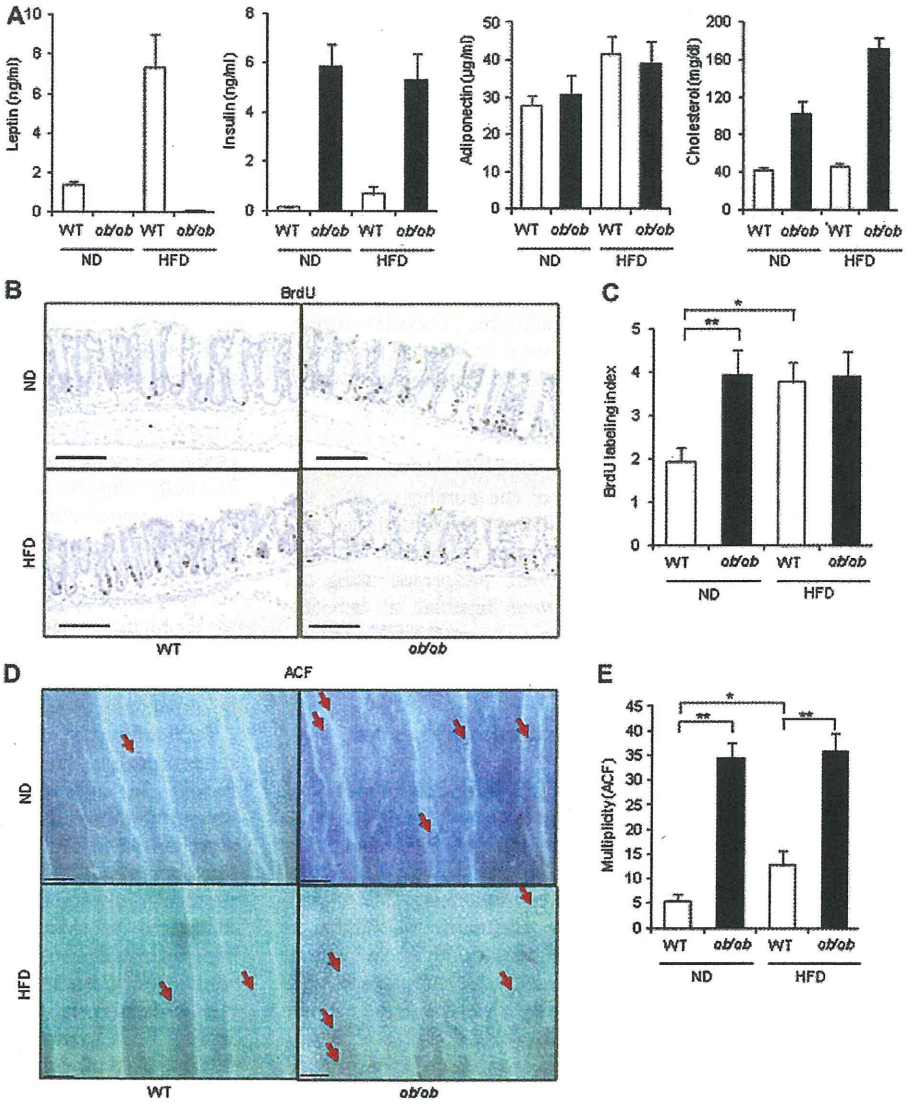

Figure 1 Leptin is not involved in the early-stage of colorectal carcinogenesis in obesity. (A) AOM-treated wild-type (WT) and *ob/ob* mice were bled and the levels of metabolic factors in serum were determined by ELISA. Results are averages ± SEM. (n=8). ND, normal diet; HFD, high-fat diet. (B) BrdU incorporation in normal colonic epithelial crypts of WT and *ob/ob* mice. Scale bars=100 μm. (C) BrdU labelling indices of colonic normal mucosa of AOM-treated WT and *ob/ob* mice. Results are averages ± SEM. (n>10). *p<0.01. **p<0.005. (D) Stereoscopic observations of ACF (arrows) in colonic tissues of WT and *ob/ob* mice. The samples were stained with 0.2% methylene blue. Scale bars=100 μm. (E) ACF multiplicity. Results are averages ±SEM. (n=10). *p<0.05. **p<0.001. ACF, aberrant crypt foci; AOM, azoxymethane; BrdU, bromodeoxyuridine; WT, wild type.

knockdown markedly reduced ObRb expression (figure 4A). We confirmed that the ObRb mRNA level was significantly reduced in the β-catenin siRNA-transfected SW480 cells (figure 4B). Reductions of ObRb protein expression by transfection of β-catenin siRNA was also observed in other colon cancer cell lines (Supplementary figure 9). It has been shown that the Wnt/β-catenin pathway can be stimulated in HEK293 cells by addition of Wnt3a.[30] We tested ObRb expression level following Wnt3a stimulation in HEK293 cells that normally contain trace amounts of nuclear β-catenin. Wnt3a-stimulated HEK293 cells showed a marked increase in ObR expression (figure 4C,D). These results are consistent with the increased expression levels of ObR in tumours as compared with those in normal mucosa (figure 3A–C), and indicate that the Wnt signalling activates ObRb expression in colonic epithelium.

Leptin activates STAT3 signalling to promote colorectal tumour growth

Phosphorylation of Tyr^{1138} in ObRb induces STAT3 activation.[31] Increased amounts of phosphorylated Tyr^{1138}-ObRb and STAT3 (p-STAT3) were observed in tumours as compared with those in normal mucosa (figure 5A). These data suggest that leptin exerts a stimulatory action on colon tumours through the ObRb/STAT3 pathway.

To determine the contribution of leptin to changes in tumour cell proliferation and survival, we analysed colon tumours of WT and *ob/ob* mice for activation of STAT3 and the expression of its target genes. Immunohistochemical analysis revealed tumour cell nuclear localisation of p-STAT3 in colon tumour cells in WT mice, while this signal was almost completely absent from similar tumours in *ob/ob* mice (figure 5C). The frequency of p-STAT3-positive cells was significantly higher in tumours of WT mice fed a HFD than in those of the mice fed a ND (figure 5C, Supplementary figure 10), closely matching the increase in serum leptin levels (figure 1A). Meanwhile, p-STAT3-positive cells were almost undetectable in normal mucosa of WT and *ob/ob* mice (figure 5B). Importantly, the lack of STAT3 activation in colonic mucosa coincided with the lack of colonic leptin signalling, namely, lack of functional leptin (figure 1A) or lack of colonic ObR expression (figure 3A–C). Therefore, our data indicate that leptin is a crucial STAT3 activator in colonic epithelium during tumour growth. Next, we analysed the STAT3-mediated proliferative response in WT and leptin-deficient tumours. To do that, we investigated the expression of

