GGGCGGTAAGCAGCAATG-3' ; probe, 5'-6FAMCTTCTTGGCCTTCGCCTCGTGCTTAMRA-3' ; ribosomal S9 forward, 5'-ATCCGCCAGCGCCATA-3' and reverse, 5'-TCAATGTGCTTCTGGGAATCC-3' ; probe, 5'-6FAMAGCAGGTGGTGAACATCCCGTCCTTTAMRA-3') using the Primer Express software (Applied Biosystems).

Fluorescent signal data were collected by the ABI Prism 7700 Sequence Detection System (Applied Biosystems). The log-linear phase of amplification was monitored to obtain the threshold cycle (defined as the fractional cycle number at which the amount of the amplified target reaches a fixed threshold) values for each RNA sample. Ribosomal S9 was used as the internal reference and was selected because it exhibits minimal variability in tissues of different origins [29]. The comparative threshold cycle method was employed to determine IGF II expression levels in each sample relative to a calibrator, in this case MCF10AT cells [29,30]. Each sample was run in triplicate.

Statistical analysis

A relationship between BrdU labeling or apoptotic indexes and co-culture with NAF or with CAF was assessed by multiple linear regression analysis to allow combining replicate experiments performed on different days. Results of ELISA and quantitative real-time PCR were compared by *t* test. Outliers were eliminated prior to analysis using a basic outlier test, where a high outlier was defined as a number greater than quartile 3 + 1.5 (interquartile range) and a low outlier was less than quartile 1 – 1.5 (interquartile range).

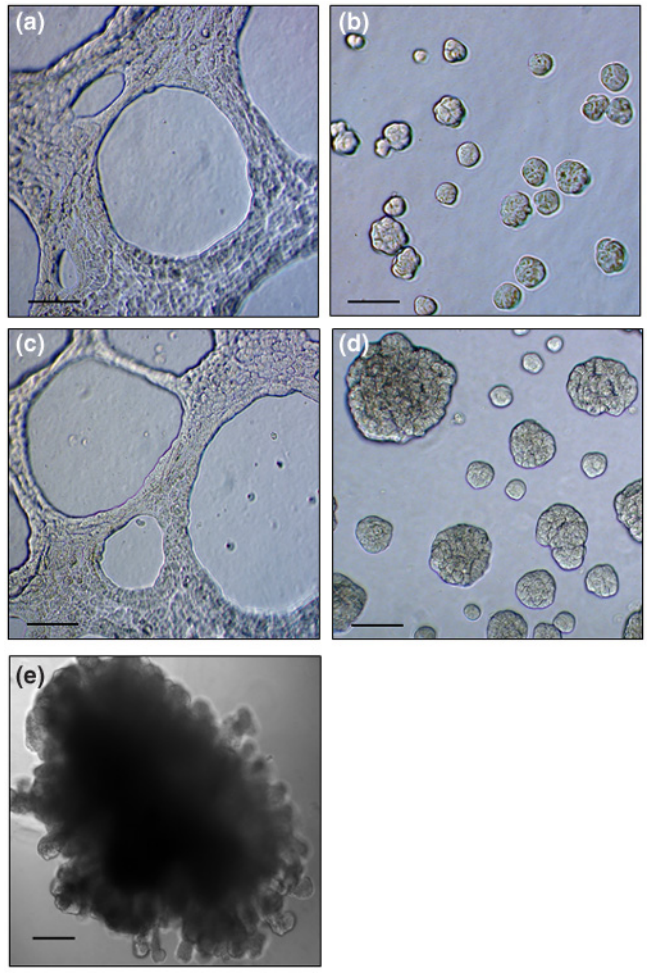
Results

Fibroblasts alter the 3D morphology of MCF10A cells and MCF10AT cells

The immunocytochemical characterization of fibroblasts used in the described experiments confirmed their stromal nature. Immunostaining for vimentin was strongly positive and staining for epithelial membrane antigen and CK 5/CK 8 was negative.

In 3D monocultures, both MCF10A cells and MCF10AT cells initially form a lattice-like network of duct-like structures. After several days, the lattice-like network is replaced by a predominance of rounded epithelial cell groups (spheroids) (Fig. 1a,1b,1c,1d). MCF10A cells form small, rounded spheroids (Figs 1b and 2a). MCF10AT cells aggregate into larger solid groups or sheets with extensive squamous metaplasia (Figs 1d and 2c). The formation of larger three-dimensional structures and the abnormal differentiation (i.e. squamous metaplasia) of MCF10AT cells supports their greater degree of transformation.

Figure 1



MCF10A cells and MCF10AT cells in monoculture and in co-culture with fibroblasts. **(a)**, **(b)** MCF10A cells and **(c)**, **(d)** MCF10AT cells in monoculture initially form a lattice/scaffold arrangement (a, c). After several days of culture, spheroidal structures become more prominent (b, d). **(e)** MCF10AT cells in co-culture with fibroblasts form three-dimensional rounded structures. Similar structures are formed by MCF10A cells in co-culture with fibroblasts (phase contrast, 100 × magnification; scale bar, 200 μm).

In 3D co-cultures with NAF or with CAF, the epithelial cells and fibroblasts form large rounded structures (Fig. 1e). In histologic sections of co-cultures with NAF or with CAF, MCF10A cells and MCF10AT cells form spheroids or sheets within Matrigel®, as seen in monocultures, and these epithelial groups surround an aggregate of fibroblasts (Fig. 2b,2d). These 3D co-cultures resemble a terminal duct-lobular unit in the normal breast or in proliferative breast disease/DCIS (Fig. 3a,3b,3c), in that epithelial cells are arranged in groups (similar to an acinus or a terminal duct) surrounded by a laminin-rich basement membrane with fibroblasts located outside the basement membrane and separating epithelial groups. The 3D *in vitro* model differs from normal breast or *in situ* breast disease *in vivo*,