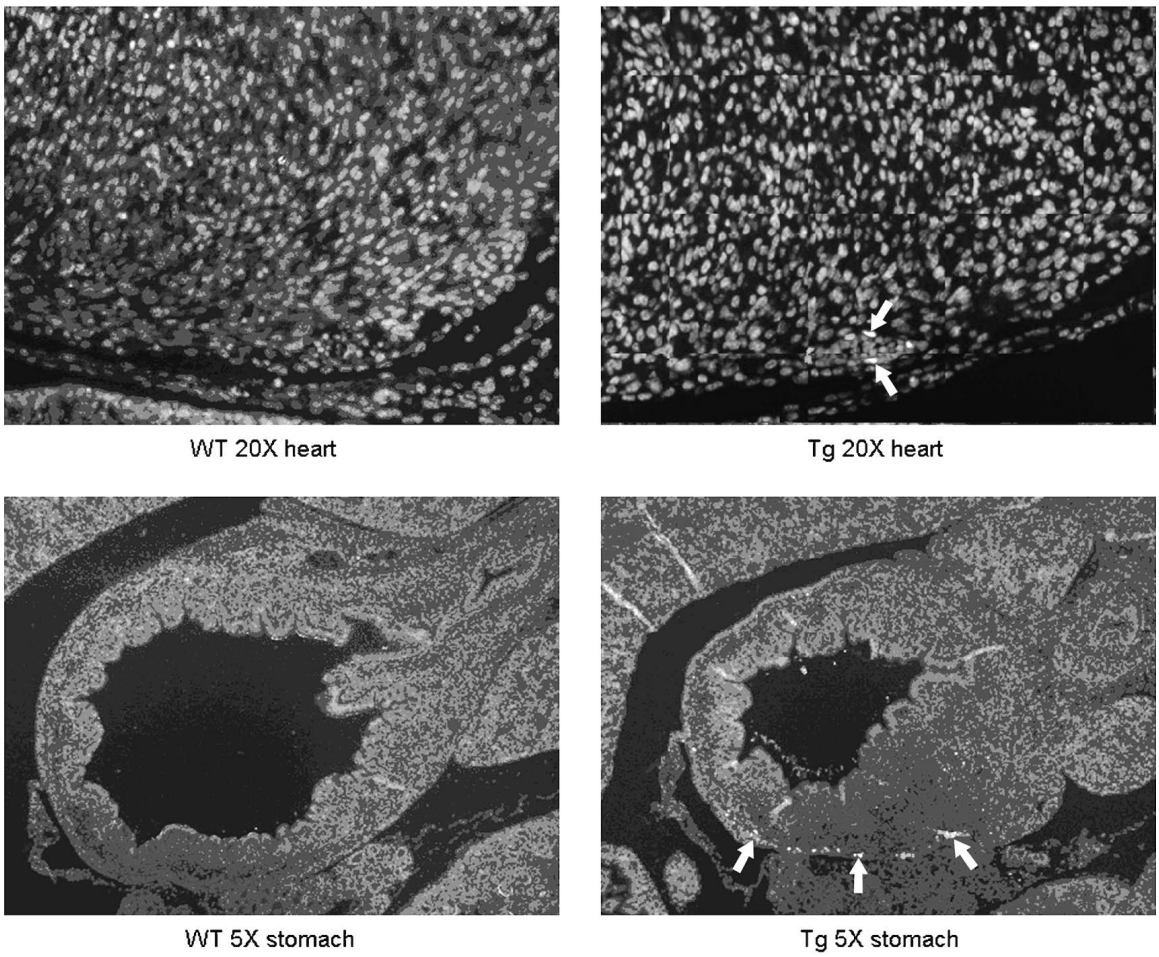

Fig. 4. FOXC2 expression (arrows) detected with a goat antibody specific for FOXC2 shows ectopic expression in embryonic day 16.5 in stomach and heart. Wt=wild-type; aP2=aP2-FOXC2 transgenic