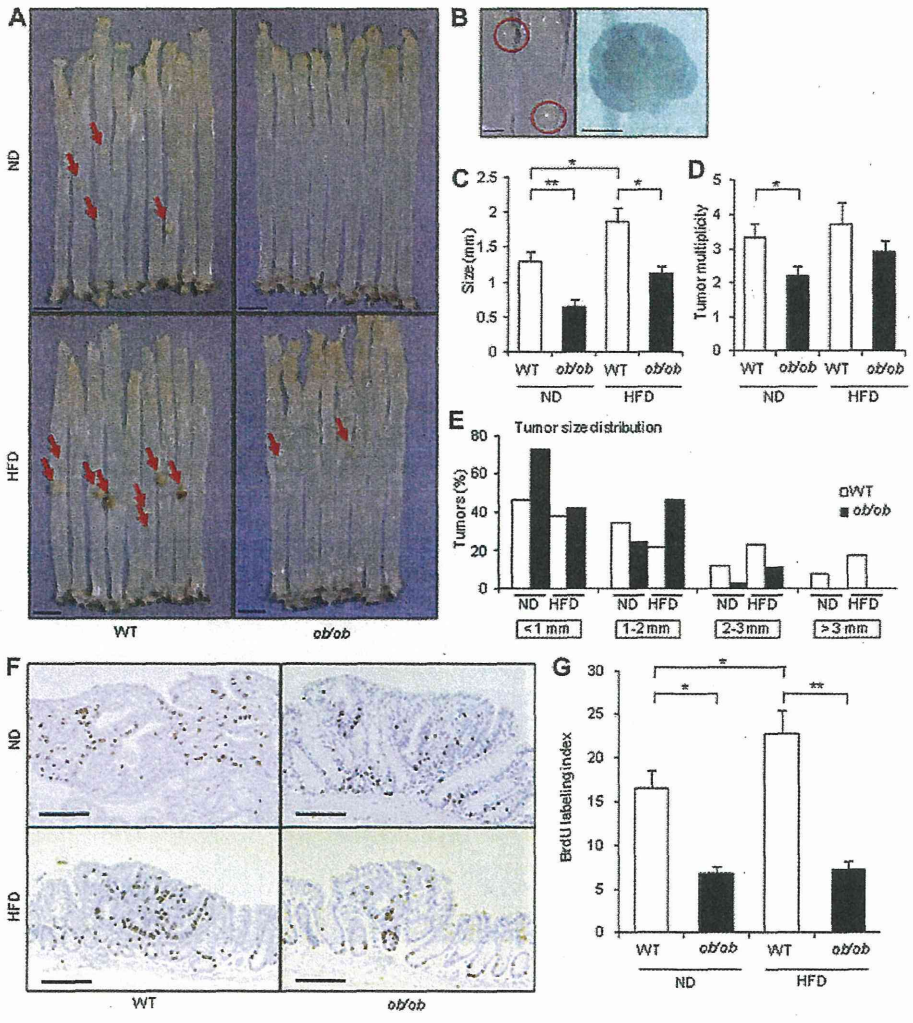

Figure 2 Leptin regulates AOM-induced colon tumour growth. (A) Macroscopic findings of colon tumours. Arrows indicate large tumours. Scale bars=1 cm. ND, normal diet; HFD, high-fat diet. (B) Macroscopic (left panel) and stereomicroscopic (right panel) findings of small colon tumours. Scale bars=1 mm (left panel) and 200 μm (right panel). (C) Tumour size. Results are averages ±SEM. (n=10). *p<0.05. **p<0.005. (D) Tumour multiplicity in WT and *ob/ob* mice fed ND or HFD. Results are averages ±SEM. (n=10). *p<0.05. (E) Histogram showing size distribution of tumours. (F) BrdU incorporation in colon tumour of WT and *ob/ob* mice. Scale bars=100 μm. (G) BrdU labelling indices in colon tumours of the AOM-treated WT and *ob/ob* mice. Results are averages ±SEM. (n>10). *p<0.05. **p<0.005. AOM, azoxymethane; BrdU, bromodeoxyuridine; WT, wild type.

cell-cycle genes and of the cyclin-dependent kinase (Cdk) inhibitor $p21^{cip}$ in tumours. We found that mRNA expressions of cyclin D1, c-Myc, cyclin B1, cyclin E and cdc2 were increased to a greater degree in WT mice than in *ob/ob* mice (figure 5D). This suggested a stimulatory effect of STAT3 on the cell cycle, and this observation was consistent with the downregulation of the Cdk inhibitor $p21^{cip}$ in tumours of WT mice. Furthermore, we observed elevated expression levels of Bcl-X_L and survivin in WT mice as compared with those in *ob/ob* mice (figure 5D). These results suggest that impaired induction of Bcl-X_L and survivin protein expression may account for the increased rate of apoptosis observed in leptin-deficient *ob/ob* mice. Collectively, these results strongly support the notion that the STAT3-associated proliferative and antiapoptotic effects are important for tumour epithelia.

Exogenous leptin compensates for suppressed tumour growth in leptin-deficient mice

We found that continuous treatment with recombinant leptin during the late-stage of CRC (figure 6A) resulted in an increase of tumour sizes (figure 6B–D), whereas tumour multiplicity was not affected (figure 6E). Supplementary table 3 summarises the histological findings of tumours. As expected, treatment with recombinant leptin resulted in elevated serum levels of leptin (figure 6F). Importantly, leptin supplementation enhanced STAT3 phosphorylation in colonic tumours (figure 6G). Thus, leptin signalling can increase tumour size without affecting tumour multiplicity, which has an impact on tumour growth.

DISCUSSION

The existence of a relationship between obesity-related factors and CRC has been speculated upon in recent years, but no definitive conclusions have been reached. The present investigation to elucidate the precise mechanisms involved was necessary because of the major clinical implications. We identified a novel mechanism to explain how leptin deficiency might suppress colon tumour growth, even in the presence of marked increase in the levels of other obesity-related factors. Our finding suggests that leptin is a crucial factor for colon tumour growth among the various obesity-related factors. We demonstrated an increase in the proliferative activity of the normal colonic epithelial cells and ACF formation in the obesity model but, unexpectedly, tumour growth was inhibited dramatically in the leptin-deficient obesity model, indicating the importance of leptin signalling for colon tumour growth. Taken in combination, our data indicate that leptin acts as growth factor for CRC at stages subsequent to cancer initiation.

Previous studies have provided much evidence of an association between metabolic factors and increased risk of colorectal carcinogenesis.[7] Therefore, we hypothesised at first that *ob/ob*

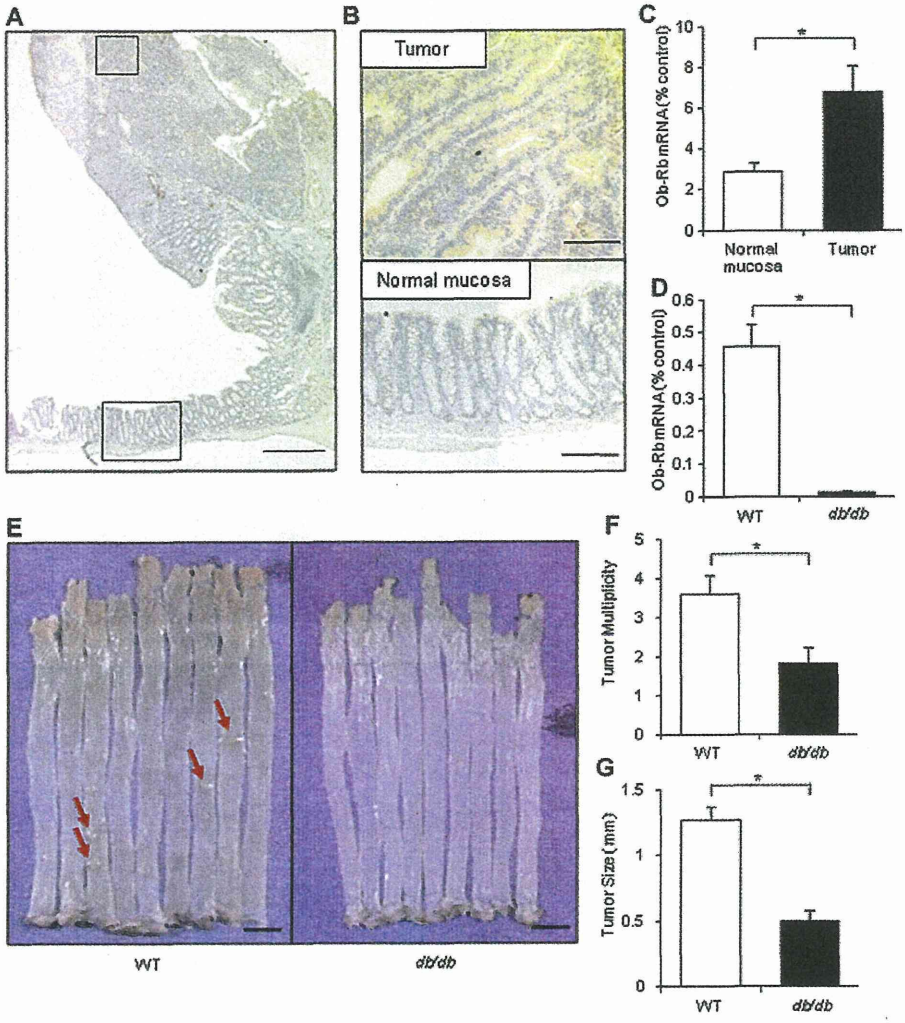

Figure 3 Leptin receptors are required for colorectal tumour growth. (A) and (B) Section of colon from an AOM-treated mouse showing a representative tumour protruding into the colonic lumen was stained with antibodies to ObR. Magnified views of the boxed area confirms the presence of ObR-positive cells in the cytoplasm of tumour cells (upper panel), but scarce expression of ObR in normal epithelial cells (lower panel) of the colonic mucosa. Scale bars=400 μm (left panel), 10 μm (right, upper panel), and 100 μm (right, lower panel). (C) Relative expression level of ObRb mRNA in normal colonic mucosa and tumours of AOM-treated mice, analysed by real-time PCR. Results are averages ±SEM. (n=5). *p<0.005. (D) Expression of ObRb in the isolated colonic mucosa from WT and leptin-receptor-deficient *db/db* mice. Results are averages ±SEM. (n=5). *p<0.005. (E) Macroscopic findings of the colon tumours. Arrows indicate large tumours. Scale bars=1 cm. (F) Tumour multiplicity in WT and *db/db* mice subjected to induction of colon tumours. Results are averages ±SEM. (n=10). *p<0.05. (G) Tumour size in WT and *db/db* mice. Results are averages ±SEM. (n=10). *p<0.0001. AOM, azoxymethane; WT, wild type.

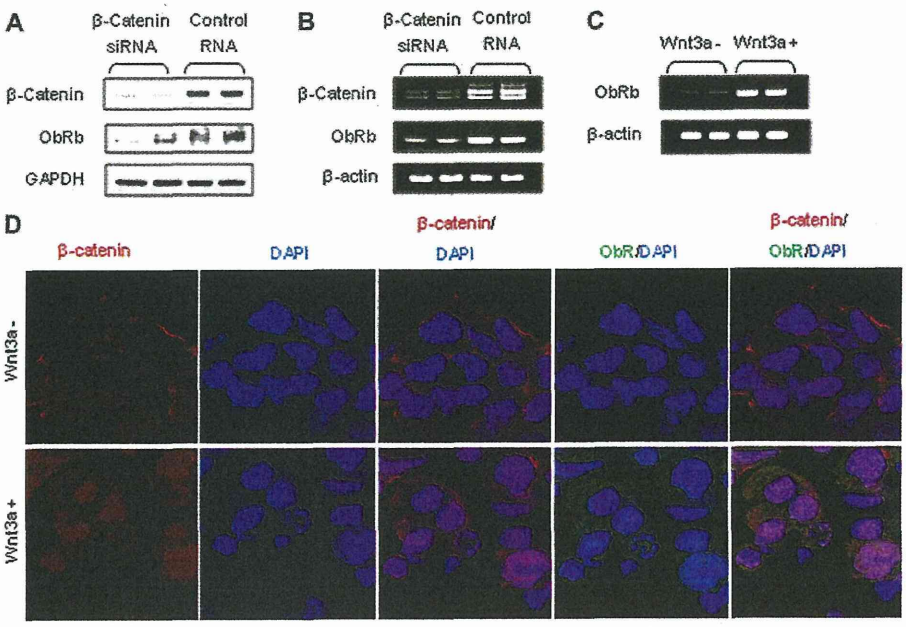

Figure 4 Wnt signalling increases the expression levels of ObRb. Western blot (A) and RT-PCR (B) analyses of ObRb expression in SW480 cells transfected with β-catenin siRNA (50 nM). Samples were prepared 48 h after transfection. ObRb mRNA and protein expression was decreased by β-catenin siRNA. GAPDH and β-actin are shown as the loading controls. (C) RT-PCR analysis of ObRb expression in HEK293 cells with and without Wnt3a stimulation. ObRb mRNA expression levels were increased by Wnt3a stimulation. β-actin is shown as a loading control. (D) Immunofluorescence of β-catenin (red) and ObR (green) in HEK293 cells treated with recombinant Wnt3a. Wnt3a induced translocation of β-catenin from the cytoplasm to the nuclei and induced the expression of ObR. DAPI, 4′-diamidine-2′-phenylindole hydrochloride; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