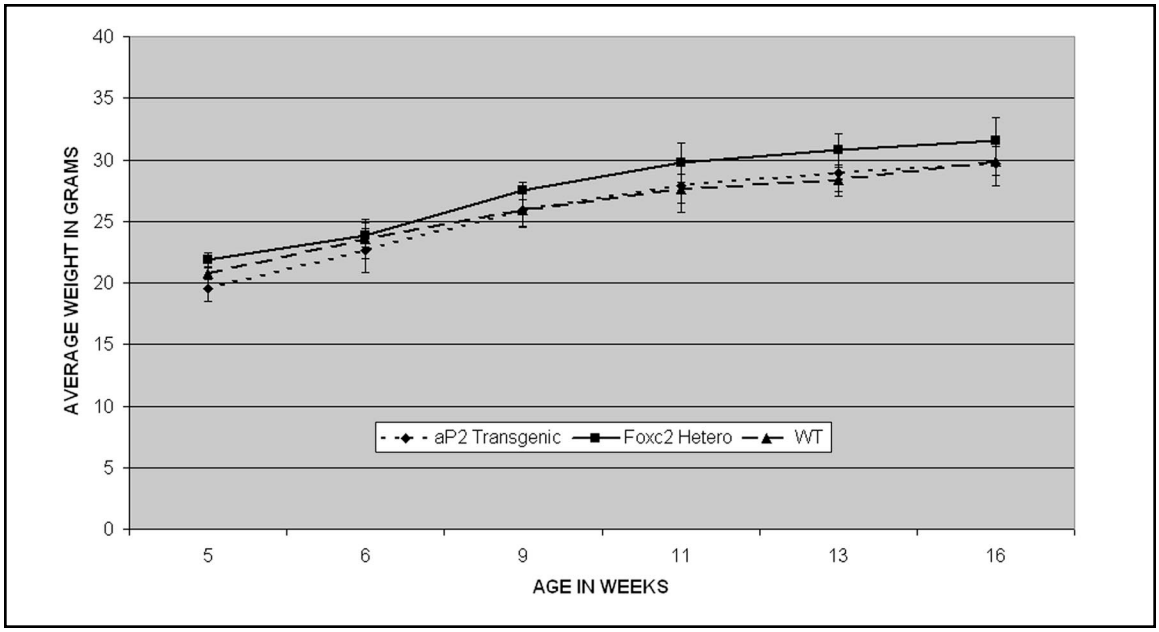

Fig. 5. Similar weight and growth curves of wild-type (WT), Foxc2+/-, and aP2 FOXC2 transgenic male mice.

lities of distichiasis, ptosis, orbital edema, ocular dysplasia, cataracts and corneal abrasions were found only in *Foxc2*$^{+/-}$ and the compound *Foxc2*$^{+/-}$, Tg but not in the aP2-*FOXC2* transgenic mice. Expression of the transgene did not alter the frequency of ocular abnormalities in the compound heterozygotes (*Table 2*).

Histology and Immunohistochemistry

Endogenous mouse Foxc2 has been shown to be normally expressed in adult lymphatic collectors of the mesentery (11,12). In adult animals, antibody studies (with an antibody that did *not* distinguish human FOXC2 from mouse Foxc2) did not detect aberrant expression of the FOXC2 transgene in lymphatic capillaries or submucosal lymphatics of the intestine. At embryonic (E) day 13.5, the expression of FOXC2/Foxc2 and a number of lymphatic markers were normal (data not shown). FOXC2/Foxc2 ectopic expression was found in embryonic day 16.5 stomach and heart (*Fig. 4*).

Metabolic Phenotype

Although aP2-*FOXC2* transgenic mice on a 4% fat diet have been reported to be markedly lean with a decrease from 30% to 10% total lipids in carcasses (13), we did not note visual differences in size on a 6% fat diet. Moreover, aP2-*FOXC2* Tg mice did not show different weights or growth curves from non-transgenic mice, either normal controls or *Foxc2*$^{+/-}$ (*Fig. 5*).