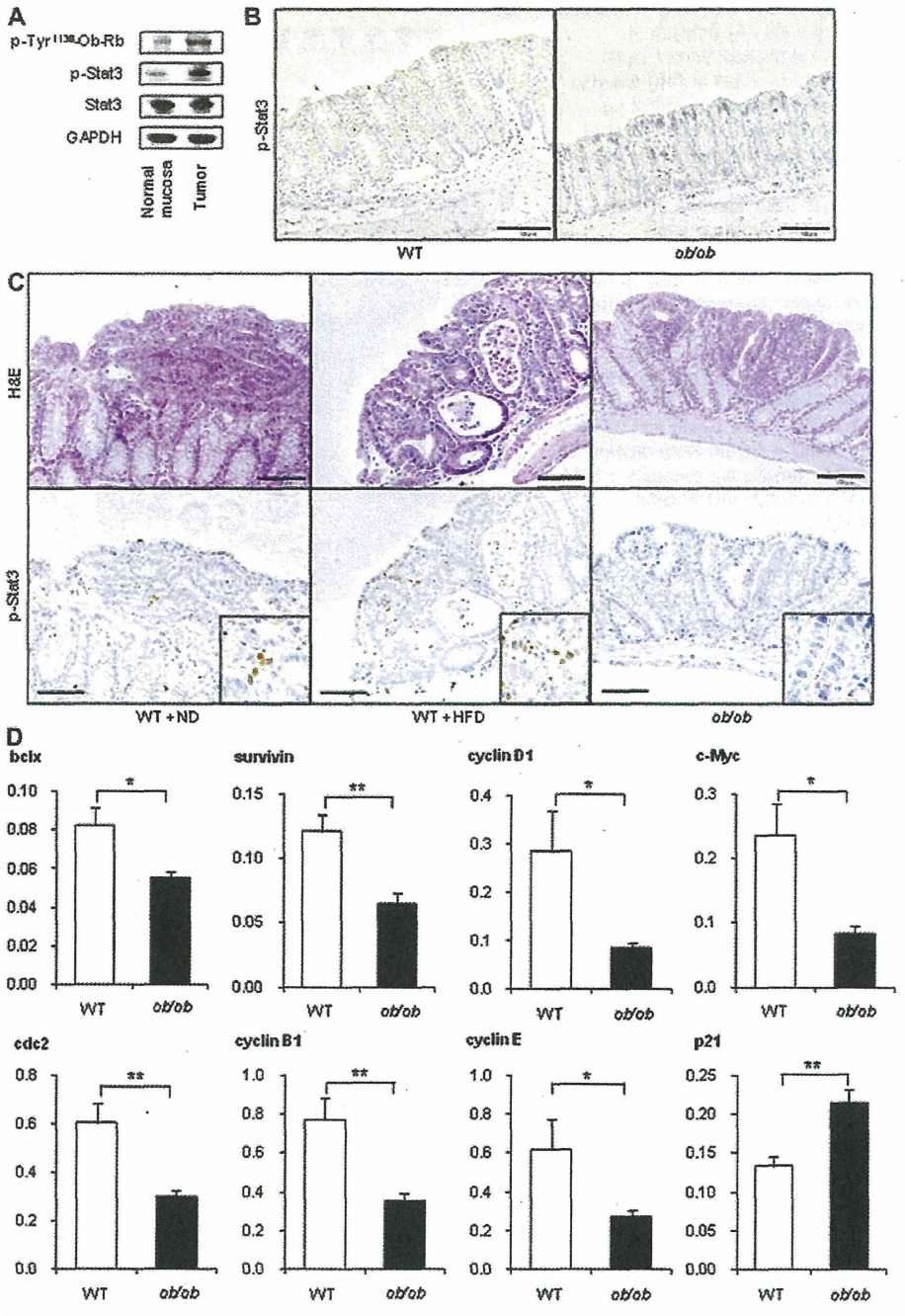

Figure 5 Leptin increases STAT3 phosphorylation in colon tumours. (A) Immunoblot analysis for Tyr^{1138}-phosphorylated ObRb and phosphorylated STAT3 (p-STAT3) in normal colonic mucosa and tumours of AOM-treated mice. (B) Paraffin-embedded sections of tumour-containing colons from WT and *ob/ob* mice were stained with H&E (upper panel) and with anti-p-STAT3 (lower panel). Insets in lower panels demonstrate nuclear localisation of p-STAT3 in WT mice, which was absent from tumours in *ob/ob* mice. Scale bars=100 μm. ND, normal diet; HFD, high-fat diet. (C) Normal colon mucosa from AOM-treated WT and *ob/ob* mice was stained with anti-p-STAT3. Scale bars=100 μm. (D) Expression of cell-cycle and apoptosis regulators in isolated colonic tumours from WT and *ob/ob* mice. Relative mRNA expression levels were determined by real-time PCR. Results are averages ±SEM. (n=6). *p<0.05. **p<0.01. AOM, azoxymethane; WT, wild type.

mice, which have obese metabolic phenotypes with elevated levels of insulin, glucose and lipid, would show increased susceptibility to CRC development as compared to their lean littermates. However, to our surprise, we found that *ob/ob* mice developed far smaller tumours than the corresponding WT mice, despite the animals exhibiting severe obesity. In contrast, administration of a HFD to WT mice resulted in increased tumour sizes, despite the finding that levels of various obesity-related metabolic factors, with the exception of leptin, in these mice were not as high as those in *ob/ob* mice. These results strongly indicate that, in vivo, leptin is important for the regulation of colon tumour growth, irrespective of obesity. Furthermore, these results also explain that CRC does not grow under leptin-deficient conditions, regardless of the serum insulin levels. Leptin-deficient mice exhibited few and small tumours despite a high intake of dietary fat. These findings suggest that leptin is a crucial factor for CRC development, regardless of dietary composition. Adiponectin has also been reported to influence colorectal carcinogenesis. Recently, we have demonstrated that adiponectin deficiency might promote the development of CRC only under HFD conditions, using adiponectin-knockout mice.[21] Furthermore, a human epidemiological study has shown that decreased levels of plasma adiponectin are associated with increased risk of CRC.[32] In a cell model study, adiponectin has been shown to block leptin-induced colon epithelial cell proliferation.[33] However, there was no significant difference in the serum adiponectin level between the WT and *ob/ob* mice in the present study. We speculate that there are