In an effort to search for potential metabolic reasons for the normal growth despite the lean phenotype, we measured metabolic indicators in serum from adult (> 6 weeks) mice (*Table 3*). As noted before (13), cholesterol levels were not altered nor were glucose, alanine aminotransferase, total protein, albumin, globulin, BUN, calcium, phosphorus, creatinine and total bilirubin (data not shown) except for amylase which was slightly reduced in the aP2-*FOXC2* Tg but not the *Foxc2*$^{+/-}$ or the compound *Foxc2*$^{+/-}$, Tg mice. Free fatty acids were mildly increased only in the *Foxc2*$^{+/-}$ mice, but we

TABLE 3
Clinical Chemistry Values in *Foxc2*$^{+/-}$ and/or aP2- FOXC2 Transgenics and Controls

Test	Units	*Foxc2*$^{+/-}$	aP2-*FOXC2* Tg	C57BL/6J
Amylase	U/L	(6)524 ± 54*	(3)447 ± 11†	(6)590 ± 89
Triglycerides	mg/dl	(7)131.4 ± 30.4	(4)131.3 ± 56.4	(5)119.52 ± 28.8
Free fatty acids	mEq/L	(7)1.35 ± 0.23†	(4)1.20 ± 0.40	(5)1.06 ± 0.13
Leptin	pg/ml	(4)135.1 ± 97.8	(3) 110.1 ± 20.3	(4) 96.1 ± 6.5
Insulin	pg/ml	(4)179.8 ± 72	(3)96.3 ± 34.7	(4)126.9 ± 43.5
Glucose	mg/dl	(18)169.6 ± 55.7	(14)174.3 ± 36.9	(12)198.5 ± 25.9
Insulin/glucose	ng/ug	(4) 9.5 ± 3.0	(3) 6.1 ± 2.0	(4) 7.3 ± 2.3

*(n) mean ± std. dev.
†$p < 0.05$ vs. control (C57BL6/J)

did not find the previously reported decrease in triglycerides (13) in the transgenic mice. Leptin and simultaneous insulin/glucose levels also did not differ.

DISCUSSION

It was surprising to find that the lymphatic system abnormalities in aP2-*FOXC2* Tg mice were very similar to those in *Foxc2*$^{+/-}$ mice since aP2 has been extensively studied as an adipocyte-specific, lipid binding protein (24,25). However, although the aP2 promoter is frequently regarded as adipocyte specific, Ross et al (26) found that the 5.4 kb promoter used by Cederberg et al (13) also leads to high lymphoid tissue expression. Specifically, the 5.4 kb 5' flanking sequence characterized as containing a functional adipose-specific enhancer was studied in 3 transgenic lines. Levels of recorder gene expression were 7-52% as high in spleen, and 10-40% as high in thymus, as they were in white adipose tissue (26). The line with the highest white adipose tissue expression (about 10-fold the other 2 lines) showed splenic levels as great as 20%, and thymic levels as much as 40%, of those in white adipose tissue. When we used antibodies against FOXC2/Foxc2 to study *FOXC2* expression in these transgenic mice, we found ectopic expression of FOXC2, especially in embryonic heart. Presumably, the hyper-dysplastic lymphatic phenotype is somehow related to ectopic expression of *FOXC2* in these and/or other tissues, although we could not clearly demonstrate this connection.

Eye abnormalities were not found in the aP2-*FOXC2* Tg mice. Although it has been shown that haploinsufficiency of the transcription factors *Foxc2* and the related *Foxc1* leads to abnormal ocular phenotypes (27,28), we did not find the ocular abnormalities seen in the *Foxc2*$^{+/-}$ mice. This difference may be due to the lack of expression of the aP2-promoter in ocular tissues, or incorrect timing or location of expression of the promoter in ocular and ocular-related tissues.