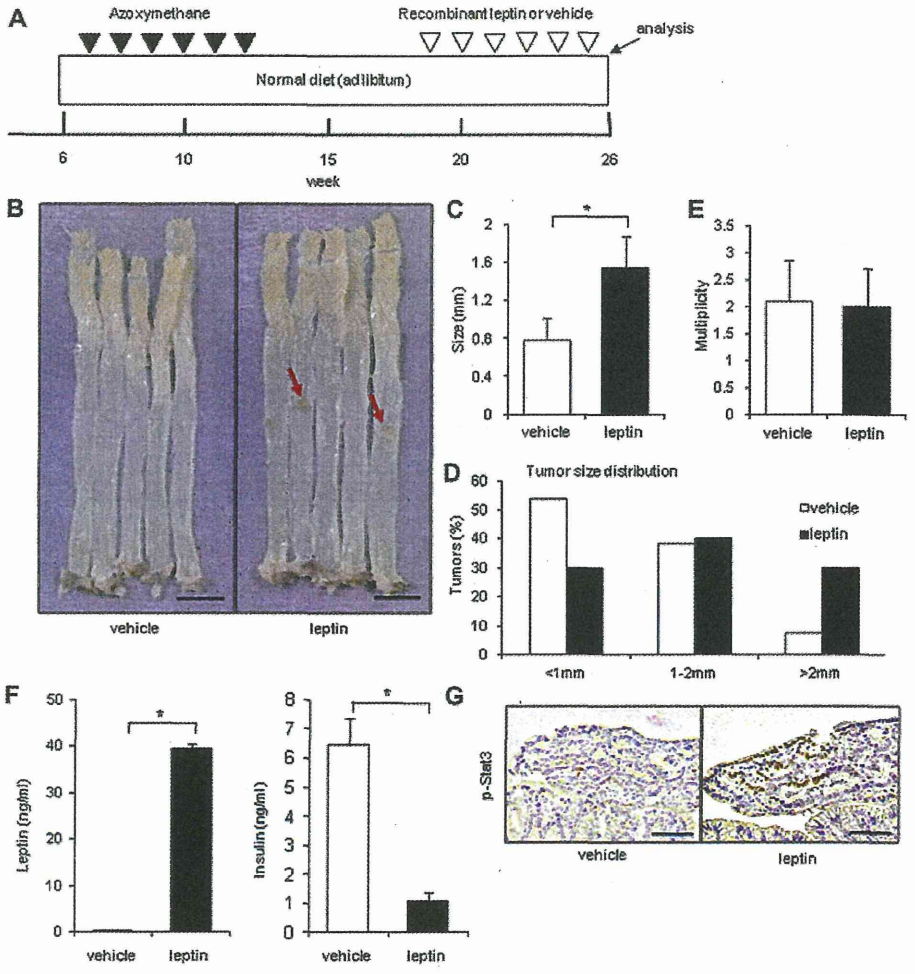

Figure 6 Leptin signalling stimulates tumour growth. (A) Scheme of treatment with recombinant leptin during the late stage of CRC growth. Mice were injected i.p. with 2 μg recombinant leptin, or control saline every day from 15 weeks after initial AOM injection. (B) Macroscopic findings of colon tumours. Arrows indicate large tumours. Scale bars=1 cm. (C) Tumour size. Results are averages ±SEM. (n=6). *p<0.05. (D) Histogram showing size distribution of colon tumours. (E) Tumour multiplicity. Results are averages ±SEM. (n=6). (F) Vehicle- or leptin-treated *ob/ob* mice were bled and the levels of leptin (left panel) and insulin (right panel) in serum were determined by ELISA. Results are averages ±SEM. (n=6). *p<0.001. (G) Paraffin-embedded sections of tumour-containing colons of vehicle- and leptin-treated *ob/ob* mice stained with anti-p-STAT3. Scale bars=100 μm. AOM, azoxymethane; CRC, colorectal cancer.

many factors that influence colon carcinogenesis in an obesity background, and adiponectin may be one of these factors. Further studies in animal CRC models are necessary to address the interaction between adiponectin and leptin.

Using genetic models, we demonstrated that leptin is an important regulator of CRC development. However, leptin signalling did not have a significant effect on promotion of premalignant lesions in the CRC model, because its absence did not alter the number of ACF. These data indicate that leptin does not act as a growth-promoting agent at an early stage of colon carcinogenesis. We observed that the number of ACF was significantly greater in *ob/ob* and *db/db* mice than in WT mice, which suggests that metabolic factors other than leptin act as promoters of early-stage colon carcinogenesis. Furthermore, leptin signalling did not promote normal colonic epithelial cell proliferation either. Why did leptin enhance tumour cell proliferation, but not induce formation of ACF or proliferation of normal colonic mucosa? Here, we noted a difference in ObR between tumours and normal mucosa. A marked increase in ObR expression level was observed in tumours as compared with that in the normal mucosa. Carcinogen-induced tumours frequently show mutation of β-catenin that leads to stabilisation and nuclear translocation of β-catenin, thereby activating the Wnt pathway. On the other hand, mutation and altered cellular localisation of β-catenin are not observed in normal mucosa or ACF.[34] Based on this evidence, we hypothesised that activation of the Wnt pathway not only triggers the formation of colon tumours, but also induces the expression of ObR in colon tumours. To elucidate the roles of Wnt signalling activation on regulation of ObR expression, we examined the effects of β-catenin knockdown on ObR expression in colon cancer cell lines, and confirmed decrease in ObR mRNA and protein levels. Furthermore, we also confirmed increased ObR expression levels in exogenous Wnt-stimulated HEK293 cells. Thus, we propose that Wnt signalling contributes to the upregulation of ObR in colonic epithelium. Based on these results, we conclude that leptin stimulates the proliferation of tumour cells that carry activating alterations in the canonical Wnt pathway (Supplementary figure 11). Furthermore, these data also suggest that leptin is not involved in early-stage colorectal carcinogenesis. Collectively, our observations provided a novel finding that leptin acts as growth factor for CRC only after the tumour initiation stage during the process of colorectal carcinogenesis (Supplementary figure 12).

Our data define a novel role for leptin signalling in the control of tumour growth in addition to its essential role in food intake and energy regulation. The role of leptin signalling is evident from the finding of increased ObR expression in colon tumours, and of such increased expression coinciding with the activation of STAT3. Furthermore, absence of leptin signalling prevented tumour growth, and suppressed STAT3 activation in these tumours. These findings demonstrated that activation of STAT3 in tumours is crucially dependent on leptin signal transduction. Finally, the leptin signalling mechanism of action was revealed

operationally by the finding that treating mice with recombinant leptin increased tumour growth. Taken together, these data provide strong evidence to indicate that leptin signalling controls tumour growth in vivo.

It has been shown previously that recombinant leptin does not stimulate cell proliferation and carcinogenesis in vivo.[16 18 20] While continuous treatment with recombinant leptin enhanced tumour growth in AOM-treated mice, the effect of exogenous leptin was not as strong as we had expected. On the other hand, there is general agreement that leptin acts as a growth factor for colon cancer cells in vitro.[15–17] These discrepancies between in vivo and in vitro studies could be explained by the complicated interaction between various hormones and cytokines. The effects of leptin in vivo are not as simple as those in vitro. Leptin is known to regulate the secretion of several hormones. Importantly, the actions of leptin involve amelioration of hyperinsulinaemia.[35 36] We observed such actions of leptin on insulin levels in mice treated with recombinant leptin. Insulin has the effect of promoting the development of chemically induced tumours in the colon.[37] Therefore, in vivo, the effects of exogenous leptin on promotion of colonic tumourigenesis might be suppressed through a decrease in insulinaemia.

In conclusion, we clearly demonstrated a relationship between leptin signalling and growth of colon tumours, using leptin-deficient or leptin-receptor-deficient mice. The dramatic suppression of colon tumour growth resulting from inhibition of leptin signalling indicates that leptin is an important growth factor for colon cancer progression. We speculate that dietary intake of excessive fat and calories might result in energy storage in the visceral and subcutaneous adipose tissue compartments, and that any surplus energy might be used for growth of CRC through leptin signalling. On the basis of the current results, it is reasonable to conceive that colon tumours might have a tendency to develop in obese individuals who over-eat and who show elevated serum leptin levels. Future study is warranted to address the importance of leptin signalling in the metastatic spread of CRC. Our data provide novel insights into leptin signalling in CRC and suggest novel therapeutic and preventive targets against colon polyps and cancers based on inhibition of leptin-dependent STAT3 signalling.

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Competing interests None.

Ethics approval All animal experiments were conducted with the approval of the institutional Animal Care and Use Committee of Yokohama City University School of Medicine.

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REFERENCES

1. **Mokdad AH,** Ford ES, Bowman BA, *et al*. Prevalence of obesity, diabetes, and obesity-related health risk factors, 2001. *JAMA* 2003;**289**:76–9.
2. **Calle EE,** Thun MJ, Petrelli JM, *et al*. Body-mass index and mortality in a prospective cohort of U.S. adults. *N Engl J Med* 1999;**341**:1097–105.
3. **Bianchini F,** Kaaks R, Vainio H. Overweight, obesity, and cancer risk. *Lancet Oncol* 2002;**3**:565–74.
4. **Flegal KM,** Graubard BI, Williamson DF, *et al*. Cause-specific excess deaths associated with underweight, overweight, and obesity. *JAMA* 2007;**298**:2028–37.
5. **Renehan AG,** Tyson M, Egger M, *et al*. Body-mass index and incidence of cancer: a systemic review and meta-analysis of prospective observational studies. *Lancet* 2008;**371**:569–78.
6. **Nam SY,** Kim BC, Han KS, *et al*. Abdominal visceral adipose tissue predicts risk of colorectal adenoma in both sexes. *Clin Gastroenterol Hepatol* 2010;**8**:443–50.
7. **Frezza EE,** Wachtel MS, Chiriva-Internati M. Influence of obesity on the risk of developing colon cancer. *Gut* 2006;**55**:285–91.
8. **Zhang Y,** Proenca R, Maffei M, *et al*. Positional cloning of the mouse obese gene and its human homologue. *Nature* 1994;**372**:425–32.
9. **Considine RV,** Sinha MK, Heiman ML, *et al*. Serum immunoreactive-leptin concentrations in normal-weight and obese humans. *N Engl J Med* 1996;**334**:292–5.
10. **Maffei M,** Halaas J, Ravussin E, *et al*. Leptin levels in human and rodent: measurement of plasma leptin and ob RNA in obese and weight-reduced subjects. *Nat Med* 1995;**1**:1155–61.
11. **Tamakoshi K,** Toyoshima H, Wakai K, *et al*. Leptin is associated with an increased female colorectal risk: a nested case–control study in Japan. *Oncology* 2005;**68**:454–61.
12. **Stattin P,** Lukanova A, Biessy C, *et al*. Obesity and colon cancer: does leptin provide a link? *Int J Cancer* 2004;**109**:149–52.
13. **Wallace AM,** Sattar N, McMillan DC. Effect of weight loss and the inflammatory response on leptin concentrations in gastrointestinal cancer patients. *Clin Cancer Res* 1998;**4**:2977–9.
14. **Arpaci F,** Yilmaz MI, Ozet A, *et al*. Low serum leptin level in colon cancer patients without significant weight loss. *Tumori* 2002;**88**:147–9.
15. **Hardwick JC,** Van Den Brink GR, Offerhaus GJ, *et al*. Leptin is a growth factor for colonic epithelial cells. *Gastroenterology* 2001;**121**:79–90.
16. **Aparicio T,** Kotelevets L, Tsocas A, *et al*. Leptin stimulates the proliferation of human colon cancer cells in vitro but does not promote the growth of colon cancer xenografts in nude mice or intestinal tumorigenesis in *Apc*$^{Min/+}$ mice. *Gut* 2005;**54**:1136–45.
17. **Rouet-Benzineb P,** Aparicio T, Guilmeau S, *et al*. Leptin counteracts sodium butyrate-induced apoptosis in human colon cancer HT-29 cells via NF-kappaB signaling. *J Biol Chem* 2004;**279**:16495–502.
18. **Chaudhary M,** Mandir N, FitzGerald AJ, *et al*. Starvation, leptin and epithelial cell proliferation in the gastrointestinal tract of the mouse. *Digestion* 2000;**61**:223–9.
19. **Hirose Y,** Hata K, Kuno T, *et al*. Enhancement of development of azoxymethane-induced colonic premalignant lesions in C57BL/KsJ- *db/db* mice. *Carcinogenesis* 2004;**25**:821–5.
20. **Aparicio T,** Guilmeau S, Goiot H, *et al*. Leptin reduces the development of the initial precancerous lesions induced by azoxymethane in the rat colonic mucosa. *Gastroenterology* 2004;**126**:499–510.
21. **Fujisawa T,** Endo H, Tomimoto A, *et al*. Adiponectin suppresses colorectal carcinogenesis under the high-fat diet condition. *Gut* 2008;**57**:1531–8.
22. **Osawa E,** Nakajima A, Wada K, *et al*. Peroxisome proliferator-activated receptor gamma ligands suppress colon carcinogenesis induced by azoxymethane in mice. *Gastroenterology* 2003;**124**:361–7.
23. **Barker N,** Huch M, Kujala P, *et al*. Lgr5^{+ve} stem cells drive self-renewal in the stomach and build long-lived gastric units in vitro. *Cell Stem Cell* 2010;**6**:25–36.
24. **Bird RP.** Observation and quantification of aberrant crypts in the murine colon treated with a colon carcinogen: preliminary findings. *Cancer Lett* 1987;**37**:147–51.
25. **Rao CV,** Hirose Y, Indranie C, *et al*. Modulation of experimental colon tumorigenesis by types and amounts of dietary fatty acids. *Cancer Res* 2001;**61**:1927–33.
26. **Giovannucci E,** Goldin B. The role of fat, fatty acids, and total energy intake in the etiology of human colon cancer. *Am J Clin Nutr* 1997;**66**:1564S–71S.
27. **Lee GH,** Proenca R, Montez JM, *et al*. Abnormal splicing of the leptin receptor in diabetic mice. *Nature* 1996;**379**:632–5.
28. **Takahashi M,** Wakabayashi K. Gene mutations and altered gene expression in azoxymethane-induced colon carcinogenesis in rodents. *Cancer Sci* 2004;**95**:475–80.
29. **Takahashi M,** Fukuda K, Sugimura T, *et al*. β-Catenin is frequently mutated and demonstrates altered cellular location in azoxymethane-induced rat colon tumors. *Cancer Res* 1998;**58**:42–6.
30. **Li J,** Wang CY. TBL1-TBLR1 and beta-catenin recruit each other to Wnt target-gene promoter for transcription activation and oncogenesis. *Nat Cell Biol* 2008;**10**:160–9.
31. **Banks AS,** Davis SM, Bates SH, *et al*. Activation of downstream signals by the long form of the leptin receptor. *J Biol Chem* 2000;**275**:14563–72.
32. **Otake S,** Takeda H, Fujishima S, *et al*. Decreased levels of plasma adiponectin associated with increased risk of colorectal cancer. *World J Gastroenterol* 2010;**16**:1252–7.
33. **Fenton JI,** Birmingham JM, Hursting SD, *et al*. Adiponectin blocks multiple signaling cascades associated with leptin-induced cell proliferation in Apc Min/+ colon epithelial cells. *Int J Cancer* 2008;**122**:2437–45.
34. **Takahashi M,** Nakatsugi S, Sugimura T, *et al*. Frequent mutation of the β-catenin gene in mouse colon tumors induced by azoxymethane. *Carcinogenesis* 2000;**21**:1117–20.
35. **Sivitz WI,** Walsh SA, Morgan DA, *et al*. Effects of leptin on insulin sensitivity in normal rats. *Endocrinology* 1997;**138**:3395–401.
36. **Kieffer TJ,** Habener JF. The adipoinsular axis: effects of leptin on pancreatic β-cells. *Am J Physiol Endocrinol Metab* 2000;**278**:E1–14.
37. **Tran TT,** Medline A, Bruce WR. Insulin promotion of colon tumors in rats. *Cancer Epidemiol Biomarkers Prev* 1996;**5**:1013–15.