The compound *Foxc2*$^{+/-}$, Tg mice displayed physical characteristics of both the *Foxc2*$^{+/-}$ and the aP2-*FOXC2* Tg mice. The lymphatic hyper-dysplasia was similar to both the haploinsufficient and transgenic mice. In addition, the ocular phenotype (including distichiasis), and the altered distribution of brown and white fat was uniformly present. It appears that *FOXC2*/*Foxc2* may have a dose dependent effect on the developing lymphatic system.

The altered lymphatic system could contribute to the metabolic phenotype of the aP2-*FOXC2* transgenic mice. Alternatively, altered adipocyte distribution or size around lymphatics could underlie the altered lymphatic phenotype. As mentioned above, FOX transcription factors are involved in many regulatory and developmental pathways. The FOXO group has particularly been implicated in "glucose metabolism, stress response, cell cycle regulation and apoptosis" (29). In mammals there are multiple FOXO family members which are involved in a shift of metabolism from glycolysis to gluconeogenesis by inducing multiple enzymes including glucose-6-phosphalase (30,31) and phosphoenol-pyruvate carboxykinase (32,33). *Drosophila* has only one *FOXO* ortholog where its crucial role in insulin signaling can be readily assessed (34). *Foxo1*$^{-/-}$ mice have impaired vascular development and Foxo1 seems to be essential for the blood vascular endothelial response to VEGF (35). The work of Cederberg et al (13) implicates *Foxc2* in these pathways. The mRNAs for insulin receptors (IR), insulin receptor substrates 1 and 2 (IRS1, IRS2), insulin-responsive glucose transporter-4 (GLUT4) and C/EBPα (CCAAT-box enhancer bindings protein-α, known to promote adipocyte differentiation) were all increased in the aP2-*FOXC2 Tg* mice (13). Foxc2 appears to be upstream of PPARγ but inhibits its promotion of adipogenic genes (36) while it appears to be downstream of insulin and TNFx (37). In addition, human FOXC2 polymorphisms have been associated with obesity, metabolic syndrome, and dyslipidemia (21,22). Although we did not find a decrease in serum triglycerides [our mice averaged 2-4 months of age compared to 4-6 months of age in the Cederberg et al study (13)] and the growth curves were normal [unlikely to be due to the slight difference in fat content of the diet, ~6% in our lab and 4% in theirs (38)], the previously described striking alterations in brown and white fat distribution were confirmed.

Since lymphatics play a major role in the absorption of dietary cholesterol (reviewed in 39), one may also consider whether the lymphatic abnormalities contribute to the altered metabolism noted in the aP2-*FOXC2* transgenics. In rats, 40% of orally administered cholesterol appears in the thoracic duct (40). In mice, variations in intestinal transport of cholesterol have been noted between various inbred strains [measured by plasma and fecal dual isotope ratio methods (41)]. However, the standard laboratory mouse chow has negligible cholesterol, and mice, unlike humans, synthesize almost all their own cholesterol. Nonetheless, altered absorption of fats and transport of chylomicrons may be a factor in the "lean" phenotype of the aP2-*FOXC2* transgenic mice. It has also been suggested that lymphatics have adipogenic activity (42).

Finally, a direct connection between lymphatic maldevelopment and obesity has recently been postulated based on the finding of adult onset obesity and metabolic abnormalities in *Prox1*$^{+/-}$ mice with lymphatic vascular patterning defects (23). *Prox1* is a crucial transcription factor in lymphatic development, and *Prox1* knockout mice die early in embryonic development with hydrops associated with failure of development of the lymphatic system (43).

Thus, it appears that Foxc2 has a dose-dependent effect on the developing lymphatic system which may contribute to or be influenced by an altered metabolic phenotype. In light of the increasing interest in interactions between the lymphatic system and adipose tissue (44) and the implications of this linkage, the consequences of Foxc2 gene imbalance deserve to be further pursued.

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