Leptin acts as a growth factor for colorectal tumours at stages subsequent to tumour initiation in murine colon carcinogenesis

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MOLECULAR CANCER

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An integrative genomic analysis revealed the relevance of microRNA and gene expression for drug-resistance in human breast cancer cells

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Abstract

Background: Acquisition of drug-resistance in cancer has led to treatment failure, however, their mechanisms have not been clarified yet. Recent observations indicated that aberrant expressed microRNA (miRNA) caused by chromosomal alterations play a critical role in the initiation and progression of cancer. Here, we performed an integrated genomic analysis combined with array-based comparative hybridization, miRNA, and gene expression microarray to elucidate the mechanism of drug-resistance.

Results: Through genomic approaches in MCF7-ADR; a drug-resistant breast cancer cell line, our results reflect the unique features of drug-resistance, including MDR1 overexpression via genomic amplification and miRNA-mediated TP53INP1 down-regulation. Using a gain of function study with 12 miRNAs whose expressions were down-regulated and genome regions were deleted, we show that miR-505 is a novel tumor suppressive miRNA and inhibits cell proliferation by inducing apoptosis. We also find that Akt3, correlate inversely with miR-505, modulates drug sensitivity in MCF7-ADR.

Conclusion: These findings indicate that various genes and miRNAs orchestrate to temper the drug-resistance in cancer cells, and thus acquisition of drug-resistance is intricately controlled by genomic status, gene and miRNA expression changes.

Keywords: aCGH, microRNA, gene expression, breast cancer, drug resistance

Background

Systemic therapy improves disease-free survival in patients with breast cancer, but does not cure patients with advanced or metastatic disease, and fails to benefit the majority of patients with localized breast cancer. Intrinsic resistance to chemotherapy is emerging as a significant cause of treatment failure, and evolving research has identified several potential causes of resistance [1]. For instance, P-glycoprotein (Pgp), the drug efflux pump encoded by the MDR-1 gene is associated with multidrug resistance in several kinds of advanced cancer. Furthermore, the multidrug resistance-associated protein MRP1 [2,3], breast cancer resistance protein (ABCG2) and other transporters [4], which act as energy-dependent efflux pumps capable of expelling a large range of xenobiotics, have been reported to be upregulated in tumor cells showing the multidrug-resistant phenotype. In addition, overexpression of anti-apoptotic proteins, such as Bcl-2 and Bcl-xL, are also associated with drug resistance and poor clinical outcome in cancer patients. It is essential to decide the molecular target to treat the advanced cancer by molecular targeted therapies such as RNA interference and antibody treatment, however, regulatory networks underlying drug resistance in cancer cells have been elusive.

MicroRNAs (miRNAs) are small non-coding RNA of 21-25nt transcripts, playing central roles in physiological and pathological processes, including cell differentiation, apoptosis, and oncogenesis by either inducing mRNA degradation or by regulating the translational efficiency of mRNA [5-7]. Recently, several research groups have

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provided evidence that some miRNA expression levels are frequently modulated by genomic aberrations, such as genomic DNA copy number gain or loss, translocations, and epigenetic regulations [8]. For example, miR-15a and miR-16-1, whose genomic regions are deleted and expressions are down-regulated in the majority of chronic lymphocytic leukemia (CLL). Furthermore, their target Bcl-2 is overexpressed in CLL at the mRNA and protein level [9]. Another study showed that the expression level of miR-34a was down-regulated by deletion of 1p36 heterozygosity in neuroblastoma and contributed to an aggressive phenotype [10]. As reported in the studies of cancer genetics in lung, leukemia, colon, breast and ovary, a large number of miRNAs are located at chromosomal fragile sites, i.e., minimal regions of loss of heterozygosity (LOH) and minimal regions of genomic amplification [11]. These reports indicated that the emphasis on a genomic analysis was due to the fact that DNA copy number alterations are associated with expression levels of miRNAs and genes.

In this study, to better understand the regulatory network underlying drug resistance in breast cancer cells, we focus on miRNAs and genes located on the genome-amplified and -deleted regions because genomic aberration is closely associated with gene expression, and this expression alteration might be constantly maintained. For the identification of molecular targets, we initially performed an integrated genomic analysis to compare the DNA copy number and expression profile of mRNA and miRNA between MCF7; a parental breast cancer cell line and MCF7-ADR; a drug-resistant breast cancer cell line [12,13]. Through the genomic analysis, we found that the genomic alterations of drug resistance-related genes, e.g. amplified genomic regions and overexpression of MDR-1 and miRNA-mediated TP53INP1 down-regulation. In addition, of 12 miRNAs whose expressions were down-regulated and genomic regions were deleted, we determined that miR-505 promotes the inhibition of cell growth in MCF7-ADR cells, by inducing apoptotic cell death in the presence of docetaxel (DOC).

Methods

Cell culture

MCF7 human mammary carcinoma cells and multidrug-resistant MCF7-ADR human mammary carcinoma cells were obtained from Shien-Lab, Medical Oncology, National Cancer Center Hospital. MCF7-ADR-Luc cells were established by transfecting with a pLuc-neo expression vector, which has the firefly luciferase GL3 cDNA cloned into the downstream of the SV40 promoter and the G418 selective marker gene. Cells were selected in a medium containing 0.6 mg/ml of G418 (Gibco BRL) and were maintained and passaged in an RPMI 1640 medium (Gibco BRL) supplemented with 10% fetal bovine serum (Gibco BRL) under 5% CO_2 in a humidified incubator at 37°C.

RNA and genomic DNA extraction

Total RNA was extracted from MCF7, MCF7-ADR, and MCF7-ADR-Luc cells using the ISOGEN solution (Nippon Gene, Tokyo, Japan) according to the manufacturer's protocol. Genomic DNA was prepared from MCF7 and MCF7-ADR. The yield and purity of the genomic DNA and total RNA were measured using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). The quality of the total RNA was verified to have an RNA Integrity Number using a Bioanalyzer and RNA 6000 LabChip Kit (Agilent Technologies).

Oligonucleotide array CGH (aCGH) Analysis

All DNA labeling reactions and hybridizations were carried out following the manufacture's protocol (Agilent Oligonucleotide Array-Based CGH for Genomic DNA Analysis, Version 4.0, Direct Method). Briefly, 3.0 μg of MCF-7, MCF7-ADR and reference DNA (Promega, female, p/n G1521) were digested with *Alu*I and *Rsa*I for 2 hours at 37°C, followed by heat inactivation at 65°C for 10 minutes. Digested DNA was then labeled using the Agilent Genomic DNA Labeling Kit Plus (p/n 5188-5309) using random primers and the exo-Klenow fragment to differentially label genomic DNA samples with fluorescently labeled nucleotides. All experimental and reference samples were labeled with Cyanine-5 dUTP and Cyanine-3 dUTP, separately, for 2 hours at 37°C to enable duplicate hybridizations with the corresponding dye reversal arrays. Experimental and reference targets for each hybridization were purified with a Microcon YM-30 column (Millipore) and validated by the NanoDrop ND-1000, respectively to ensure the yield and the specific dye incorporation activity of the labeled genomic DNA. The individual pair of labeled targets were combined together, mixed with Cot-1 DNA (Invitrogen) and 10xBlocking Agent (Agilent), and then mixed with Agilent 2xHybridization Buffer (p/n 5188-5220). Before hybridization, the combined mixtures were denatured for 3 minutes at 95°C, incubated for 30 minutes at 37°C and then applied to the Agilent Human 244A CGH arrays (G4411B). Using an Agilent microarray hybridization chambers, the hybridization was carried out for 40 hours at 65°C in a rotating oven (Agilent) at 20 r.p.m. The hybridization chambers were then disassembled and array slides were washed for 5 minutes at room temperature in Agilent Oligo aCGH Wash Buffers 1, followed by 1 minute at 37°C in Agilent Oligo aCGH Wash Buffer 2 (p/n 5188-5226) (prewarmed to 37°C overnight). The slides were removed from the wash buffer 2 slowly (5-10 seconds) after which time they were completely dry and were scanned using an Agilent DNA Microarray scanner with 5 μm resolution. The data of microarray

images were extracted by Agilent Feature Extraction Software v9.5 in which a modified Feature Extraction protocol, CGH-v4_95_Feb07 was used in conjunction with a gene list on chromosome 21 q-arm to normalize spot-intensity values and ratios to each extraction set. These ratio data along with associated error values and flagged features were imported into CGH Analytics Software v3.4 (Agilent). The dye reversal data and intra-replicate spots were then combined while the data centralization and fuzzy zero algorithms were not applied in the CGH Analytics. To make aberration calls, an aberration detection algorithm, ADM-2 [14] was used at threshold 10 and an aberration filter was set at 2 for the minimum number of probe region and 1 for minimum absolute average log2 ratio for regions in the CGH Analytics to reduce false positives.

Gene Expression Analysis

All RNA labeling reactions and hybridizations were carried out following the manufacture's protocol (Agilent One-Color Microarray-Based Gene Expression Analysis, Version 5.0.1). Briefly, polyA(+)RNA in 500 ng of total RNA was primed with an oligo (d)T-T7 primer and converted into dsDNA with MMLV-RT, then transcribed and simultaneously labeled with Cyanine 3-CTP for 2 hours at 40°C using Agilent Low RNA Input Linear Amplification Kit (p/n 5188-5339). After labeling and cRNA purification, cRNA was quantified and the specific dye incorporation activity was validated using the NanoDrop ND-1000. 1.65 μg of labeled cRNA was mixed with Agilent 10×Blocking Agent and 25×Fragmentation Buffer, then incubated at 60°C for 30 hours. After fragmentation, the cRNA mixtures were immediately mixed with Agilent 2×Hybridization Buffer (p/n 5188-5339) and applied to the Agilent Human 4×44 K whole genome microarrays (G4112F) for 17 hours at 65°C (10 r.p.m.). Array slides were washed with Agilent Gene Expression Wash Buffer 1 and 2 (p/n 5188-5327) and then scanned using the Agilent DNA Microarray scanner with 5 μm resolution and the eXtended Dynamic range setting (XDR Hi 100%, Low 10%) to avoid saturated features. The data of microarray images were extracted by Agilent Feature Extraction Software v9.5 using the GE1_v5_95 protocol. The extracted signal intensities and flagged information were imported into GeneSpring 7.3.1 software and the data sets were normalized by adjusting the intensity distribution of well-above background and unflagged features to 50th percentile to account for the interchip variability. Comparison of MCF7 and MCF7-ADR was done using duplicate array data set for each cell line.

miRNA Expression Analysis

All RNA labeling reactions and hybridizations were carried out following the manufacture's prototype protocol (Agilent miRNA Microarray system, Version 0.3, early access). Briefly, 100 ng of total RNA including fraction of small mature miRNA was dephosphorylated by calf intestine alkaline phosphatase (p/n E2250Y, Amersham Biosciences) for 30 minutes at 37°C and denatured by adding DMSO (p/n D8418, Sigma) for 8 minutes at 100°C. Ligation was then carried out with T4 RNA ligase (p/n E2050Y, Amersham Biosciences) and pCp-Cy3 (p/n 5190-0408, Agilent) for 2 hours at 16°C that allowed us to perform a quantitative direct labeling method [15]. The labeled miRNAs were desalted with Micro Bio-Spin 6 column (p/n 732-6221, Bio-Rad) and combined with Agilent 10×GE Blocking Agent and 2×Hybridization Buffer (p/n 5190-0408). The mixture was heated for 5 minutes at 100°C and immediately cooled to 0°C. Each sample was hybridized to the Agilent early access Human 8×15 K microRNA microarrays covered 470 miRNAs (AMADID 015508, early access) for 20 hours at 55°C (20 r.p.m.). Array slides were washed with 6× SSC/ 0.005% Triton X-100 for 10 minutes, then 0.1× SSC/ 0.005% Triton X-100 for 5 minutes, both at room temperature. Slides were scanned using the Agilent DNA Microarray scanner with 5 μm resolution and the eXtended Dynamic range setting (XDR Hi 100%, Low 5%) to avoid saturated features. The data were extracted by Agilent Feature Extraction Software v9.5 using the miRNA_120106 protocol which extracts intensities of multiple probes with multiple features per probe and reports the measurements and errors as the TotalGeneSignal and TotalGeneSignalError for each of the miRNAs. These values were imported to the GeneSpring GX version 7.3.1 without applying any normalization algorithm. The miRNA profiles generated on the Agilent platform were prior normalized to the amount of input total RNA in which 100 ng of total RNA were equally used for each assay and all of the labeled targets were loaded on each array. Comparison of MCF7 and MCF7-ADR was done using duplicate array data set for each cell line.

Transfection of miRNA into MCF7-ADR-Luc cells

For MCF7-ADR-Luc cells, transfection of miRNA was carried out using DharmaFECT 1 (Dharmacon) according to the manufacturer's protocol. MCF7-ADR-Luc cells were plated in growth medium 24 hours before transfection. The cells, which were grown to 50% confluence, were transfected with 20 nM miRNAs and cultured. Two or 3 days after transfection, the cells were subjected to further analyses.

Apoptosis assay measurement of caspase activity *in vitro*

Caspase-7, which plays key effecter roles in apoptosis, was detected in caspase-3-deficient MCF7-ADR-Luc cells. The arrays used in an *in vitro* growth assay were